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INVESTIGATION OF DIFFERENT BLOOD PRESSURE REGULATING SYSTEMS IN KININOGEN-DEFICIENT BNK RATS

Referees: Prof. Dr. Ulrich Hilgenfeldt

Prof. Dr. Gert Fricker

We read that the Swedish author August Strindberg was once caught blowing pipe smoke into a tub of water to see whether he could produce gold. And we say to ourselves: Look what an insane idea emerged from such a brilliant mind! But isn't it more correct to say: Wasn't it Strindberg who experimentally proved that releasing tobacco smoke into water does not produce gold? Someone had to try it. Someone had to examine that dead end of human knowledge and alert the world: Not this way, my friends!

Jara Cimrman, Czech genius

words like violence
break the science

wows are spoken
to be broken

feelings are intense
words are trivial

pleasures remain
so does the pain

words are meaningless
and forgettable

all I ever wanted
all I ever needed
is here in my arms
words are very unnecessary
they can only do harm

Thank you all,
Jiri, Sarah, Radka, Eugi and Chef

ABSTRACT

Lukasova, Martina; pharmacist oral examination on 22nd February 2006

INVESTIGATION OF DIFFERENT BLOOD PRESSURE REGULATING SYSTEMS IN KININOGEN-DEFICIENT BNK RATS

Referees: Prof. Dr. Ulrich Hilgenfeldt; Prof. Dr. Gert Fricker

The kininogen-deficient BNK rats were shown to progressively develop salt-sensitive hypertension by accumulation of Na^+. It was believed that the kininogen-deficiency is responsible for the defective NaCl excretion leading to a cardiovascular damage. In addition to the kallikrein-kinin system (KKS), endothelin (ET) and aldosterone have important implications in the regulation of salt homeostasis.

To precisely characterize the relations between the KKS, ET and aldosterone male BN and BNK rats were given standard or high salt diet in presence or absence of spironolactone for 10 days. Besides for experiments with metabolic cages the rats were characterized by tail-cuff measurements of blood pressure. Kininogens, bradykinin (BK), kallidin-like peptide (KLP), ET-1, deoxycorticosterone (DOC), corticosterone and aldosterone were measured by specific RIAs in plasma and 24h urine. Corticoids were additionally determined in brain tissue. Activity of plasma and urinary kallikrein was measured with chromogenic substrates. Expression analysis of adrenal steroidogenic enzymes 11ß-hydroxylase and aldosterone synthase was performed by real-time RT-PCR in LightCycler. Functional cardiac parameters were assessed in isolated hearts perfused according to Langendorff technique. Acute effects of 30min global ischaemia with or without previous ischaemic preconditioning (IPC) were investigated. Myocardial damage was judged by the creatine kinase activity in the coronary effluent measured with a specific kit.

BNK rats are characterized by plasma kininogen deficiency that is reflected in lower levels of plasma kallikrein activity and consequently in lower plasma BK and KLP levels. The renal KKS of BNK rats was almost identical to that of wild type BN rats. Plasma and urinary KLP was found to be the major kinin responsible for most physiological effects mediated by the B_2 receptor in both rat strains. We found that the deleterious effects of salt diet in BNK rats are in fact attributable to enhanced mineralocorticoid action. The renal mineralocorticoid receptor (MR) displayed an overexpression and several mutations that might be responsible for altered affinity and responses toward aldosterone and other ligands, e.g. DOC and corticosterone. Moreover, the BNK rats have increased levels of plasma and urinary ET-1, which may contribute to the deleterious effects of Na^+ during high salt diet. *In vitro* investigation of ischaemic preconditioning in isolated hearts revealed no significant findings. Missing plasma components are necessary for the overall protective effects of IPC. Nevertheless, long-lasting effects like attenuation of plasma kinins or antagonism of the MR seemed to be of importance for the regulation of cardiac function. The absence of plasma KLP during high salt diet was found to be responsible for the enhanced deleterious effects of Na^+ leading to cardiac hypertrophy. In BNK rat hearts aldosterone was found to be responsible for the higher heart rate and increased contractility besides for the impairment of coronary flow. Aldosterone is also involved in the acute response to ischaemia and consequently in response to IPC. In case of an enhanced mineralocorticoid action like in BNK rats aldosterone may account for the increased sensitivity to ischaemia whereby attenuating the protective effect of IPC.

We conclude that in BNK rats the higher blood pressure, increased cardiac sensitivity to ischaemia and diminished effects of IPC are not attributable to the kininogen deficiency but rather caused by the enhanced mineralocorticoid action.

ZUSAMMENFASSUNG

Lukasova, Martina; Apothekerin mündliche Prüfung am 22.02.2006

UNTERSUCHUNGEN VERSCHIEDENER BLUTDRUCKREGULIERENDER SYSTEME IN KININOGEN-DEFIZIENTEN BNK RATTEN

Gutachter: Prof. Dr. Ulrich Hilgenfeldt; Prof. Dr. Gert Fricker

Kininogen-defiziente BNK Ratten entwickeln einen progressiven salz-sensitiven Bluthochdruck und ein erhöhtes kardiovaskuläres Risiko, das durch eine verminderte NaCl Ausscheidung verursacht wird. Neben dem Kallikrein-Kinin System (KKS) sind auch Endothelin (ET) und Aldosteron an der Regulation der Salz-Wasserhomöostase beteiligt.

Um die Wechselwirkung zwischen den KKS, ET und Aldosteron genauer zu untersuchen, wurden männliche BN und BNK Ratten 10 Tage lang mit einer Standard- oder Hochsalz-Diät gefüttert und gleichzeitig mit oder ohne Spironolacton behandelt. Vor und nach Beendigung der Behandlungsperiode wurde der Blutdruck gemessen und die Ratten 24h auf Stoffwechselkäfige gesetzt. Wir bestimmten Kininogene, Bradykinin (BK), Kallidin-like Peptid (KLP), ET-1, Deoxykortikosteron (DOC), Kortikosteron und Aldosteron im Plasma und im 24h Urin mit spezifischen RIAs sowie die enzymatische Aktivität von plasmatischem und urinärem Kallikrein. Ferner konnten zusätzlich Kortikoide im Gehirn bestimmt werden. Expressionsanalysen der adrenalen 11ß-Hydroxylase und Aldosteronsynthase führten wir mit Hilfe von real-time RT-PCR im LightCycler durch. Mittels Langendorff Technik wurden im Modell des isoliert-perfundierten Rattenherzen primäre kardiale Parameter und Akuteffekte nach 30min Ischämie mit und ohne ischämische Präkonditionierung untersucht. Die Aktivität der Kreatinkinase in koronaren Perfusaten diente zur Evaluierung myokardialer Schäden.

BNK Ratten weisen eine Kininogen-Defizienz im Plasma auf, verbunden mit einer geringeren Kallikreinkonzentration und deutlich verminderten BK und KLP Konzentrationen. Das renale KKS von BNK Ratten zeigte im Vergleich mit BN Kontrollratten beinahe identische Werte. In beiden Rattenstämmen erwies sich das Kallidin-Äquivalent, KLP, als primäres Kinin, das für die meisten physiologischen Effekte verantwortlich ist, die über den B_2 Rezeptor vermittelt werden. Die negativ Effekte von Hochsalzdiät in BNK Ratten wurden vor allem durch eine verstärkte mineralokortikoide Antwort verursacht. Wir konnten zeigen, dass in BNK Ratten der Mineralokortikoid Rezeptor (MR) überexprimiert wird und mehrere Mutationen besitzt, die eine veränderte Affinität für Aldosteron und andere Liganden, wie z.B. DOC und Kortikosteron, nach sich zieht. Daher zeigten BNK Ratten auch höhere plasmatische und urinäre ET-1 Spiegel, die an den negativen Effekten der Hochsalzdiät beteiligt sind. *In vitro* Untersuchung der ischämische Präkonditionierung zeigte keine signifikante Unterschiede zwischen den beiden Rattenstämmen. Wir führen das auf das Fehlen plasmatischer Komponenten zurück, die eine wichtige Rolle bei der Vermittlung der protektiven Effekte bei der IPC spielen. Dennoch war durch die unterschiedliche Vorbehandlung die Ausgangssituation der isolierten Herzen unterschiedlich, da die kardiale Funktion durch die niedrigere plasmatische Kininkonzentration oder den Antagonismus des MR beeinflusst wird. Während eine Hochsalzdiät erhöhte der Mangel an KLP das Risiko des negativen Salzeffektes und führte zu einer kardialen Hypertrophie. Bei den BNK Ratten stand Aldosteron im Zusammenhang mit einer erhöhten Herzrate, erhöhten Kontraktilität und einem verringerten Koronarfluss. Es konnte gezeigt werden, dass Aldosteron an der Akutantwort der Ischämie und der IPC beteiligt ist.

Zusammenfassend konnten wir zeigen, dass in BNK Ratten nicht primär die Kininogen-Defizienz, sondern die verstärkte mineralokortikoide Wirkung für den erhöhten Blutdruck, die erhöhte kardiale Sensitivität bei Ischämie und die verminderte Protektion infolge einer ischämischen Präkonditionierung verantwortlich ist.

CONTENTS

ABBREVIATIONS

A	absorbance
ACE	angiotensin-converting enzyme
ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
AgCl	silver chloride
Ala	alanine
AM	aminopeptidase
Ang	angiotensin
APM	aminopeptidase M
APP	aminopeptidase P
Arg	arginine
Asp	aspartic acid
AT_1 receptor	angiotensin II AT_1 receptor
ATP	adenosine triphosphate
B	binding
B_0	maximum binding
B_1 receptor	bradykinin B_1 receptor
B_2 receptor	bradykinin B_2 receptor
BK	bradykinin
BN rat	Brown Norway rat
BN CO	control experiment in BN hearts
BN IPC	IPC experiment in BN hearts
BNK CO	control experiment in BNK hearts
BNK IPC	IPC experiment in BNK hearts
BNK rat	Brown-Norway Katholiek rat
bp	base pair
BSA	bovine serum albumine
ΔBW	change of body weight
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine 3´,5´-monophosphate
°C	degree Celsius
C	carbon
cDNA	copyDNA
CK	creatine kinase
Cl^-	chloride
CNS	central nervous system
CO_2	carbon dioxide

CP	crossing point
cpm	counts per minute
CPM	carboxypeptidase M
CPN	carboxypeptidase N
CYP11B1	gene coding for 11ß-hydroxylase
CYP11B2	gene coding for aldosterone synthase
Cys	cysteine
DAG	diacylglycerol
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	diethylpyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	mixture of nucleotides
DOC	11-deoxycorticosterone
dp/dt_{max}	maximum contraction velocity
dp/dt_{min}	maximum relaxation velocity
dsDNA	double-stranded DNA
dTTP	2'-deoxythymidine 5'-triphosphate
E	efficiency
EC	enzyme commission
ECE	endothelin-converting enzyme
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EGW	ethylene glycole/water
eNOS	endothelial nitric oxide synthase
ET	endothelin
ET_A receptor	endothelin ET_A receptor
ET_B receptor	endothelin ET_B receptor
FELASA	Federation of European Laboratory Animal Science Associations
Fig.	Figure
g	gram
gDNA	genomic DNA
Glu	glutamic acid
Gly	glycine
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
^3H	tritium
h	hour
H^+	proton

HCl	hydrochloric acid
His	histidine
HMW kininogen	high molecular weight kininogen
H_2O	water
HR	heart rate
HS	high salt diet
HS SPI	high salt diet & spironolactone
11ß-HSD2	11ß-hydroxysteroid dehydrogenase type 2
^{125}I	iodine 125
IgG	immunoglobulin G
Ile	isoleucine
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneally
IP_3	inositol triphosphate
IPC	ischaemic preconditioning
IU	international unit
K^+	potassium
KAL	kallidin
K_{ATP} channel	ATP-sensitive potassium channel
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
KH_2PO_4	dihydrogen potassium phosphate
KI	potassium iodide
KIU	kallikrein inhibitory unit
KKS	kallikrein-kinin system
KLP	kallidin-like peptide
L	litre
µl	microlitre
Leu	leucine
LMW kininogen	low molecular weight kininogen
log_2	logarithm dualis
LVDP	left ventricular developed pressure
LVEDP	left ventricular end-diastolic pressure
LVP_{max}	maximum left ventricular pressure
LVP_{min}	minimum left ventricular pressure
Lys	lysine
µM	micromolar
M	molar
MAPK	mitogen-activated protein kinase

MBP	mean blood pressure
MBq	megabecquerel
Met	methionine
mg	milligram
Mg^{2+}	magnesium ions
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minute
ml	millilitre
mm	millimetre
mmHg	millimetre of mercury
mmol	millimoles
mol	moles
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MW	molecular weight
n	number of values
Na^+	sodium
NaCl	sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	sodium bicarbonate
NaI	sodium iodide
Na^+/K^+-ATPase	sodium, potassium-adenosine triphosphatase
NaN_3	sodium azide
NaOH	sodium hydroxide
NEP	neutral endopeptidase
nm	nanometer
nM	nanomolar
NO	nitric oxide
NOS	nitric oxide synthase
NS	normal salt diet
NSB	non-specific binding
NS SPI	normal salt diet & spironolactone
O_2	oxygen
p	probability
PBGD	porphobilinogen deaminase
PCR	polymerase chain reaction
PEG	polyethylene glycol
Phe	phenylalanine
pI	isoelectric point

PI	phosphatidylinositol
PKC	protein kinase C
pKLK	plasma kallikrein
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
pNA	p-nitroaniline
Pro	praline
R	correlation coefficient
RALES	Randomized Aldactone Evaluation Study
RAS	renin-angiotensin system
RAAS	renin-angiotensin-aldosterone system
REST	relative expression software tool
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
rpm	(rounds per minute) revolutions per minute of rotor
RT	reverse transcription
s	second
s.c.	subcutaneously
Ser	serine
SHR	spontaneous hypertensive rat
Std. Dev.	standard deviation
Std. Err.	standard error
Tab.	Table
Tc	total count
tKLK	tissue kallikrein
Tm	melting temperature
Trp	tryptophan
Tyr	tyrosine
U	unit
Val	valine
VSMC	vascular smooth muscle cells

1. INTRODUCTION

1.1. DIETARY SODIUM AND CARDIOVASCULAR EFFECTS

The importance of dietary sodium chloride (NaCl) in the regulation of blood pressure has received much attention over the past few years. It is generally accepted that the role of the kidney in handling sodium (Na^+) is a key to the long-term regulation of blood pressure. The relationship between renal handling of Na^+ and blood pressure is apparently influenced by a complex combination of many factors, e.g. nutritional, genetic, neurohormonal and metabolic (Jones, 2004). Furthermore, dietary Na^+ may contribute to cardiovascular target organ injury. Recently published studies provide evidence for blood pressure-independent effects of an excess of Na^+ on blood vessels, the heart, and the kidney, such as cardiac hypertrophy and perivascular fibrosis (Jones, 2004).

Among various putative genetic markers, reduced urinary kallikrein excretion has been used as a marker of salt sensitivity in normotensive subjects as well as in essential hypertensive patients, suggesting that depressed activity of the renal **kallikrein-kinin system** (**KKS**) could contribute in the pathogenesis of salt-dependent hypertension. It was hypothesized that the enhanced blood pressure sensitivity to salt is due to Na^+ retention and total volume expansion attributed to the effects of kinins on renal filtrations and/or tubular handling of Na^+. In addition, the KKS-deficient phenotype might be particularly susceptible to develop renal damage as a consequence of modest elevation in blood pressure levels (Madeddu et al., 1997).

Importance of Na^+ accumulation in the development of hypertension and the role of the renal KKS was studied in mutant kininogen-deficient **Brown-Norway Katholiek** (**BNK**) **rats**. The mutant BNK rats have no apparent symptoms. However, they are sensitive to ingested salt, so that 2% NaCl in the diet cause an increase in the systemic blood pressure accompanied with an increased intake of water, reduced urine volume, reduced urinary Na^+, and accumulation of Na^+ in the erythrocytes. The accumulation of Na^+ in the cerebrospinal fluid causes an increase in the sympathetic discharge from the central nervous system (CNS). Elevated levels of Na^+ in the cells

increase sensitivity of the arterioles against noradrenaline and angiotensin (Ang) II. Accordingly blood pressure can raise without an increase in the plasma levels of vasoconstrictors (Majima et al., 1993).

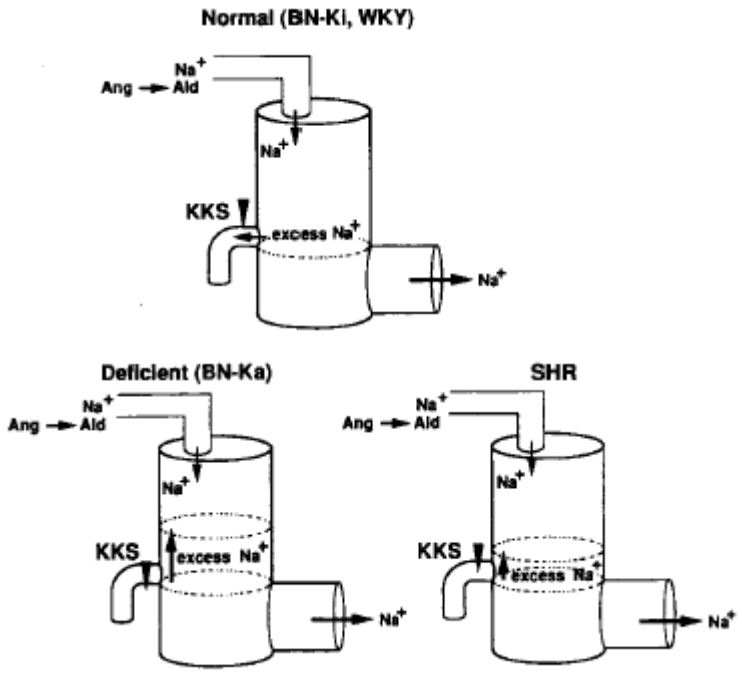


Fig.1 **Role of the kallikrein-kinin system in the kidney.** In normal rats, the KKS safely excretes Na^+ after excess salt intake or Na^+ accumulation by release of Ang or aldosterone, while in the kininogen deficient rats BNKa KKS does not work and excess Na^+ is easily accumulated. In SHR, KKS may insufficiently work and excess Na^+ will tend to be accumulated. BN-Ki, Brown Norway Kitasato rats; WKY, Wistar Kyoto rats; BN-Ka, Brown Norway Katholiek rats; SHR, spontaneous hypertensive rats; Ald, aldosterone (Katori et al., 2001).

Under normal physiological conditions the contribution of the KKS to Na^+ excretion in the renal tubules may be minimal. Once excess Na^+ is ingested or shows a tendency to be accumulated in the body, the kinin generated in the renal tubules is thought to inhibit reabsorption or accelerate secretion of Na^+. The renal KKS acts as a sort of floodgate against Na^+ retention. When this gate is completely closed, as in mutant BNK rats, the loading of Na^+ or Na^+ accumulation through release of aldosterone by Ang II may cause accumulation of Na^+ in the body. In spontaneous hypertensive rats (SHR), the reduction of kallikrein production causes hypertension with Na^+ accumulation (Fig.1). In normal rats, although renal kallikrein may be released basolaterally and kinin released in the interstitial space may play some role in

vasodilation, luminal kinin has a much more important function in preventing the development of hypertension (Majima and Katori, 1995).

Another useful biological marker for the prediction of salt-sensitive hypertension may be the urinary excretion of **endothelin** (**ET**). The kidney expresses both endothelin ET_A and ET_B receptor subtypes. ET_A is localized primarily in the renal vasculature, whereas ET_B receptors are of particular abundance in the epithelial cells of the inner medullary collecting duct (Kohan, 1997). As in peripheral blood vessels, ET-1 of local or systemic origin mediates vasoconstriction via ET_A receptor in renal vasculature causing a decrease in renal blood flow and glomerular filtration rate and subsequently diminishing Na^+ and water excretion. On the contrary, locally produced ET-1 in the medulla provokes vasodilation and promotes salt and water excretion via ET_B receptors (Abassi et al., 2001). Similar patterns of plasma and urinary ET-1 levels have been found in patients and animals with hypertension. Enhanced synthesis of this peptide, especially by the cortical renal vasculature in proximity to ET_A, may result in renal vasoconstriction, which is known to influence systemic blood pressure (Schiffrin, 1999). Despite the importance of ET-1 levels, differences in the vascular sensitivity between normotensive and hypertensive subjects may result in enhanced activity of ET-1, regardless of its concentration. The enhanced sensitivity of the renal vasculature to ET-1 may stem from increased ET_A receptor expression. Decreased renal clearance (through ET_B receptors or enzymatic) of ET-1 has also been implicated in the hypersensitivity (Markewitz and Kohan, 1995). On the contrary, reduced renal ET generation or impaired abundance of ET_B receptors in the medullary tissue diminishes salt and water excretion, leading to salt-dependent hypertension through volume-overload (Fig.2). Activation of ET_B receptors leads to a variety of intracellular events, of which nitric oxide (NO) release is the major one. NO plays an important role in the regulation of medullary blood flow (Abassi et al., 1998). Impairment of NO production may reduce intrarenal blood flow and subsequently reset the pressure-natriuresis relationship, leading to prompt Na^+ retention and contributing to high blood pressure (Abassi et al., 2001).

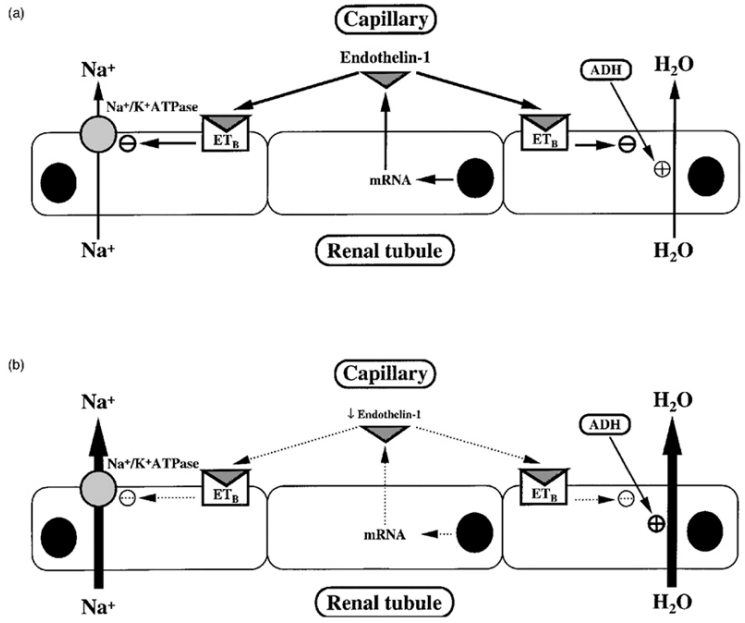


Fig.2 **Effects of renal tubular ET-1 on reabsorption of Na^+ and water in (a) normotensives and (b) hypertensives.** Under physiological circumstances, renal tubule epithelial cells generate ET-1 that acts on epithelial cell ET_B receptors to inhibit reabsorption of Na^+ (less activity of Na^+/K^+- ATPase) and reabsorption of water (less activity of ADH). In hypertension, less renal generation of ET-1 than normally occurs, which results in less tonic inhibition of tubular reabsorption of Na^+ and water and thus leads to retention of Na^+. ADH, antidiuretic hormone; Na^+/K^+-ATPase, sodium, potassium-adenosine triphosphatase (Haynes and Webb, 1998).

In addition to its direct vascular effects, ET-1 has inotropic and mitogenic properties, alters central and peripheral sympathetic activity and stimulates the renin-angiotensin-aldosterone system (RAAS). It also appears likely that ET-1 participates in the adverse cardiac and vascular remodelling of hypertension, as well as in hypertensive renal damage (Haynes and Webb, 1998).

The RAAS plays a central role in the development of hypertension and the progression of end-organ damage. **Aldosterone** has acute and long-term effects that impair endothelial function and promote necrosis and fibrosis of both the vasculature and the heart (Stier et al., 2002). As previously recognized the incidence of primary aldosteronism is more common and may be as high as 7-12% in patients with essential hypertension (Fardella et al., 2000). Most of these patients have no signs of hypokalemia, which indicates excessive mineralocorticoid action on the kidney. Although angiotensin-converting enzyme (ACE) inhibitors and Ang II receptor

antagonists can initially suppress plasma aldosterone, it is now well established that aldosterone escape may occur, whereby aldosterone levels return to or exceed baseline levels (Stier et al., 2002). Aldosterone and salt induce a proinflammatory phenotype at least in part by increasing oxidative stress. Chronic aldosterone and salt treatment increases the expression and activity of myocardial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This enzyme catalyzes the formation of superoxide anion, which can react with NO to form peroxynitrite (Sun et al., 2002). Interestingly, extraadrenal expression of aldosterone synthase in the CNS can be regulated by manipulation of Na^+ intake (Ye et al., 2003).

1.2. MYOCARDIAL ISCHAEMIC PRECONDITIONING

Ischaemic heart disease is a major cause of mortality in industrialized societies. It is characterized by insufficient blood supply to regions of the myocardium, which leads to tissue necrosis (infarction). Ischaemic heart disease may develop as a consequence of many diseases, including hypertension and atherosclerosis. Occasionally, cardiologists report that patients with at least one episode of prodromal angina showed less severe ischaemic damage after subsequent exposure to a longer period of ischaemia. In 1986, Murry et al. first documented this phenomenon experimentally in a dog model and termed it **ischaemic preconditioning** (**IPC**). They reported that brief periods of ischaemia accompanied by reperfusion occurring just prior to a sustained ischaemic episode could paradoxically lead to the protection of tissues against longer ischaemia. Subsequently, numerous studies were performed using various tissues (liver, kidney, brain, endothelial cells) and animals. All of them showed that short period(s) of ischaemia or anoxia enable tissues to survive a subsequent period of longer ischaemia that would have otherwise been lethal (reviewed by Sanada and Kitakaze, 2004).

Ischaemia rapidly produces profound metabolic, functional and morphological changes within myocardium. The severity of these changes is ultimately determined by the duration of impaired oxygenation and substrate delivery. The principal metabolic changes centre around the failure of adequate adenosine triphosphate (ATP) generation by oxidative phosphorylation and the accumulation of byproducts of anaerobic glycolysis, particularly H^+. The functional consequences of ATP depletion

are rapidly manifested as a decrease in contractility and disturbances of host homeostatic processes, including the activities of ion channels and exchangers, cell volume regulation and enzyme reactions. The electrical properties of ischaemic myocardium may be altered to the point where arrhythmogenic mechanisms can promote life-threatening tachyarrhythmias. Ultrastructural changes may be detectable within several minutes of the onset ischaemia. Without reperfusion to salvage myocardium, the most extreme manifestation of irreversible injury is tissue necrosis (myocardial infarction). Prompt reperfusion of the occluded vessel is required to save ischaemic myocardium from irreversible injury, but paradoxically, reperfusion may be associated with further cellular stress resulting in “reperfusion injury” (Baxter and Ebrahim, 2002).

At present, the definition of IPC has been expanded to any kind of protection afforded by brief periods of ischaemia against damage caused by a subsequent sustained ischaemic insult (Sanada and Kitakaze, 2004). The phenomenon of IPC has been recognized as “the strongest form of *in vivo* protection against ischaemic injury other than early reperfusion” (Kloner et al., 1998). This powerful protective effect of antecedent ischaemia was not explained by changes in coronary collateral flow, suggesting a fundamental cellular alteration in the response to ischaemia. Unfortunately, induction of ischaemia is not a realistic treatment for patients with ischaemic heart disease. The development of therapeutic strategies that can attenuate ischaemia-reperfusion injury has been a keen area of research for more than 30 years (Baxter and Ebrahim, 2002).

IPC is associated with two forms of protection: a classical form (early phase) lasting ~ 2h after the preconditioning ischaemia followed a day later by a second window of protection (late phase) lasting ~ 3 days. Both types of preconditioning are reported to share some triggers, mediators, and effectors, although different mechanisms may be involved in the mediation of cardioprotection. The early phase is dependent on reactions that occur very rapidly, such as activation of ion channels or phosphorylation of enzymes, whereas the late phase involves processes that require modulation of the genes regulating channel proteins, receptor proteins, enzymes, molecular chaperon proteins, or immune factors (Sanada and Kitakaze, 2004).

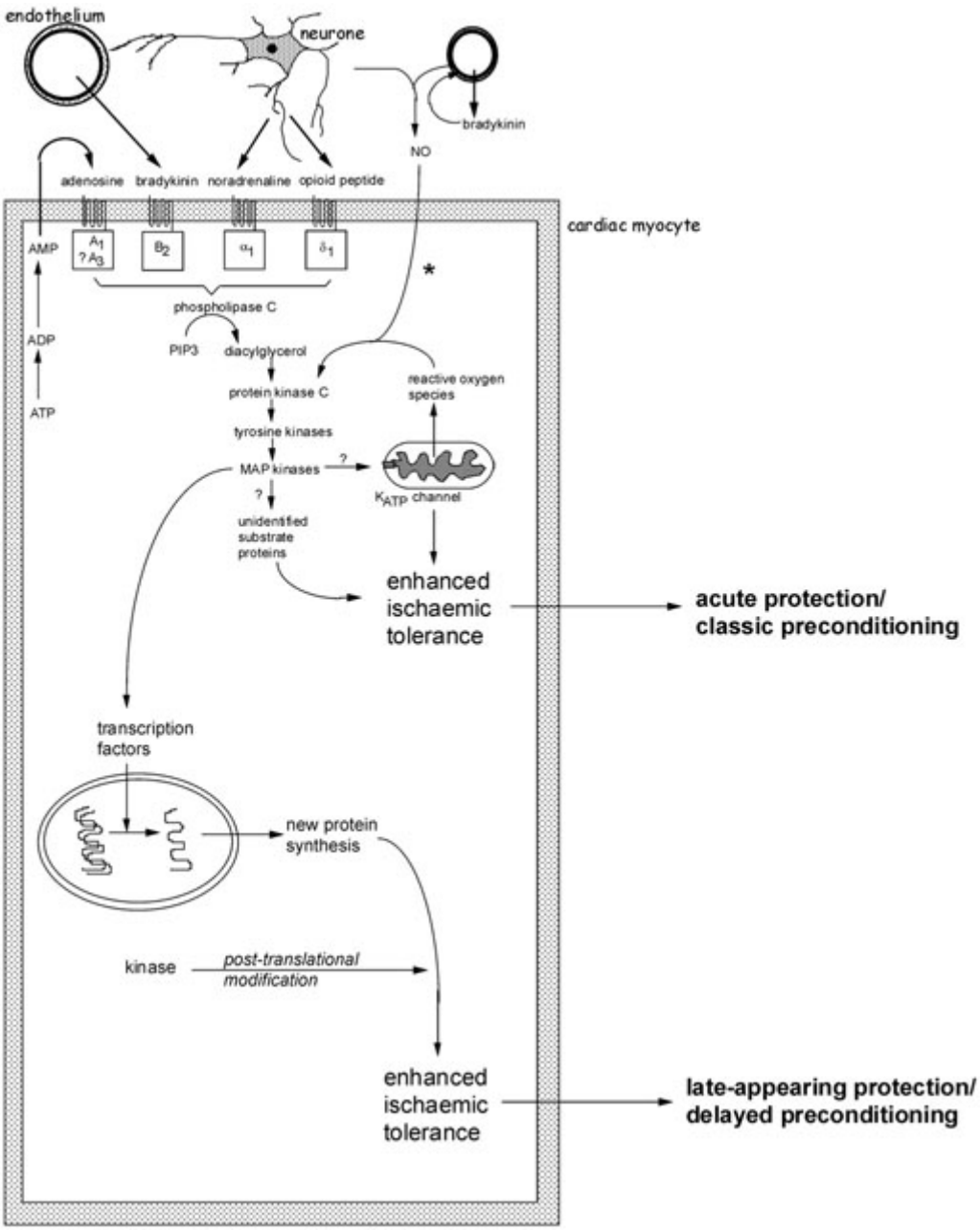


Fig.3 **Schematic representation of the major identified pathways of early and delayed forms of preconditioning** (Baxter and Ebrahim, 2002).

IPC is receptor mediated. Any G_i-coupled receptor can trigger the preconditioned state. In fact, multiple receptors work in parallel to provide redundancy to the preconditioning stimulus. During a brief ischaemic period, the heart appears to release several autocrine/paracrine triggers. Population of respective receptors then mediates the preconditioned state through activation of G_i protein resulting in the acquisition of tolerance to further ischaemia. Blockade of a single receptor type acts only to raise the ischaemic threshold required to trigger protection rather than

completely block it (Yellon and Downey, 2003). The triggers include adenosine released from myocytes during ischaemia as a result of ATP breakdown, **bradykinin** (**BK**) released from vascular endothelium and mediators of neural origin (noradrenaline and opioid peptides). Reactive oxygen species, especially superoxide anion generated as a result of mitochondrial uncoupling, may also act as upstream mediators. A complex signal cascade is activated which involves activation of protein kinase C (PKC) isoenzymes, tyrosine kinases and mitogen-activated protein kinases (MAPK). On the mitochondrial inner membrane, the phosphorylation cascade is thought to result in activation of the ATP-sensitive potassium (K_{ATP}) channel. The participation of other cytoprotective proteins has been proposed, including proteins that suppress or modulate apoptosis and proteins associated with cytoskeletal integrity (αB-crystallin and 27 kDa heat shock protein). Although endogenous NO (of endothelial or neural origin) has been linked to the trigger and end-effector phases of delayed preconditioning (possibly as a signalling intermediate downstream of BK), evidence for its role in early preconditioning is limited or even questionable. It appears that in early preconditioning, NO may lower the threshold for the protection observed, even though in itself it may not be a direct trigger of early preconditioning.

Many endpoints of ischaemic injury have been adopted to assess the extent of protection conferred by preconditioning, including development of necrosis (infarct size), severity of arrhythmias, post-ischaemic recovery of contractile function and cardiac enzyme release (reviewed by Baxter and Ebrahim, 2002).

1.3. KALLIKREIN-KININ SYSTEM

The KKS consists of the precursor kininogens, the proteolytic kallikrein enzymes, the kinin peptides (which are produced through cleavage of kininogens by kallikreins) and two G protein-coupled receptors termed B_1 and B_2 receptors that mediate the biological effects of kinin peptides (Fig.4).

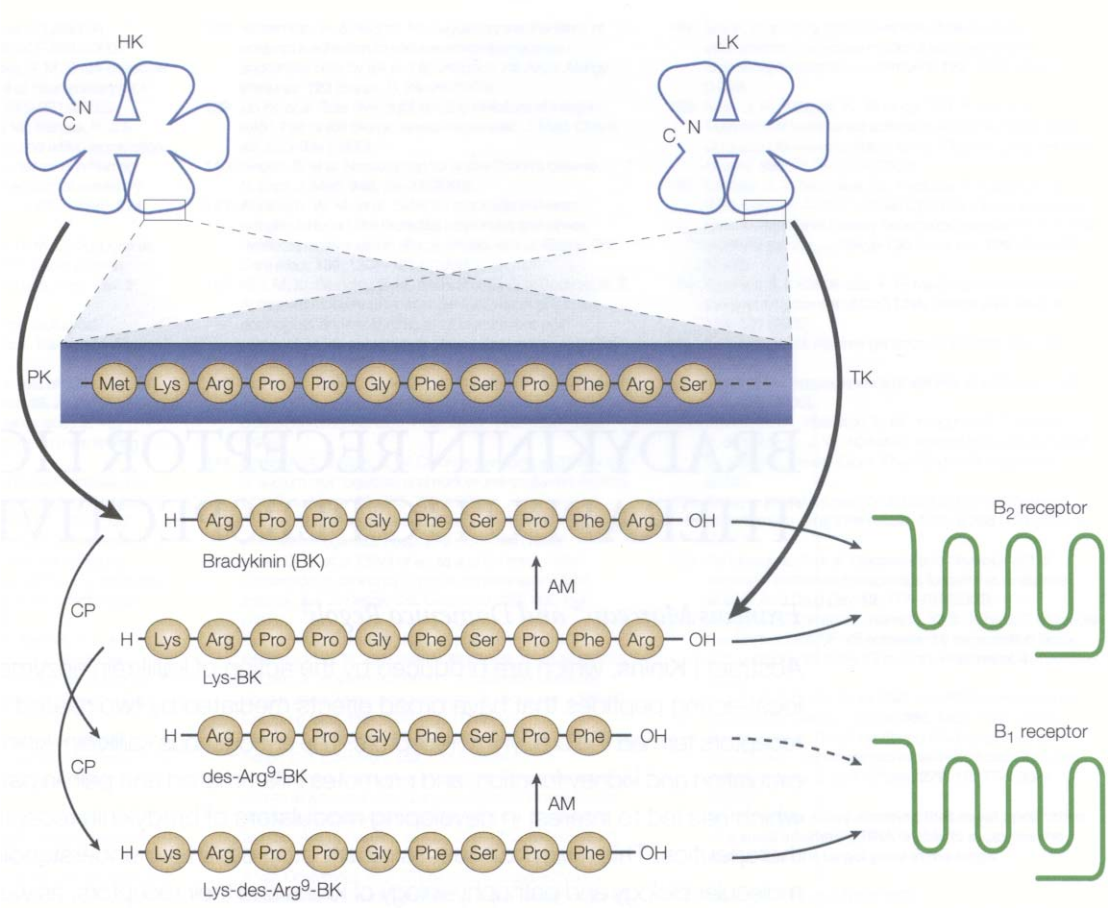


Fig.4 **Schematic representation of the kallikrein-kinin system.** AM, aminopeptidase M; CP, carboxypeptidases N and M (=kininase I); HK, high molecular weight kininogen; LK, low molecular weight kininogen; PK plasma kallikrein; TK, tissue kallikrein (Marceau and Regoli, 2004).

With respect to functional aspects, the KKS can be divided into plasma KKS and tissue (glandular) KKS. Plasma KKS generates the biologically active peptide BK, tissue KKS generates the biologically active peptide kallidin (KAL) (Hilgenfeldt et al., 1998). The tissue KKS works independently from the plasma KKS in various organs and body fluids (Katori et al., 2001).

Components of the KKS have been under investigation since 1909, when a hypotensive principle was found in urine (this was later identified as tissue kallikrein). In 1949, the active peptide BK was isolated from plasma globulins treated with trypsin. Since this time, research has shown that kinin peptides are implicated in a wide range of biological phenomena, including pain, inflammation, vasodilation, increased vascular permeability and natriuresis (Marceau and Regoli, 2004).

1.3.1. KININOGENS

Kininogens are defined as circulating proteins that include the kinin sequence, as well as domains, which possess other different functions. Circulating kininogens are primarily synthesized by hepatocytes. As typical secretory proteins they undergo posttranslational glycosylation prior to secretion into the circulation. Three types of kininogens have been described: **high molecular weight** (**HMW**) **kininogen** (present in blood), **low molecular weight** (**LMW**) **kininogen** (occur in blood and localises in various tissues) and an acute phase protein called T-kininogen that is unique to the rat. The HMW and LMW kininogens are coded by a single K gene and are produced by alternate splicing of the gene transcript (reviewed by Bhoola et al., 1992).

Kininogens are single-chain glycoproteins with a common amino-terminal heavy chain (about 64 kDa) and smaller, variable carboxy-terminal light chains with the kinin moiety intervening. Separate functions are being deduced for each domain of the molecules, e.g. HMW kininogen binds calcium (Ca^{2+}) via domain 1, inhibits important cysteine proteinases such as cathepsin and calpain via domains 2 and 3, binds to surfaces such as endothelium via domain 5, just downstream of BK in domain 4, and binds plasma prekallikrein and factor XI via domain 6 of the light chain (Margolius, 1995). After active kallikrein is formed, the HMW kininogen light chain continues to participate as an essential cofactor, promoting amplification of the activation cascade. In contrast to the light chain of HMW kininogen, which is about 45 to 58 kDa, the light chain of LMW kininogen is only 4 to 5 kDa and lacks the contact activation and prekallikrein-binding sites. After synthesis, HMW kininogen in platelets is transported to the plasma membrane. Specific receptors for HMW kininogen were found on the cell membrane of platelets, endothelial cells and neutrophils. HMW

bound to endothelial cells is a substrate of plasma kallikrein, which releases BK, a nonapeptide from domain 4 by proteolytic cleavage. Much less is known about the localization, regulation and role of LMW kininogen (reviewed by Bhoola et al., 1992).

Although rat HMW kininogen is similar in structure to the human molecule and subserves the known functions, a minor difference in amino acid sequence has been observed at the cleavage site of kallikreins. In rat HMW kininogen, at the amino-terminal sequence of BK, an Arg-Arg dipeptide replaces the Lys-Arg residue in the human sequence (Kato et al., 1985).

Mutant BNK rats show congenital deficiency of HMW and LMW kininogens and moderate absence of plasma prekallikrein. They carry negligible levels of HMW and LMW kininogens in their plasma. BNK rats produce normal levels of both kininogens in the liver, but the point mutation of Ala^{163} to Thr in the heavy chain of the molecules prevents the release of kininogens into the plasma. The defect seems to reside also in the terminal domain that complexes plasma prekallikrein; the uncomplexed form may be unstable and could account for the reduced concentration of plasma prekallikrein in the circulation of the BNK rats (Hayashi et al., 1993).

1.3.2. KALLIKREINS

The kallikreins are a group of serine proteases. They are divided into two main groups: tissue and plasma kallikrein. The two enzymes differ in their molecular weight, pI, substrate specificity, immunological characteristics, type of kinin released and functional importance. A single gene codes for plasma kallikrein, whereas tissue kallikrein is a member of a multigene family that shows different patterns of tissue specific gene expression and seems to be widely expressed.

Plasma prekallikrein is synthesized in the liver. This single-chain glycoprotein is secreted by hepatocytes as an inactive molecule that circulates in plasma as a heterodimer complex bound to HMW kininogen (Mandle et al., 1976). Both prekallikrein and coagulating factor XI are bound to domain 6 of HMW kininogen. Thus, following vascular damage, HMW kininogen settles on the endothelial and tissue surfaces by anchoring via the positively charged, histidine-rich region of

domain 5. It orientates both molecules toward Hageman factor, so that, once activated, Hageman factor can form active kallikrein and factor XI. The mature **plasma kallikrein** molecule (EC 3.4.21.34) releases BK from HMW kininogen by hydrolysis of Lys-Arg and Arg-Ser bonds to give a nonapeptide with an Arg residue at both amino- and carboxy-terminals. This serine protease is also considered to participate in the conversion of prorenin and renin (reviewed by Bhoola et al., 1992).
Tissue kallikreins belong to a large family of cell-secreted zymogens. They are synthesized as prokallikreins with an attached activation peptide sequence that must be cleaved to activate the enzyme. The tissue kallikreins are acidic glycoproteins, variably and extensively glycosylated (Margolius, 1995). The primary physiological substrate for **tissue kallikrein** (EC 3.4.21.35) is LMW kininogen. Tissue kallikrein represent a unique class of enzymes that hydrolyse one arginyl and one methionyl bond in the kininogen molecule to release KAL. Apart from its kininogenase activity, tissue kallikrein has been implicated in the processing of renin, growth factors, and peptide hormones. The cellular storage and synthesis sites of tissue kallikrein have been determined in a number of tissues. Tissue kallikrein has been characterised in pancreas, salivary glands, vascular tissue, intestinal tissues, skeletal and cardiac muscles, spleen, pituitary, ureter, adrenal glands and aorta. The presence of tissue kallikrein has been shown in a variety of body fluids, namely, saliva, urine, bile, plasma, sweat, cerebrospinal and synovial fluids and bronchoalveolar lavage fluid (reviewed by Bhoola et al., 1992). Tissue kallikrein activity can be inhibited by kallistatin, a physiological specific tissue kallikrein inhibitor that forms a covalently linked complex with tissue kallikrein. Independent of its binding to tissue kallikrein, kallistatin has also been found to have direct actions, such as reduction of blood pressure, stimulation of neointima formation and inhibition of angiogenesis and tumour growth (Chao et al., 1986).

The levels of tissue kallikrein are reduced in humans and animal models with hypertension, cardiovascular and renal disease. Recently, experiments with tissue kallikrein gene transfer or protein infusion showed beneficial effects in blood pressure reduction, attenuation of renal injury, cardiac infarction and cardiac remodelling, and reduction of stroke-induced mortality, cerebral infarction and neurological dysfunction (Chao and Chao, 2004).

1.3.3. KININS

Kinins are potent bioactive peptides formed by the enzymatic action of kallikreins on kininogens in various organs. They are detectable in secretory products (e.g. urine, saliva, sweat), interstitial fluid, and even in venous blood. Kinins influence the main mediators of inflammation as well as a number of cellular functions, including blood pressure and local blood flow, electrolyte and glucose transport and cell proliferation.

There are at least four main biochemically different kinins - **BK**, **KAL**, **des-Arg^{9}-BK** and **des-Arg^{10}-KAL** (Fig.4). It is generally accepted that KAL is released from LMW kininogen by tissue kallikrein and BK from HMW kininogen by the action of plasma kallikrein. Some conversion of KAL (Lys-BK) to BK may occur through removal of the amino-terminal Lys by aminopeptidases (Bhoola et al., 1992). The removal of the carboxy-terminal Arg of BK and KAL by carboxypeptidases leads to the formation of des-Arg metabolites that occur mostly in pathological states. Kinins are local hormones (autacoids) that are active only close to their site of formation in a paracrine manner (Marceau and Regoli, 2004). The effects of kinins in biological fluids are often very short-lived because they are rapidly destroyed by several pathways. In blood, the half-life of BK and KAL is estimated to be < 30s (Bhoola et al., 1992).

Kinin levels are very difficult to measure accurately, in part because, e.g. the blood contains all components necessary to generate and destroy these peptides *in vitro* (Marceau and Regoli, 2004). For years, kinins were overestimated by assays using inadequate enzyme inhibition or non-specific antisera (Margolius, 1995). Commercially available BK assays apply antibodies that cannot distinguish between BK and KAL, as they are directed against the identical carboxy-terminus of both peptides and crossreact also with larger and smaller analogues. Unfortunately, there are no reliable data concerning the contribution of BK and KAL to various physiological effects, although there is evidence for a clear distinction of both systems concerning their regulation and physiological properties. It is a general practice to address most kinin actions to BK. Using highly sensitive and specific antisera directed against both free carboxy- and amino-terminal ends developed in our laboratory, BK and KAL can be detected separately (Hilgenfeldt et al., 1995).

In rat, the analysis of kinins is even more complex. Until now only BK but no KAL was detected. This observation was explained by structural differences in the sequence of rat HMW and LMW kininogens containing an Arg residue instead of Lys residue in front of the amino-terminus of the BK sequence (Fig.5). Recently, a KAL equivalent, **kallidin-like peptide** (**KLP**), was isolated from rat plasma and urine and detected with a specific KAL antiserum suggesting that in rats, like in other mammals the tissue KKS mediates its physiological effects via the KLP (Hilgenfeldt et al., 2005).

HUMAN

-Leu-Met – ***Lys***^{1}-**Arg**^{2}-**Pro**^{3}-**Pro**^{4}-**Gly**^{5}-**Phe**^{6}-**Ser**^{7}-**Pro**^{8}-**Phe**^{9}-**Arg**^{10} – Ser-Ser-

Rat

-Val-Ile – ***Arg***^{1}-**Arg**^{2}-**Pro**^{3}-**Pro**^{4}-**Gly**^{5}-**Phe**^{6}-**Ser**^{7}-**Pro**^{8}-**Phe**^{9}-**Arg**^{10} – Ala-Pro-

Fig.5 **Structural differences between the human and rat kininogen with KAL and KLP sequence, respectively.**

CARDIOVASCULAR ACTIONS OF KININS

In the cardiovascular system, the classical action of kinins is vasodilation, mediated in several vascular beds by the release of NO and prostacyclin (Wirth et al., 1997). In the heart, exogenously administered BK is a potent coronary artery vasodilator, although the contribution of endogenous BK to the regulation of coronary vascular tone is unclear. Other actions of kinins in the heart include the modulation of cell growth and division in the heart and the modulation of myocardial responses to ischaemia-reperfusion (Baxter and Ebrahim, 2002).

Schoelkens et al. (1988) were the first to report the cardioprotective effects of exogenously administered BK. The ability of exogenous BK to mimic IPC has been confirmed by numerous investigators in a variety of models. Later, Wall et al. (1994) provided evidence for a primary role of endogenous BK in mediating IPC. BK released during ischaemia has been shown to primarily originate from endothelial cells but the precise molecular pathological mechanism leading to BK generation during brief ischaemia is not fully understood (Linz et al., 1996). It has been proposed

that isolated cardiac myocytes can synthesize kinins but this possibility has to be investigated in more detail (Matoba et al, 1999). The availability of an intact KKS is necessary for the achievement of cardioprotection as the rats deficient in HMW kininogen (BNK rats) were unable to develop the preconditioning response (Yang et al., 1997). Kinins are efficiently and rapidly degraded by several enzymes, especially ACE and neutral endopeptidase (NEP). Inhibition of these enzymes increases the availability of kinins at B_2 receptors on cardiac myocytes. During very brief periods of ischaemia, interstitial kinin concentrations may be insufficient to initiate the preconditioning mechanism. However, in the presence of an ACE or NEP inhibitor, augmentation of kinin concentration is sufficient to initiate preconditioning. The ability of ACE inhibitors to potentiate subthreshold ischaemic stimuli has been demonstrated for both early and delayed forms of preconditioning (reviewed by Baxter and Ebraim, 2002).

Furthermore, kallikrein gene delivery is associated with significant limitation of infarct size through attenuated apoptosis in the ischaemic zone and attenuated severity of ventricular fibrillation. These beneficial effects of kallikrein overexpression were abolished by icatibant (B_2 receptor antagonist), implying a role for the B_2 receptor (Yoshida et al., 2000). Nevertheless, the role of B_1 receptor cannot be ruled out, particularly in the late phase of preconditioning.

Surprisingly, most of the investigators suggested that BK is the only kinin mediating the cardioprotection, although it is generally accepted that in tissue KAL is the biologically active peptide. Recently, we showed that the both, BK and KLP are released during IPC from the rat heart and that the cardioprotective effect could be blocked by administration of a specific antiserum against KAL/KLP, suggesting that KLP is the cardioprotective kinin in the rat heart (Liu et al., 2005).
The mechanisms underlying the acute protective actions of kinins are not well understood. Interestingly, it is unlikely that the beneficial cardiac effect of kinins is related to their hemodynamic actions, such as increasing coronary blood flow or decreasing vascular resistance. A number of agents have been proposed to participate in the protection including NO, prostaglandin I_2, PKC and tyrosine kinases. PKC activation is thought to be central in the preconditioning phenomenon and it has been proposed that once activated, it determines the phosphorylation of distal kinase

and end effector proteins. Opening of the mitochondrial K_{ATP} channel has been proposed as distal mediator of preconditioning (reviewed by Baxter and Ebrahim, 2002).

1.3.4 KININ RECEPTORS

Two pharmacologically distinct kinin receptor subtypes have been identified and characterized, which are termed B_1 and B_2. They belong to the family of G protein-coupled receptors (GPCR). The B_2 receptor has a high affinity for "native" kinins (those generated by either plasma or tissue kallikrein), BK and KAL. The most discriminative structural determinant for high affinity binding at B_1 receptors is the removal of the carboxy-terminal Arg residue by carboxypeptidases. At the same time, des-Arg^9-BK and des-Arg^{10}-KAL are losing the affinity toward the B_2 receptor. An order of agonist potency widely valid for mammalian kinin receptors could be: B_1, des-Arg^{10}-KAL > KAL ~ des-Arg^9-BK >> BK; B_2, BK ~ KAL >> des-Arg^9-BK and des-Arg^{10}-KAL. The B_2 receptor is ubiquitous and constitutively expressed and rapidly down-regulated to a very limited extent even after prolonged agonist exposure (days) (Bachvarov et al., 2001). The B_1 receptor is expressed at a very low level in healthy tissues. It is induced in pathological states, particularly in inflammation or after exposure of tissue to noxious stimuli. Both receptor subtypes can be expressed by the same cell types: vascular cells (endothelial, smooth muscle) (Figueroa et al., 2001), nonvascular smooth muscle cells, fibroblasts, epithelial cells, nervous (afferent sensory) cells, and various tumour cells (reviewed by Leeb-Lundberg et al., 2005).

The **B_2 receptor** protein structure is typical of that of a GPCR consisting of a single polypeptide chain that spans the membrane seven times, with the amino-terminus (N-terminal domain) being extracellular and the carboxy-terminus (C-terminal domain) being intracellular, and with three extracellular loops and three intracellular loops. The B_1 receptor is homologous to the B_2 receptor (36% identity at the amino acid sequence level). Kinin receptors undergo multiple post-translational modifications including glycosylation and disulfide bridge formation in their extracellular domains as well as acylation and phosphorylation of their intracellular domains. At the cellular level, the stimulation of the B_2 receptor leads to a rapid

desensitisation of the receptor response (Blaukat et al., 2001). Kinin binding triggers a rapid redistribution of B_2 receptors to plasma membrane caveolae.

The **B_1 receptor** differs from the B_2 receptor in that it is desensitised only to a very limited degree. Furthermore, the B_1 receptor is not internalised to any appreciable extent in response to agonist exposure. The difference in the extent of desensitisation of the B_2 and the B_1 receptor may contribute to the rather distinct patterns of receptor signalling through common effector pathways in different cells (reviewed by Leeb-Lundberg et al., 2005).

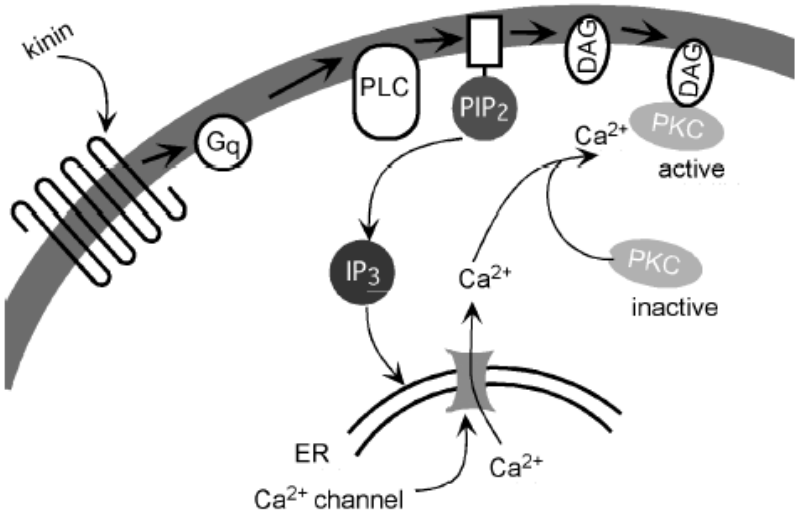


Fig.6 **Intracellular signalling cascades triggered by kinins.** Following binding of kinins to the receptor the associated heterotrimeric G protein complex dissociates. The q subunit stimulates PLC which in turn catalyses the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP2) into 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). IP3 reacts with Ca^{2+} channels in the endoplasmatic reticulum (ER) releasing Ca^{2+} into the cytosol. The increase in intracellular Ca^{2+} levels activates protein PKC, which translocates to the plasma membrane, anchoring to DAG and phosphatidylserine (Offermanns and Rosenthal, 2003).

The B_2 receptor is generally described as signalling through G_q (Fig.6), even though this receptor interacts with several other G proteins as well including G_i, and G_s. Besides of kinin-stimulated Gq-sensitive phospholipase C (PLC), stimulation of a phospholipase A_2 (PLA_2) activity appears to occur through Ca^{2+}- and

phosphorylation-dependent activation of the cytosolic isoform of PLA_2, whereas phospholipase D (PLD) activation can be mediated via Ca^{2+} influx, and PKC activation (reviewed by Leeb-Lundberg et al., 2005). Kinins are efficacious stimulators of endothelial nitric oxide synthase (eNOS) and NO production through Ca^{2+}-mediated mechanisms in endothelial cells (Fig.7) (Busse and Fleming, 1995). Depending on the cell type, BK induces proliferative or antiproliferative responses. The proliferative response involves many of the typical growth-promoted pathways (stimulation of MAPK, transactivation of the epidermal growth factor receptor or the combined actions of the protein tyrosine kinases) (Blaukat et al., 2000). The antiproliferative actions of BK may be prostaglandin-mediated, involve activation of a tyrosine phosphatase, or occur via further downstream mechanisms. BK activates multiple transcription factors that regulate the induction of several cytokines. These are involved in tissue injury and inflammation as well as in B_1 receptor induction.

The B_1 receptor directly interacts with G_q and G_i through which it mediates agonist stimulation of many of the same signalling pathways as the B_2 receptor. Although the B_1 and B_2 receptors seem to couple to similar cellular signal transduction pathways, the patterns of signalling are different. In vascular smooth muscle cells, the stimulation of B_2 receptor leads to a transient increase in phosphatidylinositol (PI) hydrolysis that exhibits little dependence on extracellular Ca^{2+}, whereas B_1 receptor stimulation is more sustained and significantly dependent on extracellular Ca^{2+}. Furthermore, the B_2 receptor elicits a sustained signal, which is characterized by a plateau of elevated Ca2+ or baseline oscillation, that are dependent on extracellular Ca^{2+} influx.

Most circulatory effects of kinins are determined by the stimulation of endothelial cells from which secondary mediators are released to affect the vascular smooth muscle. Kinins are one of the agonists that release NO (Fig.7). NO is metabolically derived from L-arginine by eNOS. NO-dependent relaxation is a prominent role of kinin action. NO diffuses from the endothelium to the smooth muscle where it activates guanylate cyclase. Prostaglandin I_2 (prostacyclin) is another secondary mediator frequently released by kinins from the endothelium. Prostacyclin stimulates cyclic adenosine 3´,5´-monophosphate (cAMP) production in the smooth muscle cells (reviewed by Leeb-Lundberg et al., 2005). Other mechanisms are also assumed,

such as the activation of a number of NO-independent ion channels located in the smooth muscle cells that may account for “endothelium-dependent hyperpolarization” (Batenburg et al., 2004).

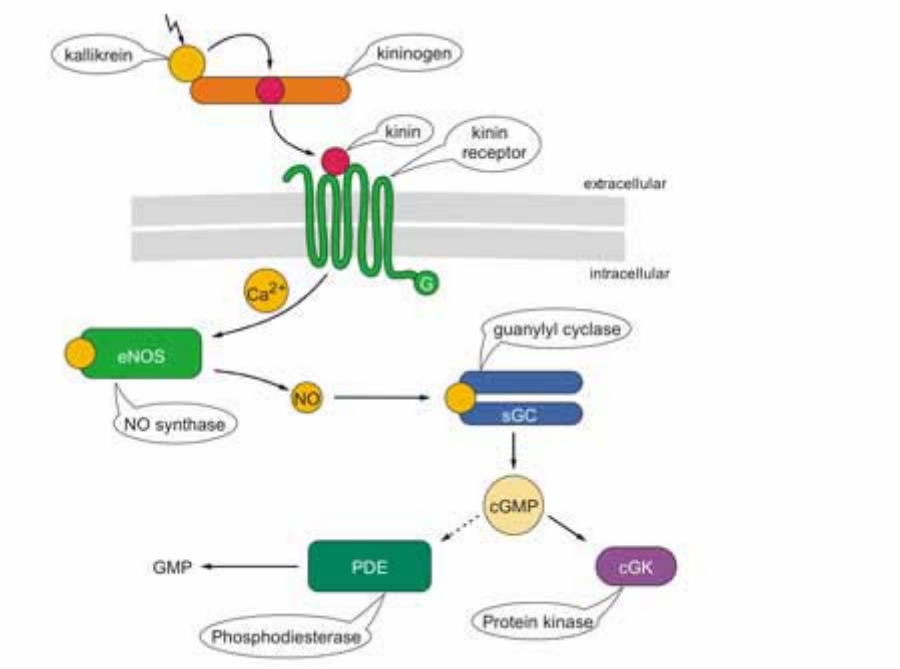


Fig.7 **Molecular mechanisms governing the kinin-NO-cGMP pathway in mammalian cells.** cGMP, cyclic guanosine monophosphate

1.3.5. KININASES

The turnover of kinins depends on both, the rate of formation and the rate of destruction. Peptidases that hydrolyse kinins are known as kininases, although none are known to be specific for kinins. The relative importance of each of the peptidases in controlling kinin levels varies with species, type of biological fluid, and tissue site of formation of the peptide. The kininase I family comprises carboxypeptidase N (CPN) and carboxypeptidase M (CPM) and the kininase II family ACE and NEP. Additional kinin-hydrolysing enzymes are aminopeptidases.

Carboxypeptidases cleave the carboxy-terminal Arg of BK and KAL, resulting in the formation of des-Arg^9-BK and des-Arg^{10}-KAL, B_1 receptor agonists. **CPN** is synthesised by the liver, is secreted into the circulation. **CPM** is a cell membrane enzyme that is located in kidney, lung and fibroblasts, endothelial cells of pulmonary

arteries and the placenta. Both CPN and CPM are not capable of degrading the kinin molecule further. The release of Arg by CPN and CPM either close to or within the endothelial cells or in synaptic clefts could provide the primary substrate for the formation of NO by nitric oxide synthase (NOS) (reviewed by Bhoola et al., 1992).

The two peptidylpeptidases ACE and NEP hydrolyse the Pro-Phe bond at the carboxy-terminus of the kinin molecule. ACE appears to be the most important within the cardiovascular system or kidney, with the exception of rat urine where NEP is the major kinin-destroying enzyme. In contrast to humans, ACE is the most potent kinin-degrading enzyme in rat plasma (Margolius, 1995). **ACE** (EC 3.4.15.1) is a single-chain transmembrane zinc metallopeptidase that cleaves carboxy-terminal dipeptides from several peptides. A soluble form of the enzyme is found in plasma, which is presumably derived from the membrane-bound form by proteolytic cleavage. ACE is expressed in great amount in vascular endothelial cells (Linz et al., 1999). The enzyme occurs in abundance at the brush border of the inner cortical proximal renal tubules (Schulz et al., 1988). ACE hydrolyses two separate bonds on the carboxy-terminal end of the kinin molecule. First, it removes the dipeptide Phe-Arg and next splits the Ser-Pro bond, which leads to fragments that are inactive on either receptor type (Erdös, 1990). ACE plays a major role in the regulation of the vascular tone by converting the biological inactive decapeptide Ang I into the vasoconstrictor and proliferative octapeptide Ang II. In experiments using isolated ischemic rat hearts, beneficial effects were observed with ACE inhibitors on reperfusion arrhythmias, cardiac functions and metabolism. These functional improvements were believed to be due to both, accumulation of endothelium-derived kinins and the inhibition of Ang II formation (Linz et al., 1999). Furthermore, ACE is also thought to directly interact with the B_2 receptor in the membrane (Hecker et al., 1994). Like ACE, **NEP** (EC 3.4.24.11) inactivates kinins by removing the carboxy-terminal Phe-Arg dipeptide. NEP is present in high concentrations in the brush border of proximal tubules of the kidney. Further sites of location are in the intestine, lung, skin, placenta and brain. Although NEP is generally embedded in cell membrane, it also seems to occur in biological fluids (reviewed by Bhoola et al., 1992).

Another important pathway of kinin inactivation in the tissue is provided by aminopeptidases. Two aminopeptidases are mentioned concerning the kinin

metabolism, aminopeptidase M (APM) and aminopeptidase P (APP). Proud et al. (1987) have reported that **APM** is able to cleave the amino-terminal Lys of des-Arg^{10}-KAL and KAL into des-Arg^{9}-BK and BK, respectively. APM represents an inactivation pathway for the potent natural B_1 receptor agonist des-Arg^{10}-KAL since its metabolite, des-Arg^{9}-BK, is a much less potent B_1 receptor agonist (Pelorosso et al., 2005). APM is a transmembrane zinc metallopeptidase, found in endothelium and smooth muscle cells (Palmieri et al., 1989). **APP** is the only peptidase known to remove Arg from the amino-terminal end of BK and contributes to the degradation of intravascular as well as interstitial BK (Dendorfer et al., 1997). APP has been detected in soluble form in serum, and contributes as a membrane-bound enzyme to the degradation of BK in the lung (Ward et al., 1992; Pesquero et al., 1992). The distribution of APP has been established in various organs. In the rat heart, nearly identical kinin-degrading activities of ACE and APP have been demonstrated (Dendorfer et al., 2000). APP is known to reside as an extracellular enzyme on endothelial membranes. It colocalizes in membrane domains with ACE, and possibly also with B_2 receptors (Ryan et al., 1996).

1.4. ENDOTHELIN SYSTEM

The endothelins are group of vasoconstrictor peptides derived from vascular endothelial cells that acts as autocrine/paracrine regulators. Three ETs have been identified, **ET-1**, -2, and -3, all consisting of 21 amino acids (Fig.8).

ET peptides are produced within the cells from large precursors, preproendothelins (~200 residues), which undergo two proteolytic cleavages by NEP to form intermediate-inactive big ETs (37-39 amino acids). The last step in the biosynthesis of mature/active ET requires the conversion of big ET into ET, which is catalyzed by one or more zinc-binding membrane bound metalloproteinases, endothelin-converting enzymes (ECE).

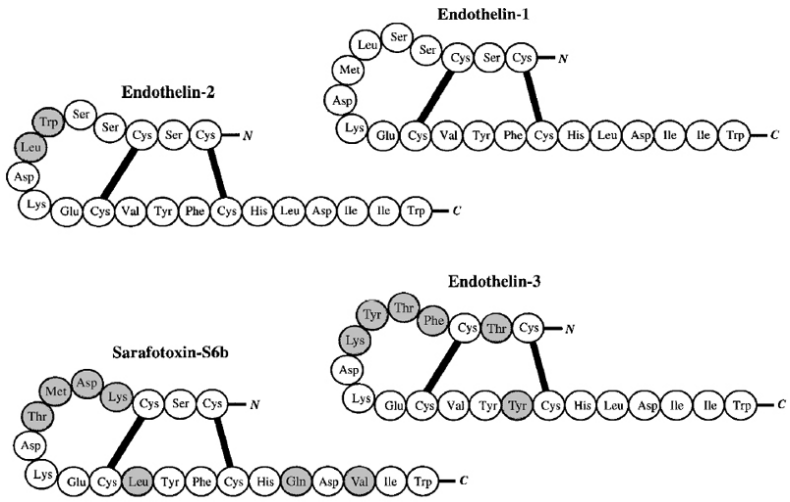


Fig.8 **Amino acid sequences of the three members of the ET family** and of the structurally related snake venom toxin sarafotoxin. Each isoform contains two intra-chain disulphide bridges linking paired cysteine amino acid residues, thus producing an unusual semi-conical structure. Shaded circles indicate where amino acids differ from those of ET-1 (Haynes and Webb, 1998).

ET-1 is the most potent natural mammalian vasoconstrictor yet discovered. Mature ET-1 acts on the cardiovascular system and other target organs by binding to two types of receptors, ET_A and ET_B. Although ET_A has affinity primarily to ET-1, ET_B has equal affinity to all ETs. High abundance of ET_A receptors has been detected in the aorta, heart and the kidney, whereas ET_B receptors are expressed mainly in the endothelial cells. Activation of ET_A receptors on vascular smooth muscle cells (VSMC) increases intracellular Ca^{2+} levels, leading to prolonged vasoconstriction and cell proliferation. In contrast, activation of ET_B receptors, present on endothelial cells, induces the release of NO and prostaglandins, thus provoking transient vasodilation (Fig.9) (Levin, 1995).

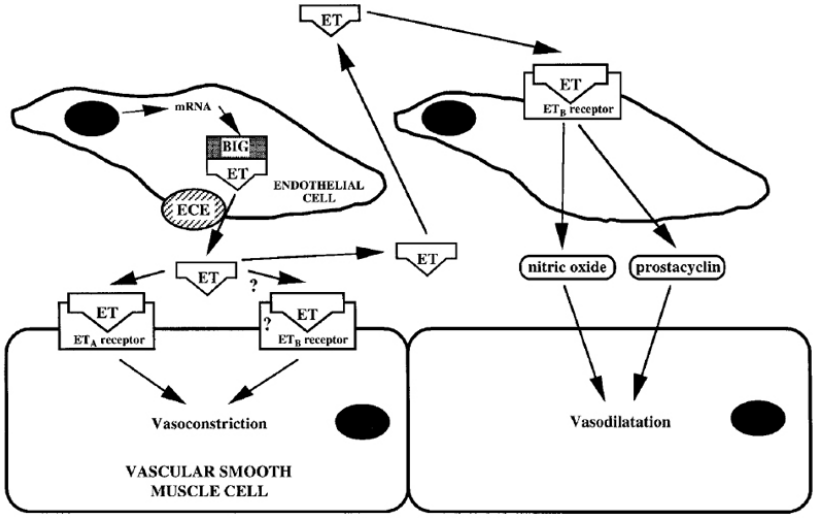


Fig.9 **Vascular actions of ET** (Haynes and Webb, 1998).

The ET receptors are classical GPCRs. Binding of ET to ET_A receptor leads to activation of the G proteins G_s and G_q, and ET binding to ET_B receptor leads to G_q and G_i activation. G_q activates the PLC to cause hydrolysis of PI and generation of cytosolic inositol-triphosphate (IP_3) and membrane-bound diacylgylcerol (DAG). IP_3 causes an early rapid increase in Ca^{2+} through its release from intracellular stores. DAG activates PKC, increasing the sensitivity of the contractile apparatus to Ca^{2+} as well as inducing intracellular signalling mechanisms that promote long-term cellular responses (proliferation and migration) through the MAPK cascade. In addition, ET activates PLD and PLA_2, the latter increasing the production of arachidonic acid and hence cyclooxygenase products (prostaglandins and thromboxanes) and lipooxygenase products (leukotriens and lipoxines) (Remuzzi et al., 2002).

Although ET-1 is produced predominantly by the endothelial cells, remarkable amounts of this peptide are generated in several tissues/organs such as kidney, heart, brain, and VSMC (Levin, 1995). ET-1 exerts a broad range of actions on these tissues, aimed modulating blood pressure and controlling extracellular fluid volume. Independent of its direct effect on the vasculature that contributes to the regulation of vascular tone and blood pressure, ET may affect blood pressure indirectly by modulating renal hemodynamics and excretory functions of the kidney (Abassi et al., 2001). ET-1 has positive chronotropic and inotropic effects and is a potent constrictor

of coronary vessels, causing myocardial ischaemia and fatal ventricular arrhythmias (Ezra et al., 1989) (Fig.10).

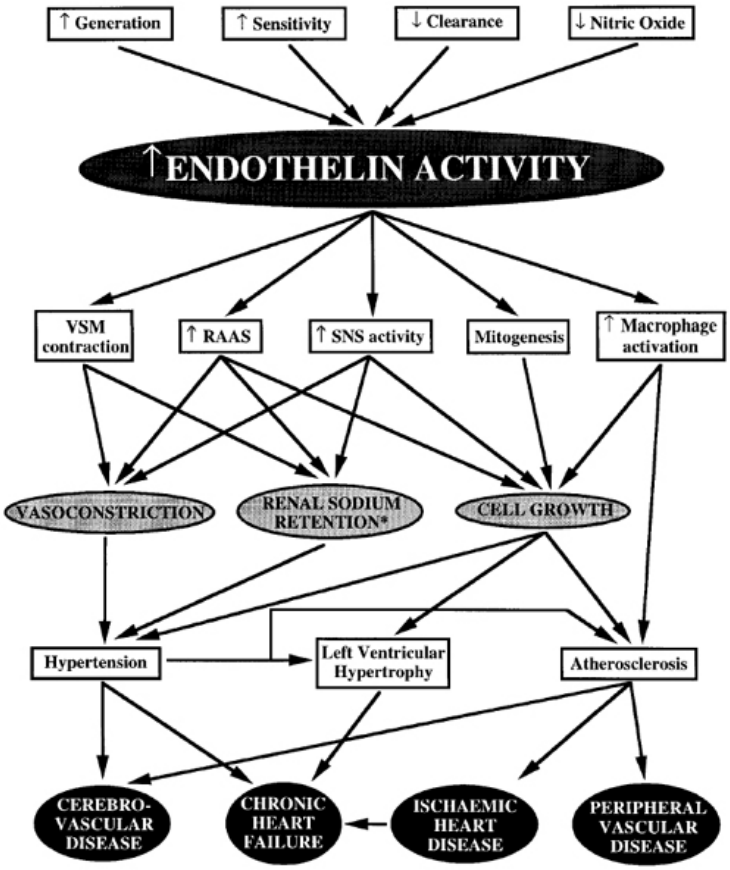


Fig.10 **Potential pathways by which ET-1 might contribute to the pathophysiology of hypertension or its complications.** *An increase in vascular activity of ET-1 could cause retention of Na^+ through ET_A receptor-mediated renal vasoconstriction. There is also persuasive evidence that there is a deficiency of tubular generation of ET-1 in hypertension that could attenuate ET_B receptor-mediated facilitation of tubular excretion of Na^+ and water. VSM, vascular smooth muscle; SNS, sympathetic nervous system (Haynes and Webb, 1998).

ETs are known to be involved in the functional regulation of neuroendocrine axes, among which is the hypothalamo-pituitary-adrenal axe, acting on either their central or peripheral branch (Fig.11). ET genes and their receptors are expressed in adrenal glands, the function of which they variously modulate. Locally synthesized ETs are involved in the autocrine/paracrine control of the secretion and growth of steroid-secreting tissues. ETs stimulate both aldosterone and glucocorticoid secretion by the adrenals, probably acting on early and late steps of steroid synthesis. This effect

appears to be mediated by both ET_A and ET_B receptors and may occur through direct and indirect mechanisms. ET binding to its adrenocortical receptor activates multiple signalling mechanisms, including PLC/PKC cascade, opening of Ca^{2+} channels, and stimulation of tyrosine kinase and Na^+/K^+-ATPase. ET affects the secretory activity of the cortex by eliciting the release of catecholamines by medullary chromaffin cells. Catecholamines in turn stimulate steroid secretion in a paracrine manner, and/or by modulating intra-adrenal blood flow. This latter effect seems to be mediated by both PKC-coupled ET_A- and NOS-coupled ET_B-receptors, which raise and lower intraglandular vascular resistances, respectively (Nussdorfer et al., 1999).

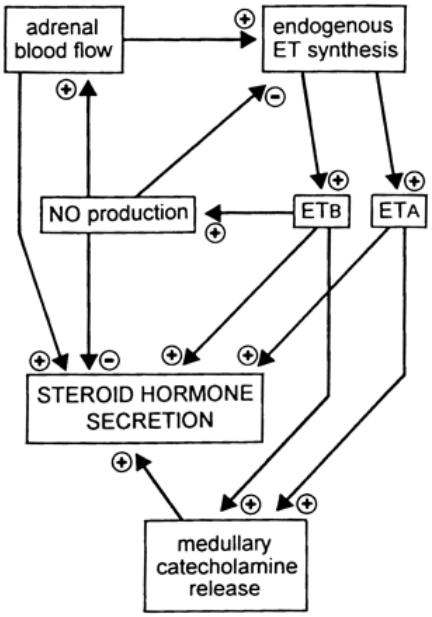


Fig.11 **Main feedback mechanisms currently thought be involved in the ET-mediated autocrine/paracrine tuning of adrenocortical secretion (**Nussdorfer et al., 1999).

ETs enhance basal aldosterone production without changes in the activity of the renin-angiotensin-system (RAS). ET-1 was found to potentiate the aldosterone response to adrenocorticotropic hormone (ACTH), but not to Ang II and potassium (K^+). Moreover, evidence indicates that ETs inhibit rather than stimulate the renin release by kidney juxtaglomerular cells. ET-1 did not change plasma corticosterone concentration (reviewed by Nussdorfer et al., 1999).

1.5. ALDOSTERONE

Aldosterone, which is the most physiologically important mineralocorticoid, is primarily responsible for electrolyte transport across epithelia, particularly in the kidney, but also in other tissues, such as salivary glands and colon. Aldosterone increases Na^+ (and consequently water) resorption and K^+ excretion directly or indirectly by increasing the activity of epithelial Na^+ channels and Na^+/K^+-ATPase (White, 1994). Aldosterone is secreted by the zona glomerulosa of the adrenal cortex in response to the activation of the RAS and/or increased plasma K^+ levels (Firsov and Muller, 2003). Spironolactone, aldosterone antagonist, has been successfully used for more than 30 years as K^+ sparing diuretics in the therapy of hypertension. Interestingly, the long clinical use of aldosterone antagonists raised new questions about its role in pathological states such as myocardial infarction or congestive heart failure. RALES trial - Randomized Aldactone Evaluation Study - showed that patients with severe heart failure have a 30% reduction in morbidity and mortality when given spironolactone, in addition to conventional therapy of an ACE inhibitor, digoxin and furosemide. The dose of spironolactone used in this study (25mg) had no incremental effect on blood pressure, suggesting a direct cardioprotective effect (Pitt et al., 1999).

The most remarkable effect of aldosterone on the cardiac tissue is perivascular fibrosis of small arteries and arterioles with associated interstitial fibrosis. However, other effects such as myocardial necrosis, vascular stiffening and injury, and production of cardiac arrhythmias were also attributed to aldosterone (Stier et al., 2002). The increased stiffness of large arteries is the main cause of the increase of systolic and pulse pressures, important independent cardiovascular risk markers (Benetos et al., 1997). Interestingly, aldosterone effect on cardiac fibrosis is dependent on Na^+ load, thus indicating that aldosterone may promote the entry of Na^+ into cardiac cells. Furthermore, aldosterone can induce cardiac fibrosis without involvement of the RAS (reviewed by Firsov and Muller, 2003). In ventricular cardiomyocytes, aldosterone increases Ca^{2+} current, which is mediated through **mineralocorticoid receptor** (**MR**). Increased intracellular Ca^{2+} might ultimately cause cardiac hypertrophy by increasing the expression of calcineurin, a calcium/calmodulin-dependent protein phosphatase that is well documented to cause

cardiac hypertrophy. Indeed, aldosterone increases calcineurin mRNA and activity. Aldosterone stimulates ACE and increases AT_1 Ang II receptor expression. Increased ACE activity should increase local levels of Ang II, which is known to cause cardiac hypertrophy and fibrosis (reviewed by White, 2003).

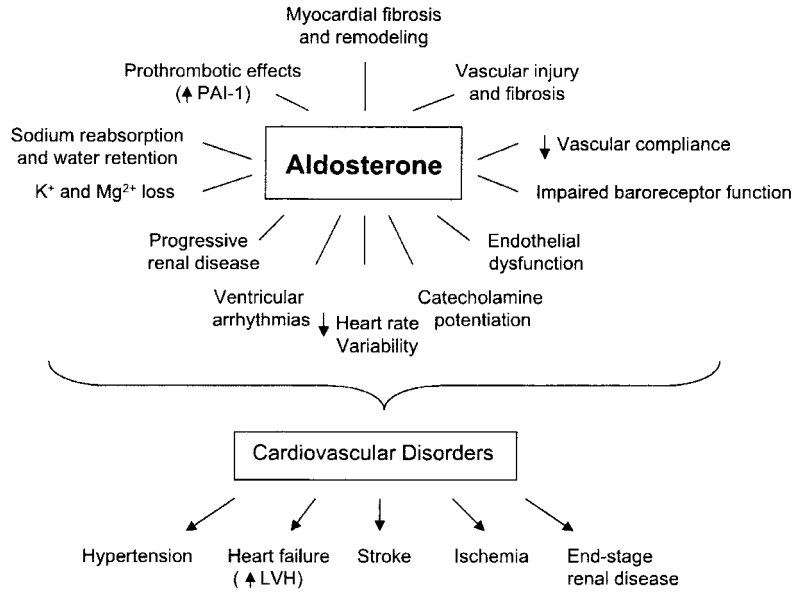


Fig.12 **Multiple mechanisms by which aldosterone dysregulation may contribute to cardiovascular disease.** LVH, left ventricular hypertrophy; PAI-1, plasminogen activator inhibitor-1 (Struthers and MacDonald, 2004).

Aldosterone also inhibits NO formation and inducible NOS (iNOS) mRNA in VSMC. Besides its vasodilatory effect, NO may also diminish mitochondrial respiration and cardiac oxygen metabolism resulting in a decrease in heart work and, consequently, myocardial oxygen demand. Thus, aldosterone's abrogation of NO formation or its actions would impair not only vascular but also myocardial function. Aldosterone may also be involved in enhanced mitochondrial oxidative phosphorylation and oxygen free radical generation by markedly stimulating myocardial citrate synthase, a Krebs cycle enzyme, or Na^+/K^+-ATPase. Aldosterone causes hypokalemia, which can directly stimulate free radical formation (reviewed by Stier et al., 2002). Furthermore, aldosterone affects the electrophysiology of the myocardium. Although aldosterone up-regulates Na^+/K^+-ATPase in kidney, the Na^+/K^+ pump current is decreased in cardiomyocytes, apparently due to a decrease in the Na^+ affinity of the pump.

Aldosterone slowly increases the force of contraction (positive inotropic effect). It prolongs monophasic action potentials indicating inhibitory effect on myocardial repolarization which may increase the risk of arrhythmia in congestive heart failure (reviewed by White, 2003). Furthermore, the myocardial action of aldosterone may be mediated, in part, via stimulation of the ß-adrenergic pathway. Mineralocorticoids have been reported to sensitize markedly the myocardium of rats to the arrhythmogenic action of ß-adrenergic stimulation. Aldosterone has also been reported to decrease myocardial noradrenaline uptake by 24% (Stier et al., 2002).

1.5.1 ALDOSTERONE ACTIONS

Aldosterone is supposed to mediate genomic and non-genomic mechanisms of action. The genomic action of aldosterone requires binding to the MR, a member of the steroid/thyroid/retinoid family of nuclear receptors. This binding happens in cytoplasm. The aldosterone-MR complex is then translocated into the nucleus where it acts as ligand-dependent transcriptional factor. Changes in transcriptional rate of aldosterone-target genes and their physiological effects are detectable from ~30 min to several hours after aldosterone stimulation (Farman, 1999). The MR is present in kidney, colon, brain, heart and lung. In heart, it occurs in myocytes, VSMC, and in the endothelial wall of large arteries (i.e. aorta and pulmonary artery) but not in smaller vessels (Farman and Rafestin-Oblin, 2001).

MR has an equivalent high affinity for corticosterone, cortisol and aldosterone. Moreover, plasma aldosterone levels range from 0,1 to 1nM, 100-1000-fold lower that those of the glucocorticoid hormones (0,1-1µM). In classical aldosterone target tissues, the MR is protected from the illicit glucocorticoids' occupancy by 11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD2), an enzyme that converts cortisol into cortisone (or corticosterone to 11-dehydrocorticosterone in rat). Cortisone (or 11-dehydrocorticosterone) has a low affinity to MR and is thus unable to induce significant MR transactivation (Firsov and Muller, 2003). The colocalization of 11ß-HSD2 and MR in cells is crucial for aldosterone's specificity of action and besides in the kidney, it is also present in the heart (Heymes et al., 2004). However, the enzymatic activity of cardiac 11ß-HSD2 is far too low to protect the MR from glucocorticoids (Farman and Rafestin-Oblin, 2001).

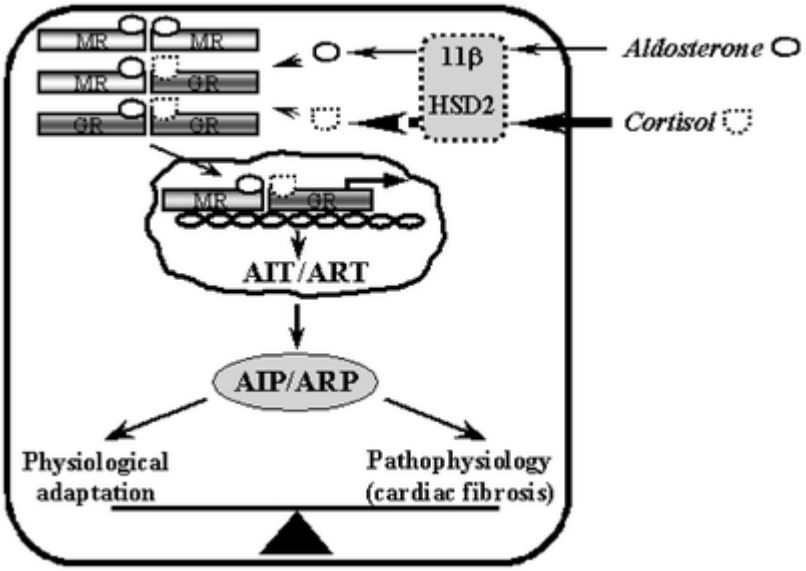


Fig.13 **Model of aldosterone action in cardiac cells.** Aldosterone binds intracellular MR and/or glucocorticoid receptor (GR). Transactivation of MR/GR receptor results in change of transcription rate of aldosterone-induced transcripts (AITs) and aldosterone-repressed transcripts (ARTs). Protein products of these transcripts (aldosterone-induced proteins, AIP; and aldosterone-repressed proteins, ARP) are responsible for the long-term effects of aldosterone in cardiac tissue (Firsov and Muller, 2003).

In the brain, MRs are expressed at high levels only in distinct regions, e.g. in the hippocampus where they seem to play a role in the control of thirst and Na^+ intake (Heymes et al., 2004). On the contrary, glucocorticoid receptors (GR) are expressed at high levels throughout the brain. Glucocorticoids can influence cognition and have either neuroprotective or neurodegenerative properties depending on the local conditions via both MR and GR. Furthermore, the MR occupancy is nonselecetive for the most part of adult brain, because the 11ß-HSD2 displays high expression only in distinct regions, which are important for the cardiovascular control (Ye et al., 2003).

Interesting question of the differentiation between protected and unprotected MRs is whether the physiological glucocorticoids act as agonists or antagonists upon MR binding. In kidney and VSMC glucocorticoids seem to act as agonists, since their action is indistinguishable from that of aldosterone. In contrast, in most non-epithelial tissues, the physiological glucocorticoids act as aldosterone antagonists at MRs. This between-tissue distinction in glucocorticoid action is presently not fully understood.

Finally, in a variety of tissues, including VSMC and cardiomyocytes, rapid, non-genomic effects of aldosterone have emerged. Such actions are of < 1min latency, and characteristically reach plateau levels within 5min of aldosterone application. These effects were ascribed to an action via a putative membrane receptor, distinct from the classical MR. Surprisingly, at least some rapid, non-genomic effects are mediated via classical MRs, both protected by 11ß-HSD2 (VSMC) and non-protected (cardiomyocytes) as in these cells aldosterone acts rapidly (< 15min). This effect can be blocked by water-soluble MR antagonist K^+ canrenoate (spironolactone metabolite) (Young and Funder, 2002). Post-receptor mechanisms established include the activation of PKC, and increases in intracellular cAMP and Ca^{2+}, leading to the activation of Na^+-hydrogen exchange in VSCM, for example, and of $Na^+/K^+/2Cl^-$ cotransport in cardiomyocytes (Funder, 2001).

1.5.2. STEROIDOGENIC ENZYMES

Fig.14 illustrates all of the enzymes involved in the biosynthesis of the adrenal steroid hormones, corticosterone, cortisol and aldosterone; and the gonadal steroid hormones, progesterone, estradiol, and testosterone. These enzymes fall into two major classes of proteins: the cytochrome P450 heme-containing proteins and the hydroxysteroid dehydrogenases. In the process of biosynthesis of steroid hormones, cytochrome P450 enzymes catalyse the hydroxylation and cleavage of the steroid substrate. They function as monooxygenases utilizing reduced NADPH as electron donor for the reduction of molecular oxygen.

Distinct enzymes are involved in the adrenal biosynthesis of corticosterone and cortisol and the biosynthesis of aldosterone. Curnow et al. (1991) identified a second isoform of CYP11B in human, CYP11B2. This enzyme was found to be the only enzyme catalysing both 11ß-hydroxylation and 18-hydroxylation and thus being essential for the biosynthesis of aldosterone.

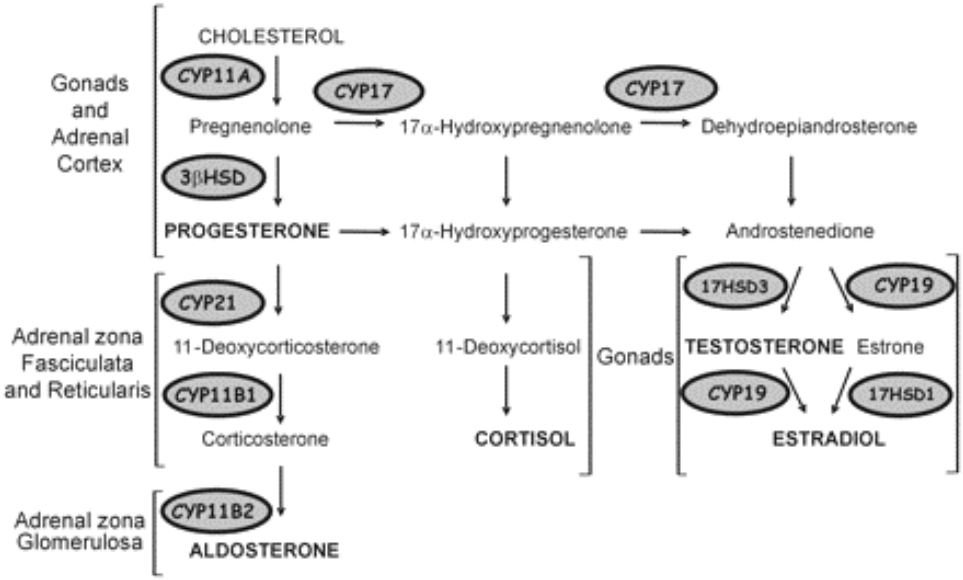


Fig.14 **Biosynthesis of steroid hormones in adrenal glands and gonads** (Payne and Hales, 2004).

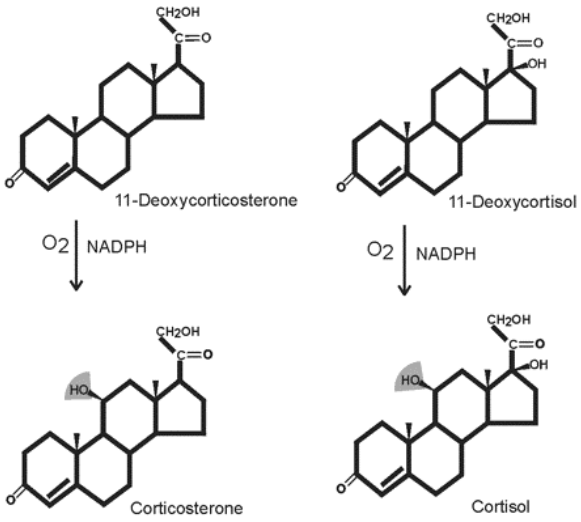


Fig.15 **Enzymatic reaction catalyzed by 11ß-hydroxylase** (Payne and Hales, 2004).

CYP11B1 (**11ß-hydroxylase**) and **CYP11B2** (**aldosterone synthase**) are located in the inner mitochondrial membrane. 11ß-hydroxylase catalyzes the 11ß-hydroxylation of 11-deoxycortisol yielding corticosterone or cortisol, respectively (Fig.15). 11ß-hydroxylase also has the capacity to hydroxylate C_{18} of 11-deoxycorticosterone (DOC) or corticosterone to form 18-hydroxycorticosterone. However, 11ß-hydroxylase cannot catalyze the oxidation of the 18-hydroxy group to form

aldosterone. Aldosterone synthesis from DOC is catalyzed by aldosterone synthase, which catalyzes three sequential reactions, each utilizing one molecule of NADPH and one molecule of oxygen and the mitochondrial electron transfer system. The three sequential reactions are: the 11ß-hydroxylation of DOC, the hydroxylation of C_{18}, followed by oxidation of the C_{18} hydroxyl group to yield the C_{18} aldehyde group resulting in the formation of aldosterone (Fig.16). In rat, third CYP11B form was characterized, CYP11B3, however it lacks the 18-oxidase activity and cannot synthesize aldosterone (Mellon et al., 1995).

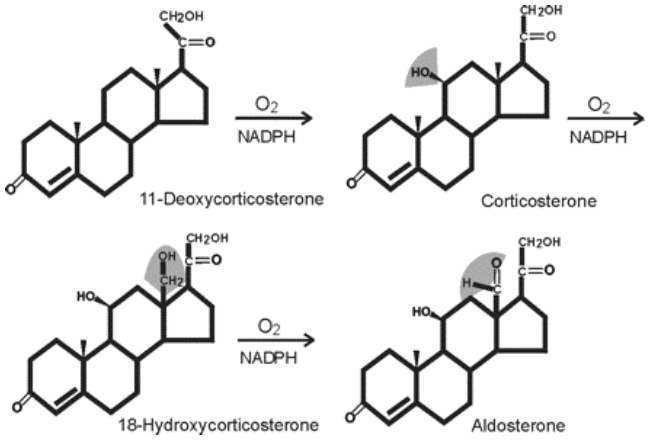


Fig.16 **Enzymatic reaction catalyzed by aldosterone synthase** (Payne and Hales, 2004).

The two genes are highly homologous, with nucleotide sequences of CYP11B1 and CYP11B2 exhibiting 95% identity in the coding sequence in human and 88% in rat (Mornet et al., 1989). In adrenals, CYP11B2 appears to be expressed exclusively in adrenal zona glomerulosa, whereas the major site of CYP11B1 expression is in the zona fasciculata/reticularis. Some expression of CYP11B1 is also observed in the mitochondria of the zona glomerulosa. Expression of CYP11B1 appears to exceed several-fold that of CYP11B2 (Curnow et al., 1991). Chronic stimulation by pituitary ACTH in the adrenal zona reticularis and zona fasciculata is acting via GPCRs. It activates adenylate cyclase thereby increasing cAMP, which in turn, leads to increased synthesis of steroidogenic P450 enzymes specific for these cells. The expression of CYP11B2 in the adrenal cortex is mainly controlled by body Na^+ status via the RAS. The transcription of CYP11B2 is enhanced by Na^+ deficiency and K^+ ions that act by increasing the intracellular concentration of Ca^{2+} (Ye et al., 2003). It was reported that cAMP can independently increase CYP11B2 transcription. In

contrast, CYP11B1 expression is determined by physiological variations of ACTH reviewed by Payne and Hales, 2004).

Recent studies provided evidence that aldosterone is also produced in extra-adrenal tissues such as cardiac tissue, blood vessels and brain. Aldosterone has been demonstrated in the perfusate of the *ex situ* rat heart and heart homogenates; however the origin of aldosterone in the heart is controversial. Some authors report a primary role for extraadrenal synthesis within the heart, and other report that all of the aldosterone in the heart is sequestered form the circulation (Gomez-Sanchez et al., 2004). In 1998 Silvestre et al. first demonstrated that aldosterone synthase is expressed in rat cardiac tissue, albeit at levels that are ~500-fold lower than that of the adrenal glands. Mizuno et al. (2001) have shown that aldosterone productions are increased in the failing human hearts. These observations led to a highly attractive “intracrine” hypothesis of aldosterone action in the heart. It has been proposed that locally produced aldosterone exerts its influence on cardiac cells without being released into the extracellular space.

The expression of mRNA for all enzymes of aldosterone synthesis has been described in the heart and vascular system. In normal adult humans, the enzymes required to synthesize aldosterone are probably expressed in the vasculature, but not in the heart. However, their expression may increase in pathological states such as congestive heart failure and myocardial infarction (White, 2003). The role of Na^+ is crucial since moderate increase of Na^+ intake decreases plasma aldosterone but stimulates cardiac aldosterone synthesis. This paradoxical response might contribute to the development of cardiac hypertrophy from salt-loading that occurred independently of blood pressure (Takeda et al., 2000). In rat species, strain and pathophysiological differences have been partially responsible for confusion concerning the existence of aldosterone synthase in the heart. Aldosterone synthase mRNA is expressed in some strains of rat. Wistar and SHR rat hearts express aldosterone synthase mRNA under basal and stimulated conditions. It was not found in Sprague Dawley rat hearts.

In addition to the source of active steroid hormones derived from the circulation there are several reports suggesting that the heart is capable of synthesizing significant

amounts of aldosterone, yet the heart cannot replace the failing or absent adrenal gland. The concentration of mRNA for all steroidogenic enzymes in rat heart and vascular tissue is much lower than that in the adrenal. The combined mass of the heart, endothelial and VSMC is very large, certainly much larger than the cell mass of the zona glomerulosa. However, conversion rates of DOC to aldosterone and corticosterone in heart homogenates were shown as high as those in zona glomerulosa, even though expression of aldosterone synthase is 1000-fold less (reviewed by Gomez-Sanchez et al., 2004).

Evidence for expression of steroidogenic enzymes in brain includes also CYP11B1 and CYP11B2. To date, there is no evidence for the expression of CYP21 in the brain indicating that the *de novo* synthesis of adrenal steroid hormones does not take place in the nervous system. However, this does not rule the possibility that circulating DOC or deoxycortisol could function as substrate for neural CYP11B1 or CYP11B2 (Mellon and Griffin, 2002). The levels of both enzyme transcripts detected in the brain were consistently much lower than those in the adrenal cortex. It was suggested that this reflects high expression in a very few cells within special brain regions, representing a very small fraction of the total cerebellar mass. With this pattern of expression, extraadrenal hormones may act in a paracrine or an autocrine manner on the abundant GR and MR within the brain. CYP11B1 and CYP11B2 also colocalize within the CNS. This does not occur in the adrenal cortex due to its strictly separated zonation. This colocalization raises some important questions whether CYP11B1 and CYP11B2 compete for the DOC as a substrate and whether the product of CYP11B1 can be used by CYP11B2, which was demonstrated *in vitro*. Local expression of CYP11B2 in the CNS can be regulated by alteration of Na^+ intake (Ye et al., 2003).

2. OBJECTIVES

The aim of this work was to characterize the relations between the KKS, ET and aldosterone. These hormonal systems have important physiological and pathological implications in the cardiovascular system, especially during high dietary salt intake.

We have focused on:

1. precise characterization of kininogen-deficient BNK rats in comparison to wild type BN rats under standard diet.
2. characterization of basic effects of spironolactone (aldosterone antagonist) in BN and BNK rats under standard diet.
3. investigation of the handling and actions of Na^+ in BN and BNK rats under high salt diet
4. characterization of spironolactone effects in BN and BNK rats under high salt diet

Besides basic physiological characteristics and hemodynamics, we have focused on the characterization of the components the KKS, ET-1 and corticoid hormones in plasma, urine and relevant tissues under all experimental conditions.

Additionally, basic functional cardiac parameters were assessed in Langendorff experiments of isolated rat hearts. We have investigated acute effects of 30min global ischaemia with or without previous ischaemic preconditioning in hearts of BN and BNK rats.

3. MATERIAL & METHODS

3.1. MATERIAL

3.1.1. EXPERIMENTAL ANIMALS

Brown-Norway (BN) rats, males, 200 - 250 g (Charles River Laboratories Germany, Sulzfeld)
Brown-Norway Katholiek (BNK) rats, males, 200 – 250 g (University of Kiel)
Standard diet, 0,5% NaCl (Altromin, Lage)
High salt diet, 5% NaCl, C1051 (Altromin, Lage)

3.1.2. INSTRUMENTS

Analytical balance, 1364MP (Sartorius, Goettingen)
Analytical balance (Mettler, Zürich, Switzerland)
Blood pressure monitor, 208001 with pressure cuff, inner diameter 13mm
and pulse transducer, inner diameter 8mm (TSE Systems, Bad Homburg)
Centrifugal vacuum concentrator, Jouan RC 10.22
with refrigerated condensation trap, Savant RT100 (GMI, USA)
and vacuum pump, vacuubrand (vacuubrand, Wertheim)
Gamma counter, Berthold LB 2111 (EG&G Berthold, Bad Wildbach)
Gel Doc 2000 System (Bio-Rad, Munich)
Incubator for microtiter plates, thermocult (Clinicon Int., Keltern)
Light Cycler System 1.0 with LightCycler Software 3.5 (Roche Diagnostics, Mannheim)
Liquid scintillation counter, Wallac 1410 (Perkin Elmer, Milano, Italy)
Magnetic mixer, temperature controlled (IKA, Staufen)
Megafuge 1.OR (Heraeus, Hanau)
Metabolic cages for rats over 300g (Techniplast, Varese, Italy)
Microanalytical balance, 708501 (Sartorius, Goettingen)
PCR Minicycler PTC 150 (MJ Reaserch Bio-Rad, Munich)
pH Meter 766 Calimatic (Knick, Berlin)
Power Supply LKB 2002 (LKB, Bromma, Sweden)
RIA Decanter (fine mechanics dept., University of Heidelberg)
RIA Roller (fine mechanics dept., University of Heidelberg)

RIA Shaker (fine mechanics dept., University of Heidelberg)
Spectrophotometer NanoDrop ND-1000
with software V3.1.0 (Nanodrop, Wellington Delware, USA)
Spectrophotometer Spectramax 250
with SOFTmaxPRO software (Molecular Devices, Munich)
Table centrifuge, 4515D (Eppendorf, Hamburg)
Test tube thermostat TCR 100 (Carl Roth, Karlsruhe)
Ultrasonic bath, Bandelin Sonorex RK 102P (Schalltec, Mörfelden-Wall)
Ultra-turrax disperser, TP 10-10 (IKA, Staufen)
Vacuum manifold (Varian, Darmstadt)
Vacuum pump and compressor, Laboport (neoLab, Heidelberg)
Vortex (Heidolph Instruments, Heidelberg)
Water bath, thermomix 1440 (B.Braun, Melsungen)
Wide Mini-SUB Cell GT for electrophoresis (Bio-Rad, Munich)

LANGENDORFF APPARATUS (PERFUSION EQUIPMENT)

ECG Electrodes (Ingenieurbüro Jäckel, Hanau)
Iso-DAM8 & Bridge8 amplifier (World precision Instruments, Berlin)
Latex balloon, size 3, 73-3478 (HSE-Harvard Apparatus, March-Hugstetten)
Organ chamber (glassblowing dept., University of Heidelberg)
Oxygenating chamber (glassblowing dept., University of Heidelberg)
Perfusion chamber, temperature controlled (fine mechanics dept., University of Heidelberg)
Perfusion Control BMT 9032 (BMT Messtechnik, Berlin)
Peristaltic pump Masterflex, 7015 (Masterflex, Gelsenkirchen)
Statham Pressure transducer with cardiac catheter (World precision Instruments, Berlin)
Water bath, thermomix 1460 (B.Braun, Melsungen)

SOFTWARE

BeMon and AMon Software 3.4 (Ingenieurbüro Jäckel, Hanau)
GeneFisher, interactive PCR primer design (University of Bielefeld)
Microsoft Office – Word, Excel, PowerPoint, Internet Explorer (Microsoft, USA)
REST-384 (Technical University, Munich)
SigmaPlot 8.0, SigmaStat 2.03 (Systat Software, USA)
Statistica 5.5(StatSoft, USA)

3.1.3. MATERIALS

Biozym pipets (Biozym, Hessisch Oldendorf)
Cannulas 27G, 23G, Microlance 3 (BD GmbH, Heidelberg)
Combitipps (Brand, Wertheim)
Eppendorf pipets (Eppendorf, Hamburg)
Filter holder FR 050/0 (Schleicher& Schuell, Dassel)
Filter pipette tips, low-retention (nerbe-plus, Winsen/Luhe)
Glass reaction tubes for RIA (neoLab, Heidelberg)
Glass tubes, round bottom, for homogenisation (neoLab, Heidelberg)
Hand dispenser Handy Step (Brand, Wertheim)
LightCycler capillaries (Roche Diagnostics, Mannheim)
LightCycler cooling block (Roche Diagnostics, Mannheim)
Membrane filters ME27, diameter 0,8µM (Schleicher& Schuell, Dassel)
Microtiter plates, 96-well (Carl Roth, Karlsruhe)
Parafilm (Brand, Wertheim)
PCR reaction tubes, thin-walled (Eppendorf, Hamburg)
Pipette tips, low-retention (nerbe-plus, Winsen/Luhe)
Polyethylene centrifuge tubes, 15, 50 ml (Greiner, Frickenhausen)
Rat operation table (fine mechanics dept., University of Heidelberg)
RIA test tubes with caps (Hormuth, Heidelberg)
Safe-lock reaction tubes, low retention, 0,6;1,5;2,0 ml (nerbe-plus, Winsen/Luhe)
Scintillation tubes (neoLab, Heidelberg)
Sep-Pak C_{18} cartridges (Waters, Eschborn)
Silicone tubing (Masterflex, Gelsenkirchen)
Surgical tools - tweezers, scissors, forceps, clamp, scalpel, aortic cannula
Syringe, 1ml, Plastipak (BD GmbH, Heidelberg)
Syringe, luer lock, 5ml, Plastipak (BD GmbH, Heidelberg)
T-connector with stopcock (neoLab, Heidelberg)

3.1.4. CHEMICALS

Acetic acid (JTBaker, Deventer, Holland)

Acetonitril, 1605 (AppliChem, Darmstadt)

Agarose, electrophoresis grade, 15510-027 (Invitrogen, Karlsruhe)

Aldosterone, A2136 (Sigma-Aldrich, Taufkirchen)

3^{H}-aldosterone (Amersham Biosciences, Freiburg)

Aldosterone antiserum (Steroid lab, University of Heidelberg)

Anti-Endothelin, E1645 (Sigma-Aldrich, Taufkirchen)

Anti-rabbit IgG, R0881 (Sigma-Aldrich, Taufkirchen)

Beriglobin, human immunoglobulin (Aventis, Frankfurt)

Boric acid (JTBaker, Deventer, Holland)

Bovine serum albumine, A7906 (Sigma-Aldrich, Taufkirchen)

Bradykinin (Bachem, Weil am Rhein)

Bradykinin antiserum (Lab Hilgenfeldt, University of Heidelberg)

Calcium chloride (JTBaker, Deventer, Holland)

Carbogen (Theoretikum, University of Heidelberg)

Celite 545 AW coarse, 22141 (Sigma-Aldrich, Taufkirchen)

Chloramine T, C9887 (Sigma-Aldrich, Taufkirchen)

Chloroform (JTBaker, Deventer, Holland)

Corticosterone, C2505 (Sigma-Aldrich, Taufkirchen)

3^{H}-corticosterone (Amersham Biosciences, Freiburg)

Corticosterone antiserum (Steroid lab, University of Heidelberg)

dATP, U1201 (Promega, Mannheim)

dCTP, U1225 (Promega, Mannheim)

Deoxycorticosterone, D7000 (Sigma-Aldrich, Taufkirchen)

3^{H}- deoxycorticosterone (Amersham Biosciencies, Freiburg)

Deoxycorticosterone antiserum (Steroid lab, University of Heidelberg)

DEPC, A0881 (AppliChem, Darmstadt)

Dextran T70 (Serva, Heidelberg)

D-Glucose, 8342 (Merck, Darmstadt)

dGTP, U1211 (Promega, Mannheim)

Dichloromethane (Riedel-de Haen, Hanover)

DNA Loading Dye 6x, R0611 (Fermentas, St.Leon-Roth)

DNA-OFF, A2860 (AppliChem, Darmstadt)

Dry ice (Theoretikum, University of Heidelberg)

dTTP, U1231 (Promega, Mannheim)

EDTA, 8417 (Merck, Darmstadt)

Endothelin-1, E7764 (Sigma-Aldrich, Taufkirchen)

Ethanol absolute (Riedel-de Haen, Hanover)

Ethidium bromide, 15585-011 (GIBCO BRL Div. of Invitrogen, Karlsruhe)

Ethylacetate (Merck, Darmstadt)

Ethylene glycole (Merck, Darmstadt)

Gelafundin, 4% (B.Braun, Melsungen)

Heparin-Natrium B.Braun 25 000 I.E. (B.Braun, Melsungen)

Hydrochloric acid, 9057 (Merck, Darmstadt)

Hyperladder I, DNA Marker, 555868 (Bioline, Luckenwalde)

Isooctane (Acros, Geel, Belgium)

Liquid nitrogen (Theoretikum, University of Heidelberg)

Lysozyme, L6876 (Sigma-Aldrich, Taufkirchen)

Kallidin (Bachem, Weil am Rhein)

Kallidin antiserum (Lab Hilgenfeldt, University of Heidelberg)

Kallidin-like peptide (Lab Metzler-Nolte, University of Heidelberg)

Kallikrein from human plasma, K2638 (Sigma-Aldrich, Taufkirchen)

Kaolin, K7375 (Sigma-Aldrich, Taufkirchen)

Magnesium sulphate, 0168 (JTBaker, Deventer, Holland)

ß-Mercaptoethanol (Acros, Geel, Belgium)

Methanol absolute (Riedel-de Haen, Hanover)

Narcoren, sodium pentobarbital (Merial, Hallbergmoos)

Norit A, charcoal, 30890 (Serva, Heidelberg)

Normal rabbit IgG carrier, 3050 (Linco Res; Biotrend, Cologne)

Olive oil, 75348 (Sigma-Aldrich, Taufkirchen)

o-Phenanthroline, P9375 (Sigma-Aldrich, Taufkirchen)

Polyethylene glycol, MW 8000 (Sigma-Aldrich, Taufkirchen)

Potassium chloride, 4936 (Merck, Darmstadt)

Potassium iodide, 30315 (Riedel-de Haen, Hanover)

Potassium metabisulfite, P2522 (Sigma-Aldrich, Taufkirchen)

Potassium phosphate, 30407 (Riedel-de Haen, Hanover)

Primers for PBGD, CYP11B1, CYP11B2 (Invitrogen, Karlsruhe)

Rat tissue kallikrein (BioAss, Diessen)

RNAlater, 76104 (Qiagen, Hilden)

RNase-OFF, A2861 (AppliChem, Darmstadt)

S-2266, chromogenic substrate for glandular kallikrein (Chromogenix, Milano, Italy)

S-2302, chromogenic substrate for plasma kallikrein (Chromogenix, Milano, Italy)

Scintillation reagent, Ultima Gold (Perkin Elmer, Milano, Italy)

Silicone solution, 35130 (Serva, Heidelberg)

Sodium azide, S2002 (Sigma-Aldrich, Taufkirchen)

Sodium carbonate, 31437 (Riedel-de Haen, Hanover)

Sodium chloride, 6404 (Merck, Darmstadt)

Sodium citrate, 6447 (Merck, Darmstadt)

Sodium hydroxide, 9137 (Merck, Darmstadt)

Sodium iodide, 3129 (Riedel-de Haen, Hanover)

Sodium-^{125} I (Hartmann Analytic, Braunschweig)

Sodium phosphate dibasic, S0876 (Sigma-Aldrich, Taufkirchen)

Sodium phosphate monobasic, S0751 (Sigma-Aldrich, Taufkirchen)

Sodium pyruvate, 15220 (Serva, Heidelberg)

Spironolactone, S3378 (Sigma-Aldrich, Taufkirchen)

Taq DNA polymerase 5U/µl,

with 10x reaction buffer and 25mM $MgCl_2$, D4545 (Sigma-Aldrich, Taufkirchen)

Trasylol ®, 500 000 KIU (Bayer, Leverkusen)

Triethylamine, T0886 (Sigma-Aldrich, Taufkirchen)

Tris, 4855 (Carl Roth, Karlsruhe)

Trypsin agarose, T1763 (Sigma-Aldrich, Taufkirchen)

Tyr^8-BK (Bachem, Weil am Rhein)

Tyr^6-KLP (PolyPeptideLaboratories, Wolfenbüttel)

ASSAY KITS

CK NAC, activated (Rolf Greiner BioChemica, Flacht)

LightCycler Fast Start DNA Mater SYBR Green I, 12239264001 (Roche Diagnostics, Mannheim)

Quantitect Reverse Transcription Kit, 205311 (Qiagen, Hilden)

RNeasy Mini Kit, 74104 (Qiagen, Hilden)

3.2. METHODS

3.2.1. ANIMAL EXPERIMENT

All experiments were performed in accordance with the FELASA guidelines for animal experimentation. All rats were kept under standard conditions with free access to food and water.

Male **Brown-Norway (BN) rats** (a total of 56 rats) and **kininogen-deficient Brown-Norway-Katholiek (BNK) rats** (a total of 56 rats) of 300–350g were used. Animals of each strain were randomly divided into four experimental groups and underwent following treatment for 10 days:

1. NS - normal salt diet (standard diet - 0,5% NaCl)
2. NS SPI - normal salt diet (0,5% NaCl) + spironolactone (20mg/day s.c.)
3. HS - high salt diet (5% NaCl)
4. HS SPI - high salt diet (5% NaCl) + spironolactone (20mg/day s.c.)

Spironolactone (100mg) was suspended in 1ml olive oil and before each application properly mixed in ultrasonic bath and vortexed. Rats received a subcutaneous injection of 200µl of spironolactone per day for 10 days.

Following experiments with metabolic cages were performed with 32 BN rats and 38 BNK rats. These rats underwent blood pressure and heart rate measurements and were used for the Langendorff heart experiments. Additional 24 BN rats and 18 BNK rats were used for the acquisition of plasma and supplementary organs.

In the beginning of the experiment, the rats were weighed, so that differences in body weight change after each treatment could be determined. During the experiment, the rats were gradually trained in individual **metabolic cages**. On the 9^{th} day, they were put into metabolic cages for 24h. The next day, body weight, food and water intake, and urine volume were recorded. During the 24h, urine was collected into urine collection tubes that were cooled with an ice bath. The tubes as well as the lower part of metabolic cages were treated with silicone solution to avoid peptide adsorption.

Urine was then centrifuged (6000rpm, 15min, 4°C) to remove food contamination. Aliquots of urine in special low-retention tubes were stored at -20°C until assayed. Afterwards, the animals had one day to recover before they were used for terminal blood pressure measurement and final heart perfusion.

3.2.2. BLOOD PRESSURE AND HEART RATE MEASUREMENTS

Mean blood pressure and heart rate were measured simultaneously by means of a tail cuff method in conscious animals. The rats were trained for the measurements during the whole experiment. Each pressure and heart rate value was obtained by averaging four to six individual readings.

For measurements, the animals were held in hands, always by one person. The tails were passed through the pressure cuff that was placed at the base of the tail in front of the pulse sensor (transducer). Both sensor and pressure cuff were connected to the control unit. This unit featured an integrated pressure generator for cuff inflation. Alterations in the diameters of the arteries caused by variations in blood pressure resulted in an altered mechanical force, which was recognized by the piezo-transducer and converted to electrical signal. The heart frequency was calculated form the pulse signal.

3.2.3. SURGERY

The **first set** of rats, used for the **heart perfusion**, was anaesthetized and heparinized with a mixture of pentobarbital sodium (60mg/kg) and heparin (500IU), administered intraperitoneally. Pentobarbital was washed out from the myocardium within the first minutes of perfusion, so that it did not impair measurements. Heparin was used to prevent blood clotting, which would injure cardiac tissue.

Surgery was not started until the rats completely lost consciousness. Animals were then fixed on an operating field filled with ice. The skin was incised in the middle of the abdomen by a transversal cut. The diaphragm was incised at the edges and was cut out from the ribs. The pericardium was incised from the bottom up to the top. At this time, the thoracic cavity was filled with ice-cold saline solution to cause

bradycardia and reduce energy and oxygen consumption. Thereafter, the thorax was opened by long cuts at the right and left side so that the complete anterior thoracic wall was turned upwards. In the region of the ascending aorta, the rest of the pericardium and thymus were removed. By use of forceps, the ascending aorta was gently separated from the pulmonary artery and connective tissue. The aorta was undermined and a thread was positioned around the aorta with a prepared surgical knot (Fig.17). Next, the aorta was incised transversely as far cranially as possible and the aortic cannula (3mm outer diameter) was inserted. The cannula was connected to an auxiliary reservoir of cold perfusion solution. Prior to the insertion, the stopcock of the perfusing system was opened so that there was already some flow through the aortic cannula, which helped to avoid air bubbles entering the coronary arteries. After insertion, the knot was tightened and flow from the auxiliary perfusion system was completely opened. Now the heart was retrogradely perfused at constant pressure (the hydrostatic pressure of the auxiliary system) and all remaining blood was washed out. The heart was then cut off and removed from the body. It was immediately transferred to the Langendorff apparatus and connected via the aortic cannula to the stopcock. The whole preparation was performed within 2min (starting with the incision of the diaphragm until positioning of the heart on the apparatus). Spontaneously beating started within few seconds of reperfusion with warmed and oxygenized perfusion medium. Now, the remaining tissues such as excessive connective tissue, remains of vessels, lungs or fat were removed.

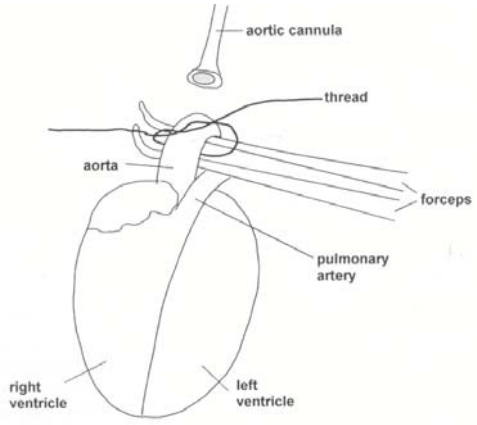


Fig. 17 **Preparation of the heart.** The scheme shows the insertion of the aortic cannula (Dhein et al., 2005)

After the heart was removed, other **organs** were quickly harvested. Firstly, the adrenals were separated and immediately put into pre-chilled tube with RNA stabilization reagent (RNAlater). The right kidney was removed and cut into pieces before it was submerged in RNAlater. The left kidney was removed, weighed and immediately frozen in liquid nitrogen. The brain was excised, weighted and quickly frozen in liquid nitrogen. The frozen samples were then stored in -40°C till processing. To ensure absolute permeation of RNAlater through the tissue, the samples in RNAlater were first overnight incubated at 2-8°C and then transferred to -20°C for archival storage. The heart weight was determined after the heart perfusion.

In the **second series**, rats were used for **plasma** and organs acquisition. For narcosis, only sodium pentobarbital (60mg/kg i.p.) was used. Heparin was omitted because it would influence plasma quality.

Animals were anaesthetized until a complete loss of consciousness. They were fixed on a flexible operational table used for exsanguinations. The skin in the upper part of throat was excised with scissors. By use of tweezers, the tissue was spread to sides to expose the left carotid artery. It was then carefully undermined with forceps and slightly lifted. A pre-chilled siliconized plastic tube with 1ml 3,8% sodium citrate was placed under the artery, so that after cutting, the blood could directly run into it. The blood (around 10ml) was then properly mixed with the citrate to prevent coagulation and immediately centrifuged (6000rpm, 10min, 4°C). Plasma supernatant was then aliquoted into low-retention tubes and stored in -20°C until assayed.

Immediately after the rats were killed by bleeding, additional **organs** were harvested. Adrenal glands were separated and put into pre-chilled tubes with RNAlater. The right kidney was removed, cut into pieces and submerged in RNAlater. The left kidney was removed, weighed and quickly frozen in liquid nitrogen. The heart was carefully excised and washed in DEPC treated water (RNase free) to remove remaining blood. Then the atria were separated and submerged in cold RNAlater. The ventricle was cut into smaller pieces and put into pre-chilled tube with RNAlater. The brain was excised, weighted and frozen in liquid nitrogen. The frozen samples were stored in -40°C till processing. The samples in RNAlater were overnight incubated at 2-8°C and then transferred to -20°C for further storage.

3.2.4. LANGENDORFF PERFUSION OF ISOLATED RAT HEART

This method is based on the basic investigation of Oscar Langendorff who in 1895 developed a method for investigation of the isolated heart. The basic principle is to maintain cardiac activity by perfusing the heart via the coronary arteries using an aortic cannula inserted into the ascending aorta. Perfusion solution is delivered to the heart in a retrograde manner via this cannula. This retrograde perfusion closes the aortic valve and flows into the coronary arteries during the diastole (Fig.18). After passing through the coronary circulation, the perfusate enters the right atrium via the coronary sinus and is driven out via the right ventricle and the pulmonary artery.

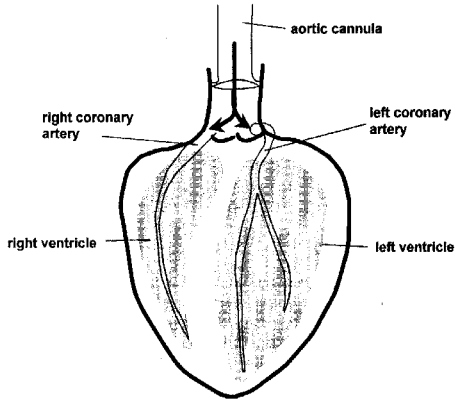


Fig. 18 **Scheme of the isolated perfused heart according to Langendorff.** Perfusion solution is flowing retrogradely within the aorta and then orthogradely within the coronary arteries during diastole. A prerequisite is that the aortic valve is closed by the perfusion pressure of the perfusion solution (Dhein et al., 2005).

The key components of a **Langendorff apparatus** (Fig.19) were represented by a glass double-walled heat exchanger and oxygenating chamber in one and a perfusion controller. Perfusion controller transported the perfusion solution to the heart via the aortic cannula and acted as a feedback system to regulate perfusate flow. The aortic cannula with the heart was fixed to the stopcock at the end of the tubing of the perfusion controller.

The oxygenating chamber was formed from 4 linked spheres that provided maximal surface area for warming and oxygenating the nutrient solution. The solution was infused at the top of the oxygenator from a reservoir via additional peristaltic pump.

The bottom of the chamber, where the solution accumulated, had an inlet for carbogen that bubbled through the solution. Perfusion controller combined a peristaltic pump and a pressure transducer which was directly connected to the heart via a T-connector at the level of the aortic cannula. Both, constant perfusion flow or constant perfusion pressure were achieved by the perfusion controller. Coronary perfusion pressure (indicator of coronary resistance) was measured continuously and as the coronary vessels autoregulated (dilated or constricted) and the vascular resistance changed, the changes in coronary pressure were registered and the perfusion controller regulated the function of the pump, so that constant perfusion pressure was maintained. For the investigation of complex situation of ischaemia and reperfusion, constant perfusion pressure was advantageous as ischaemia enhanced the energy and oxygen consumption and so the flow rate could be adjusted to additional supply of oxygen and energy to the heart. In case of crystals or contamination in the perfusion solution that could injure the heart tissue, the medium passed through 0,8µm filter before entering the heart. Around the heart there was a second double-walled chamber in the form of a cylinder which provided a warm and moisturised atmosphere around the heart.

Fig.19 **A complete Langendorff apparatus.** Temperature controlled perfusion chamber maintains inner temperature of 38°C.

The **perfusion solution** consisted of a modified Krebs-Henseleit buffer (Tab.1). The solution was always fresh prepared and aerated with a 95% O_2 + 5% CO_2 mixture and equilibrated for pH 7,4 and 38°C.

Chemicals	*MW* (g/mol)	Concentration (mmol/l)
1. NaCl	58,44	118
2. $NaHCO_3$	84,01	24
3. KCl	74,56	4
4. KH_2PO_4	136,09	1,2
5. $MgSO_4$	246,47	1
6. D-Glucose	180,16	11
7. Sodium Pyruvate	110,00	2
8. $CaCl_2$	147,02	2,5

Tab.1 **Krebs-Henseleit medium for ischaemia experiments in Langendorff heart perfusion.**

As described above, the heart was rapidly excised and mounted on the Langendorff apparatus (Fig.20). At the beginning, the heart was allowed to equilibrate as it was perfused at a constant flow rate of about 10-12ml/min.

Fig. 20 **Perfused isolated rat heart on the Langendorff apparatus, with intraventricular balloon catheter and ECG electrodes.**

For the **isovolumetric measurement of force**, a balloon was inserted via the left atrium into the cavity of the left ventricle (Fig.21). The balloon size fitted the size of the left ventricular cavity and gave an impression of the developed force of the whole left ventricle. The balloon was compressed during the systole. Since during diastole the aortic valve was closed and during systole the intraventricular pressure was higher than the perfusion pressure, there was no flow fluid into the ventricular cavity.

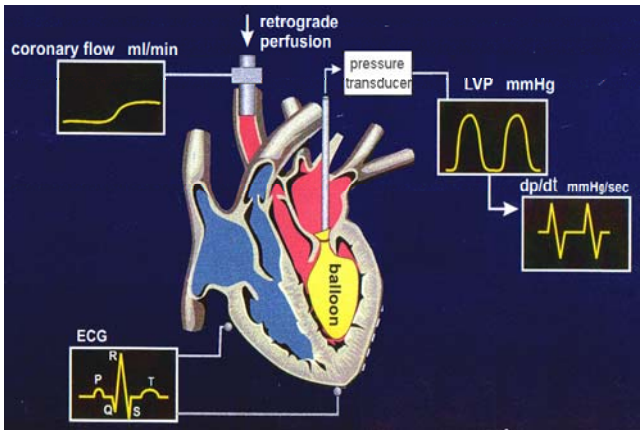


Fig.21 **Measurements of functional cardiac parameters in Langendorff heart perfusion** (Aventis).

Before insertion into the ventricle, the balloon was evacuated. In the right position, it was filled with distilled water until the desired end-diastolic pressure was achieved. It had to be free from air bubbles which would affect and falsify the measurement. Filling of the balloon was realized via a syringe connected via a three-way stopcock to a Statham pressure transducer connected to a bridge amplifier.

The correct balloon pressure was determined by measurement of the Frank-Starling mechanism. For that purpose, the balloon pressure (LVP_{min}, diastolic pressure) was increased in small steps of about 5mmHg. It was seen that as the LVP_{min} increased the maximum left ventricular pressure (LVP_{max}, systolic pressure) also increased up to a certain point. Further increase in balloon pressure now resulted in no more increase in LVP_{max}. The balloon pressure with the maximum developed force of the ventricle indicated the optimal preload (left ventricular end-diastolic pressure (LVEDP)).

Epicardial measurement of the electric activity of the heart (electrocardiogram (**ECG**)) was achieved by using a bipolar circuit. For the recording of ECG an amplifier was used. One electrode (AgCl) was positioned at the left ventricle (Fig.20), the other electrode at the right ventricle. An amplifier was used for the recordings. Steel aortic cannula helped electrical grounding.

After the stabilization period, the flow rate was adjusted to obtain a coronary perfusion pressure of approximately 65mmHg and was held constant, except of ischaemic periods during which the flow was stopped (no-flow ischaemia).

HEMODYNAMIC MEASUREMENTS

Data of the coronary flow rate were obtained indirectly as they reflected the action of peristaltic pump of the perfusion controller. As mentioned above the function of the pump was adjusted to the momentary demand of the heart according to the constant perfusion pressure of 65mmHg measured by a pressure transducer attached to the side arm of aortic cannula.

The intraventricular balloon was used for measurements of the left ventricular developed pressure (LVDP) and left ventricular end-diastolic pressure (LVEDP). LVEDP is represented by LVP_{min} and left ventricular developed pressure (LVDP) is defined as the maximum left ventricular pressure (LVP_{max}) minus LVP_{min} (LVEDP). The LVP signal was applied to the inputs of a differentiator and displayed the derived parameters maximum contraction and relaxation velocity (dp/dt_{max} and dp/dt_{min}, respectively). The heart rate was derived from the left ventricular pressure trace.

ECG parameters served for a real time control of stable cardiac function.

The analogue signals from transducers and electrodes were amplified and then processed by a BeMon32 V3.4 software and stored for subsequent analysis with the AMon32 software.

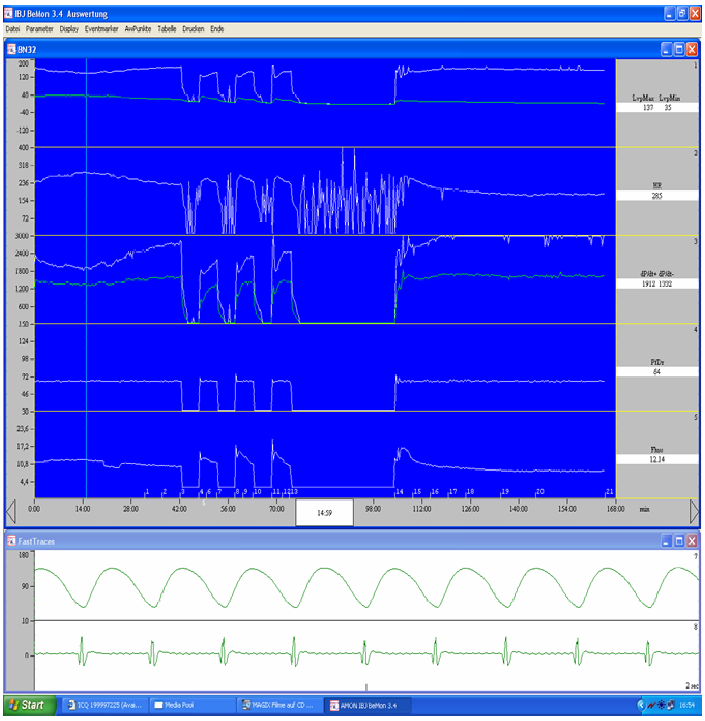


Fig.22 **BeMon software.** Important functional cardiac parameters were displayed during the heart perfusion. In the upper panel down from top: LVP_{max} and LVP_{min}, heart rate (HR), dp/dt_{max} and dp/dt_{min}, perfusion pressure and coronary flow. In the lower panel: the pulse wave and ECG signals.

EXPERIMENTAL PROTOCOL

Each pre-treatment group was randomly subdivided into controls (without preconditioning) and preconditioned hearts. The hearts in both groups were subjected to 30min equilibration period.

The **control group** was then subjected to a 30min sham period of normal perfusion followed by 30min of global ischaemia (no-flow) before a 60min reperfusion period.

In the **preconditioned group**, the hearts were first exposed to three cycles of 5min global ischaemia separated by three reperfusion cycles of 5min duration. Three cycles of IPC were employed, since it has been documented, that multiple cycles of preconditioning are needed to achieve protection. After 30min of global ischaemia (no-flow) the hearts were reperfused for another 60min.

Coronary venous effluent (à 2ml) was collected continuously during the perfusion, except of the no-flow periods, as indicated in Fig.23. The samples were stored at 4°C until assayed.

Coronary effluent was used for the measurement of the creatine kinase (CK) activity that served for evaluation of the cardiac damage caused by ischaemia. Before sample collection following ischaemia, a short washout period was performed to avoid collecting of the CK accumulated in the heart during the no-flow period. Later, all evaluated hemodynamic data were chosen to be matched to the same time points as the collection of the coronary effluent which also excluded the immediate reflective respond of the heart to ischaemia.

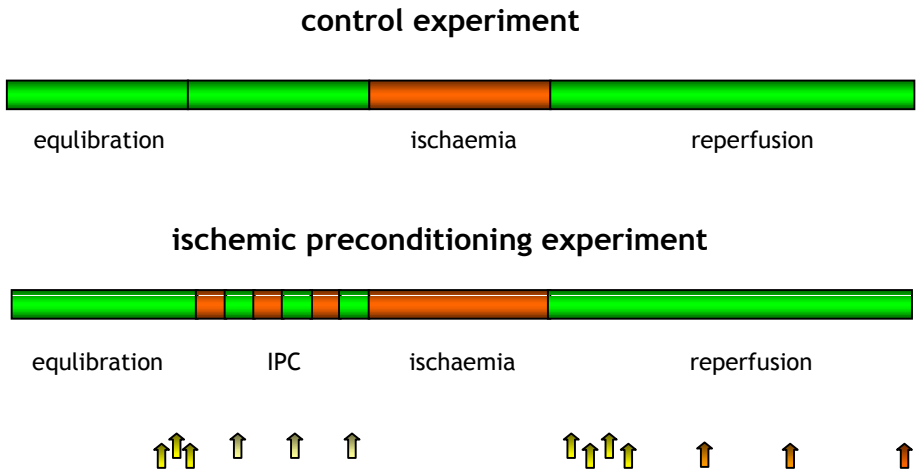


Fig.23 **Experimental protocol**. Green fields indicate perfusion, red fields indicate global ischaemia (no-flow). Coronary effluent started to be collected at the end of equilibration (20min after the beginning of the experiment), a mean value of all three measurements was used as initial value. During the IPC effluent was collected in the middle of each reperfusion cycle. After ischaemia, it was first collected four times after 5min of reperfusion; later after 10min and finally after 20min. The hemodynamic data respond to the same time scheme.

3.2.5. RADIOIMMUNOASSAYS

Radioimmunoassay (**RIA**) is a high sensitive, competitive protein-binding method where antibodies are used as specific reagents that selectively bind the substance to be measured in a native and radioactively labeled form. It is used for measurements of hormones and other substances present in minute quantities in biological fluids. It is possible to determine quantity as small as 10^{-14}g (10^{-12}M) with good precision. The technique was introduced in 1960 by Berson and Yalow as an assay for the concentration of insulin in plasma.

Specific antibody is directed against the peptide to be measured. Tracer (the same peptide labeled with radionuclide) and measured peptide are added to the incubation mixture. During the incubation step, the reactants come to equilibrium, as there is a competition between the measured peptide and tracer for a specific binding site. The bound antigens are separated from the unbound ones and the radioactivity of each is determined in a radiation counter. Separation methods are mostly based on differences in immunologic determinants, solubility or adsorption to solid material (Thorrel and Larson, 1978).

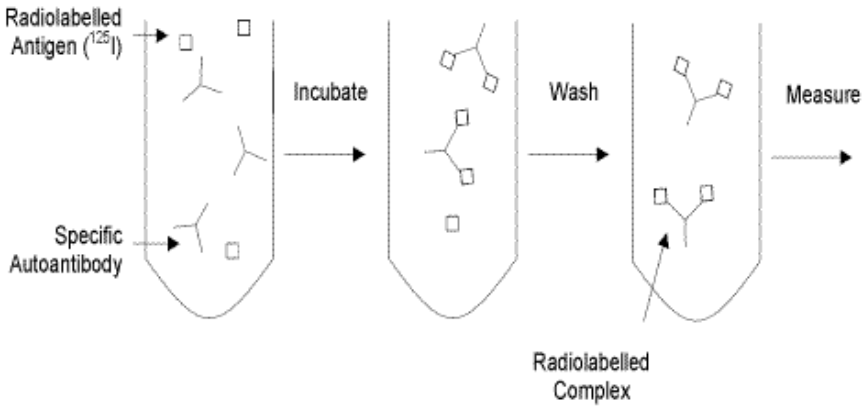


Fig.29 **Principle of radioimmunoassay.**

Standard curve is created by running few samples at known concentrations of measured antigen. At increasing concentrations of unlabeled antigen, an increasing amount of tracer is displaced from the antibody molecules and after separating, the radioactivity of each is measured. The ratio of bound and free antigen (B/B_0) in each

standard is plotted against logarithm of respective concentration. The samples to be assayed are run in parallel. After determining the ratio of bound to free antigen in each unknown, the antigen concentrations can be read directly from the standard curve.

3.2.5.1. MEASUREMENT OF PLASMA KININOGENS

The residual levels of kininogens in plasma were measured according to a modified method published by Uchida and Katori in 1979. The assay is based on kininogen's enzymatic breakdown by plasma kallikrein and trypsin. By this process generated BK was measured by RIA.

For **HMW kininogen**, plasma was incubated with kaolin suspension in the presence of o-phenanthroline (kininase inhibitor). HMW kininogen was converted to kinin by activation of plasma prekallikrein through activation of coagulation factor XII. Released BK was measured by RIA.

Digestion was performed in low-retention plastic tubes to reduce the surface adsorption of proteins and peptides. Mixture of 160µl 0,06M Tris-HCl buffer, pH 7,8 + 0,6 mg/ml o-phenanthroline, 40µl kaolin suspension (2,5 g/l kaolin, 9 g/l NaCl, 0,2 g/l NaN_3 in distilled water), and 40µl citrate plasma was incubated for 30min at 37°C. Afterwards, proteins were precipitated with 880µl ethanol absolute and the mixture was cooled on ice. After centrifugation (10min, maximum speed), supernatant with BK was transferred into new test tube. Pellet was resuspended with 440µl ethanol absolute and centrifuged 10min at maximum speed. Both supernatants were then collected and carefully evaporated under vacuum. Samples were resuspended in 1000µl RIA buffer (0,1M Tris-acetate buffer, pH 7,4 containing 0,1% Gelafundin) and stored at -20°C until assayed. For RIA, triplicates of 50µl were used for measurement of BK in samples of kininogen-deficient BNK rats and triplicates of 50µl after dilution of 1:50 in samples of BN control rats.

For **LMW kininogen**, plasma was incubated with kaolin suspension in absence of o-phenanthroline. Thus kinins converted from HMW kininogen by plasma kallikrein were destroyed by kininases in plasma. The HMW kininogen-depleted plasma was

acidified to pH 2,0 (kininase inactivation) and was incubated with trypsin after neutralisation. BK released from LMW kininogen by trypsin were assayed by RIA.

Digestion was performed in low-retention plastic tubes. Mixture of 160µl 0,06M Tris-HCl buffer, pH 7,8; 40µl kaolin suspension (2,5 g/l kaolin, 9 g/l NaCl, 0,2 g/l NaN_3 in distilled water), and 40µl citrate plasma was incubated for 30min at 37°C. Afterwards, the mixture was acidified with 10µl 1M HCl and further incubated for 15min at 37°C. The sample was neutralised with 10µl with 1M NaOH and mixed. A volume of 20µl of insoluble trypsin (1,6 U/ml Tris-HCl buffer) was added and the mixture was incubated for further 60min at 37°C. Subsequently 1ml ethanol absolute was added and the mixture was incubated next 10min at 70°C to efficiently inactivate and precipitate proteins. Sample was cooled on ice and centrifuged 10min at maximum speed. Supernatant with kinins was transferred into a new test tube. Pellet was resuspended with 440µl ethanol absolute and centrifuged 10min at maximum speed. Both supernatants were then collected and carefully evaporated under vacuum. Samples were resuspended in 1000µl RIA buffer (0,1M Tris-acetate buffer, pH 7,4 containing 0,1% Gelafundin) and stored at -20°C until assayed. For RIA, triplicates of 50µl were used for measurement of BK in samples of kininogen-deficient BNK rats and triplicates of 50µl after dilution of 1:25 in samples of BN control rats.

3.2.5.2. MEASUREMENT OF URINARY KININOGEN

For urinary **LMW kininogen**, urine was incubated with trypsin. Released BK was determined by RIA.

Digestion was performed in low-retention plastic tubes. Mixture of 180µl 0,06M Tris-HCl buffer, pH 7,8; 20µl urine and 10µl of insoluble trypsin (1,6 U/ml Tris-HCl buffer) was incubated for 60min at 37°C. Subsequently 1ml ethanol absolute was added and the mixture was incubated next 10min at 70°C to efficiently inactivate and precipitate proteins. Sample was cooled on ice and centrifuged 10min at maximum speed. Supernatant with kinins was transferred into a new test tube. Pellet was resuspended with 440µl ethanol absolute and centrifuged 10min at maximum speed. Both supernatants were then collected and carefully evaporated under vacuum. Samples were resuspended in 500µl RIA buffer (0,1M Tris-acetate buffer, pH 7,4 containing

0,1% Gelafundin) and stored at -20°C until assayed. For RIA, triplicates of 150µl were used for measurement of BK in all samples.

3.2.5.3. RADIOIMMUNOASSAY FOR THE AMOUNT OF BRADYKININ AND KALLIDIN-LIKE-PEPTIDE IN BIOLOGICAL FLUIDS

PREPARATION OF SPECIFIC ANTIBODIES

In the preparation of specific BK and KAL antibodies, both Cys^6-BK and Cys^7-KAL derivatives were first conjugated to a bovine serum albumin. The coupling products containing the kinin with both free amino- and carboxy-terminal ends were used as immunogens in rabbit. Obtained antisera were simultaneously directed against both free ends and therefore highly specific and sensitive. Both antisera displayed very low crossreactivity with other various kinins (Hilgenfeldt et al., 1995). KAL antiserum displayed approximately 80% crossreactivity with KLP which enabled reliable measurements of KLP in rat (Liu et al., 2005).

PREPARATION OF THE TRACER MOLECULES – IODINATION OF TYR^8-BK AND TYR^6-KLP

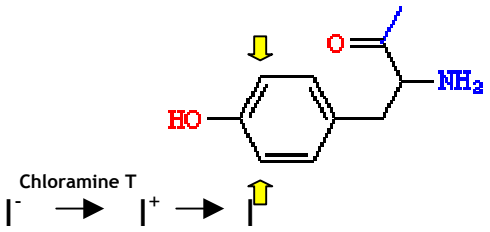


Fig.25 **Principle of iodination.** Iodide is oxidized to the positive form with chloramine-T and enters the ring structure of the tyrosyl residue of the peptide chain. Reaction is stopped by addition of reducing agent sodium metabisulfite. (modified after Thorrel and Larson, 1978).

Because kinin molecules do not contain Tyr, whose ring structure may easily incorporate one or two atoms of iodine, Tyr^8 derivative of BK and Tyr^6 derivative of KLP were used for tracer molecules. Iodination was performed according to the chloramine-T oxidation method of Greenwood and Hunter from 1961 (Fig.25).

2,5µg peptide (Tyr^8-BK or Tyr^6-KLP), in a low-retention test tube, was dissolved in 25µl 0,1M sodium phosphate buffer, pH 7,4. 10µl $Na^{125}I$ (37MBq) was added. The reaction was started by oxidation with 1µl chloramine-T (2,5µg/µl 0,1M sodium phosphate buffer, pH 7,4) and was stopped after 20s by addition of 5µl of a reducing agent potassium metabisulfite (5µg/µl 0,1M sodium phosphate buffer, pH 7,4). Unreacted iodide was removed from the labeled peptide by use of a C_{18} Sep-Pak cartridge. The cartridge was equilibrated with methanol absolute and loaded with an excess of KI (0,1% in 0,1M sodium phosphate buffer, pH 7,4 containing 0,1% BSA). In the presence of unlabeled iodide, free $Na^{125}I$ was separated from ^{125}I-Tyr^8-BK and ^{125}I-Tyr^6-KLP within the first fraction of elution with 3ml of 0,1% KI in 0,1M sodium phosphate buffer, pH 7,4 containing 0,1% BSA . After washing with 20% methanol in phosphate buffer, the purified labeled peptide was eluted into siliconized tubes within two main 0,5ml fractions with 60% methanol in phosphate buffer (Hilgenfeldt et al., 1995). The quality of the new tracer was assessed in a binding test where also the appropriate antiserum dilution was determined. The highest assay sensitivity was achieved with a dilution by which approximately 50% of the tracer in absence of unlabeled peptide was bound (B_0, maximum binding). The sensitivity was found to be 0,5pg BK and 5pg KLP per tube.

ASSAY PERFORMANCE

Assay was performed at low temperature to minimize proteolysis. Deep frozen reagents were carefully thawed on ice. Initially, triplicates of total amount of added radioactivity (Tc, total count), maximum binding of tracer to antiserum (B_0,) and blank tubes without antiserum (NSB, non-specific binding) preceded. Triplicates of reaction mixtures as seen in Tab.2 were prepared in siliconized RIA tubes. After all reagents were simultaneously pipetted, each tube was mixed for few seconds and then covered with parafilm. Assay tubes were incubated overnight in ice-water bath at 4°C. Afterwards, 150µl properly mixed charcoal suspension (5g Norit A in 100ml 0,1M Tris-acetate buffer, pH 7,4 containing 50ml 4% Gelafundin) was added to the reaction mixture and mixed. Free kinins were adsorbed to charcoal, whereas antibody-bound kinins remained in solution. After centrifugation (15min at 4°C, 6000rpm) the supernatant from single tubes was removed by means of a vacuum pump and the free charcoal-bound tracer was measured with a gamma counter. For

standard curve, data were plotted as the ratio of bound activity to bound activity in the zero standard (B/B_0) against the logarithm of concentration. After determining this ratio in each unknown, the antigen concentration was read directly from the standard curve. All samples within an experiment were measured in the same assay.

Reagents	Volume (µl)	
	BK	KLP
I. Standards or BK: 0,5; 1; 2; 4; 8; 16; 32; 64; 128 pg KLP: 5; 10; 20; 40; 80; 160; 320; 640; 1280 pg	50	50
I. Sample: Plasma or	5	5
HMW kininogen plasma digest or	50	-
LMW kininogen plasma digest or	50	-
LMW kininogen urine digest or	150	-
Urine	20	20
RIA buffer (if needed to complete sample volume) 0,1M Tris-acetate buffer, pH 7,4; containing 0,1% Gelafundin	ad 50	ad 50
II. Tracer (5000cpm) ^{125}I-Tyr^{8}-BK ^{125}I-Tyr^{6}-KLP	50	50
III. Antiserum BK: working dilution 1:96000 KAL: working dilution 1:60000	500	500

Tab.2 **Reaction mixtures in BK and KLP RIA.** Gelafundin was used as protective colloid against undesirable adsorption of peptides. All reagents were diluted in RIA buffer. Samples, tracer, aliquots of standard curve, and dialysed antiserum in dilutions 1:100 were stored at -20°C.

3.2.5.4. RADIOIMMUNOASSAY FOR THE AMOUNT OF ENDOTHELIN IN BIOLOGICAL FLUIDS

PREPARATION OF THE TRACER MOLECULE - IODINATION OF ET-1

Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-**Tyr**-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp

Fig.26 **Molecule of ET-1.** Tyr in position 13 allows direct iodination.

ET-1 was iodinated according to a protocol for iodination of kinins with slight modification in the purification steps. After the free $Na^{125}I$ was separated, the cartridge was washed with 20% methanol in water. The labeled ET-1 was then eluted with 60% methanol in water. The assay sensitivity was found to be 50pg ET per tube.

ASSAY PERFORMANCE

ET assay was performed similarly to kinin RIA. Second antibody technique was used for the separation of bound (antigen-antibody complex) and free radioactivity. Commercial antiserum Anti-Endothelin (developed in rabbit) did not distinguish between rat ET-1, ET-2 and ET-3.

Triplicates of reaction mixtures were prepared according to the scheme in Tab.3. After the overnight incubation at 4°C, 2^{nd} antibody Anti-Rabbit IgG (developed in goat) was added. After mixing, test tubes were covered with parafilm and allowed to incubate for another 3h at room temperature. Upon completion the second incubation step, normal rabbit carrier (secondary antibody precipitating system) and 10% PEG 8000 solution was added. The antibody-antigen complex containing labeled and unlabeled ET precipitated. The tubes were properly mixed and centrifuged at 4800rpm for 15min at 4°C. Supernatant was carefully removed from each tube and the amount of radioactivity present in the precipitate was counted in a gamma counter. All samples within an experiment were measured in the same assay.

Reagents	Volume (µl)				
	Tc	B_0	NSB	Standards	Samples
I. Standards ET-1: 0,05, 0,1; 0,2; 0,4; 0,8; 1,6; 3,2; 6,4; 12,8 ng	-	-	-	50	-
Samples plasma / urine	-	-	-	-	50
Na-P Puffer 0,1M sodium phosphate buffer, pH 7,4	-	500	500	450	450
Anti-Endothelin working dilution 1:16 250	-	100	-	100	100
RIA Puffer 0,1M sodium phosphate buffer, pH 7,4 + 0,1% BSA	-	-	100	-	-
Tracer (5000cpm) ^{125}I-ET-1	50	50	50	50	50
II. Anti-Rabbit IgG reconstituted in 0,1M sodium phosphate buffer, pH 7,4	-	100	100	100	100
III. Normal Rabbit Carrier reconstituted in 3% PEG 8000 in 0,1M sodium phosphate buffer, pH 7,4	-	100	100	100	100
10% PEG 8 000 in 0,1M sodium phosphate buffer, pH 7,4	-	1 000	1 000	1 000	1 000

Tab.3 **Reaction mixtures in ET RIA.** BSA was used as protective colloid against undesirable adsorption. Standards, Anti-Endothelin and tracer were diluted in RIA buffer. Samples, tracer, aliquots of standard curve, antibodies and carrier were stored at -20°C. Tc, total count; B_0, maximum binding; NSB, non-specific binding; Na-P Puffer, sodium phosphate buffer; BSA, bovine serum albumin; PEG 8000, polyethylene glycole, MW 8000.

3.2.5.5. RADIOIMMUNOASSAY FOR THE AMOUNT OF DEOXYCORTICOSTERONE, CORTICOSTERONE AND ALDOSTERONE IN BIOLOGICAL FLUIDS AND BRAIN TISSUE

Production of antisera with adequate specificity to measure DOC, corticosterone and aldosterone in presence of similar compounds is usually not achievable, as the steroid derivatives display only small differences in their structure.

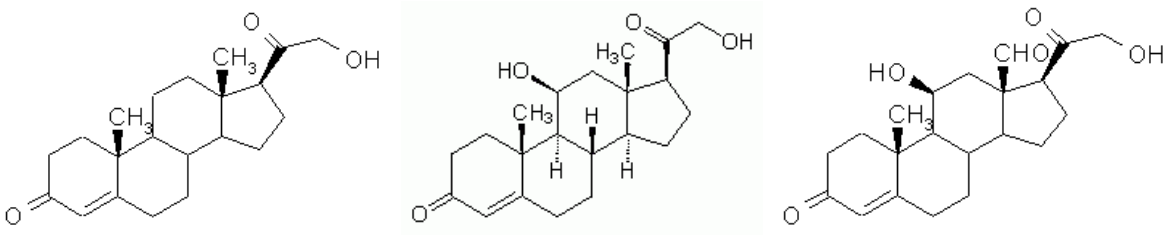


Fig.26 **Structural similarities in corticoid hormones.**

The RIA required several preparatory steps; solvent extraction and chromatographic purification to isolate the compound to be measured from those causing undesired cross-reactions.

SAMPLE PREPARATION

All procedures were performed in glass test tubes to avoid steroids' adsorption to plastic.

A volume of 500µl **plasma / urine** was diluted in 500µl water containing 0,2% ethylene glycole (EGW). 100µl (3000cpm) of each tritiated steroid (^{3}H-DOC, ^{3}H-corticosterone and ^{3}H-aldosterone, generated commercially) were added to the sample before it was subjected to extraction to correct for losses during extraction (recovery). Steroids were then 1h extracted in 3ml 10% chloroform in ethylacetate containing 0,1% triethylamine in slowly rolling tubes. Afterwards, the tubes were left at -20°C for 30min. The liquid organic phase was separated from the frozen lower water phase by decanting. The organic extract was evaporated and properly reconstituted in 1ml 10% ethylacetate in isooctane containing 0,1% triethylamine during 1h shaking.

The **brain** was properly homogenized, two-times, each in 3ml of methanol by means of an Ultra-Turrax disperser. During homogenisation the tube was held on ice. After centrifugation (10min at 3500rpm) the supernatants were collected and 100µl (3000cpm) of each tritiated steroid (^{3}H-DOC, ^{3}H-corticosterone and ^{3}H-aldosterone) were added to calculate recovery. Brain homogenate was then evaporated and reconstituted in 4ml EGW. Steroids were then extracted in 5ml dichloromethane containing 0,1% triethylamine during 30min tubes' rolling. The organic phase was dried and during 30min shaking redissolved in 1ml 10% ethylacetate in isooctane containing 0,1% triethylamine.

PURIFICATION BY PARTITION CHROMATOGRAPHY

DOC, corticosterone and aldosterone were separated in a 600 mg Celite 545 AW column pre-washed with 300µl 80% ethylene glycole/water under vacuum. The organic extracts were slowly passed through the cartridge during which the steroids bound to the matrix. First, **DOC** was eluted with 8ml of 10% ethylacetate in isooctane containing 0,1% triethylamine. Subsequently, **corticosterone** was eluted with 10ml 30% ethylacetate in isooctane containing 0,1% triethylamine. Lastly, **aldosterone** was eluted with 7ml 70% dichloromethane in isooctane containing 0,1% triethylamine. The eluates were evaporated. DOC was reconstituted in 3ml EGW, corticosterone was redissolved in 6ml 5% ethanol/water and aldosterone was resuspended in 1ml EGW. The method recovered approximately 88% of DOC, 60% of corticosterone and 47% of aldosterone.

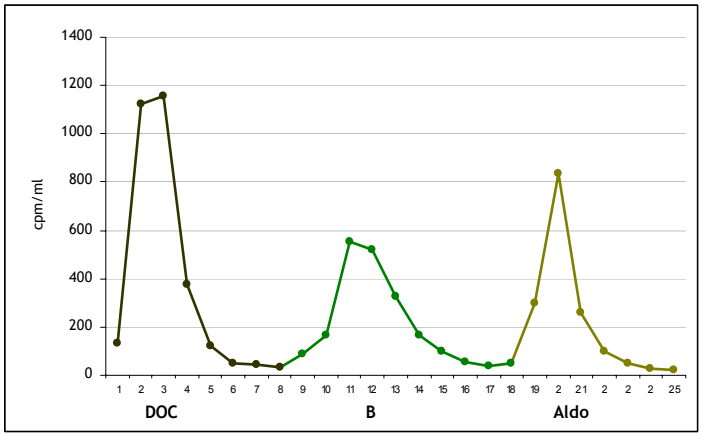


Fig. 27 **Elution profile of DOC, corticosterone and aldosterone from Celite column.**

ASSAY PERFORMANCE

Levels of aldosterone, corticosterone, and deoxycorticosterone were determined in duplicates by RIA using rabbit polyclonal antibodies. The cross-reactivity of DOC antiserum with corticosterone was 9%. Corticosterone antiserum displayed 6,7% cross-reaction with DOC. The cross-reactivity of aldosterone antiserum with corticosterone was determined to be 0,15%. The sensitivity of the RIA for DOC was 1pg / tube; for corticosterone, it was 2,5pg / tube and for aldosterone, it was 1pg / tube.

Reaction mixtures were prepared according to the Tab.4 at room temperature. The tubes were mixed and covered with parafilm. After overnight incubation at 4°C, the charcoal suspension was added (Tab.4). During the tubes were properly shaken by hand, free steroids were adsorbed to charcoal. Following 10min centrifugation at 6000rpm the supernatant with antibody-bound steroids was decanted into a scintillation tube. A scintillation reagent (Tab.4) was added and after mixing, the tubes were counted in a beta-counter. Data were plotted as the ratio of bound activity to bound activity in the zero standard (B/B_0) against the logarithm of concentration. Value of each sample was corrected with respective recovery. The antigen concentration in unknown samples was read from the standard curve. All samples within an experiment were measured in the same assay.

Reagents	Volume (µl)			
	Tc	Standards	Samples	Sample Recovery
Standards DOC, corticosterone, aldosterone (each): 0; 1; 2,5; 5; 10; 25; 50; 100; 250; 500; 1000 pg	-	100	-	-
DOC, aldosterone: EGW **corticosterone:** 5% ethanol/water	100	100	-	-
Samples plasma / urine / brain homogenate	-	-	200	DOC, aldost.: 50 B: 200
Tracer (3000 cpm) ^{3}H-DOC ^{3}H-corticosterone ^{3}H-aldosterone	100	100	100	-
Antiserum DOC: end-dilution 1: 42500 corticosterone: end-dilution 1: 37500 aldosterone: end-dilution 1: 140000	-	200	200	-
Charcoal suspension 10g Norit A, 600mg dextran T70 in 640ml 0,1M borate-KCl buffer, pH 7,4 containing 0,63% Beriglobin	-	100	100	-
Scintillation reagent	2500	2500	2500	2500

Tab.4 **Reaction mixtures in DOC, corticosterone and aldosterone RIA.** Samples were pre-diluted 1:50 for DOC measurement in brain homogenate, 1:10 for corticosterone measurement in plasma and 1:100 for corticosterone measurement in brain homogenate. DOC and aldosterone standards were diluted in EGW. Corticosterone standards were diluted in 5% ethanol/water. Tracers and antibodies were diluted in LBP buffer (0,1% lysozyme in 0,1M borate-KCl buffer, pH 7,4) Standards, tracer and antibody dilution were stored at 4°C. Tc, total count.

3.2.6. ENZYME ASSAYS

3.2.6.1. DETERMINATION OF KALLIKREIN-LIKE ACTIVITY IN PLASMA

The plasma kallikrein-like activity catalyses the splitting of p-nitroaniline (pNA) from the substrate H-D-Pro-Phe-Arg-pNA (S-2302) (Fig.28). The rate of pNA formation, i.e. the increase in absorbance per second at 405nm, is proportional to the enzymatic activity. After stopping the reaction with acetic acid, the absorbance is determined with a photometer. The method for the determination of activity is based on the differences in absorbance between the pNA formed and the original substrate.

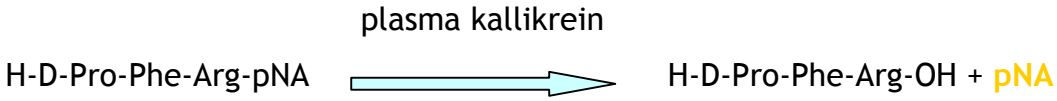


Fig.28 **Principle of measurement of kallikrein-like activity in plasma.**

ASSAY PERFORMANCE

Citrate plasma, stored at -20°C, was thawed on ice. For standard curve, kallikrein from human plasma in concentrations 1,5; 3; 6; 12,5; 25; 50 and 100mU/ml 0,05M Tris - 0,113M NaCl - HCl buffer, pH 7,8 was used.

A sample (standard or citrate plasma in duplicates) volume of 20µl was incubated with 50µl 0,05M Tris - 0,113M NaCl - HCl buffer, pH 7,8 and 20µl S-2302 (25mg dissolved in 20ml sterile water, stored at 4°C) for 10 min at 37°C in a 96-well microplate. The reaction was stopped with 20µl acetic acid, 20%. Plasma blanks were prepared by adding the reagents in reverse order without incubation. The absorbance of the sample was read against its blank in a photometer at 405nm. The kallikrein-like activity in plasma samples was calculated from the standard curve.

3.2.6.2. DETERMINATION OF KALLIKREIN ACTIVITY IN URINE

Kallikrein in urine splits the substrate H-D-Val-Leu-Arg-pNA (S-2266) and the rate of pNA formation increases linearly with increasing of kallikrein (Fig.29). By adding aprotinin (Trasylol®), a potent inhibitor of glandular kallikrein, to the same blank, protease activities not inhibited by aprotinin as well as the colour from the urine itself can be subtracted. After stopping the reaction with acetic acid, the absorbance at 405nm is determined with a photometer.

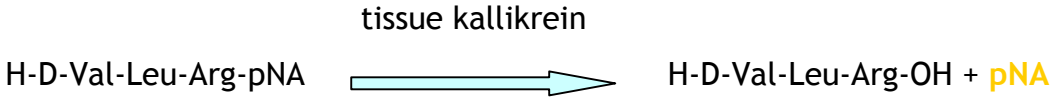


Fig.29 **Principle of measurement of kallikrein activity in urine.**

ASSAY PERFORMANCE

As the kallikrein concentration in urine may vary during the day, the total volume collected during 24h was pooled. After centrifugation, the supernatant was stored at -20°C. Before analysis, the urine was thawed on ice and 1:6 pre-diluted in 0,2M Tris-HCl buffer, pH 8,2. For standard curve, rat tissue kallikrein in concentrations 16, 32, 63, 125, 250, 500 and 1000mU/ml 0,2M Tris-HCl buffer, pH 8,2 was used.

A volume of 40µl sample (standard or urine in duplicates) was incubated with 50µl 0,2M Tris-HCl buffer, pH 8,2 and 20 µl S-2266 (25mg dissolved in 28,8ml sterile water, stored at 4°C) for 30 min at 37°C in a 96-well microplate. The reaction was stopped by acidification with 10 µl acetic acid, 50%. Blanks were prepared by adding 50µl of Trasylol® (aprotinin, 20KIU/ml 0,2M Tris-HCl buffer, pH 8,2) prior to the incubation. The absorbance of the sample was read against its blank in a photometer at 405nm. The activity in samples was read from the standard curve. The activity of kallikrein in urine excreted during 24h was calculated by multiplying with the 24h urine volume.

3.2.6.3. MEASUREMENT OF CREATINE KINASE ACTIVITY IN CORONARY EFFLUENT

Measurement of CK activity was used for assessment of the severity of myocardial damage. Creatine kinase (EC 2.7.3.2.) is located in the heart muscle. Cardiac muscle injury following myocardial infarction results in a rise in CK activity. CK activity was measured in the coronary effluent using a specific kit from Rolf Greiner BioChemica.

CK catalyzes the reaction between creatine phosphate and adenosine diphosphate (ADP), forming creatine and ATP. The ATP formed is utilized to phosphorylate glucose, producing glucose-6-phosphate in the presence of hexokinase. Subsequently, glucose-6-phosphate is oxidised to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide phosphate (NADP). This reaction is catalysed by glucose-6-phosphate dehydrogenase. During this oxidation, an equimolar amount of NADP is reduced to NADPH increasing the absorbance at 340nm (Fig.30). The rate of change in absorbance is directly proportional to CK activity.

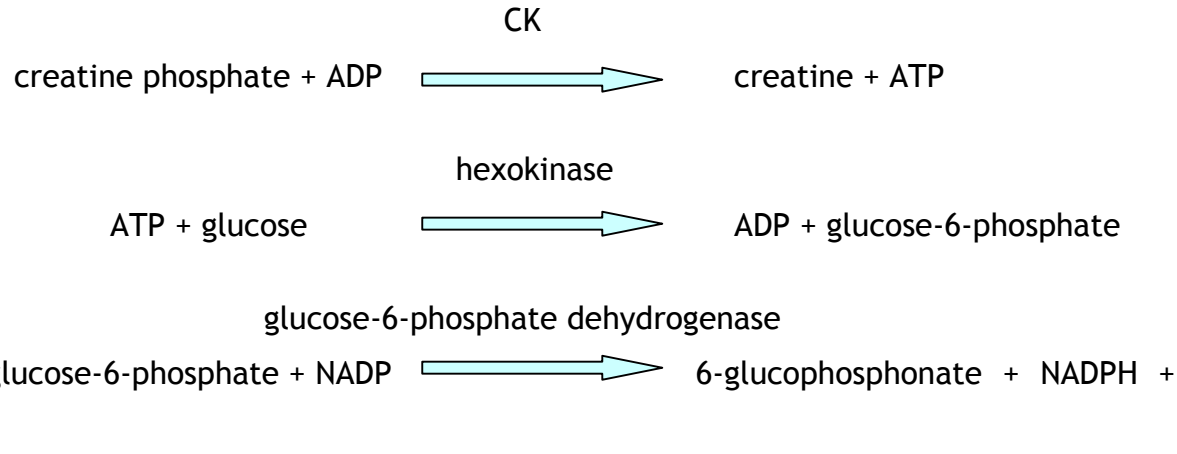


Fig.30 **The enzymatic reactions involved in the assay.**

ASSAY PERFORMANCE

The vial of enzyme/substrate was reconstituted with 3ml assay buffer and was warmed to 30°C. A volume of 100µl of coronary effluent was added to the 96-well quartz microplate and was brought to incubation temperature of 30°C. Afterwards, 100µl of working reagent was added to the effluent and the microplate was pre-

incubated 2min at 30°C in a temperature controlled chamber of the spectrophotometer. Initial absorbance (A) at 340nm was read against water as reference. The incubation continued at 30°C and absorbance was recorded at 30s intervals for a period of 180s to check the linearity of the reaction rate. The final absorbance was read and the ΔA was calculated. The CK activity was calculated by use of millimolar absorptivity of NADPH, which is 6,22 at 340nm. The activity of the sample was calculated according to the formula:

CK (U/l) = (ΔA x total volume of 0,2ml x 1000) / (6,22 x sample volume of 0,1ml)

3.2.7. MOLECULAR BIOLOGY

For expression analysis of 11ß-hydroxylase and aldosterone synthase total RNA was isolated from adrenal gland. mRNA was reverse transcribed into copyDNA (cDNA). With use of specific primers for respective CYP11B1 and CYP11B2 genes, the expression was quantitative determined by means of real-time - polymerase chain reaction (PCR) in LightCycler®.

3.2.7.1. RNA STABILIZATION

After the adrenals were harvested, they were immediately put into pre-chilled 2ml tubes with 0,3ml **RNAlater**. RNAlater is a tissue storage reagent that rapidly permeates tissues and stabilizes and protects cellular RNA. To ensure absolute permeation of RNAlater through the tissue, the samples in RNAlater were first overnight incubated at 4°C and then transferred to -20°C for archival storage. This process efficiently preserves the expression profile of the sample at the time of harvesting to ensure reliable gene-expression analysis.

3.2.7.2. RNA ISOLATION AND PURIFICATION

The total RNA was isolated with the Qiagen **RNeasy Mini Kit**. The samples were first lysed and homogenized. Ethanol was added to the lysate to provide ideal binding conditions. The lysate was then loaded onto the RNeasy silica-gel membrane of a spin column. RNA was selectively adsorbed to the silica-gel membrane in presence of chaotropic salts, which removed water from hydrated molecules in solution and

separated RNA within certain size parameters. Polysaccharids and proteins were not adsorbed and were removed. After a wash step, pure and concentrated RNA was eluted under no-salt condition in a small volume of water.

DISRUPTION AND HOMOGENISATION OF ADRENAL TISSUE

The adrenals in RNAlater were shortly thawed on ice, further steps were quickly performed at room temperature. One adrenal gland, weighing approximately 30mg, was homogenized in 600µl lysis buffer (1%14,3M ß-mercaptoethanol in RLT buffer) in appropriate RNase-free glass tube with an Ultra-Turrax homogeniser. The tissue was 1min thoroughly disrupted and simultaneously homogenized. To achieve complete disruption of cells walls and plasma membranes of cells and organelles, tissue lysates were additionally homogenized using a syringe and needle. The lysates were 10 times passed through a 27-gauge needle, attached to a sterile plastic syringe.

RNA ISOLATION AND PURIFICATION

The lysate was centrifuged for 3min at maximum speed and the supernatant was mixed with 600µl 70% ethanol. Sample aliquots were subsequently applied to an RNeasy mini column placed in a 2ml collection tube and were shortly centrifuged. The flow-through was discarded after each centrifugation step. The column was washed with 700µl RW1 buffer, shortly centrifuged and the flow-through was discarded. The column was placed on a new 2ml collection tube and was washed twice with 500µl RPE buffer. After centrifugation, the flow-through was discarded. The RNeasy silica-gel membrane was dried from residual ethanol by 2min centrifugation. The tube was transferred to a new 1,5ml collection tube. A volume of 70µl RNase-free water was pipetted directly on the membrane and RNA was eluted by 1min centrifugation at 10000rpm.

RNA QUANTIFICATION AND QUALITY CONTROL

The concentration of RNA was determined by measuring the absorbance at 260nm (A_{260}) in NanoDrop® spectrophotometer using 1µl of RNA solution. Water was used

to zero the spectrophotometer. An absorbance of 1U at 260nm corresponds to 40µg of RNA per ml. The concentration of RNA sample was calculated according to the formula:

$$RNA\ (ng/\mu l) = 40 \times A_{260}.$$

Three readings were performed for each sample and an average RNA concentration was calculated. The RNA concentrations ranged from approximately 600 - 1100ng/µl. The yield of the isolation from 30mg adrenal tissue was about 40 - 80µg total RNA.

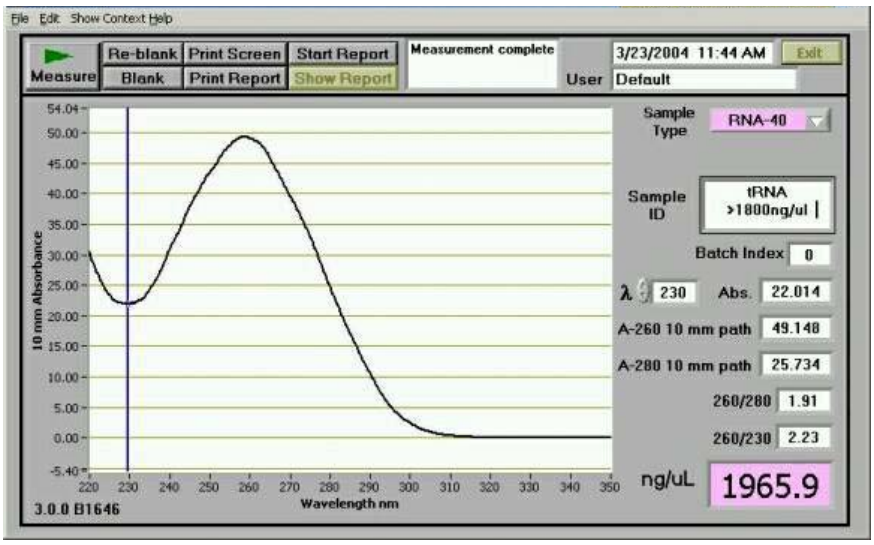


Fig.31 **An example of RNA analysis with NanoDrop®.** A secondary measure of RNA purity in 260/230 ratio indicate absence of co-purified contaminants.

The ratio of absorbance at 260 and 280nm was used to assess the protein contamination in the RNA preparation. The values were usually about 2,15 indicating high purity of RNA preparation.

STORAGE OF RNA

Purified RNA was stored at -70°C in water until further downstream application.

3.2.7.3. cDNA SYNTHESIS

cDNA is a single-stranded DNA copy synthesized from mRNA. The mRNA composes approximately 1 - 5% of the total RNA preparation. The enzyme used is reverse transcriptase, an RNA-dependent DNA polymerase isolated from a retrovirus. As with other polymerases a short double-stranded sequence is needed at the 3' end of the mRNA which acts as a start point for the polymerase. This is provided by the poly(A) tail found at the 3' end of mRNA to which a short complementary synthetic oligonucleotide (oligo dT primer) is hybridized. Together with all 4 deoxynucleotide triphosphates (random primers), magnesium ions and at neutral pH, the reverse transcriptase synthesises a single-stranded complementary DNA on the mRNA template (Fig.32).

Qiagen **QuantiTect Reverse Transcription Kit** was used for the cDNA synthesis with integrated removal of genomic DNA (gDNA) contamination for use in real-time, two step reverse transcription (RT) - PCR. Quantiscript Reverse Transcriptase is a novel blend of Omniscript and Sensiscript Reverse Transcriptases.

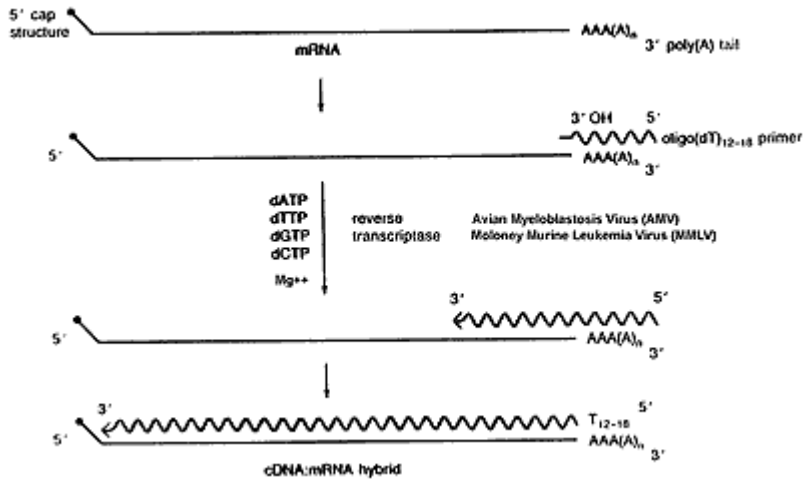


Fig.32 **Synthesis of the first strand of cDNA using an oligo(dT) primer and reverse transcriptase.**

ELIMINATION OF GENOMIC DNA AND REVERSE TRANSCRIPTION

To obtain accurate results in downstream quantitative real-time RT-PCR gene expression assays, it is important that only cDNA is amplified and detected. It is essential that the starting RNA sample is free of gDNA.

Reverse transcription was performed with Quantiscript Reverse Transcriptase, Quantiscript Reverse Transcription Buffer, and Reverse Transcription Primer Mix. The RT Primer Mix contained a mix of oligo-dT and random primers that enabled full-length cDNA synthesis from all regions of RNA transcripts, even from 5' regions. For *in vitro* reverse transcription, two activities of reverse transcriptase were utilized to produce single-stranded cDNA. First, the RNA-dependent DNA-polymerase activity transcribed cDNA from an RNA template (reverse transcription) and second, RNase H activity specifically degraded only the RNA in RNA-DNA hybrids.

PERFORMANCE

The template RNA and kit reagents were thawed on ice. All reactions were set up on ice. The volume of 2µg RNA was calculated according to the respective RNA concentration in each sample.

Initially gDNA was eliminated by 2min incubation of 2µl of gDNA Wipeout Buffer, 2µg RNA and RNase-free water, ad 14µl total volume at 37°C. Afterwards, the tubes were immediately placed on ice. The RNA sample was then used directly in reverse transcription.

14µl of template RNA were added to the tube containing the master mix prepared from 1µl of Quantiscript Reverse Transcriptase, 4µl of Quantiscript Reverse Transcription Buffer, and 1µl of Reverse Transcription Primer Mix. The tube was mixed and incubated for 15min at 37°C. Finally, the Quantiscript Reverse Transcriptase was inactivated at 95°C for 3min.

Synthesized cDNA was stored at -20°C until PCR.

3.2.7.4. AMPLIFICATION OF cDNA - POLYMERASE CHAIN REACTION

The PCR is an *in vitro* method for enzymatically synthesizing defined sequences of DNA (Fig.33). The reaction uses two oligonucleotide primers that hybridise to opposite strands and flank the target sequence that is to be amplified. The elongation of the primers is catalyzed by a heat-stable DNA polymerase. A repetitive series of cycles involving template denaturation, primer annealing, and extension of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment (Mullis et al., 1986).

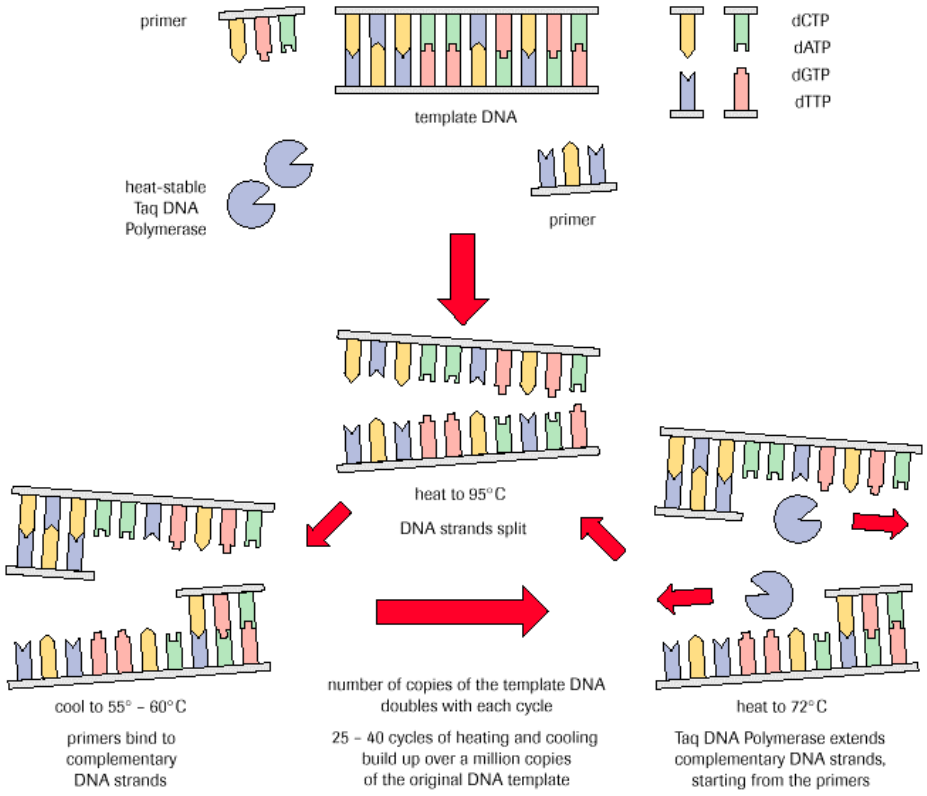


Fig. 33 **Schematic diagram of PCR** (Roche).

RNA cannot serve as a template for PCR, so it must be first reverse transcribed into cDNA (e.g. with reverse transcriptase from Moloney murine leukaemia virus or avian myeloblastosis virus). In 1987 Powell et. al., first described a combined technique, commonly known as RT-PCR, in which reverse transcription is coupled with PCR amplification of the resulting cDNA.

cDNA QUALITY CONTROL - PBGD PCR

The quality of synthesized cDNA was tested in a PCR with primers for a rat gene for porphobilinogen deaminase (**PBGD**); genebank accession number X06827. PBGD is a housekeeping gene with constant expression (Technical Notes No. LC 15/2002, Roche).

The primers for PBGD were designed with GeneFisher software. Lyophilized primers (synthesized commercially) were dissolved in RNase-free water to final concentration of 100µM (stock solution). Aliquots of 5µM working solution were prepared and both were stored at -20°C. Working solutions of dNTPs (100µl mixture of 125mM of each nucleotide) and 25mM $MgCl_2$ were also stored at -20°C. Mg^{2+} ions form soluble complexes with dNTPs and template DNA to produce the actual substrate that the polymerase recognizes.

Reagent	Volume (µl)	Final concentration
10x PCR reaction buffer 100mM Tris-HCl; pH 8,3; 500mM KCl	5	1x
dNTP mix, 12,5mM each dTTP, dGTP, dATP, dCTP in water	2	50µM each
$MgCl_2$, 25mM	6	3mM
PBGD reverse primer (5' to 3'), 5µM GCA ACA CAC CCA CTA GGT CCA AG, position 720 - 688	1	0,1µM
PBGD forward primer (5' to 3'), 5µM GGA GTT CAG TGC CAT TAT CCT GGC, position 548 - 571	1	0,1µM
Template cDNA 1:10 dilution in water	3	-
Taq DNA polymerase	0,5	0,05U/µl
H_2O	31,5	-
Total volume	50	

Tab.5 **Pipetting scheme for 1 reaction in PBGD PCR.** Reagents were pipetted into thin-walled PCR tubes, gently vortexed and briefly centrifuged. The samples were placed into thermal cycler and the program was started.

	Temperature	Time	Cycle number
Initial denaturation	94°C	4min	1
Denaturation	94°C	45s	40
Primer annealing	60°C	45s	
Elongation	72°C	45s	
Final elongation	72°C	4min	1
Cooling	4°C	for ever	1

Tab.6 **Temperature and time profile for PBGD PCR.**

The PCR results were analysed on 1% agarose gel using 1x TAE (40mM Tris-acetate, 1mM EDTA, pH 8,0) as running buffer. The gel was stained with ethidium bromide and the PCR products were estimated by comparing the product size with that of the DNA molecular weight marker (Fig.34).

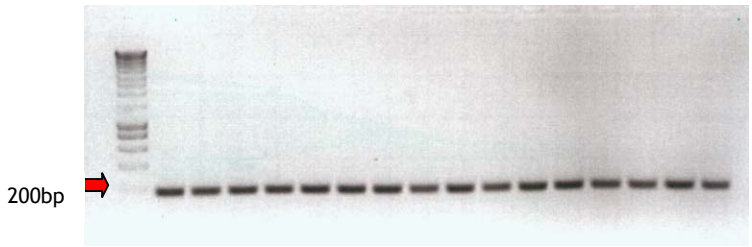


Fig.34 **An example of PBGD PCR with cDNA samples from adrenal glands of few BN rats.** PBGD PCR product size – 172bp.

Detection of Genes Coding for 11ß-Hydroxylase and Aldosterone Synthase - CYP11B1 and CYP11B2 PCR

The primers specific for rat **CYP11B1** and **CYP11B2** genes (genebank accession numbers - D14091 and D14097) were synthesized according to the sequence published by Gomez-Sanchez in 2004 (Tab.7). Lyophilised primers were dissolved in RNase-free water to final concentration of 100µM. Aliquots of 5µM working solution were prepared and stored at -20°C. Primers were tested in PCR performed equally to that of PBGD.

Gene	Primer (5' to3')		PCR product size
	Reverse	Forward	
CYP11B1	GAT GGC ATC CAT TGA CAG AGT A	AAG AAC ACT TTG ATT CCT GGG ATA	148bp
position	909 - 888	761 - 784	
CYP11B2	AGT CAA GCT TCT GGG TAA GAA CAG	TAT AGA AGC CAG CA ACTT TGC AC	147bp
position	712 - 735	588 - 610	

Tab.7 **Sequences of primers for 11ß-hydroxylase and aldosterone synthase** (Gomez-Sanchez et al., 2004).

3.2.7.5. QUANTITATIVE REAL-TIME PCR WITH LIGHTCYCLER®

The real-time PCR is based on simultaneous amplification and detection of specific nucleic acid sequences via fluorescence-detecting thermocyclers.

The **LightCycler® System** combines two instruments in one: a PCR thermal cycler with high speed cycling capabilities and an integrated fluorescence detection device that allows fluorescence monitoring either continuously or once per cycle, as well as on-line computer analysis of results. The instrument offers a broad dynamic range and superior sensitivity. The LightCycler® instrument can detect from 10 to 10^{10} copies in a single run. Detection sensitivity using SYBR Green I dye in 2-step RT-PCR is 0,1pg total RNA.

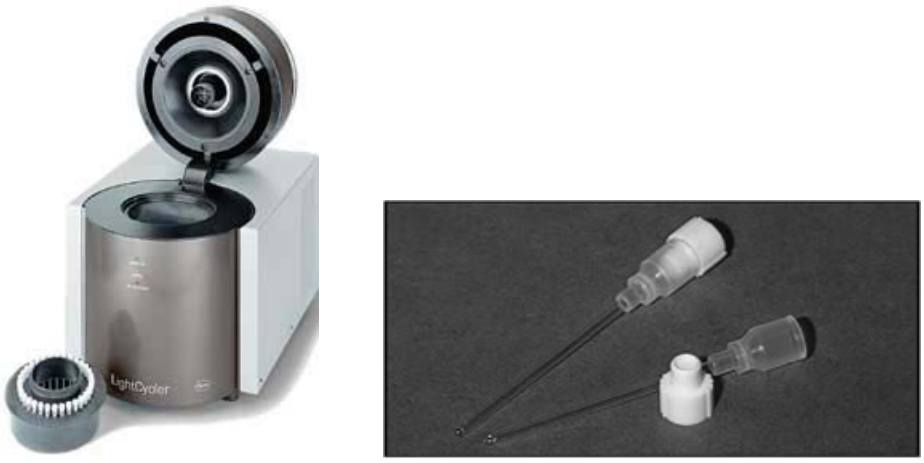


Fig.35 LightCycler® Instrument with sample carousel. PCR occurs in specially designed borosilicate glass capillaries (Roche).

The PCR is monitored with the SYBR Green I DNA binding dye. The SYBR Green I dye emits a fluorescence signal at 530nm when bound to double-stranded DNA (dsDNA) (Fig.36). Fluorescence emission is measured at the end of each elongation phase throughout a PCR. The fluorescence signal is proportional to the increasing amount of dsDNA in the sample.

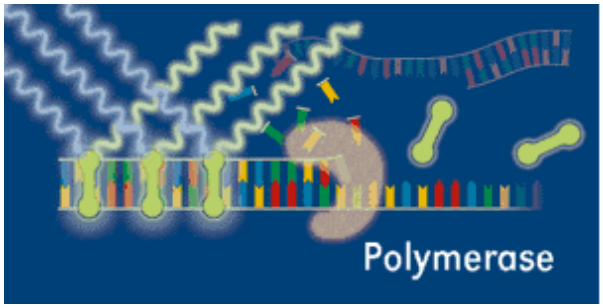
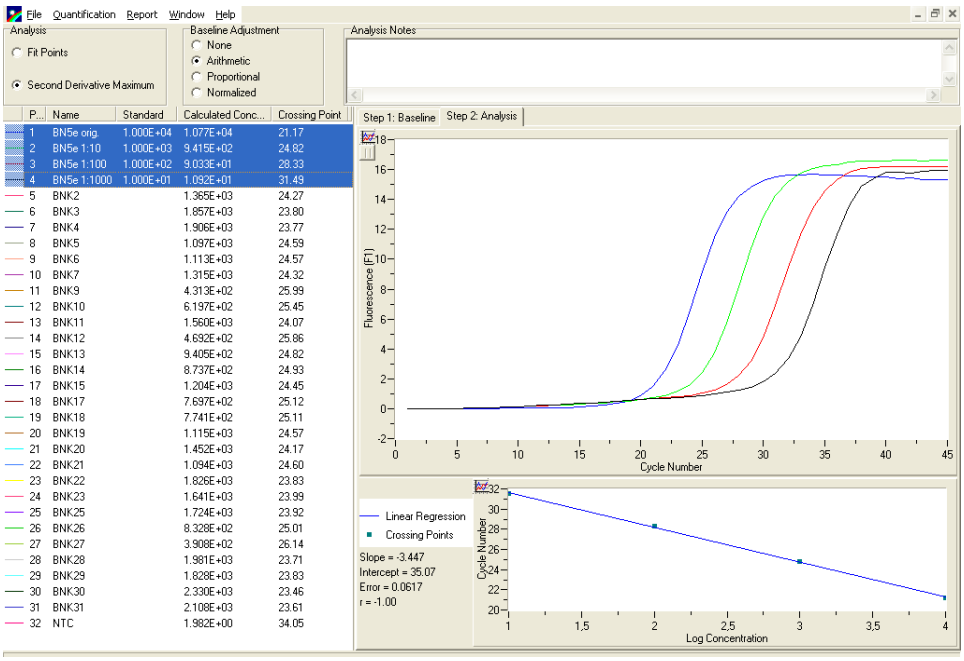


Fig.36 **SYBR Green I** (Roche).

QUANTIFICATION



P...	Name	Standard	Calculated Conc...	Crossing Point
1	BN5e orig.	1.000E+04	1.077E+04	21.17
2	BN5e 1:10	1.000E+03	9.415E+02	24.82
3	BN5e 1:100	1.000E+02	9.033E+01	28.33
4	BN5e 1:1000	1.000E+01	1.092E+01	31.49
5	BNK2		1.365E+03	24.27
6	BNK3		1.857E+03	23.80
7	BNK4		1.906E+03	23.77
8	BNK5		1.097E+03	24.59
9	BNK6		1.113E+03	24.57
10	BNK7		1.315E+03	24.32
11	BNK9		4.313E+02	25.99
12	BNK10		6.197E+02	25.45
13	BNK11		1.560E+03	24.07
14	BNK12		4.692E+02	25.86
15	BNK13		9.405E+02	24.82
16	BNK14		8.737E+02	24.93
17	BNK15		1.204E+03	24.45
18	BNK17		7.697E+02	25.12
19	BNK18		7.741E+02	25.11
20	BNK19		1.115E+03	24.57
21	BNK20		1.452E+03	24.17
22	BNK21		1.094E+03	24.60
23	BNK22		1.826E+03	23.83
24	BNK23		1.641E+03	23.99
25	BNK25		1.724E+03	23.92
26	BNK26		8.328E+02	25.01
27	BNK27		3.908E+02	26.14
28	BNK28		1.981E+03	23.71
29	BNK29		1.828E+03	23.83
30	BNK30		2.330E+03	23.46
31	BNK31		2.108E+03	23.61
32	NTC		1.982E+00	34.05

Fig.37 **Quantification of PBGD in rat adrenal cDNA.** Standard curve (highlighted) was performed with different starting amount of one rat adrenal cDNA. Crossing points (cycle numbers) were plotted against the logarithmic concentration of the standard (lower panel). Left panel displays crossing points of log-linear correlations with the baseline and calculated concentrations of the samples.

The amount of PCR product increases logarithmically in the first few PCR cycles. Identifying the first cycle of the PCR run, in which the log-linear signal can be distinguished from the background, makes it possible to quantify the initial target concentration. After completion of PCR, the LightCycler® software sets a baseline x-axis that intersects these cycles. The x-axis **crossing point** (number of cycle) of each standard is determined and plotted against the logarithm of concentration to produce a standard curve. The concentrations of target sequence in samples are extrapolated from the standard curve.

MELTING CURVE ANALYSIS

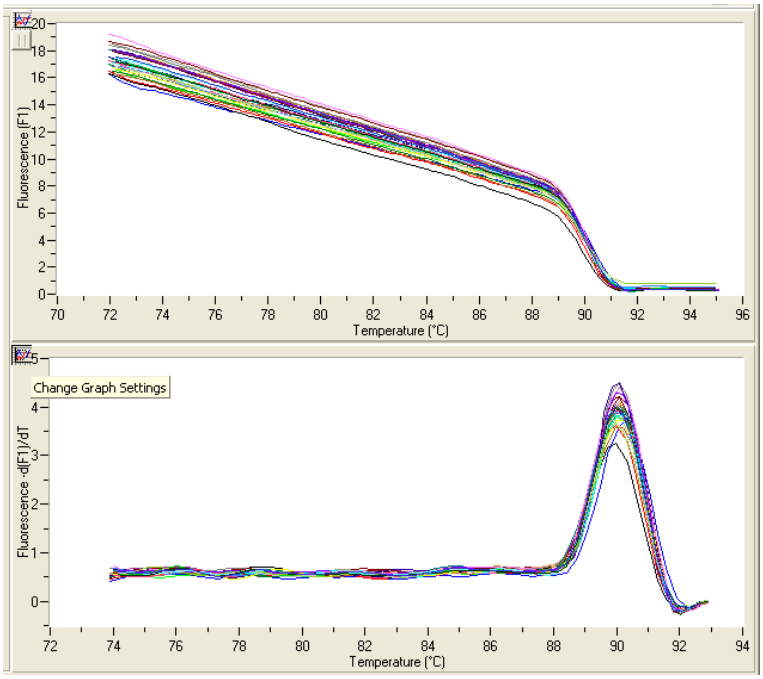


Fig.38 **Melting curve analysis of PBGD PCR product.** The melting temperature of this fragment is visualizes by taking the first negative derivative (-dF/dT) of the melting curve. The turning point of the melting curve results in a peak which permits easy identification of the fragment-specific T_m.

Each dsDNA product has its own specific **melting temperature** (T_m), which is defined as the temperature at which 50% of the DNA becomes single stranded and depends on its sequence, length and GC content. Determination of T_m of PCR products allows confirmation of the PCR product identity and differentiation of specific PCR product from non-specific ones, such as primer-dimers.

At the end of the PCR, the temperature in the thermal chamber is slowly raised. During this process the fluorescence in each tube is measured in continuous mode. As soon as the dsDNA starts to denature, the SYBR Green I dye is released, resulting in a decrease of fluorescence.

RELATIVE QUANTIFICATION OF mRNA EXPRESSION LEVELS

Each cDNA sample may exhibits an individual variation, caused by e.g. tissue harvesting, total RNA isolation and reverse transcription that influence the final absolute quantification of the target gene. Compensation of sample to sample variation is achieved by the relative quantification. Target concentration is expressed relative to the concentration of a reference (housekeeping gene) in the same sample omitting the need for a standard with known concentrations.

Relative quantification normalises the expression of the target gene by an endogenous non-regulated reference gene expression, derived from housekeeping genes. The Relative Expression Software Tool - 384 (**REST©**) software computed an expression ratio, based on the PCR efficiencies (E) and the crossing point deviation (ΔCP) of a group of unknown samples versus a control group according to the following formula:

$$\text{Ratio} = \frac{(E_{target})^{\Delta CP_{target}\,(\text{mean control} - \text{mean sample})}}{(E_{reference})^{\Delta CP_{reference}\,(\text{mean control} - \text{mean sample})}}$$

The PCR efficiencies were determined from the slope of the standard curve given in the LightCycler® 3.5 software. The efficiency was calculated according to the equation:

$$E = 10^{(-1/\text{slope})}.$$

The theoretical maximum and optimum efficiency is 2 (each template is duplicated) which correspond to a slope of -3,32. Investigated transcripts showed PCR efficiency rates for PBGD (E=1,89), CYP11B1 (E=1,89) and CYP11B2 (E=2,03). Subsequently, differences in expression between control and treated samples were tested for significance by a randomisation test.

PERFORMANCE OF REAL-TIME PCR IN LIGHTCYCLER®

Conditions for real-time PCRs were optimised in a gradient cycler with regard to primer concentrations, $MgCl_2$ concentrations (2,5 - 4,0mM) and various annealing temperatures (55-65°C). PCR products were separated on a 1% agarose gel electrophoresis visualized with ethidium bromide. Optimised conditions were transferred to the following LightCycler® real-time PCR protocol.

Reagents	PBGD	CYP11B1	CYP11B2
H_2O PCR grade	8,8µl	11,2µl	9,6µl
$MgCl_2$ 25mM	3,2µl (5mM)	2,4µl (4mM)	2,4µl (4mM)
Reverse primer 5µM	2µl (0,5µM)	1,2µl (0,3µM)	2µl (0,5µM)
Forward primer 5µM	2µl (0,5µM)	1,2µl (0,3µM)	2µl (0,5µM)
FastStart DNA Master SYBR Green I	2µl	2µl	2µl
cDNA template	2µl	2µl	2µl

Tab.8 **Reaction mixtures for real-time PCR** (volumes per reaction). LightCycler® FastStart Master SYBR Green I was prepared by pipetting LightCycler® FastStart Enzyme to LightCycler® FastStart Reaction Mix SYBR Green I. It contained FastStart Taq DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of sTTP), SYBR Green I dye, and 10mM $MgCl_2$.

A master mix was prepared from all reagents except of the cDNA template according to the Tab.8. A volume of 18µl of master mix was filled in the glass capillaries and 2µl of cDNA were added as PCR template. Adrenal cDNA from BN control rat was used for standards. Four sequential 10-fold cDNA dilutions were used to construct a standard curve. cDNA from unknown samples was used in 1:10 dilution. A negative control (without cDNA template) was included in each run. Capillaries were closed, centrifuged and placed into a carousel in LightCycler. A four-step experimental run protocol was used as described in Tab.9. A single fluorescence measurement was performed at the end of each elongation phase. Specificity of PCR products was documented with melting curve by continuous fluorescence measurement. Analysis

resulted in specific T_m of 90°C for PBGD (Fig.37), 84°C for CYP11B1 (Fig.38) and 86°C for CYP11B2 PCR product. No primer-dimer formation was generated during any of the PCR.

	PBGD		CYP11B1		CYP11B2	
	Temperature (°C)	Time (s)	Temperature (°C)	Time (s)	Temperature (°C)	Time (s)
Denaturation	95	600	95	600	95	600
Amplification						
Denaturation	95	10	95	10	95	10
Annealing	69	10	60	5	60	10
Elongation	72	7	72	6	72	6
Add. segment	-	-	83	1	-	-
Melting curve						
Segment 1	95	0	95	0	95	0
Segment 2	72	15	65	15	65	15
Segment 3	95	0	95	0	95	0
Cooling	40	30	40	30	40	30

Tab.9 **LightCycler experimental protocols for PBGD, CYP11B1 and CYP11B2 PCR.**

Melting curve analysis revealed non-specific CYP11B1 PCR product. To improve SYBR Green I quantification, a fluorescence measurement at higher temperature at the end of additional fourth segment was performed. The non-specific PCR products were melted before the fluorescence signal was detected which ensured accurate quantification of the desired PBGD PCR product (Fig.39).

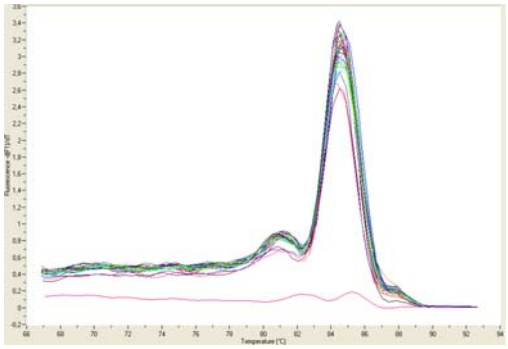


Fig.39 **Melting curve analysis of CYP11B1 PCR product.** A non-specific PCR product with T_m of 81°C was detected.

For the data evaluation we used the recently established REST© software that computed an expression ratio based on a real-time PCR efficiency and the crossing point deviation of an unknown sample versus a control (Pfaffl et al., 2002). For CP determination, the second derivative maximum method was performed using the LightCycler 3.5 software. The relative expression of CYP11B1 and CYP11B2 was normalized by the expression of the housekeeping gene PBGD.

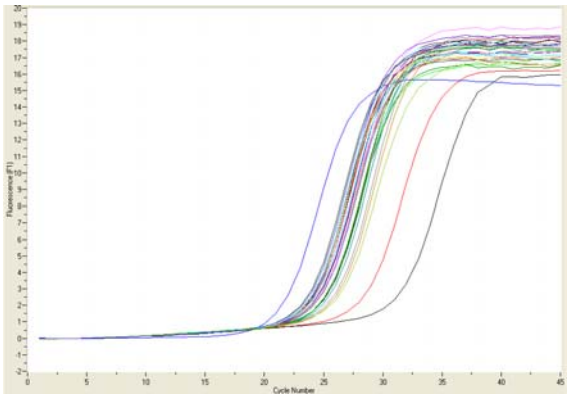


Fig.40 **Detection of PBGD PCR product amplified form rat adrenal cDNA in LightCycler.** The samples yielded crossing points a in the range of 23-26 cycles, confirming the constant expression of a housekeeping gene.

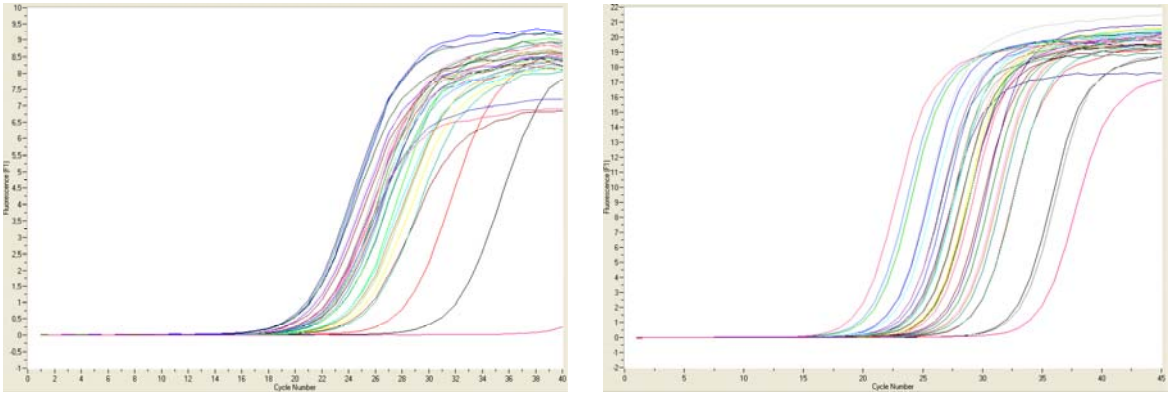


Fig.41 **Detection of CYP11B1 (left) and CYP11B2 (right) PCR products amplified form rat adrenal cDNA in LightCycler.** Most samples yielded crossing points a in the range of 20-25 cycles in CYP11B1 PCR and 19-30 in CYP11B2 PCR.

3.2.8. RESULTS EXPRESSION AND DATA ANALYSIS

The effect of treatment and differences between BN control rats and kininogen-deficient BNK rats were analysed with the use of SigmaStat 2.03 statistical software except of the LightCycler data that were analysed with REST© software, as mentioned above.

Statistical significance of differences in groups of BN and BNK rats was determined with either a one way analysis of variance (ANOVA), if normality test passed or a Kruskal-Wallis ANOVA on ranks, if the normality test failed. All Pairwise Multiple Comparison Procedures within one strain were determined with Dunn's Method after a significant difference was identified with Kruskal-Wallis ANOVA on ranks. All Pairwise Multiple Comparison Procedures within one strain were performed with the use of Tukey Test after a significant difference was identified by an ANOVA. Student's t-test was used to compare mean values of groups between both strains if normality test passed. Mann-Whitney Rank Sum Test was used to compare groups between both strains if normality test failed. Pearson's correlation test was used to determine correlations between experimental groups. Graphic presentation was performed either with Statistica, SigmaPlot 8.0 or Microsoft Office Excel 2003 software. Data are presented as mean ± SEM or as median and quartiles according to the distribution of values which was evaluated in normality test. Probability values (p) smaller than 0,05 were considered to be statistically significant.

4. RESULTS

The experiments were carried out with BN control rats and kininogen-deficient BNK rats. The animals received standard or high salt diet in presence or absence of spironolactone (20mg/day s.c.) for 10 days.

General animal characteristics are described in chapter 4.1., followed by hemodynamic data (4.2.). The components of KKS, ET and aldosterone with its precursors are defined in plasma (4.3.) and urine (4.4). The steroids are characterized also in brain tissue. The expression of two steroidogenic enzymes in adrenal glands is described in chapter 4.5. Basic cardiac parameters investigated during experiments of IPC in Langendorff heart perfusion are presented in chapter 4.6.

Generally, first the effects of treatment (standard diet ± spironolactone, high salt diet ± spironolactone) in each strain are shown in separated diagrams followed by graphic comparison of both strains.

4.1. ANIMAL CHARACTERISTICS

The rats used in the experiment were described by general physiological characteristics. Data of body weight, gain or loss of body weight, food and salt consumption, and water balance were obtained in experiments with metabolic cages. In addition, the final weight of the heart and left kidney is reported.

4.1.1. BODY WEIGHT

The effect of treatment on final body weight was investigated. The initial body weight was identical within each strain. Reported values represent final body weight determined in the last day of the experiment. The rats were weighed before sacrifice.

rat strain	Treatment			
	normal salt (NS)	normal salt & spironolactone (NS SPI)	high salt (HS)	high salt & spironolactone (HS SPI)
BN	297 ± 2,95	303 ± 6,01	296 ± 3,64	293 ± 6,11
BNK	354 ± 8,31	358 ± 11,6	370 ± 6,57	365 ± 9,29

Tab.10 **Body weight** (g) in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=14 in all BN groups and BNK HS, n=20 in BNK NS, and n=11 in BNK NS SPI and BNK HS SPI.

Tab.10 shows the final body weight of rats used in each experimental group. In both strains, neither high salt diet nor treatment with spironolactone influenced the body weight. Experiments were conducted with BN rats, weighing about 300g, and BNK rats, weighing approximately 350g. Because of this relative different initial body weights, groups of BN and BNK rats were not compared.

4.1.2. CHANGE IN BODY WEIGHT

The effect of ten days treatment on body weight gain or loss (ΔBW) in BN and BNK rats is shown in Fig.42. The rats were weighed in the beginning of the experiment and after ten days of treatment and/or diet.

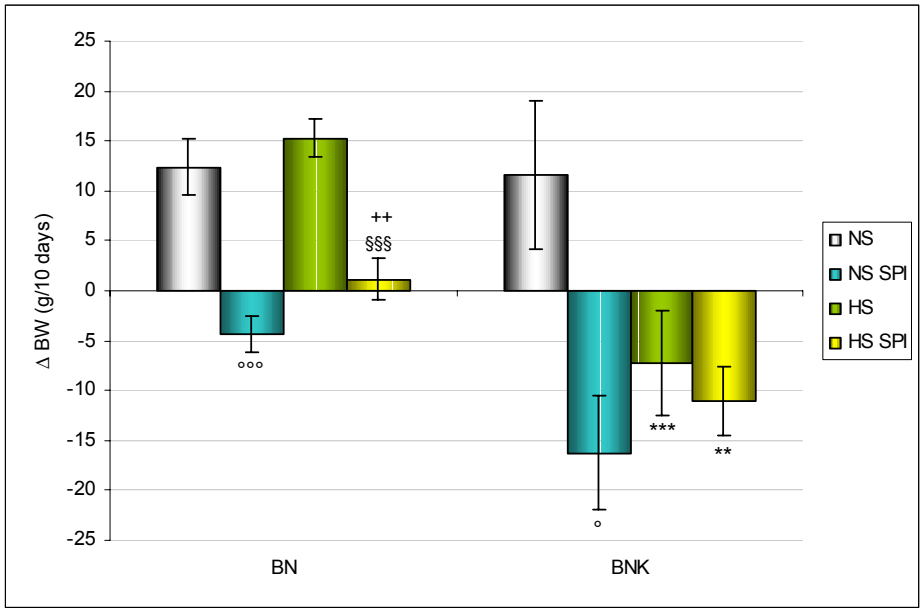


Fig.42 **Changes in body weight** in BN control rats and kininogen-deficient BNK rats after ten days of normal or high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=14. **** p<0,01; *** p<0,001 BNK rats vs. BN rats**; ° p<0,05; °°° p<0,001 NS SPI vs. NS in rats of the same strain; $^{++}$ p<0,01 HS SPI vs. NS in rats of the same strain; §§§ p<0,001 HS SPI vs. HS in rats of the same strain.

Under standard diet all rats gained weight of about 12g (Fig.42). Following ten days of high salt diet, BN control rats gained weight of approximately 15g. On the contrary, kininogen-deficient BNK rats lost weight of about 7g. This effect could be explained neither by the loss of appetite nor by the loss of body fluids. Food intake did not change in any group (data not shown) and water balance appeared identical in both strains (Fig.43c and Tab.11).

The treatment with spironolactone significantly reduced body weight in all groups, independently of salt diet. This effect was possibly derived from the diuretic activity of spironolactone. In BN rats, spironolactone did not influence salt appetite. In BNK rats, spironolactone reduced food intake (data not shown), which contributed to the greater body weight loss.

4.1.3. URINE VOLUME

The effect of high salt diet and spironolactone on daily urine production of BN and BNK rats was investigated. Rats were placed into separate metabolic cages. After 24h, the volume of collected urine was registered.

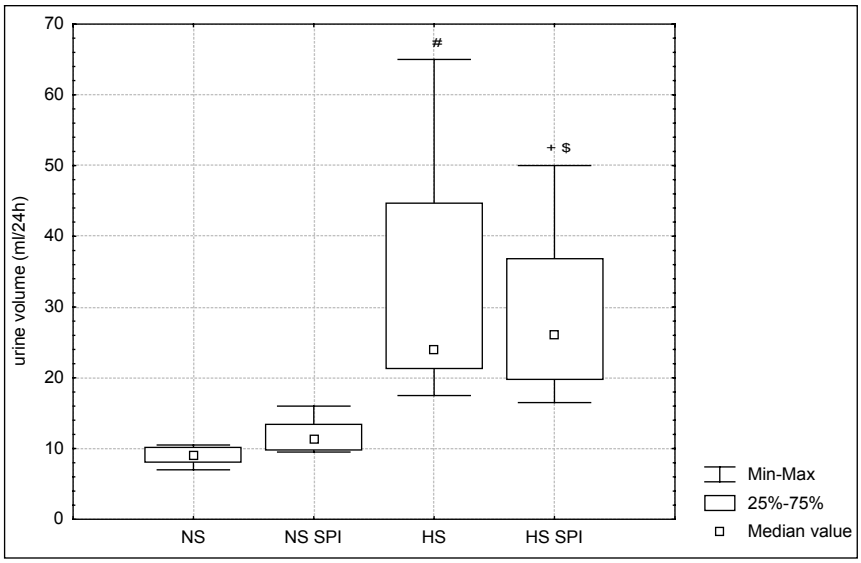


Fig.43a **Urine volume** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. # p<0,05 HS vs. NS; + p<0,05 HS SPI vs. NS; $ p<0,05 HS SPI vs. NS SPI.

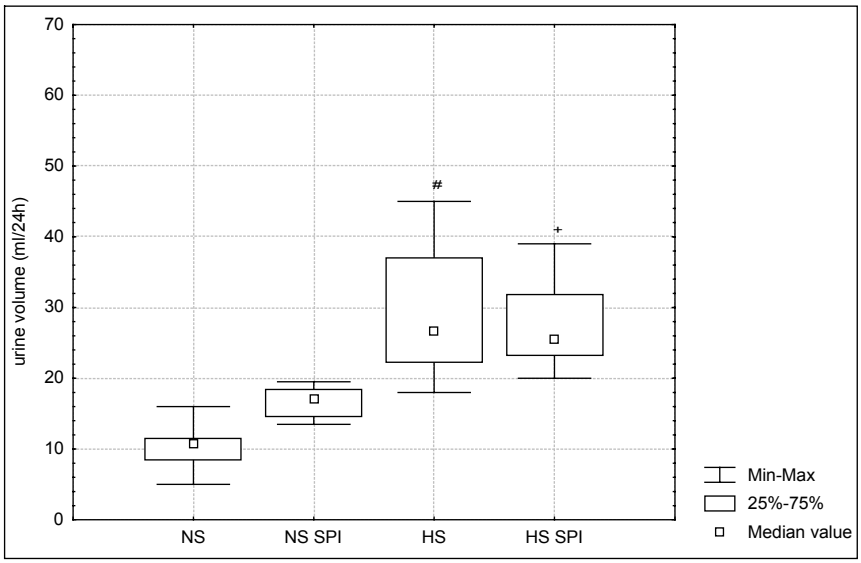


Fig.43b **Urine volume** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. # p<0,05 HS vs. NS; + p<0,05 HS SPI vs. NS.

COMPARISON OF BN AND BNK RATS

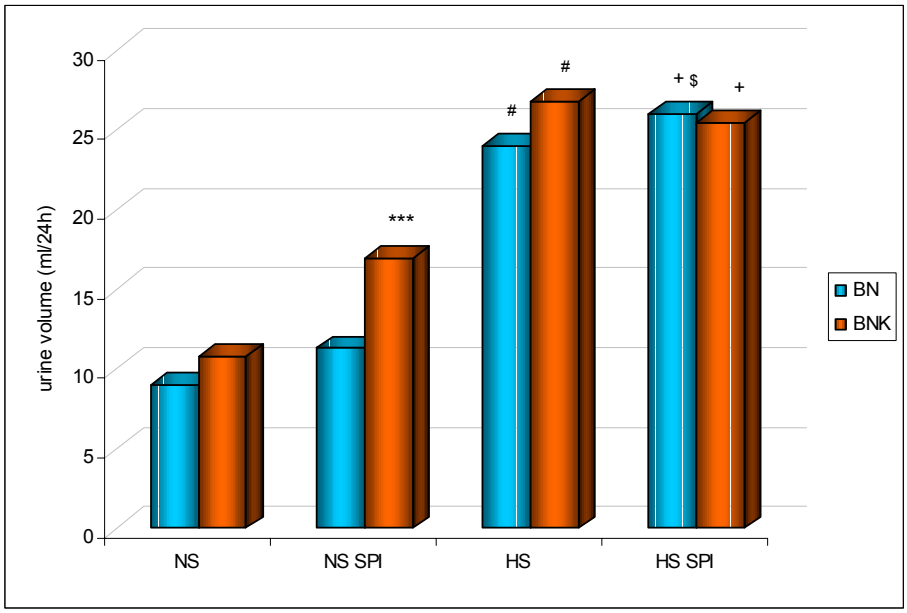


Fig.43c **Urine volume** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the median, n=8 in all groups except of BNK NS, where n=14. ***** p<0,001 BNK rats vs. BN rats**; # p<0,05 HS vs. NS in rats of the same strain; + p<0,05 HS SPI vs. NS in rats of the same strain ; $ p<0,05 HS SPI vs. NS SPI in rats of the same strain.

High salt diet increased thirst and water intake that consequently caused an increase in urine production in both strains (Fig.43c). The diuretic effect of spironolactone was more effective in BNK rats than in BN rats fed with standard diet. This effect could have contributed to the larger decline of body weight in BNK rats (Fig.42). Spironolactone could not exert further diuretic effects in rats fed with high salt diet. The antagonising effect of spironolactone was attenuated due to minimal aldosterone levels (Fig.65c).

4.1.4. WATER INTAKE

The effect of treatment on daily water intake in BN and BNK rats is shown in Tab.11. Animals stayed 24h in individual metabolic cages. At the end, water consumption was registered.

rat strain	Treatment			
	normal salt (NS)	normal salt & spironolactone (NS SPI)	high salt (HS)	high salt & spironolactone (HS SPI)
BN	5,5	5,5	5,5	10,0
BNK	16,5	11,5	7,5	5,5

Tab.11 **Water intake** (ml/24h) in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the median, n=8 in all groups except of BNK NS, where n=14.

Water intake displayed no significant changes, possibly due to the relatively large variances in single rats.

4.1.5. HEART WEIGHT

The effect of high salt diet and spironolactone on heart weight of BN and BNK rats was investigated. The hearts were weighed after the termination of the heart perfusion.

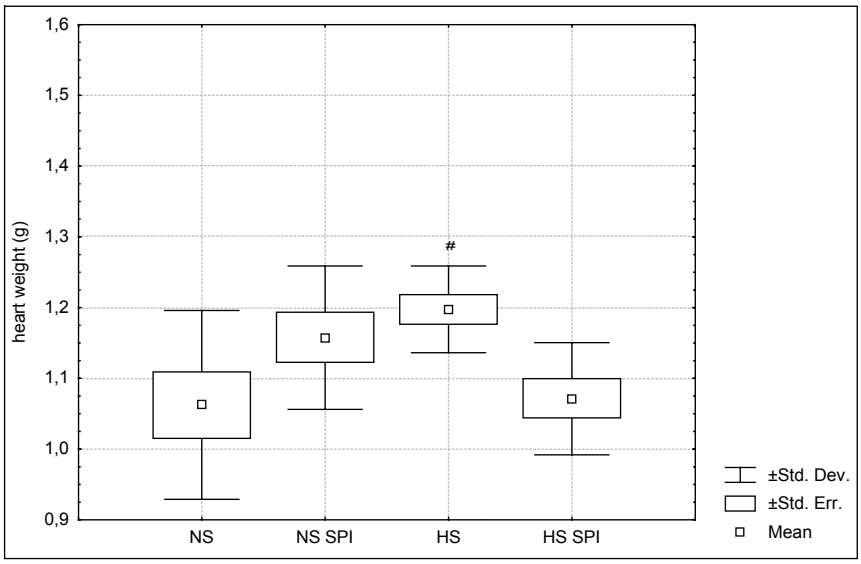


Fig.44a **Heart weight** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. # p<0,05 HS vs. NS.

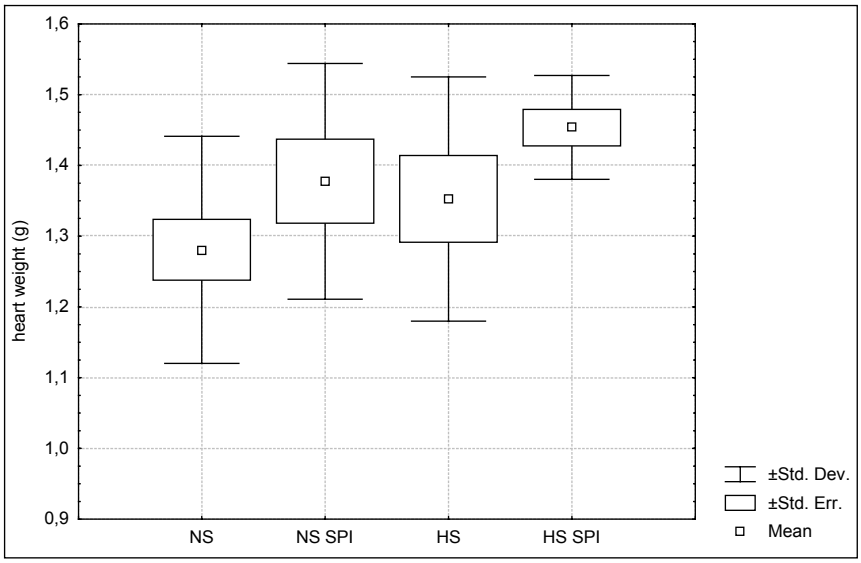


Fig.44b **Heart weight** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14.

The basal heart weight reflected the different initial body weight in each rat strain (Tab.11). Accordingly, groups of BN and BNK rats were not compared. In BN control rats, the heart weight was significantly higher after ten days of high salt diet (Fig.44a). This change may refer to the slightly increased body weight in BN rats fed with high salt diet (Fig.42). In BNK rats, the heart weight was not significantly altered by any experimental conditions (Fig.44b).

4.1.6. LEFT KIDNEY WEIGHT

The effect of high salt diet and spironolactone on the left kidney weight of BN and BNK rats was investigated. The kidneys were weighed immediately after harvesting.

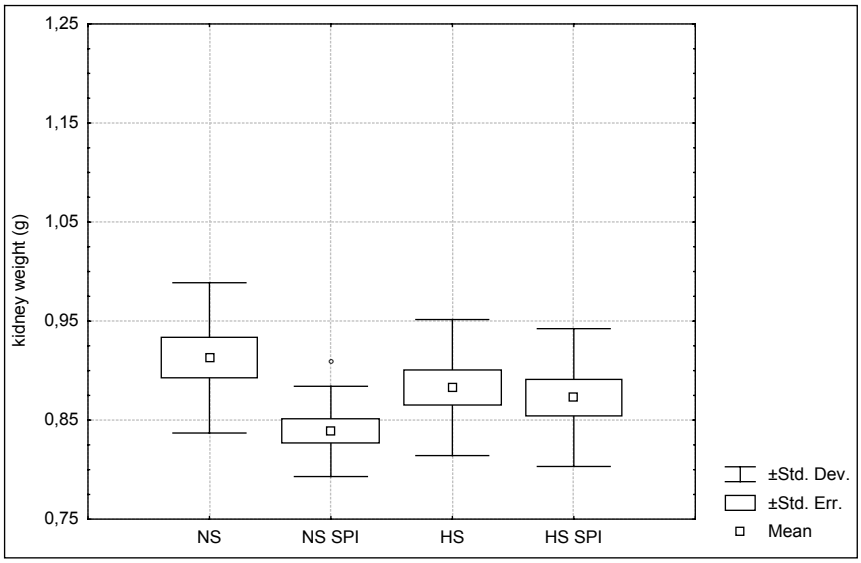


Fig.45a **Left kidney weight** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=14. ° p<0,05 NS SPI vs. NS.

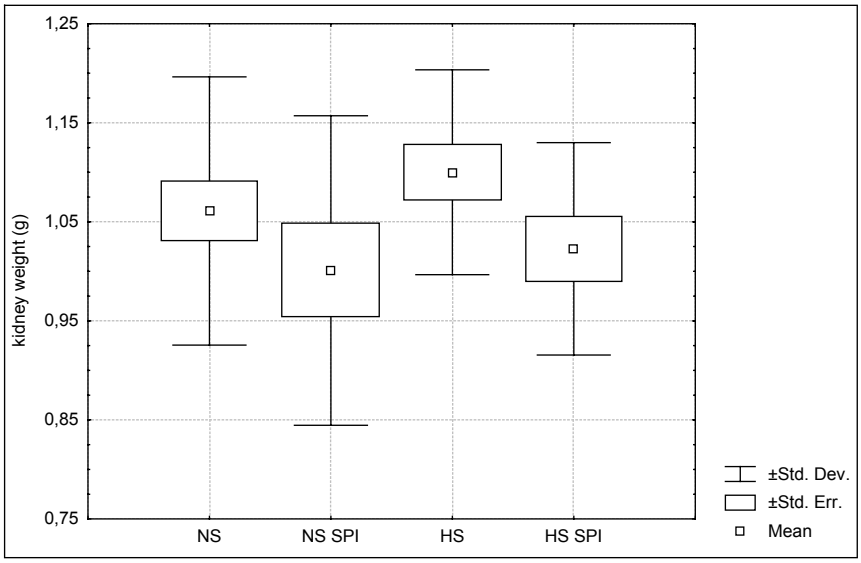


Fig.45b **Left kidney weight** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=20 in NS, n=14 in HS, and n=11 in NS SPI and HS SPI.

The final left kidney weight reflected the differences in initial body weight in both rat strains (Tab.11). Because of the different initial body weight of BN and BNK rats, the strains were not compared. In BN control rats, the left kidney weight was significantly lower after ten days of spironolactone treatment (Fig.45a). This change may derive from the body weight loss in this group. In BNK rats, the left kidney weight was not significantly altered by any experimental conditions (Fig.45b).

SUMMARY OF ANIMAL CHARACTERISTICS

During the ten days of treatment with spironolactone, the body weight significantly decreased in both strains. This effect could be explained by the diuretic action of spironolactone that was confirmed by an increased urine volume. BN control rats lost about 5g and kininogen-deficient BNK rats about 15g of body weight. This difference between the strains originated in the action of spironolactone. The potency of spironolactone to increase urine volume was significantly greater in BNK rats.

Following ten days of high salt diet, body weight increased in BN rats but decreased in BNK rats. Water balance, described by water intake and urine volume, displayed no differences between both strains. Spironolactone treatment caused a decrease in body weight also in rats fed with high salt diet. This effect did not originate in the diuretic action of spironolactone because no further increase in urine volume occurred. Spironolactone influenced salt appetite of BNK rats, which augmented the loss in body weight.

4.2. HEMODYNAMICS

Mean blood pressure (MBP) and heart rate (HR) were investigated in BN and BNK rats fed with standard or high salt diet in presence or absence of spironolactone. Both, mean blood pressure and heart rate, were measured simultaneously by tail cuff method in conscious animals.

4.2.1 MEAN BLOOD PRESSURE

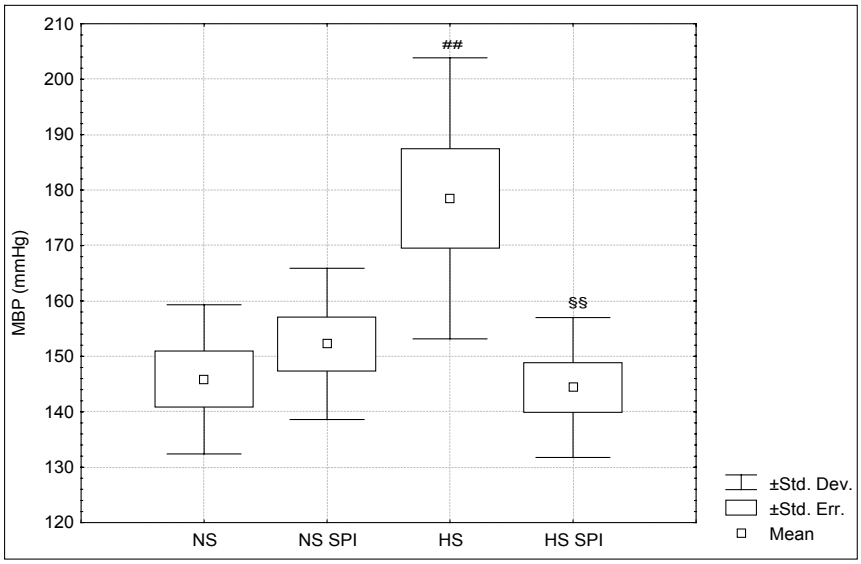


Fig.46a **Mean blood pressure** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. ## p<0,01 HS vs. NS; §§ p<0,01 HS SPI vs. HS.

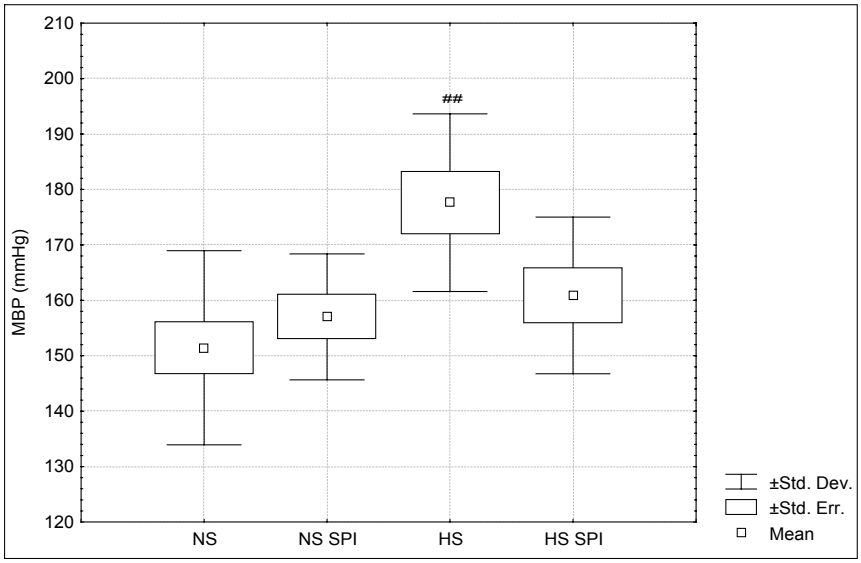


Fig.46b **Mean blood pressure** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. ## p<0,01 HS vs. NS.

COMPARISON OF BN AND BNK RATS

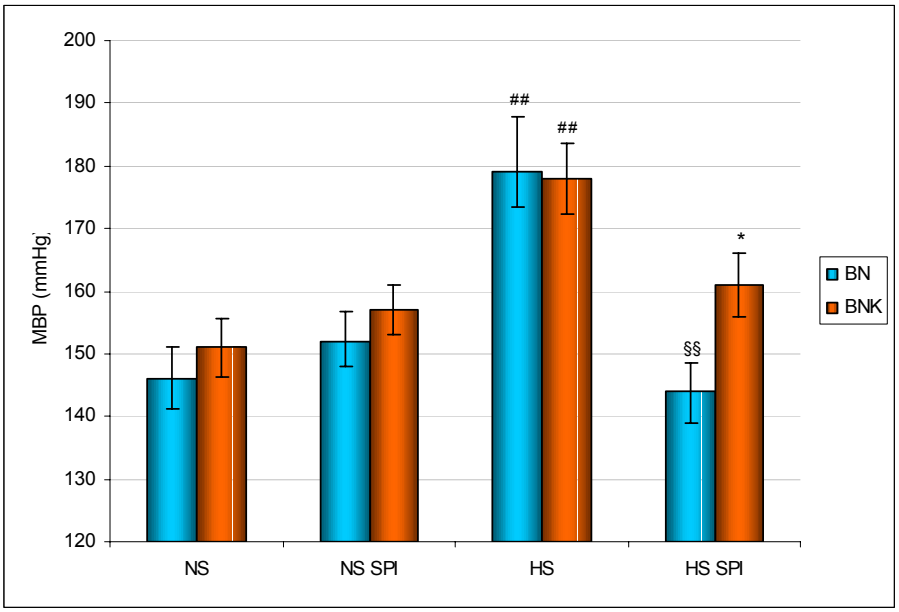


Fig.46c **Mean blood pressure** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=14. *** p<0,05 BNK rats vs. BN rats**; ## p<0,01 HS vs. NS in rats of the same strain; §§ p<0,01 HS SPI vs. HS in rats of the same strain.

Basal mean blood pressure was similar in BN control rats and kininogen-deficient BNK rats (Fig.46c). Spironolactone, currently used as antihypertensive drug, did not change blood pressure in rats fed with standard diet. Ten days of high salt diet significantly increased mean blood pressure in both, BN and BNK rats. Blood pressure elevation did not originate in an increase of body fluids. Animals in this experimental group did not gain body weight and water balance remained constant. The elevated blood pressure, caused by high salt diet, was reduced by spironolactone. In BNK rats, the reduction was not as effective as in BN rats, where blood pressure decreased to basal levels. The reduction of blood pressure was not caused by spironolactone's diuretic activity, since urine volume did not increase.

4.2.2 HEART RATE

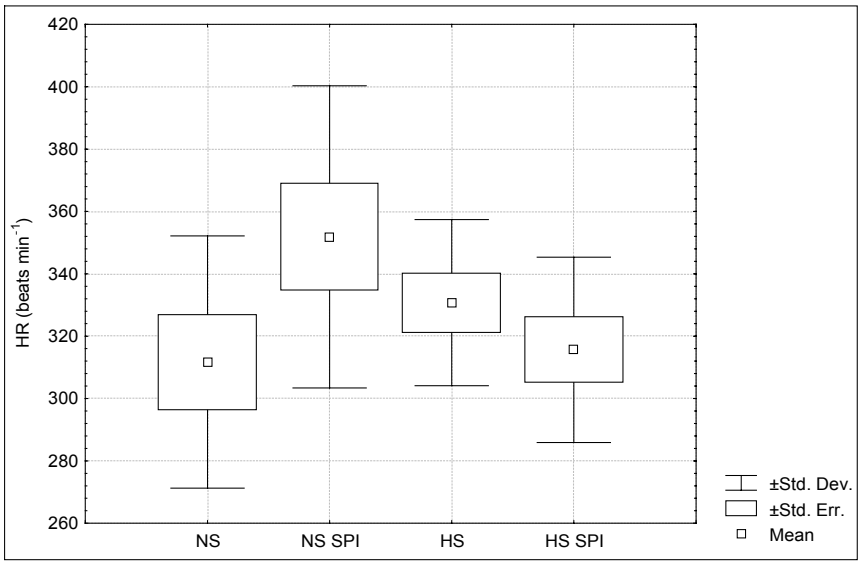


Fig.47a **Heart rate** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8.

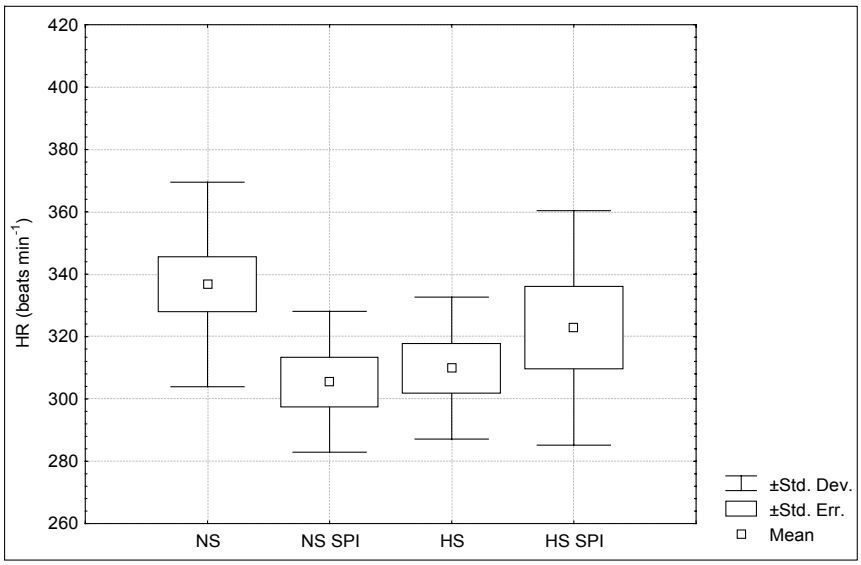


Fig.47b **Heart rate** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14.

COMPARISON OF BN AND BNK RATS

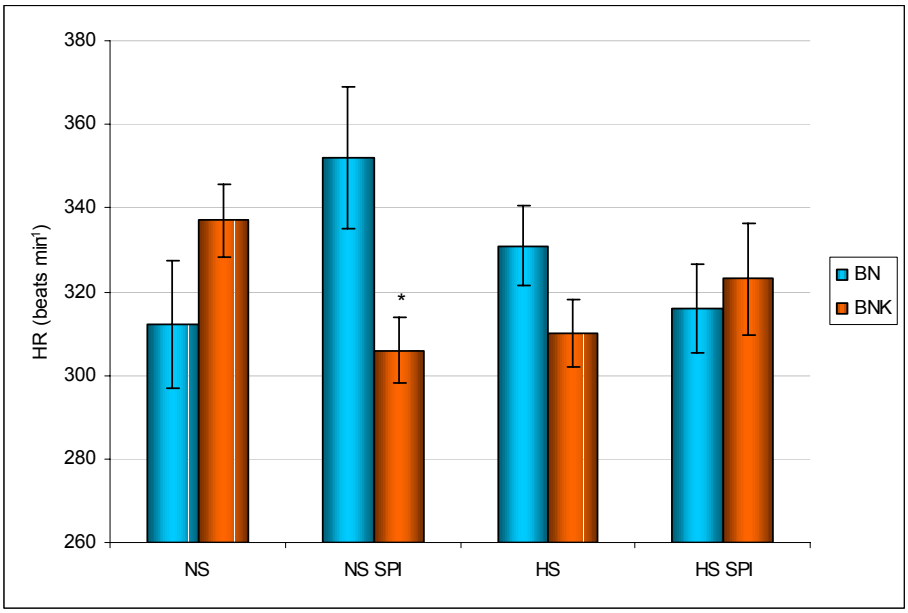


Fig.47c **Heart rate** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=14. *** p<0,05 BNK rats vs. BN rats**.

Basal heart rate was similar in both, BN and BNK rats (Fig.47c). Following treatment with spironolactone, heart rate increased in BN control rats and decreased in kininogen-deficient BNK rats.

SUMMARY OF HEMODYNAMIC PARAMETERS

Both, basal blood pressure and heart rate were slightly higher in kininogen-deficient BNK rats than in BN control rats. In rats fed with standard diet, treatment with spironolactone did not alter blood pressure. Spironolactone mediated an opposite effect on heart rate in both strains; an increase in BN rats and a decrease in BNK rats. In both, BN and BNK rats, high salt intake caused a significant increase in blood pressure that was assigned to increased Na^+ concentration. No changes occurred in the heart rate. Under the conditions of high Na^+ intake, spironolactone was able to reduce blood pressure, more effectively in BN rats. Among the classical action of aldosterone in the kidney these data suggest local direct actions of aldosterone in the cardiovascular system.

4.3. PLASMA ANALYSIS

The components of KKS, ET-1, aldosterone and its precursors were characterized in plasma of BN control rats and kininogen-deficient BNK rats. The effects of standard or high salt diet in presence or absence of spironolactone on the plasma KKS and corticoid hormones were analysed in both strains. For the measurement of ET-1 only supplementary plasma samples from control animals were available, therefore any effect of treatment could be determined.

4.3.1. PLASMA KALLIKREIN-KININ- SYSTEM

The main components of the KKS, namely plasma HMW and LMW kininogen, plasma kallikrein, and plasma kinins, BK and KLP, were analysed. These measurements should reveal differences between both rat strains derived from the kininogen-deficiency of BNK rats. The effects of standard or high salt diet in presence or absence of spironolactone were determined in both, BN control rats and kininogen-deficient BNK rats.

4.3.1.1. PLASMA HMW KININOGEN

In particular, measurements of HMW kininogen in plasma should confirm the kininogen deficiency in BNK rats. Plasma digestion preceded the measurement. BK was released from HMW kininogen after activation of plasma kallikrein. BK was then determined by a specific RIA. Results are presented in ng BK equivalent per ml plasma.

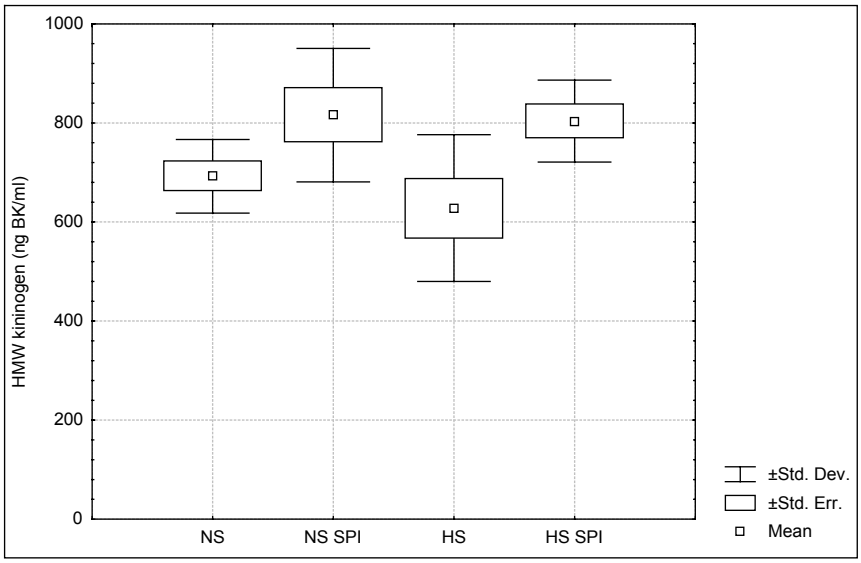


Fig.48a **Plasma HMW kininogen in BK equivalent** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6.

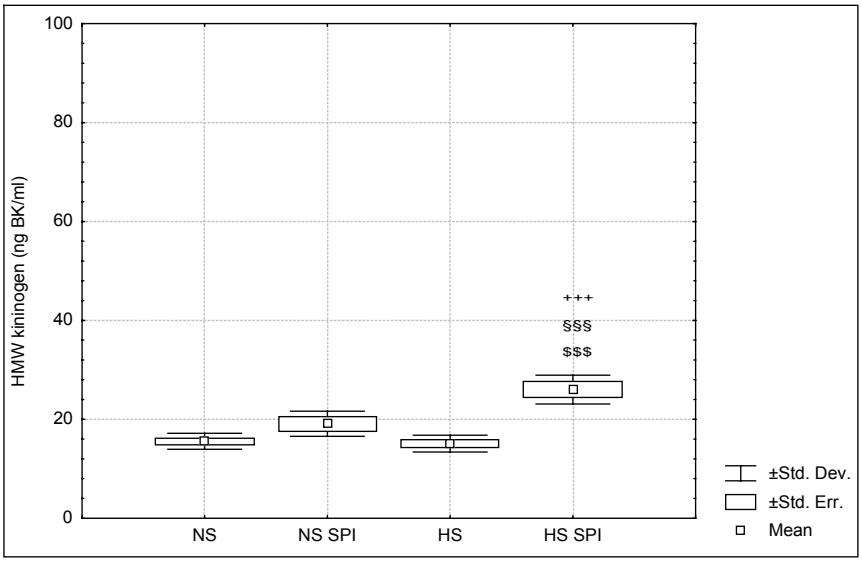


Fig.48b Pl**asma HMW kininogen in BK equivalent** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6 in NS and HS, and n=3 in NS SPI and HS SPI. $^{+++}$ p<0,001 HS SPI vs. NS; §§§ p<0,001 HS SPI vs. HS; $^{$$$}$ p<0,001 HS SPI vs. SPI.

COMPARISON OF BN AND BNK RATS

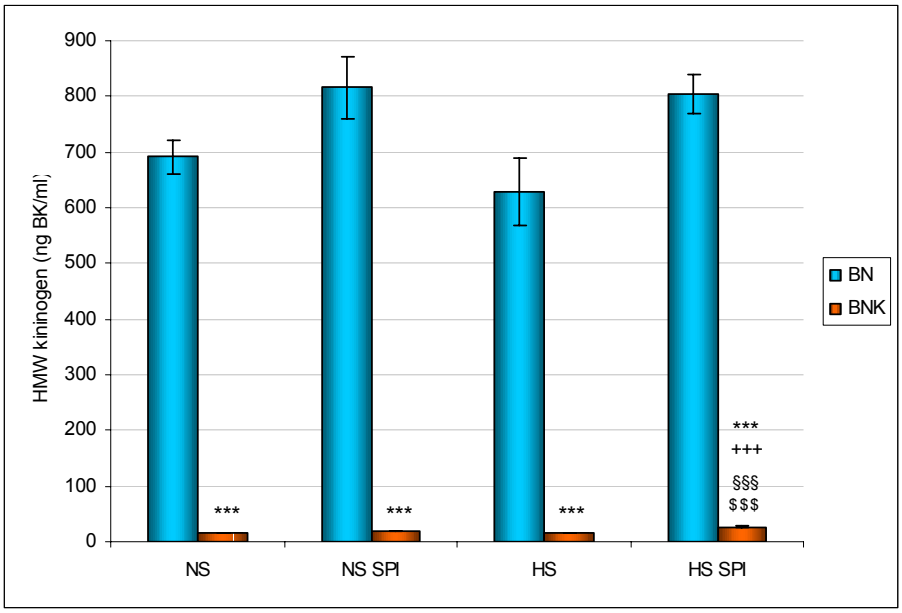


Fig.48c **Plasma HMW kininogen in BK equivalent** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=3-8. ***** p<0,001 BNK rats vs. BN rats**; $^{+++}$ p<0,001 HS SPI vs. NS in rats of the same strain; §§§ p<0,001 HS SPI vs. HS in rats of the same strain; $^{\$\$\$}$ p<0,001 HS SPI vs. SPI in rats of the same strain.

Determination of HMW kininogen in plasma confirmed the kininogen deficiency of BNK rats (Fig.48c). The HMW kininogen in plasma was approximately 35-fold lower in BNK rats than in BN rats (20ng BK equivalent vs. 700ng BK equivalent per ml plasma). In BN control rats, treatment with spironolactone tended to increase HMW kininogen in plasma (Fig.48a). In kininogen-deficient BNK rats fed with high salt diet in presence of spironolactone, plasma HMW kininogen significantly increased (Fig.48b). Nevertheless, the levels remained significantly lower (30-fold) than in BN control rats.

4.3.1.2. PLASMA LMW KININOGEN

Determination of plasma LMW kininogen should describe differences between BN control rats and kininogen-deficient BNK rats. Measurement of LMW kininogen was preceded by plasma digestion. BK was released from LMW kininogen by trypsin

added to the incubation reaction. BK was then determined by a specific RIA. Values are presented in ng BK equivalent per ml plasma.

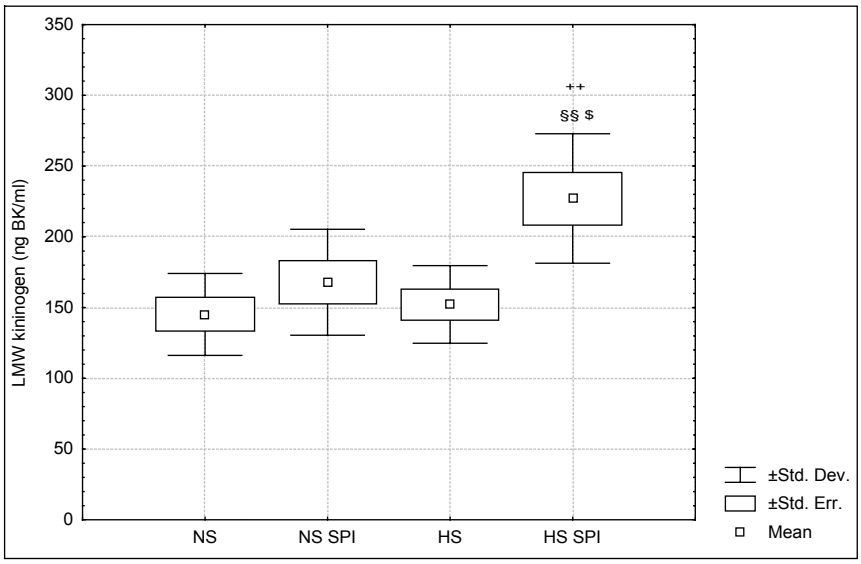


Fig.49a **Plasma LMW kininogen in BK equivalent** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6. $^{++}$ p<0,05 HS SPI vs. NS; §§ p<0,01 HS SPI vs. HS; $^{\$}$ p<0,05 HS SPI vs. SPI.

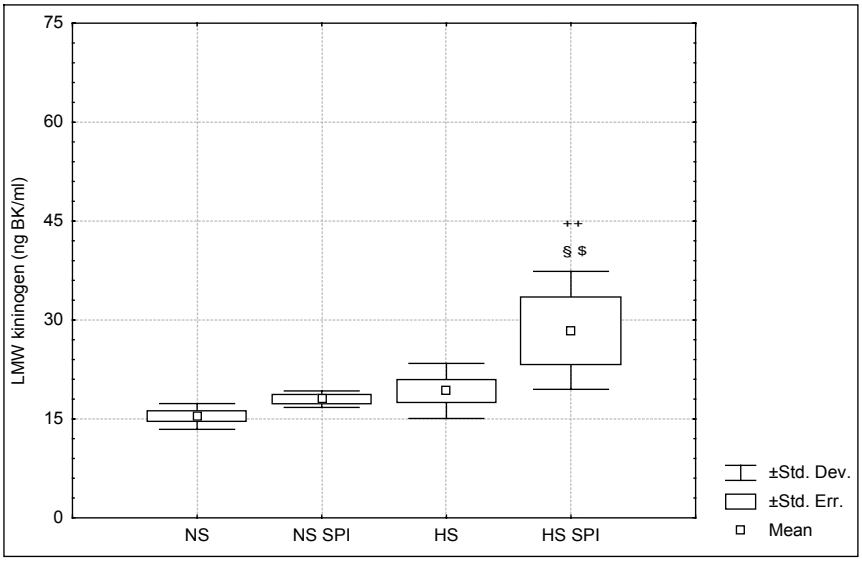


Fig.49b **Plasma LMW kininogen in BK equivalent** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6 in NS and HS, and n=3 in NS SPI and HS SPI. $^{++}$ p<0,05 HS SPI vs. NS; § p<0,05 HS SPI vs. HS; $^{\$}$ p<0,05 HS SPI vs. SPI.

COMPARISON OF BN AND BNK RATS

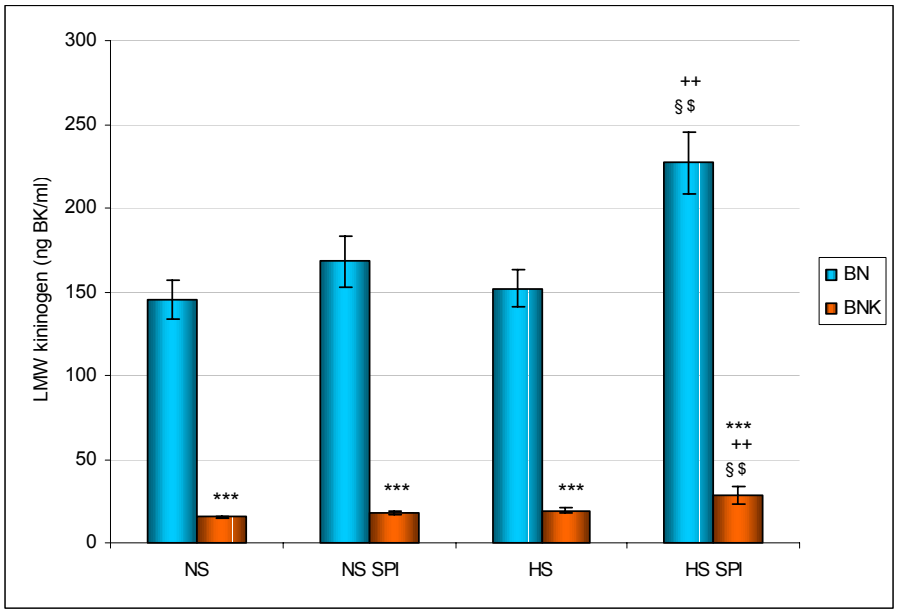


Fig.49c **Plasma LMW kininogen in BK equivalent** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=3-8. ***** p<0,001 BNK rats vs. BN rats**; $^{++}$ p<0,01 HS SPI vs. NS in rats of the same strain; § p<0,05 HS SPI vs. HS in rats of the same strain; $^{\$}$ p<0,05 HS SPI vs. SPI in rats of the same strain.

Determination of LMW kininogen in plasma acknowledged kininogen deficiency of BNK rats (Fig.49c). The LMW kininogen in plasma was about 10-fold lower in BNK rats than in BN rats (15ng BK equivalent vs. 150ng BK equivalent per ml plasma). In both strains, combination of high salt diet and spironolactone significantly increased plasma LMW kininogen. Nevertheless, in BNK rats the levels remained significantly lower.

4.3.1.3. PLASMA KALLIKREIN ACTIVITY

Plasma kallikrein (pKLK) precursor circulates bound to HMW kininogen. Therefore, activity of plasma kallikrein should also reflect the kininogen deficiency. Measurements reveal differences between BN control rats and kininogen-deficient BNK rats. Plasma kallikrein activity was determined amidolytiallly using a selective chromogenic substrate. Absorbance was then measured in a spectrometer.

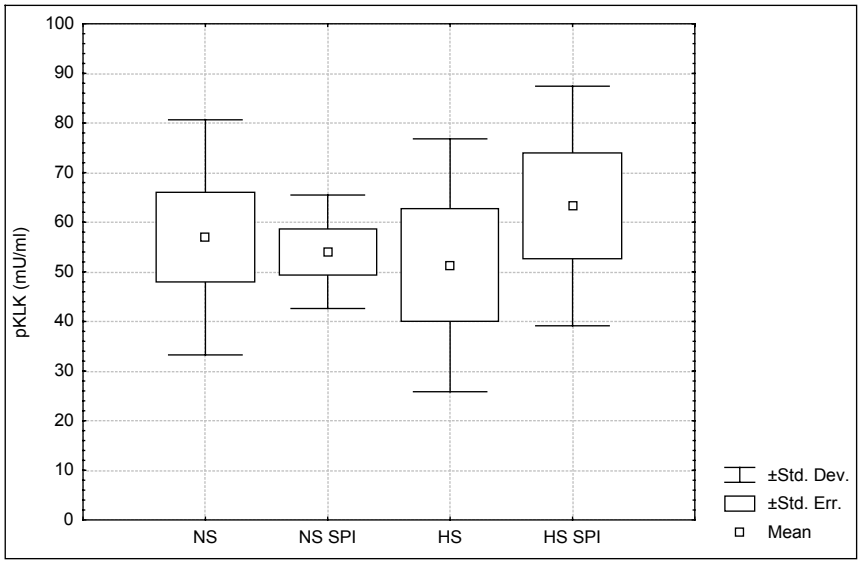


Fig.50a **Plasma kallikrein activity** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=7 in NS, n=6 in NS SPI and HS, and n=5 in HS SPI.

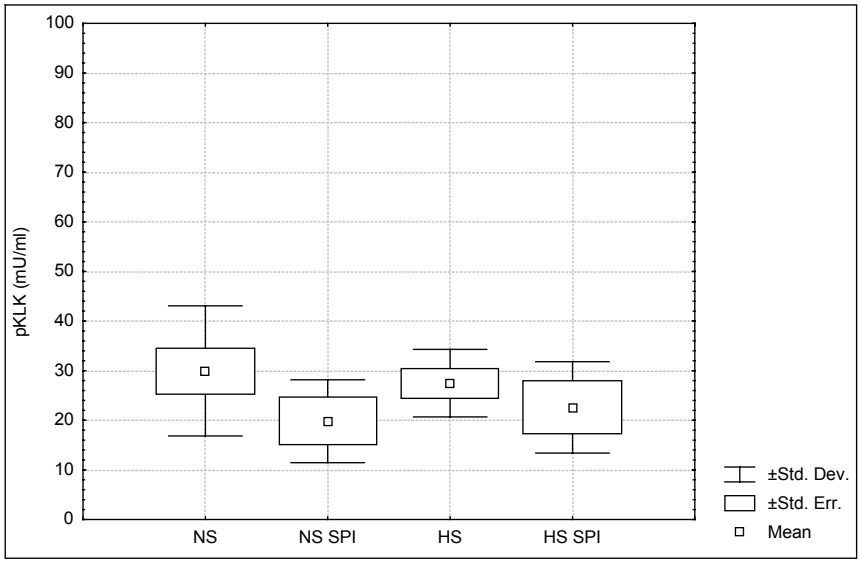


Fig.50b **Plasma kallikrein activity** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 in NS, n=5 in HS, and n=3 in NS SPI and HS SPI.

COMPARISON OF BN AND BNK RATS

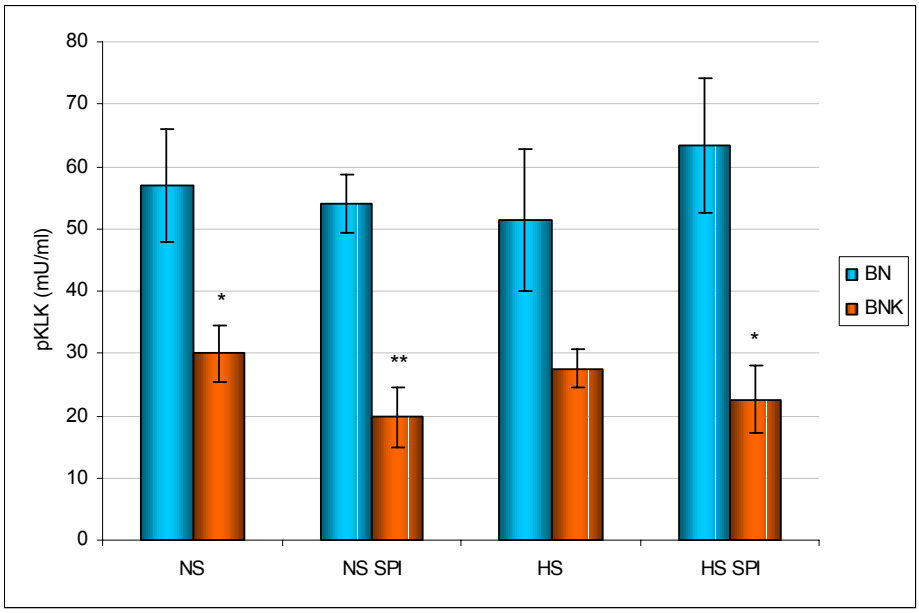


Fig.50c **Plasma kallikrein activity** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=3-8. *** p<0,05; ** p<0,01 BNK rats vs. BN rats**.

Measurement of plasma kallikrein activity confirmed that kininogen deficiency influences also the plasma concentration of plasma kallikrein. In BNK rats, the activity of plasma kallikrein was about 2-fold lower in BNK rats than in BN rats (Fig.50c). Neither high salt diet nor spironolactone influenced the activity of plasma kallikrein in both strains. The activity of plasma kallikrein seemed to be highly conserved and not involved in responds to high salt diet and treatment with spironolactone.

4.3.1.4. PLASMA BRADYKININ

It is believed that BK is cleaved from HMW kininogen through plasma kallikrein. Measurements of plasma bradykinin should reveal potential differences between BN control rats and kininogen-deficient BNK rats. BK levels in plasma were measured by means of a specific RIA.

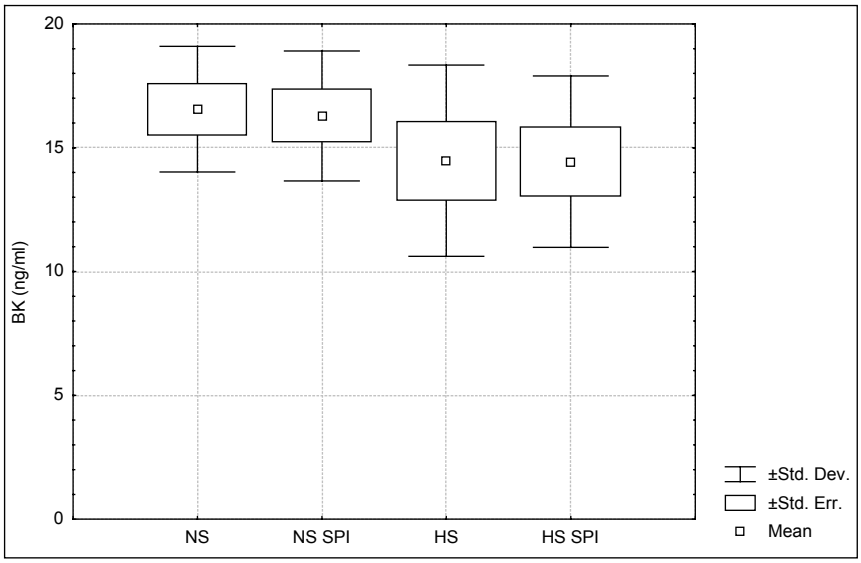


Fig.51a **Plasma BK** levels in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6.

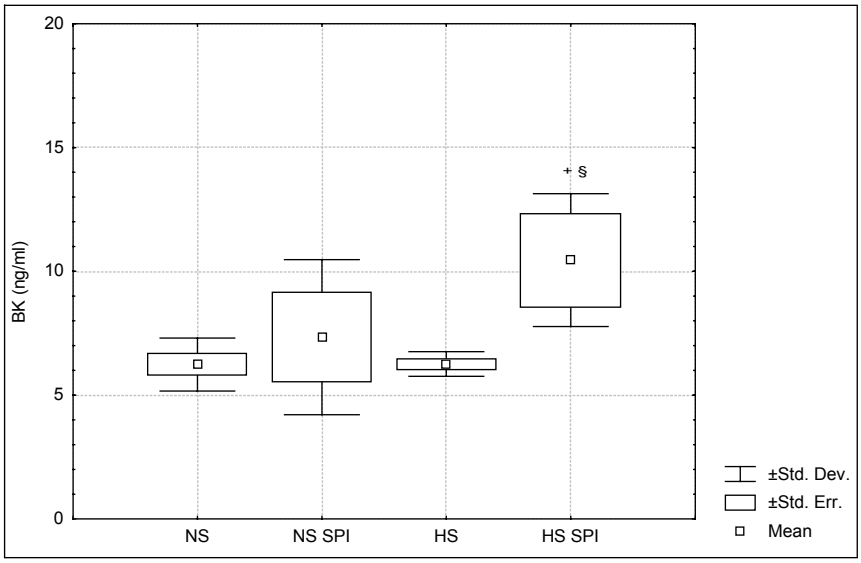


Fig.51b **Plasma BK** levels in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6 in NS and HS, and n=3 in NS SPI and HS SPI. $^{+}$ p<0,05 HS SPI vs. NS; § p<0,05 HS SPI vs. HS.

COMPARISON OF BN AND BNK RATS

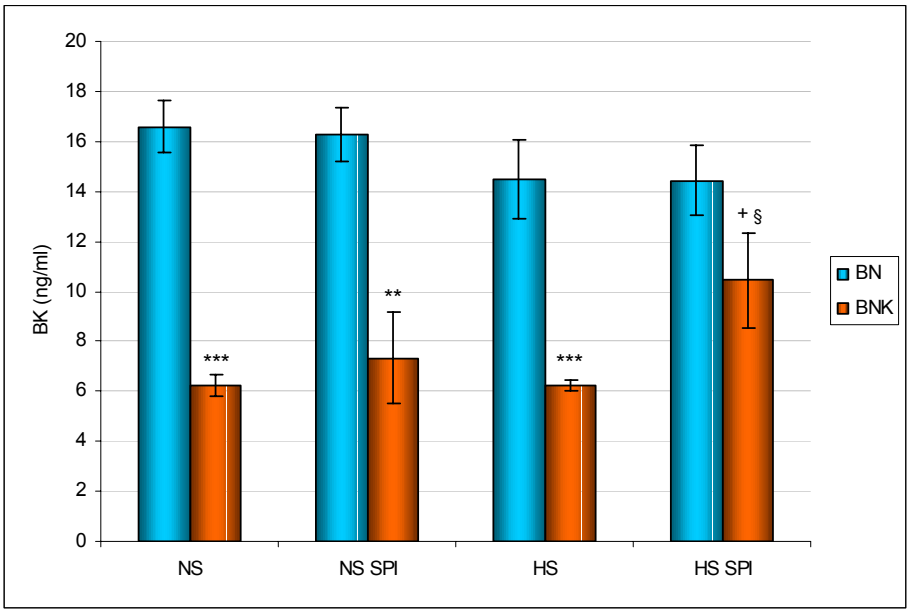


Fig.51c **Plasma BK** levels in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=3-6. **** p<0,01; *** p<0,001 BNK rats vs. BN rats**; $^{+}$ p<0,05 HS SPI vs. NS in rats of the same strain; § p<0,05 HS SPI vs. HS in rats of the same strain.

Plasma BK levels reflected kininogen deficiency of BNK rats (Fig.51c). BK concentration in plasma was approximately 2,5-fold lower in BNK rats than in BN control rats (6ng vs. 16,5ng BK per ml plasma). The difference in plasma BK levels between BN and BNK rats was notably smaller than the difference in both kininogens. BK levels in plasma correlated with the activity of plasma kallikrein that was about 2-fold lower in BNK rats. Neither high salt diet nor spironolactone altered plasma BK levels. In BNK rats, plasma BK levels significantly increased following high sat diet and spironolactone. The same increase was seen in both, HMW and LMW kininogen levels (Fig.48c and 49c) but not in plasma kallikrein activity (Fig.50c).

4.3.1.5. PLASMA KALLIDIN-LIKE-PEPTIDE

Until quite recently, KLP (KAL equivalent in rat) has been found. Generally, it is believed that KAL is cleaved from LMW kininogen by tissue kallikrein. First, measurements of plasma KLP should characterize this kinin in rat. Second, they

should reveal potential differences between BN control rats and kininogen-deficient BNK rats. Third, the effect of treatment should zoom into its functional importance. Plasma KLP was determined with a specific RIA for KAL/KLP.

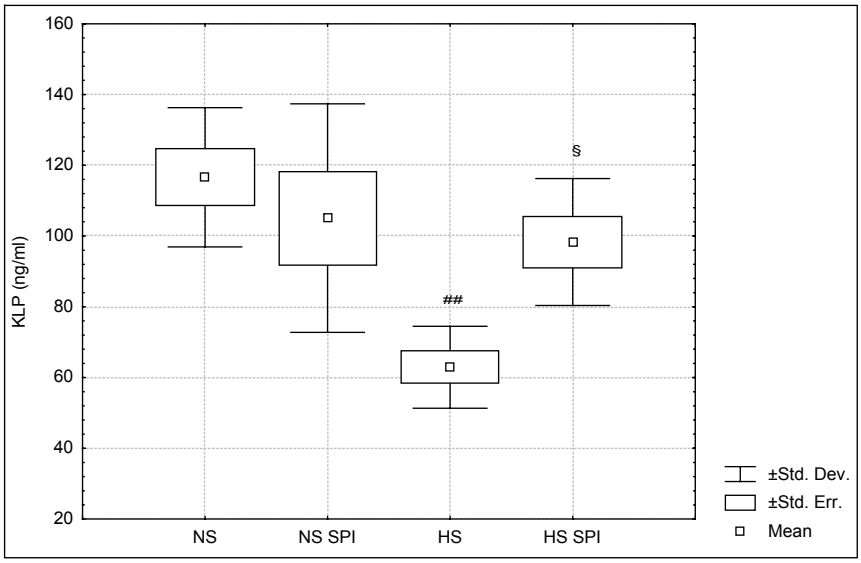


Fig.52a **Plasma KLP** levels in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6. ## p<0,01 HS vs. NS; § p<0,05 HS SPI vs. HS.

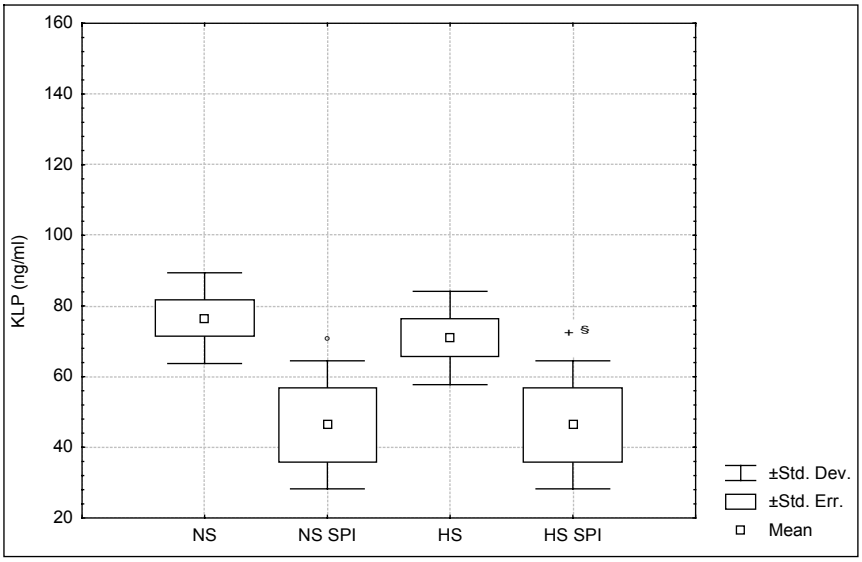


Fig.52b **Plasma KLP** levels in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6 in NS and HS, and n=3 in NS SPI and HS SPI. ° p<0,05 NS SPI vs. NS; + p<0,05 HS SPI vs. NS; § p<0,05 HS SPI vs. HS.

COMPARISON OF BN AND BNK RATS

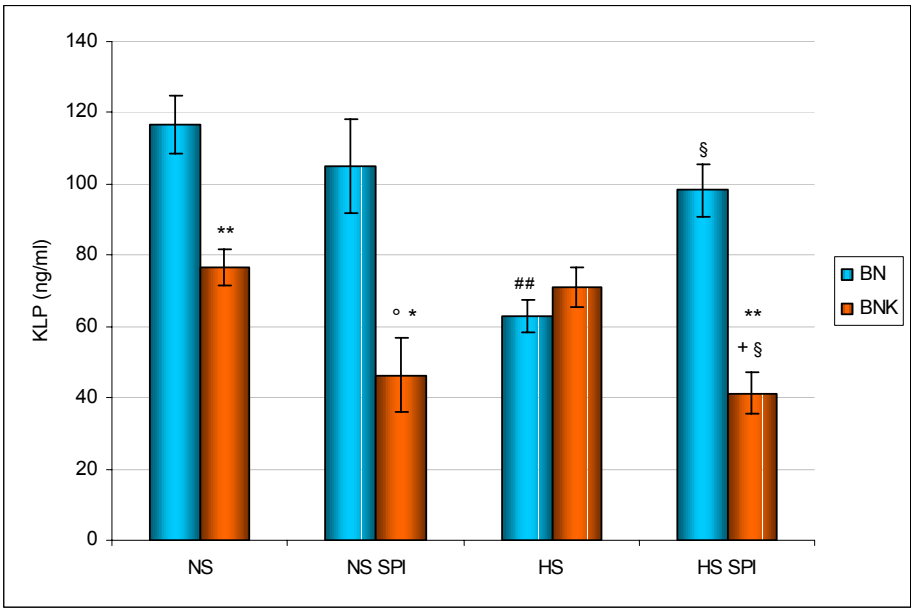


Fig.52c **Plasma KLP** levels in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=3-6. *** p<0,05; ** p<0,01 BNK rats vs. BN rats**; ° p<0,05 NS SPI vs. NS in rats of the same strain; ## p<0,01 HS vs. NS in rats of the same strain; + p<0,05 HS SPI vs. NS in rats of the same strain; $ p<0,05 HS SPI vs. NS SPI in rats of the same strain.

Measurement of KLP confirmed newly published data, that rats are capable in production of this peptide. The kininogen-deficient BNK rats displayed significantly lower levels of KLP in plasma than the BN control rats (Fig.52c). The difference in plasma KLP concentration was less pronounced than that of BK (Fig.51c). In BNK rats, treatment with spironolactone, independently of salt diet, significantly reduced plasma KLP levels. This effect was not seen in BN rats. In BN rats fed with high salt diet, plasma KLP levels significantly decreased to those of BNK rats.

4.3.2. PLASMA ENDOTHELIN-1

Basal ET-1 levels in BN control rats and kininogen-deficient BNK rats were investigated. Plasma concentrations of ET-1 were determined by means of a specific RIA.

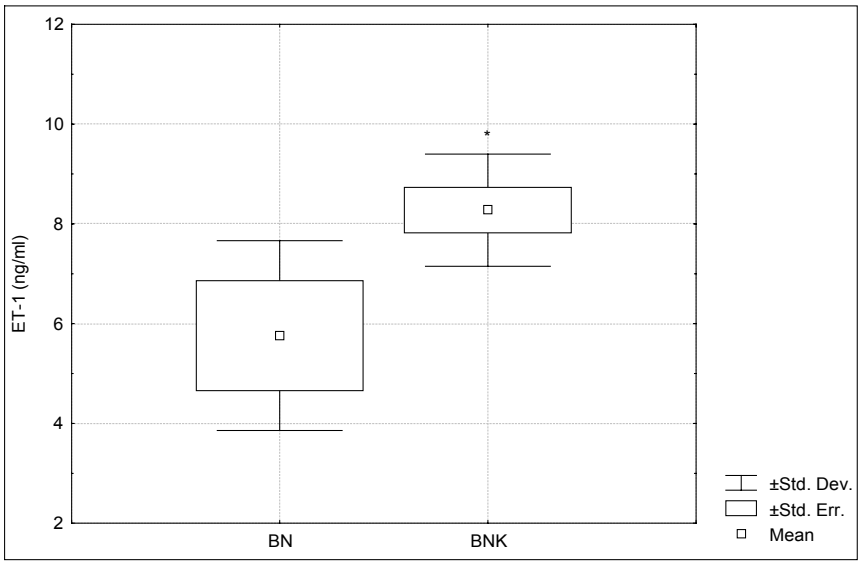


Fig.53 **Plasma ET-1** levels in BN control rats (n=3) and kininogen-deficient BNK rats (n=6). * p<0,05.

Kininogen-deficient BNK rats displayed significantly higher plasma ET-1 levels than BN control rats (Fig.53). The effect of salt diet or spironolactone on plasma ET-1 was not analysed.

4.3.3. ALDOSTERONE AND ITS PRECURSORS IN PLASMA

Aldosterone is formed from deoxycorticosterone (DOC). The conversion involves three consecutive reactions and is catalyzed by aldosterone synthase. In parallel, DOC is converted to corticosterone by the action of 11ß-hydroxylase. Plasma concentrations of aldosterone, DOC and corticosterone were investigated in BN control rats and kininogen-deficient BNK rats. The effects of salt diet or spironolactone in presence or absence of spironolactone on these corticoid hormones were determined in both rat strains.

4.3.3.1. PLASMA DEOXYCORTICOSTERONE

DOC is a mineralocorticoid hormone and a substrate for 11ß-hydroxylase and aldosterone synthase that catalyse the formation of corticosterone and aldosterone, respectively. DOC levels were determined by RIA after its selective extraction from plasma.

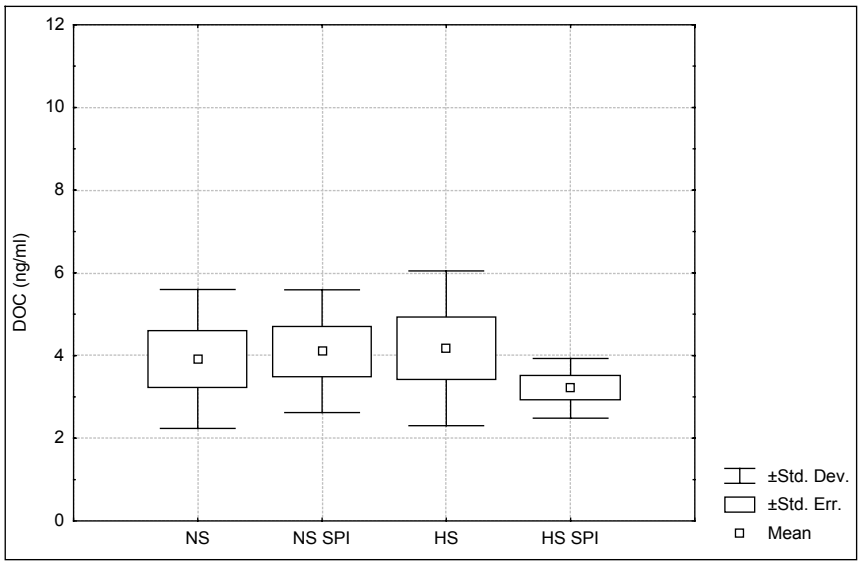


Fig.54a **Plasma deoxycorticosterone** levels in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6.

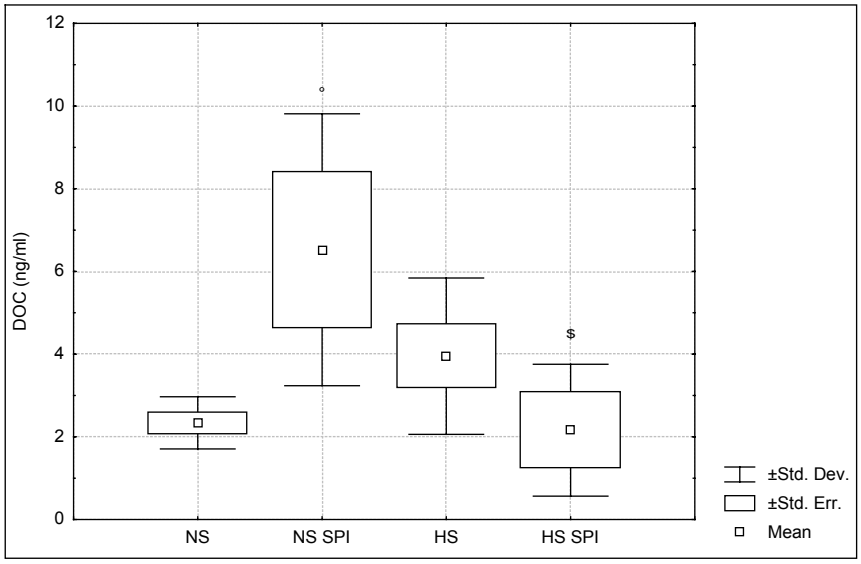


Fig.54b **Plasma deoxycorticosterone** levels in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=3-6. ° $p<0,05$ NS SPI vs. NS; $^{\$}$ $p<0,05$ HS SPI vs. NS SPI.

COMPARISON OF BN AND BNK RATS

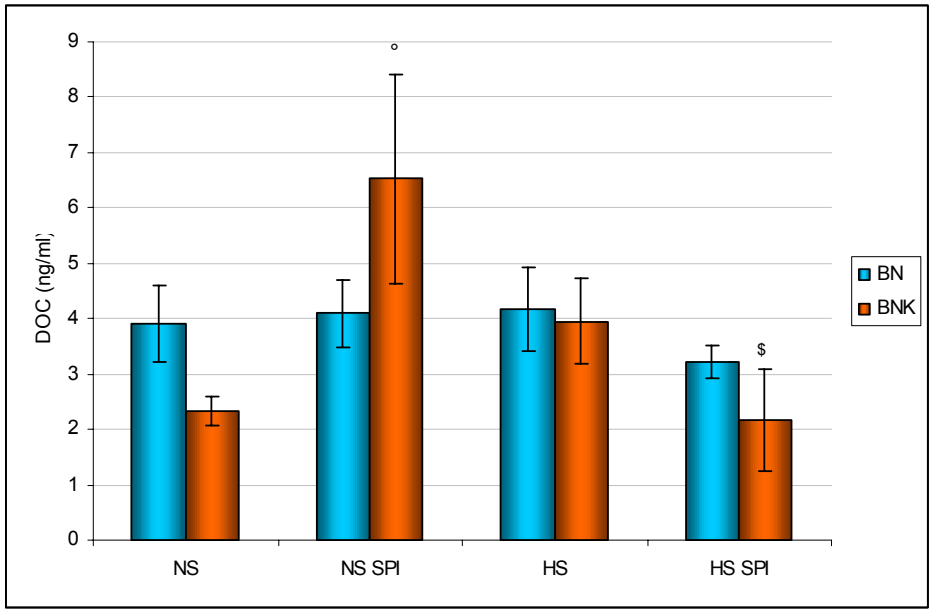


Fig.54c **Plasma deoxycorticosterone** levels in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=3-6. ° p<0,05 NS SPI vs. NS in rats of the same strain; $ p<0,05 HS SPI vs. NS SPI in rats of the same strain.

Basal plasma DOC levels were lower in kininogen-deficient BNK rats than in BN control rats (Fig.54c). In BN rats, neither spironolactone nor high salt diet altered DOC levels in plasma (Fig.54a). In BNK rats, treatment with spironolactone significantly increased plasma DOC levels. High salt diet slightly elevated DOC concentration in plasma that reached the levels of BN control rats. In rats fed with high salt diet, spironolactone decreased elevated plasma DOC (Fig.54b).

4.3.3.2. PLASMA CORTICOSTERONE

Corticosterone is the main glucocorticoid hormone in rat. Corticosterone is an end-product of 11ß-hydroxylation and an intermediate during aldosterone synthesis. Corticosterone concentrations were determined by RIA after its selective extraction from plasma.

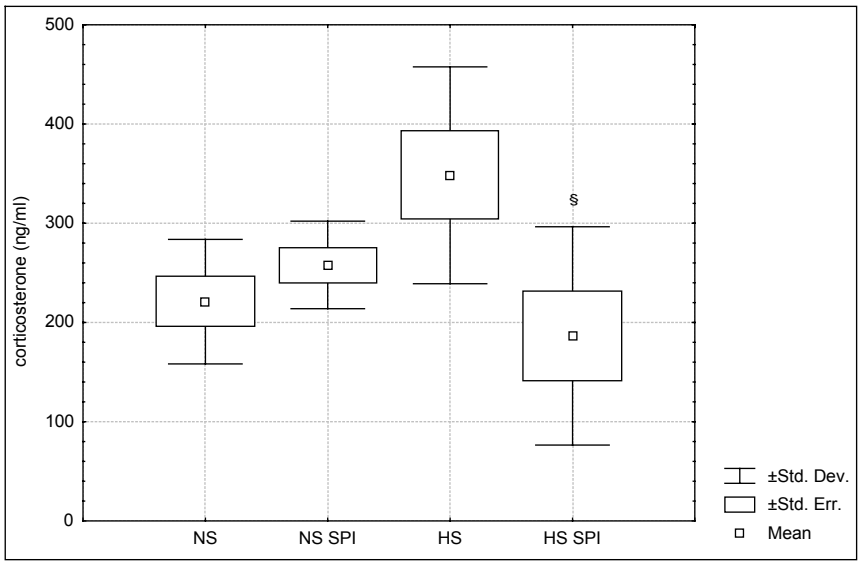


Fig.55a **Plasma corticosterone** levels in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6. § p<0,05 HS SPI vs. HS.

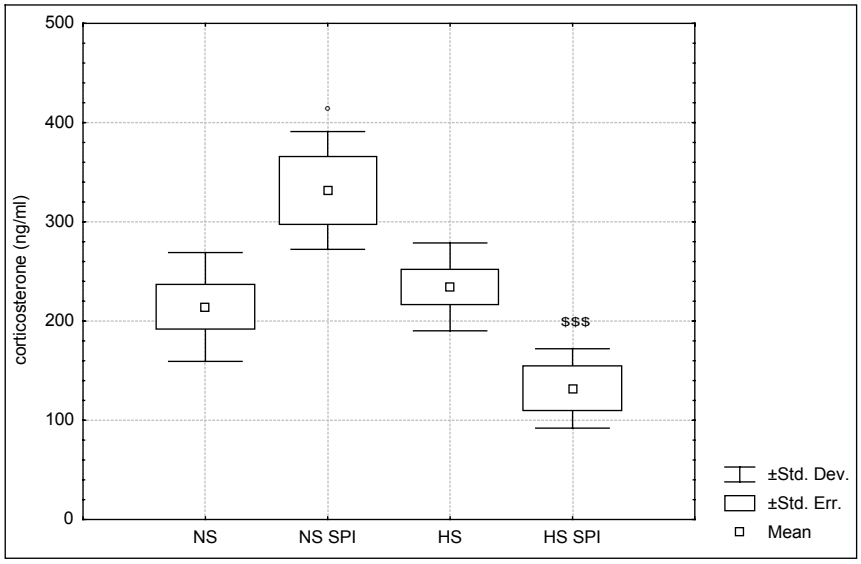


Fig.55b **Plasma corticosterone** levels in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=3-6. ° p<0,05 NS SPI vs. NS; $^{\$\$\$}$ p<0,001 HS SPI vs. HS.

COMPARISON OF BN AND BNK RATS

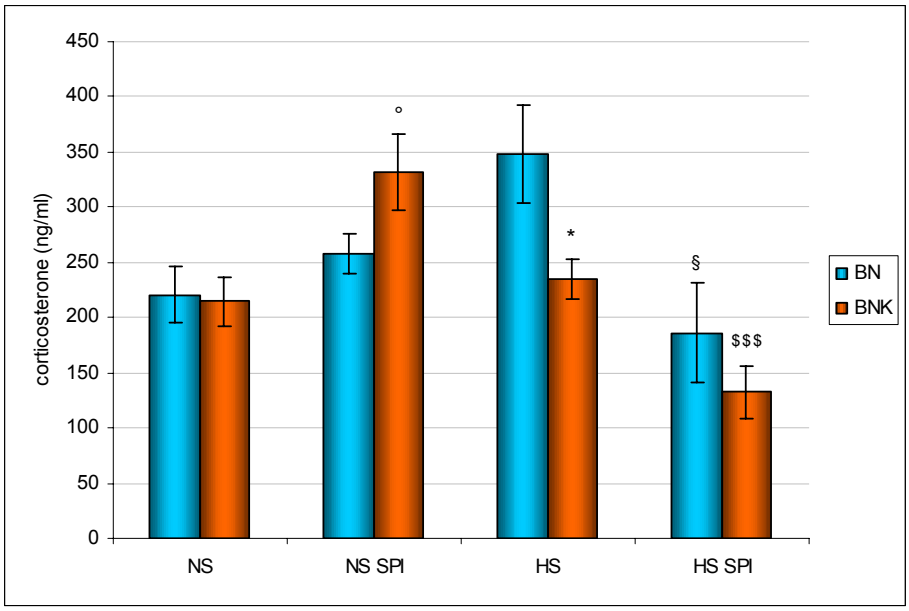


Fig.55c **Plasma corticosterone** levels in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=3-6. *** p<0,05 BNK rats vs. BN rats**; ° p<0,05 NS SPI vs. NS in rats of the same strain; § p<0,05 HS SPI vs. HS in rats of the same strain; $^{\$\$\$}$ p<0,001 HS SPI vs. NS SPI in rats of the same strain.

Basal plasma corticosterone levels were identical in BN and BNK rats (Fig.55c). Corticosterone, as the main glucocorticoid and an end-product of the 11ß-hydroxylation, displayed much higher plasma concentrations than DOC and aldosterone. Treatment with spironolactone caused a significant increase in plasma corticosterone levels in BNK but not in BN rats. On the contrary, high salt diet increased plasma corticosterone in BN but not in BNK rats. In BN and BNK rats fed with high salt diet, spironolactone significantly decreased elevated corticosterone concentrations.

4.3.3.3. PLASMA ALDOSTERONE

Aldosterone is the main mineralocorticoid hormone. The formation of aldosterone from DOC is catalysed by aldosterone synthase. Aldosterone levels were measured by RIA after its selective extraction from plasma.

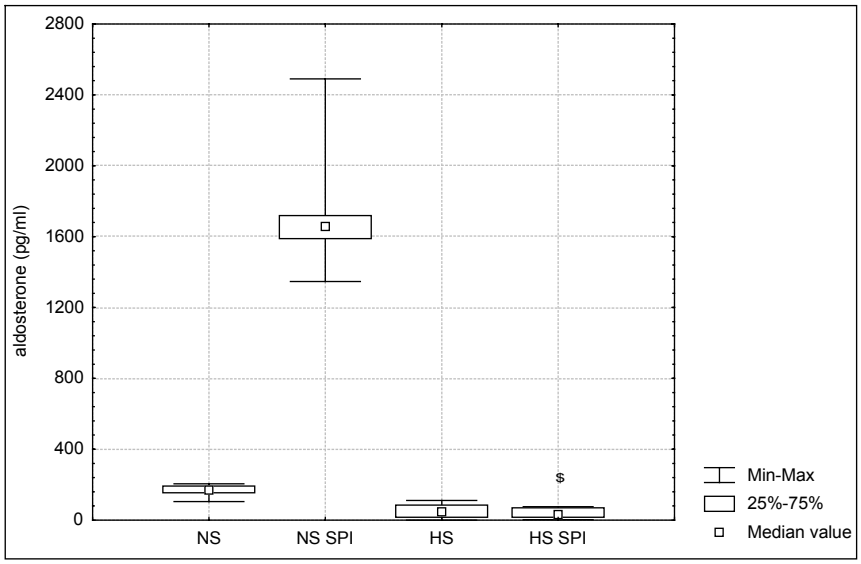


Fig.56a **Plasma aldosterone** levels in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=6. $ p<0,05 HS SPI vs. NS SPI.

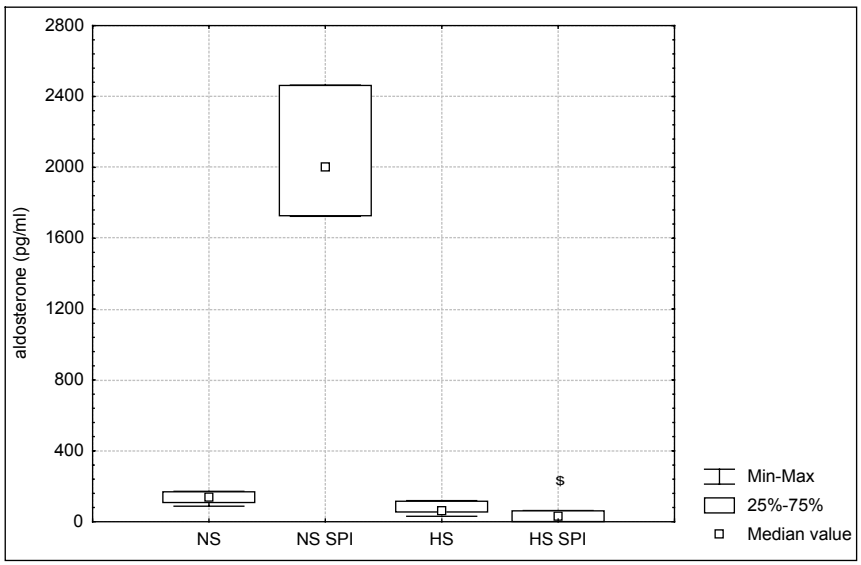


Fig.56b **Plasma aldosterone** levels in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=3-6. $ p<0,05 HS SPI vs. NS SPI.

COMPARISON OF BN AND BNK RATS

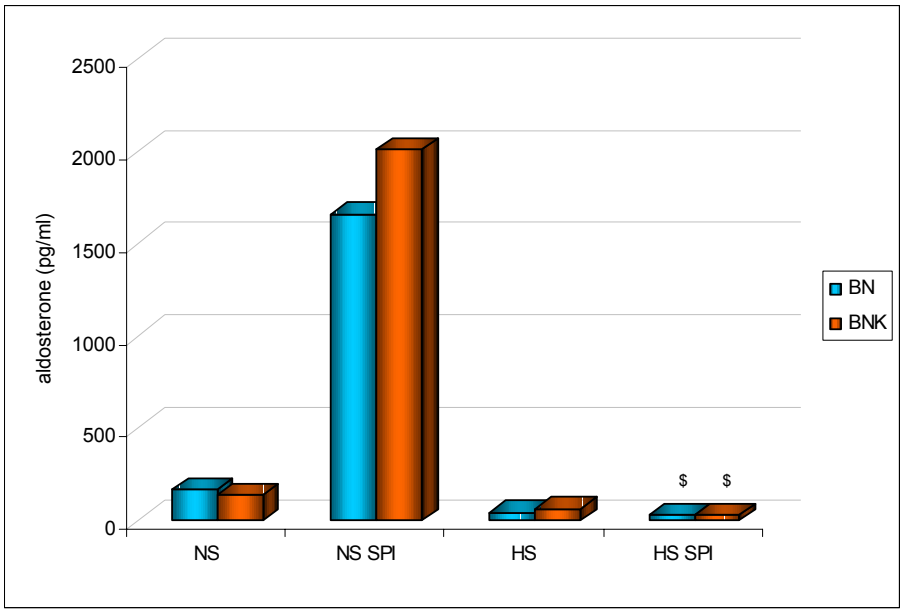


Fig.56c **Plasma aldosterone** levels in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=3-6. $ p<0,05 HS SPI vs. NS SPI in rats of the same strain.

Basal aldosterone levels in plasma were identical in both strains (Fig.56c). In both strains, spironolactone displaced aldosterone form the mineralocorticoid receptor (MR), which led to a substantial increase of free aldosterone in plasma. In BNK rats, this free plasma aldosterone was higher than that of BN rats. Following high salt diet, plasma aldosterone was markedly reduced in both rat strains. Consequently, in rats fed with high salt diet spironolactone did not increase plasma aldosterone.

SUMMARY OF PLASMA PROFILE

The kininogen deficiency of BNK rats was confirmed by measurements of all components of the KKS. In BNK rats, all components of the KKS were significantly reduced when compared with BN control rats. The most pronounced difference was found in the plasma HMW kininogen. The BNK rats displayed approximately 35-fold lower levels than BN rats. The LMW kininogen concentration in plasma was 10-fold lower in BNK rats than in BN rats. The kininogen deficiency influenced also the concentration of plasma kallikrein as confirmed by its 2-fold lower activity in plasma

of BNK rats. The activity of plasma kallikrein was not altered by any of the experimental conditions. Consequently, the plasma concentration of BK and KLP was significantly reduced in BNK rats. BK levels correlated with the activity of plasma kallikrein. The measurements revealed that plasma KLP levels were approximately 10-fold higher than those of BK.

The levels of both kininogens and BK were not altered by high salt diet or spironolactone. In BNK rats, the combination of high salt diet and spironolactone increased the plasma levels of HMW kininogen and consequently also the BK concentration in plasma. LMW kininogen increased in both strains but the elevation was not reflected in KLP levels. In BN rats, high salt diet caused a significant decrease in plasma KLP levels. In contrast, they were not altered in BNK rats. In both strains, treatment with spironolactone tended to decrease plasma KLP levels.

Differences between BN control rats and kininogen-deficient BNK rats were found also in plasma concentrations of ET-1. Plasma levels of ET-1 were significantly higher in BNK rats than in BN rats.

Similarly, measurements of plasma corticoid hormones revealed differences between kininogen-deficient BNK rats and BN control rats. In BNK rats, basal plasma levels of DOC were lower than those of BN rats. Plasma corticosterone and aldosterone were identical in both strains.

In BNK rats, spironolactone treatment significantly increased plasma DOC and corticosterone levels. On the contrary, in BN rats these corticoids remained unchanged. In both strains, spironolactone significantly increased the free fraction aldosterone in plasma, which demonstrated spironolactone’s binding to the MR. In BN rats fed with high salt diet, plasma DOC levels remained unchanged but the corticosterone levels increased. Opposite effects were found in BNK rats. High salt diet slightly increased DOC plasma levels and corticosterone concentration was not altered. In both strains, high salt diet decreased aldosterone concentration. In rats fed with high salt diet, spironolactone treatment slightly decreased plasma DOC and corticosterone levels. Aldosterone concentration remained unaltered.

4.4. URINE ANALYSIS

Analogous to plasma, the KKS, ET-1, aldosterone and its precursors were characterized in urine of BN control rats and kininogen-deficient BNK rats. The effects of standard diet or high salt diet in presence or absence of spironolactone were analysed in both strains. Urine from individual rats was collected on ice bath during the experiments with metabolic cages. With aid of the information about urinary volume, the results are presented as daily excretion of the respective substance.

4.4.1. RENAL KALLIKREIN-KININ- SYSTEM

Renal KKS is independent from plasma KKS. It is believed that kininogens or kallikreins present in plasma are not filtered into primary urine to a reasonable extend. Filtered kinins are rapidly destroyed in the brush border of the proximal tubule by various peptidases. The amount of LMW kininogen, tissue kallikrein activity and concentrations of KLP and BK in urine should describe in particular the activity of the renal KKS. Measurements of these parameters should reveal potential differences between BN control rats and kininogen-deficient BNK rats. The effects of standard diet or high salt diet in presence or absence of spironolactone were determined in both, BN and BNK rats.

4.4.1.1. URINARY LMW KININOGEN

Determination of urinary LMW kininogen should describe potential differences between BN control rats and kininogen-deficient BNK rats. Measurement of LMW kininogen was preceded by urine digestion. BK was released from LMW kininogen by trypsin added to the incubation reaction. BK was then determined by a specific RIA. Values are presented in ng BK equivalent per 24h.

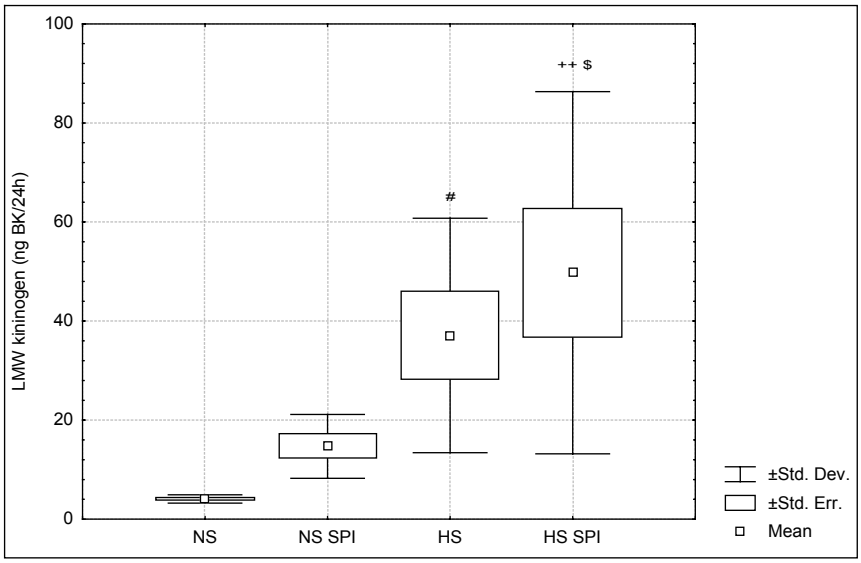


Fig.57a **Urinary LMW kininogen in BK equivalent** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. # p<0,05 HS vs. NS; ++ p<0,05 HS SPI vs. NS; $ p<0,05 HS SPI vs. SPI.

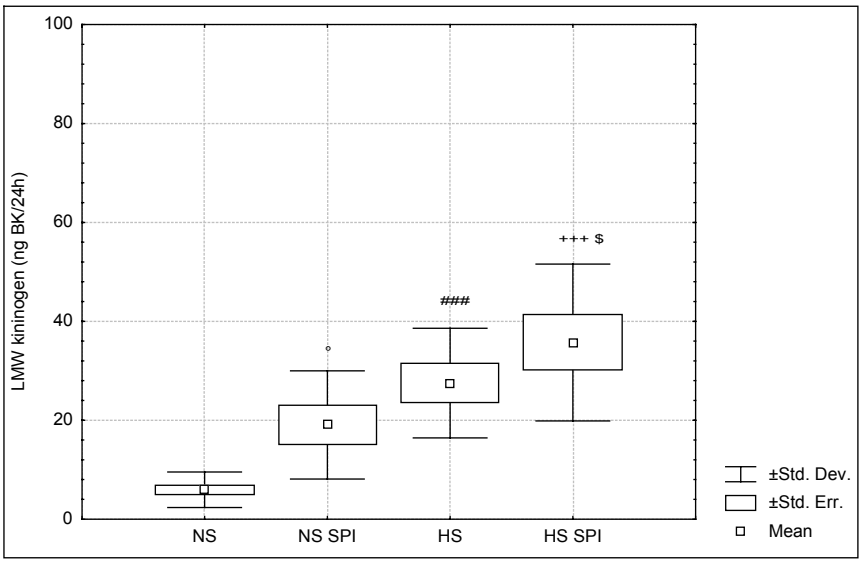


Fig.57b **Urinary LMW kininogen in BK equivalent** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. ° p<0,05 NS SPI vs. NS; ### p<0,001 HS vs. NS; +++ p<0,001 HS SPI vs. NS; $ p<0,05 HS SPI vs. SPI.

COMPARISON OF BN AND BNK RATS

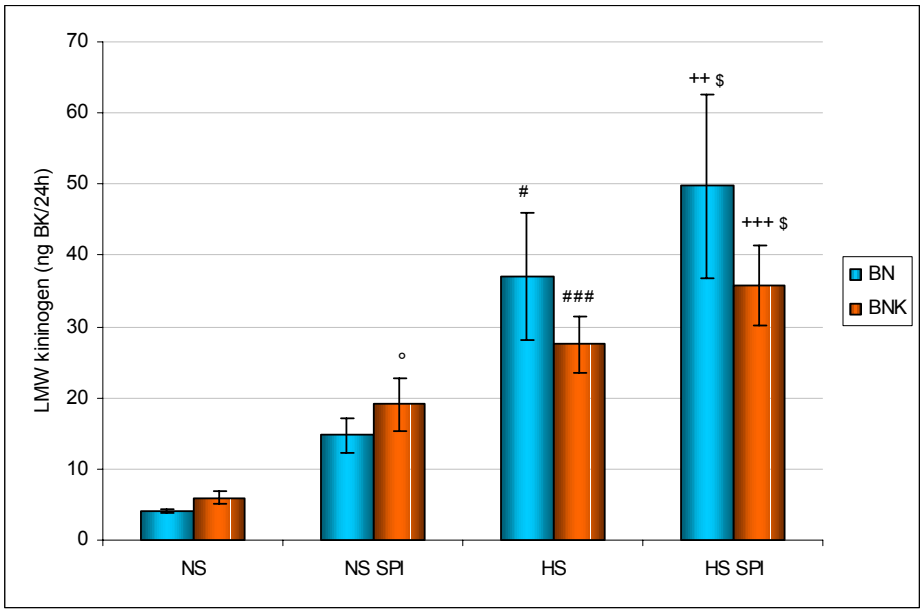


Fig.57c **Urinary LMW kininogen in BK equivalent** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=14. ° p<0,05 NS SPI vs. NS in rats of the same strain; # p<0,05; ### p<0,001 HS vs. NS in rats of the same strain; ++ p<0,01; +++ p<0,001 HS SPI vs. NS in rats of the same strain; $ p<0,05 HS SPI vs. NS SPI in rats of the same strain.

Determination of LMW kininogen in urine revealed no significant differences between BN control rats and kininogen-deficient BNK rats (Fig.57c). In both strains, the excretion of urinary LMW kininogen increased following treatment with spironolactone. Also high salt diet led to a significant increase in urinary LMW kininogen levels. The urinary LMW excretion was further enhanced in rats fed with high salt diet and simultaneously treated with spironolactone. In BNK rats, the elevation of LMW kininogen excretion following high salt diet, independently of spironolactone, was less pronounced than that of BN rats.

4.4.1.2. URINARY KALLIKREIN ACTIVITY

Urinary kallikrein (tKLK) represents renal tissue kallikrein that is secreted into urine where it is generating urinary kinins. It is believed that tissue kallikrein generates KLP

from the renal LMW kininogen. Tissue kallikrein activity in urine was measured amidolytically using a selective chromogenic substrate. Absorbance was then measured in a spectrometer.

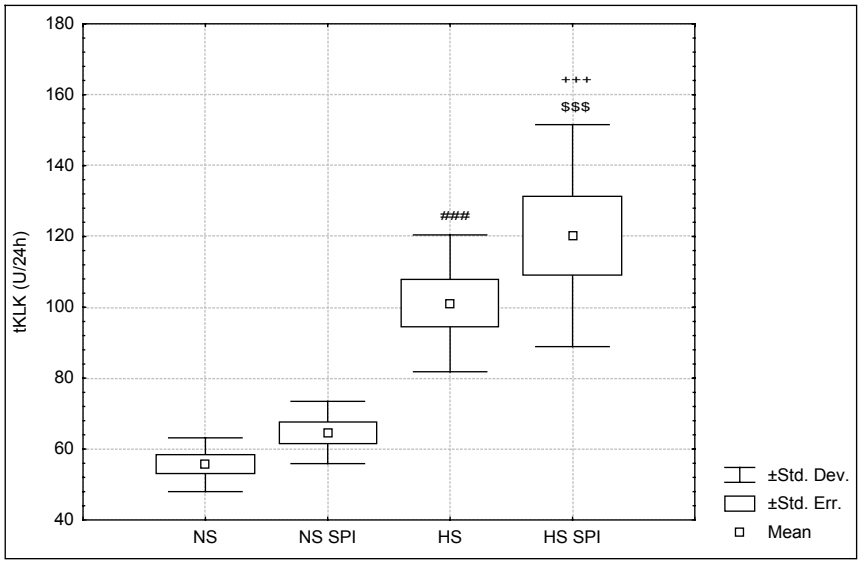


Fig.58a **Urinary kallikrein activity** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. ### p<0,001 HS vs. NS; +++ p<0,001 HS SPI vs. NS; $$$ p<0,001 HS SPI vs. NS SPI.

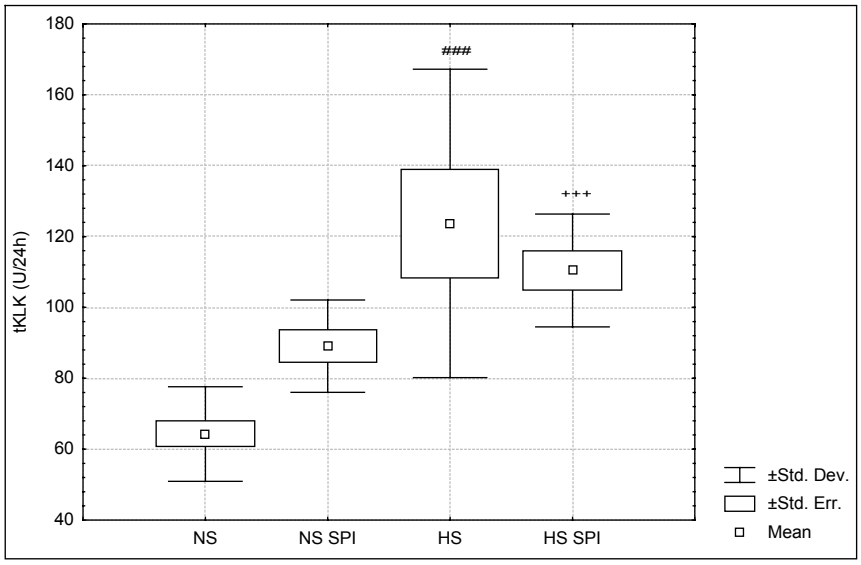


Fig.58b **Urinary kallikrein activity** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. ### p<0,001 HS vs. NS; +++ p<0,001 HS SPI vs. NS.

COMPARISON OF BN AND BNK RATS

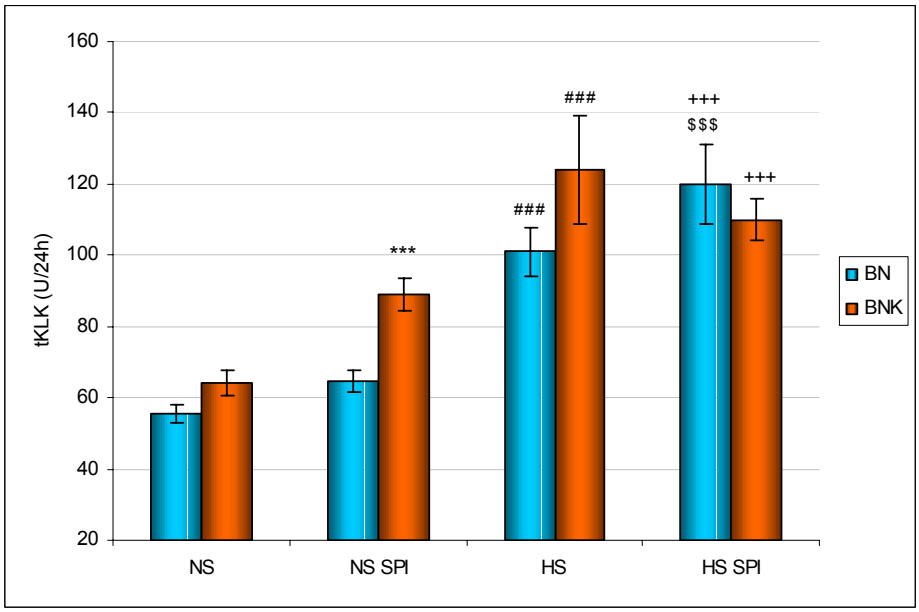


Fig.58c **Urinary kallikrein activity** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=14. ***** p<0,001 BNK rats vs. BN rats**; ### p<0,001 HS vs. NS in rats of the same strain; +++ p<0,001 HS SPI vs. NS in rats of the same strain; $$$ p<0,001 HS SPI vs. NS SPI in rats of the same strain.

The results of daily tissue kallikrein activity (U/24h) were calculated by multiplying the measured activity (U/ml) with urine volume (ml/24h). Daily activity of urinary kallikrein (Fig.58c) remarkably agreed with daily urine production (Fig.43c).

A correlation between daily tissue kallikrein activity and 24h urine volume is shown in Fig.59. Pearson's correlation test calculated the correlation coefficient (r) of 0,95 and p value of 1,080E-036; n=70. It confirmed that both, daily tissue kallikrein activity and 24h urine volume increased together (the more urine volume, the more urinary kallikrein).

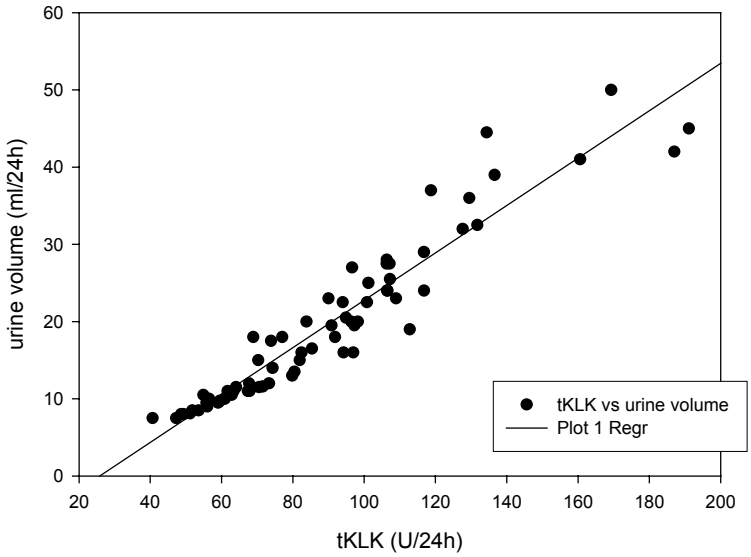


Fig.59 Correlation of daily urinary kallikrein activity and 24h urine volume with a regression line.

It can be assumed that the urinary activity represented the concentration of tissue kallikrein in urine. From this point of view, it seemed that the renal tissue kallikrein was produced at a constant rate and its urinary secretion was influenced by the urinary flow.

4.4.1.3. URINARY KALLIDIN-LIKE-PEPTIDE

As already stated, the description of KLP existence in rat is very recent. It is believed that KLP is cleaved from LMW kininogen by tissue kallikrein. Measurements of urinary KLP should describe this new rat kinin and reveal potential differences between BN control rats and kininogen-deficient BNK rats. Characterisation of the effects of treatment will describe its importance in the regulation of renal functions. Urinary KLP was determined with a specific RIA.

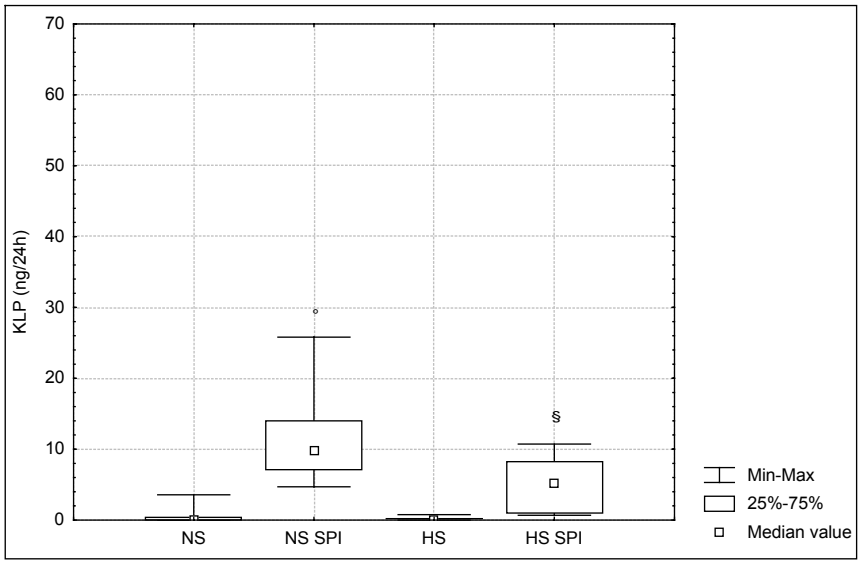


Fig.60a **Urinary KLP excretion** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. ° p<0,05 NS SPI vs. NS; § p<0,05 HS SPI vs. HS.

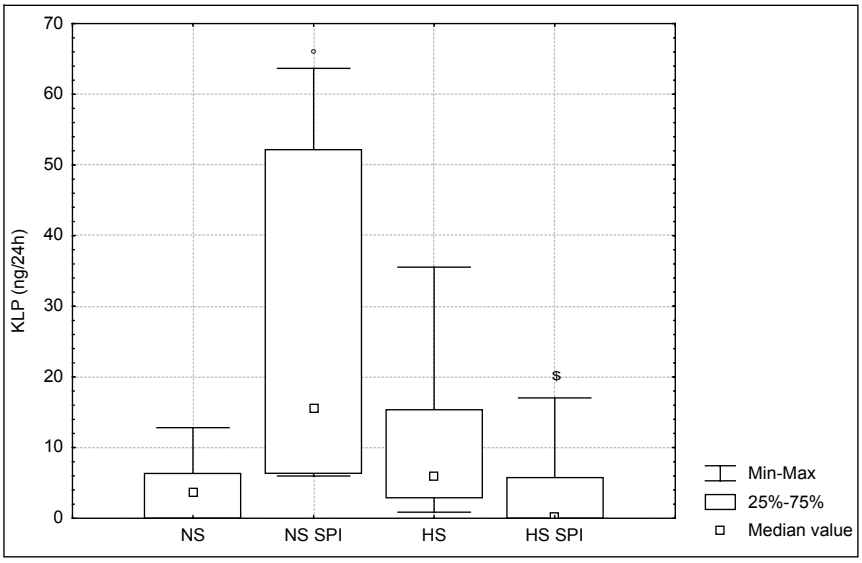


Fig.60b **Urinary KLP excretion** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. ° p<0,05 NS SPI vs. NS; $ p<0,05 HS SPI vs. NS SPI.

COMPARISON OF BN AND BNK RATS

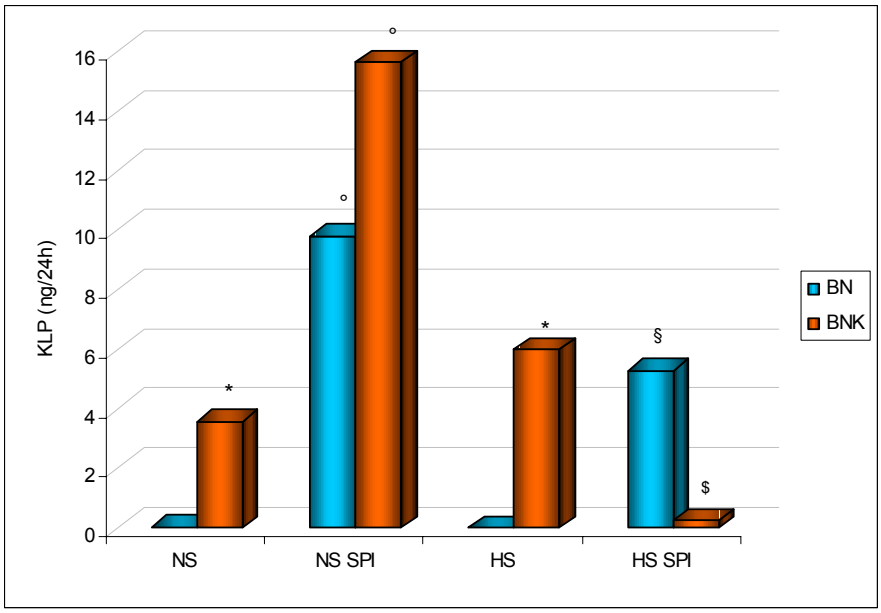


Fig.60c **Urinary KLP excretion** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the median, n=8 in all groups except of BNK NS, where n=14**. * p<0,05 BNK rats vs. BN rats**; ° p<0,05 NS SPI vs. NS in rats of the same strain; § p<0,05 HS SPI vs. HS in rats of the same strain; $ p<0,05 HS SPI vs. NS SPI in rats of the same strain.

Under standard conditions, the KLP was detectable only in urine of BNK rats. In BN rats, the KLP concentration in urine was below the detection limit of the assay. Treatment with spironolactone caused a significant elevation of urinary KLP levels in both strains. Spironolactone's effect was more pronounced in BNK rats. In BN rats fed with high salt diet, the KLP levels in urine stayed below the detection limit. On the contrary, urinary KLP concentration slightly increased in BNK rats. In rats fed with high salt diet and simultaneously treated with spironolactone, the urinary KLP levels increased in BN rats but decreased in BNK rats (Fig.60c).

4.4.1.4. URINARY BRADYKININ

The origin of BK in rat urine is unclear. It is believed that tissue kallikrein cleaves KLP from LMW kininogen. Hence it seems likely that urinary BK is derived from KLP by the action of urinary aminopeptidases. Measurements of urinary bradykinin should

define the amount of this kinin and reveal potential differences between BN control rats and kininogen-deficient BNK rats. BK levels in urine were measured by means of a specific RIA.

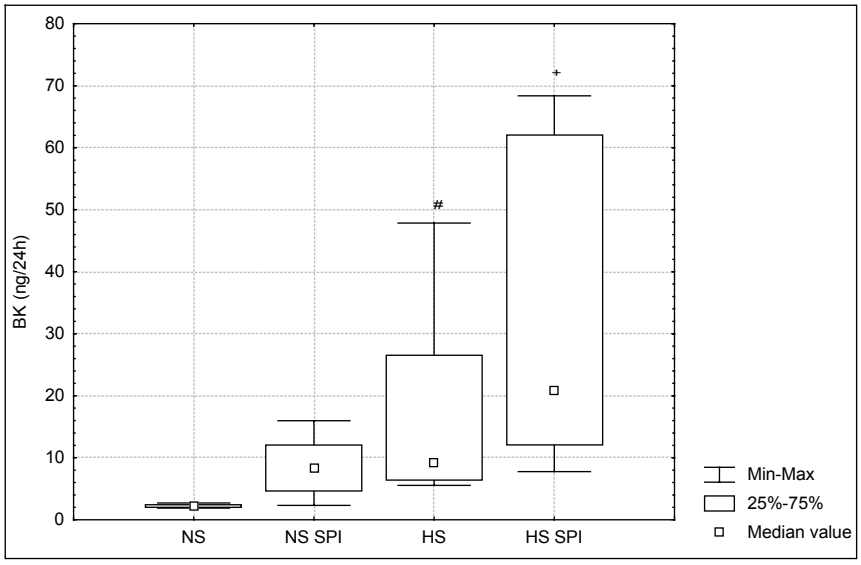


Fig.61a **Urinary BK excretion** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. # p<0,05 HS vs. NS; + p<0,05 HS SPI vs. NS.

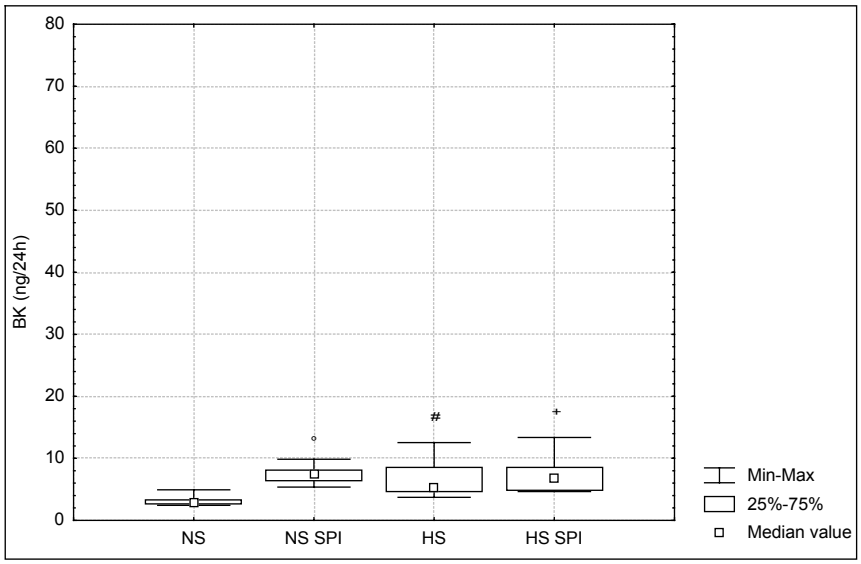


Fig.61b **Urinary BK excretion** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. ° p<0,05 NS SPI vs. NS; # p<0,05 HS vs. NS; + p<0,05 HS SPI vs. NS.

COMPARISON OF BN AND BNK RATS

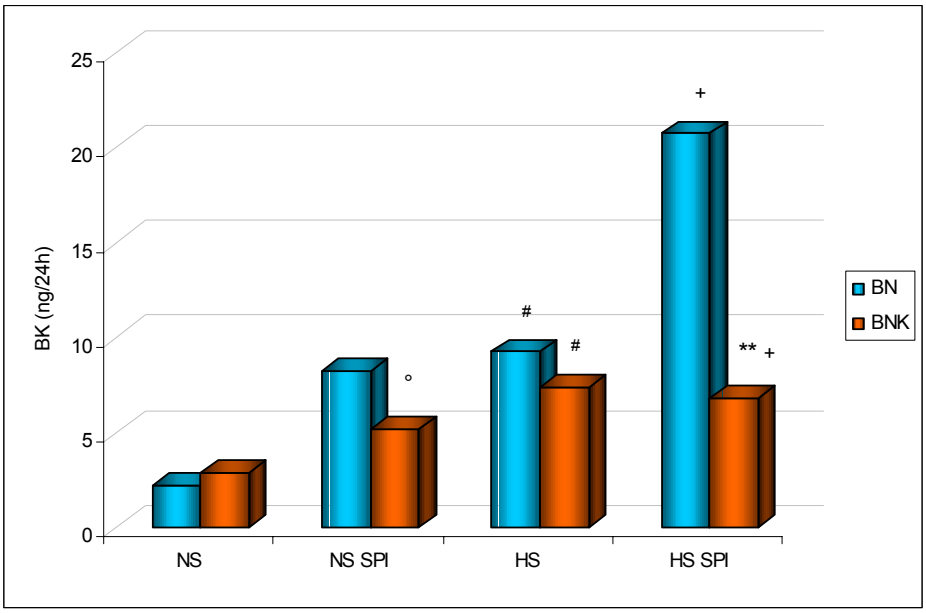


Fig.61c **Urinary BK excretion** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the median, n=8 in all groups except of BNK NS, where n=14. **** p<0,01 BNK rats vs. BN rats**; ° p<0,05 NS SPI vs. NS in rats of the same strain; # p<0,05 HS vs. NS in rats of the same strain; + p<0,05 HS SPI vs. NS in rats of the same strain.

The basal excretion of urinary BK was similar in BN and BNK rats (Fig.61c). In both strains, treatment with spironolactone elevated urinary BK levels. Similarly, BK excretion significantly increased in rats fed with high salt diet. These effects were more pronounced in BN rats than in BNK rats, which was in contrast to urinary KLP. Similarly to urinary excretion of KLP, spironolactone treatment during high salt diet further increased BK concentration in urine in BN but not in BNK rats.

4.4.2. URINARY ENDOTHELIN-1

Basal urinary levels of ET-1 in BN control rats and kininogen-deficient BNK rats were investigated. Measurements should reveal relations between ET, KKS and aldosterone, following standard diet or high salt diet in presence or absence of spironolactone. Concentrations of ET-1 in urine were determined by a specific RIA.

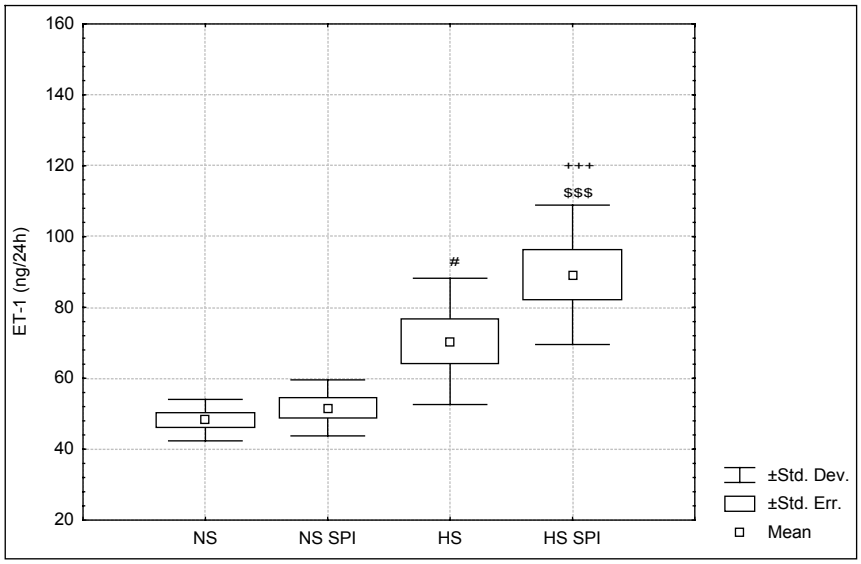


Fig.62a **Urinary ET-1 excretion** in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. # p<0,05 HS vs. NS; +++ p<0,001 HS SPI vs. NS; $$$ p<0,001 HS SPI vs. NS SPI.

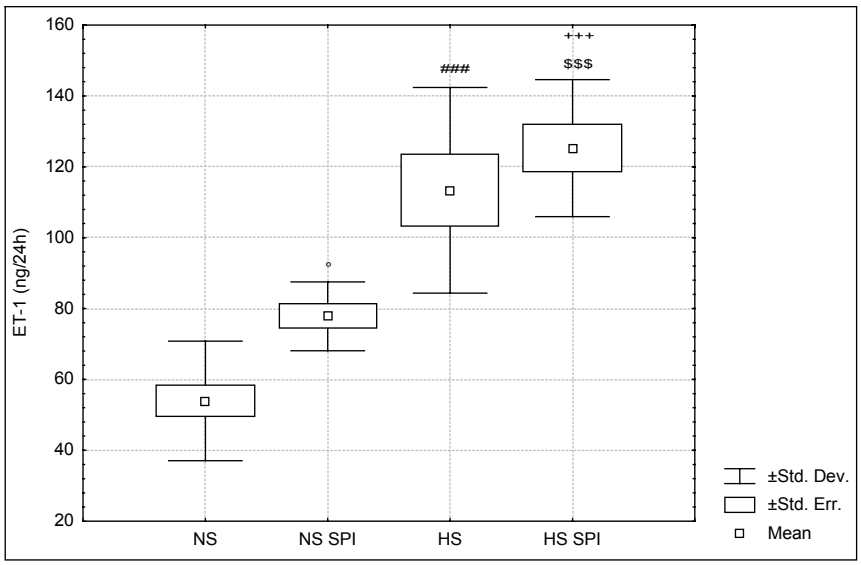


Fig.62b **Urinary ET-1 excretion** in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. ° p<0,05 NS SPI vs. NS; ### p<0,001 HS vs. NS; +++ p<0,001 HS SPI vs. NS; $$$ p<0,001 HS SPI vs. NS SPI.

COMPARISON OF BN AND BNK RATS

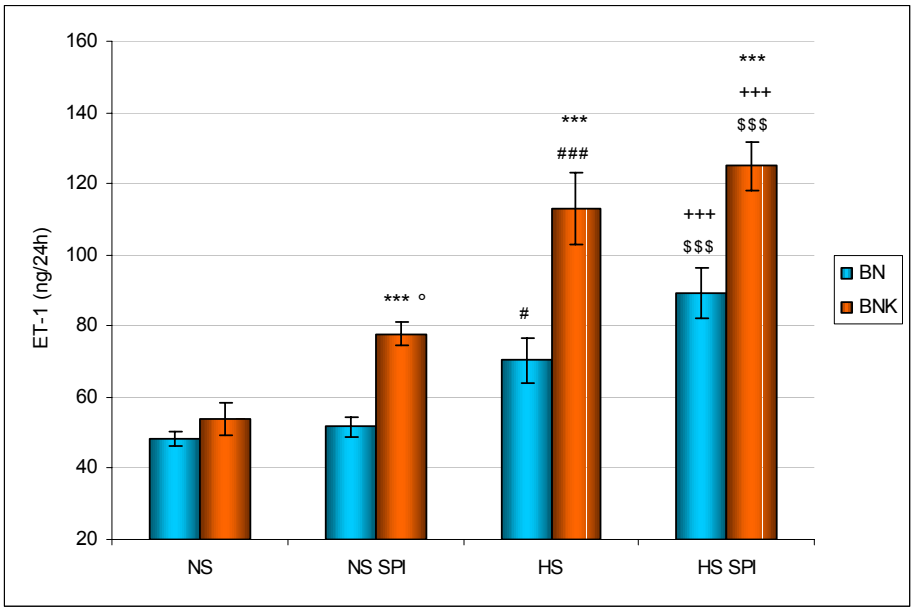


Fig.62c **Urinary ET-1 excretion** in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=14. ***** p<0,001 BNK rats vs. BN rats**; ° p<0,05 NS SPI vs. NS in rats of the same strain; $^{\#}$ p<0,05; $^{\#\#\#}$ p<0,001 HS vs. NS in rats of the same strain; $^{+++}$ p<0,001 HS SPI vs. NS in rats of the same strain; $^{\$\$\$}$ p<0,001 HS SPI vs. NS SPI in rats of the same strain.

Basal urinary ET-1 levels were similar in BN control rats and kininogen-deficient BNK rats (Fig.62c). In all experimental groups, urinary ET-1 was significantly higher in BNK rats than in BN rats. In BNK rats, treatment with spironolactone significantly elevated urinary ET-1 levels suggesting that aldosterone may inhibit urinary ET-1 secretion. In BN rats, urinary ET-1 levels did not change from their respective basal levels. High salt diet significantly increased urinary ET-1 excretion. In BNK rats, ET-1 levels in urine were higher than following spironolactone and almost twice as high as in BN rats. In BN and BNK rats fed high salt diet in presence of spironolactone, urinary ET-1 levels further increased.

4.4.3. ALDOSTERONE AND ITS PRECURSORS IN URINE

Aldosterone is formed from DOC. The conversion involves three consecutive reactions and is catalyzed by aldosterone synthase. In parallel, DOC is also converted to corticosterone by the action of 11ß-hydroxylase. Basal corticoid concentrations in BN control rats and kininogen-deficient BNK rats were investigated. The effects of standard salt diet or high salt diet in presence or absence of spironolactone were determined in both strains.

4.4.3.1 URINARY DEOXYCORTICOSTERONE

DOC is a mineralocorticoid hormone and a substrate for 11ß-hydroxylase and aldosterone synthase that catalyse the formation of corticosterone and aldosterone, respectively. DOC levels were determined by RIA after its selective extraction from urine.

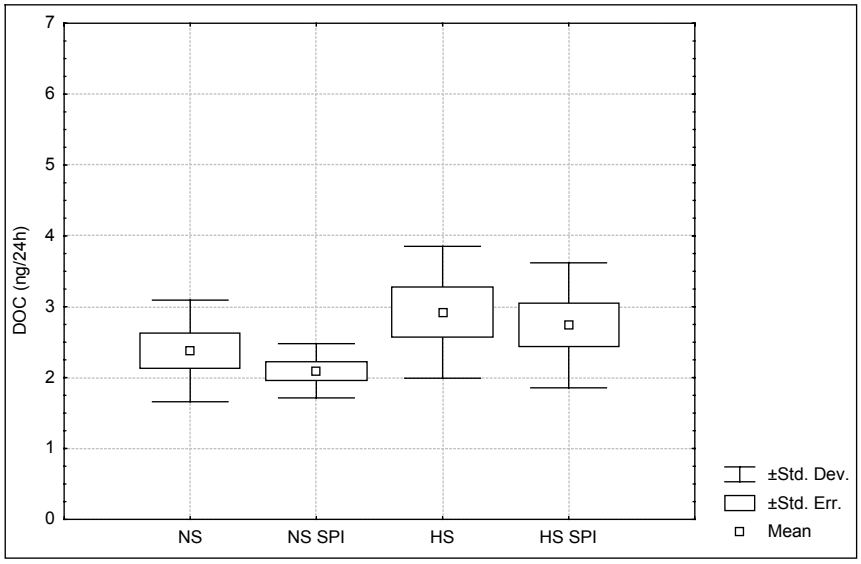


Fig.63a **Urinary deoxycorticosterone** levels in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8.

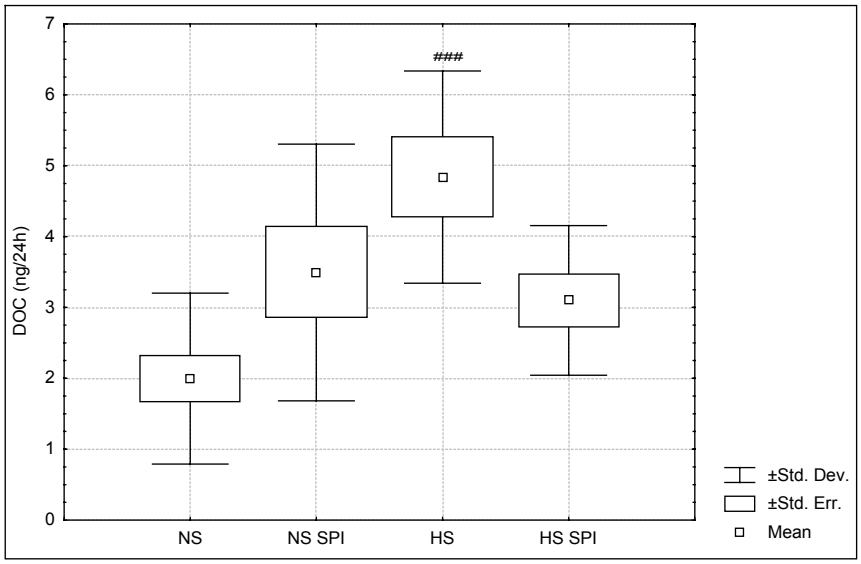


Fig.63b **Urinary deoxycorticosterone** levels in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. ### p<0,001 HS vs. NS.

COMPARISON OF BN AND BNK RATS

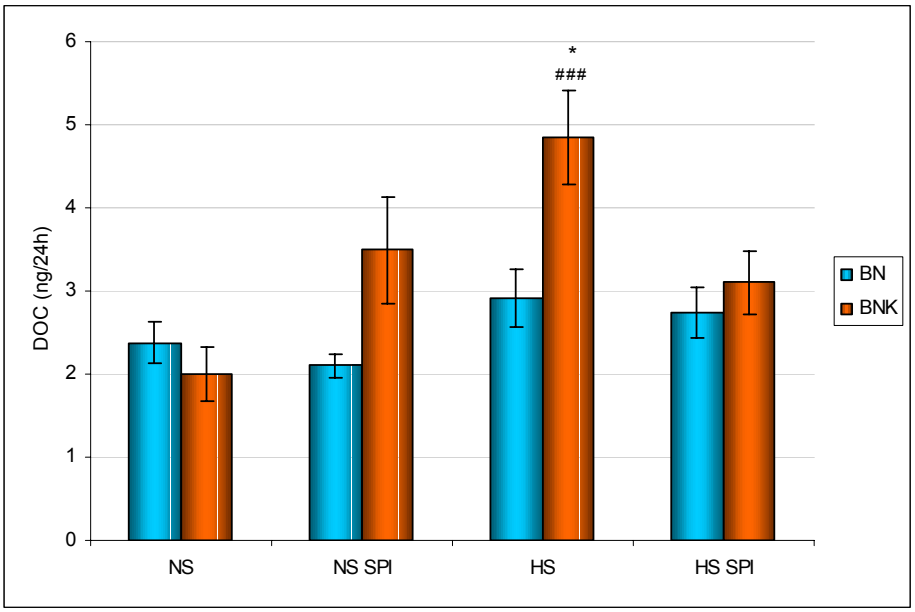


Fig.63c **Urinary deoxycorticosterone** levels in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=14. *** p<0,05 BNK rats vs. BN rats**; ### p<0,001 HS vs. NS in rats of the same strain.

Basal DOC levels in urine were similar in BN control rats and kininogen-deficient BNK rats (Fig.63c). In BNK rats, spironolactone elevated urinary DOC levels. High salt diet significantly increased urinary DOC levels. Combination of both, high salt diet and spironolactone restored elevated DOC levels back to basal values (Fig.63b). In BN rats, neither spironolactone nor high salt diet altered urinary DOC levels (Fig.63a).

4.4.3.2. URINARY CORTICOSTERONE

Corticosterone is the main glucocorticoid hormone in rat. Corticosterone is an end-product of 11ß-hydroxylation and an intermediate during aldosterone synthesis. Corticosterone levels were determined by RIA after its selective extraction from urine.

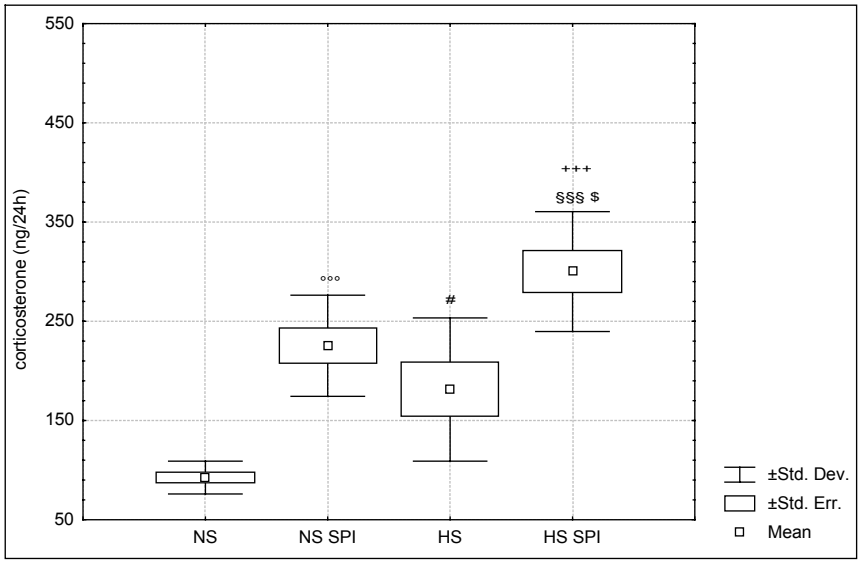


Fig.64a **Urinary corticosterone** levels in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. °°° p<0,001 NS SPI vs. NS; # p<0,05 HS vs. NS; +++ p<0,001 HS SPI vs. NS; §§§ p<0,001 HS SPI vs. HS; $ p<0,05 HS SPI vs. NS SPI.

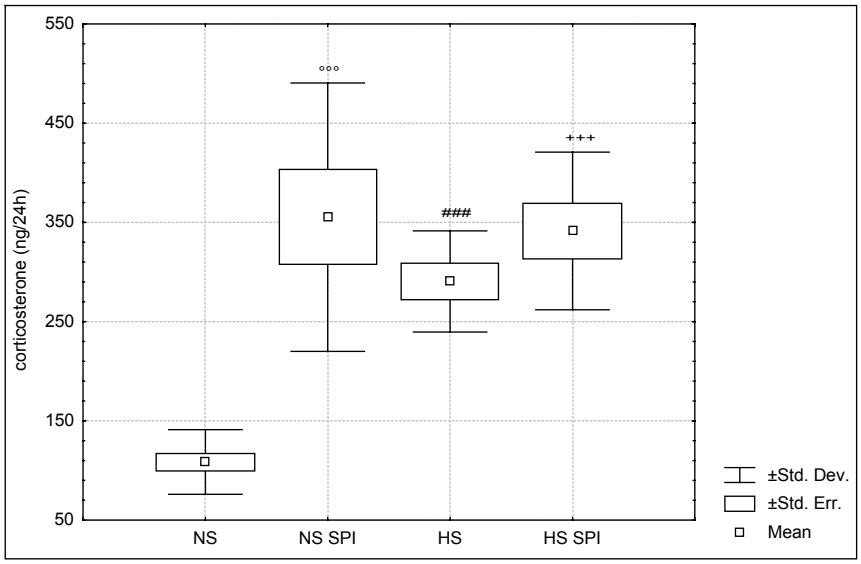


Fig.64b **Urinary corticosterone** levels in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. °°° p<0,001 NS SPI vs. NS; # p<0,05; ### p<0,001 HS vs. NS; +++ p<0,001 HS SPI vs. NS.

COMPARISON OF BN AND BNK RATS

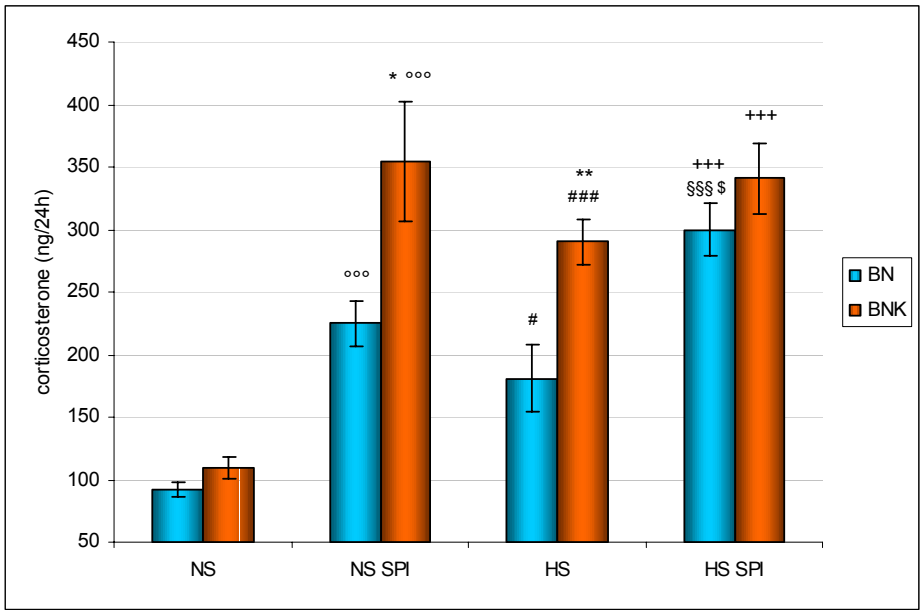


Fig.64c **Urinary corticosterone** levels in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=14. *** p<0,05; ** p<0,01 BNK rats vs. BN rats**; °°° p<0,001 NS SPI vs. NS in rats of the same strain; # p<0,05; ### p<0,001 HS vs. NS in rats of the same strain; +++ p<0,001 HS SPI vs. NS; §§§ p<0,001 HS SPI vs. HS; $ p<0,05 HS SPI vs. NS SPI.

Basal corticosterone levels in urine were almost identical in BN control rats and kininogen-deficient BNK rats (Fig.64c). Treatment with spironolactone and high salt diet caused a significant increase in corticosterone levels in both rat strains. These changes were significantly more pronounced in kininogen-deficient BNK rats.

4.4.3.3. URINARY ALDOSTERONE

Aldosterone is the main mineralocorticoid hormone. The formation of aldosterone from DOC is catalysed by aldosterone synthase. Aldosterone levels were determined by RIA after its selective extraction from urine.

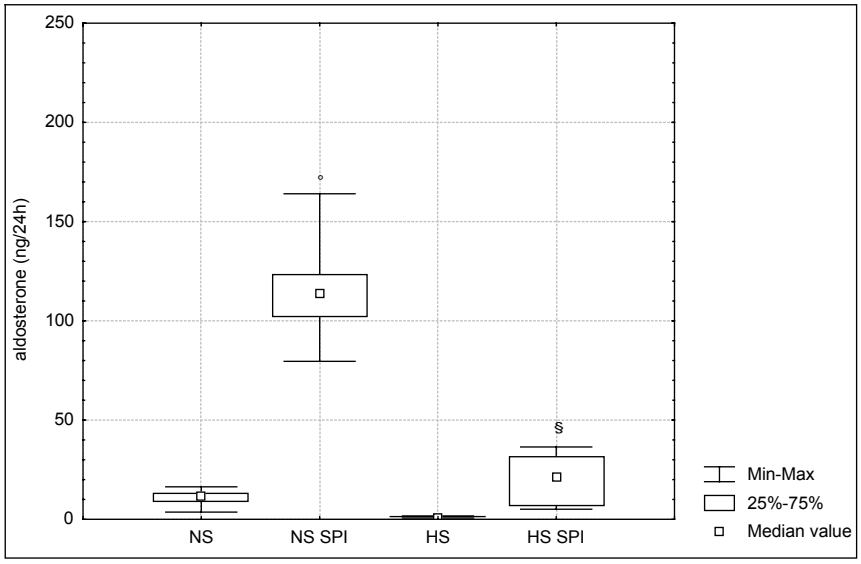


Fig.65a **Urinary aldosterone** levels in **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. ° $p<0,05$ NS SPI vs. NS in rats of the same strain; § $p<0,001$ HS SPI vs. HS.

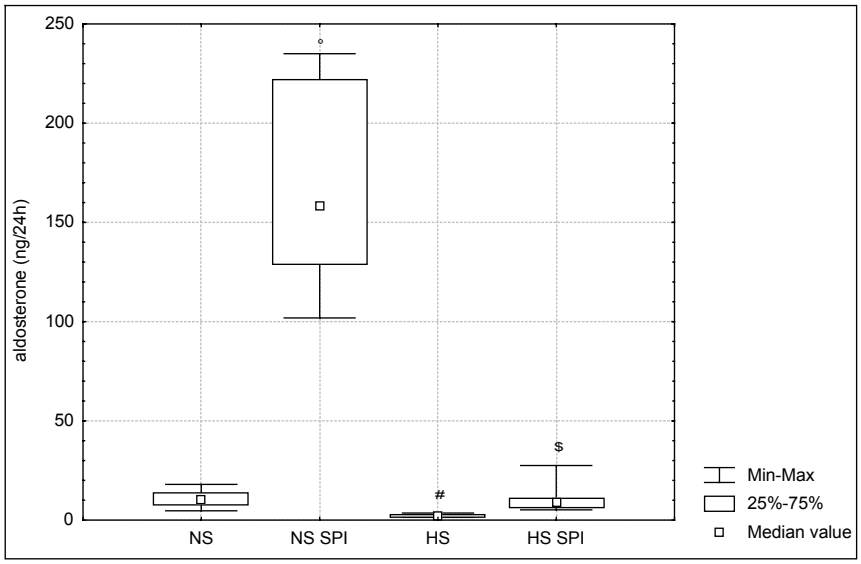


Fig.65b **Urinary aldosterone** levels in **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=14. ° p<0,05 NS SPI vs. NS; # p<0,05 HS vs. NS; $ p<0,05 HS SPI vs. NS SPI.

COMPARISON OF BN AND BNK RATS

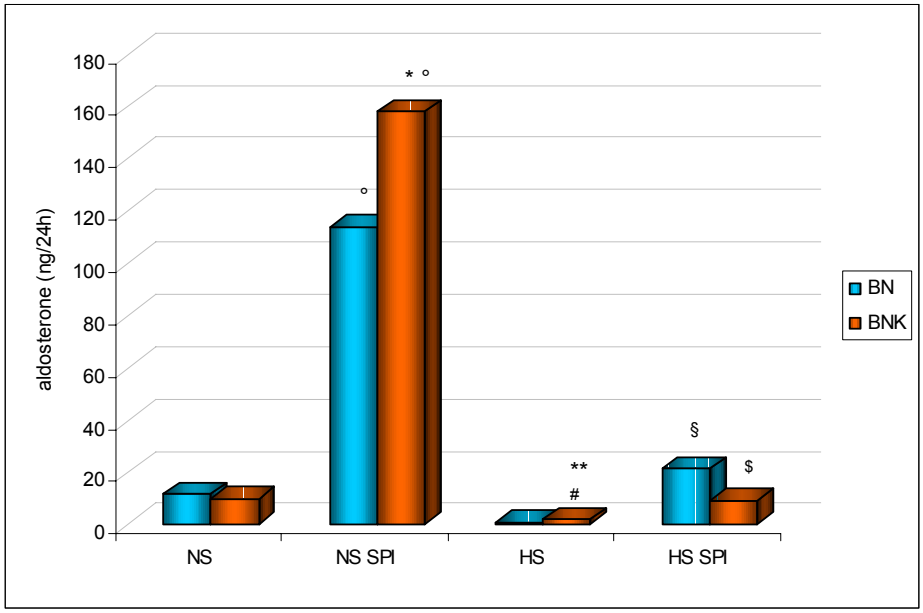


Fig.65c **Urinary aldosterone** levels in BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the median, n=8 in all groups except of BNK NS, where n=14. *** p<0,05; ** p<0,01 BNK rats vs. BN rats**; ° p<0,05 NS SPI vs. NS in rats of the same strain; # p<0,05 HS vs. NS in rats of the same strain; § p<0,001 HS SPI vs. HS in rats of the same strain; $ p<0,05 HS SPI vs. NS SPI in rats of the same strain.

Basal urinary aldosterone levels were identical in BN control rats and kininogen-deficient BNK rats (Fig.65c). In both strains, spironolactone markedly increased urinary aldosterone levels showing that spironolactone replaced aldosterone at its receptor binding sites. The increase in urinary aldosterone levels was significantly greater in BNK rats than in BN rats. In both strains, high salt diet decreased urinary aldosterone levels to a minimum. In BNK rats, aldosterone levels were still significantly higher than in BN rats. In rats fed with high salt diet in presence of spironolactone, the measurements of urinary aldosterone revealed that even under conditions of high Na^+ intake, the MR is occupied by residual aldosterone.

SUMMARY OF URINE PROFILE

Analysis of urine revealed no significant between-strain differences in the activity of renal KKS with the exception of KLP. The basal urinary excretion of LMW kininogen was similar in both strains. Spironolactone treatment as well as high salt diet caused an increase in urinary LMW kininogen levels in both, BN and BNK rats. Also the activity of urinary kallikrein was almost identical in both rat strains. The excretion of renal kallikrein was primarily influenced by the urine volume. Spironolactone and high salt diet increased urine volume that consequently led to an increase in the urinary kallikrein activity. Under standard conditions, the urinary KLP was undetectable in BN rats in contrast to BNK rats. Spironolactone treatment significantly increased the renal excretion of KLP in both strains. High salt diet elevated urinary KLP levels in BNK rats but not in BN rats. In rats fed with high salt diet, treatment with spironolactone caused an increase in KLP excretion in BN rats and a decrease in BNK rats. In both strains, the basal BK excretion was similar. Spironolactone elevated BK urinary levels, to a less extent than those of KLP. Also in rats fed with high salt diet, urinary BK excretion significantly increased. Combination of high salt diet and spironolactone further increased BK excretion in BN rats but not in BNK rats.

Urinary excretion of ET-1 was analogous in both rat strains. Spironolactone treatment caused a significant increase in urinary ET-1 levels in BNK rats but not in BN rats. In both strains, high salt diet increased the ET-1 levels in urine, in BNK rats to a significantly greater extent than in BN rats.

The urinary excretion of all three corticoid hormones, DOC, corticosterone and aldosterone, was identical in both rat strains. In BN rats, the DOC levels in urine remained unchanged in all experimental groups. In BNK rats, spironolactone as well as high salt diet increased the urinary excretion of DOC. Similarly, urinary corticosterone increased following spironolactone and high salt diet in both strains. This increase was more pronounced in BNK rats. Treatment with spironolactone substantially increased urinary levels of aldosterone in both strains. In BNK rats, spironolactone replaced significantly more aldosterone from the MR. In both strains, high salt diet decreased aldosterone levels to a minimum. Nevertheless, urinary aldosterone was significantly higher in BNK rats than in BN rats. In rats fed with high salt diet, residual aldosterone was replaced from the MR by spironolactone.

4.5. TISSUE ANALYSIS

In the brain tissue, a local steroid synthesis was described with a role in the salt sensitive hypertension. Therefore the concentrations of aldosterone and its precursors in the brain were investigated. The expression of 11ß-hydroxylase and aldosterone synthase was investigated in adrenal gland. Both enzymes catalyse the terminal stages of corticosterone and aldosterone production. This investigation should reveal potential differences in the synthesis of aldosterone between BN control rats and kininogen-deficient BNK rats. Brains and adrenals were harvested after sacrificing the animals. Brains were immediately frozen in liquid nitrogen and stored at -20°C until analysed. Adrenal glands were collected into RNA stabilising solution and stored frozen until processed.

4.5.1. BRAIN ALDOSTERONE AND ITS PRECURSORS

Measurements of DOC, corticosterone and aldosterone should characterize potential differences between both strains of rats and describe effects of treatment with salt diet and spironolactone. Steroids were extracted from brain homogenates and after selective extraction measured by RIA.

4.5.1.1. BRAIN DEOXYCORTICOSTERONE

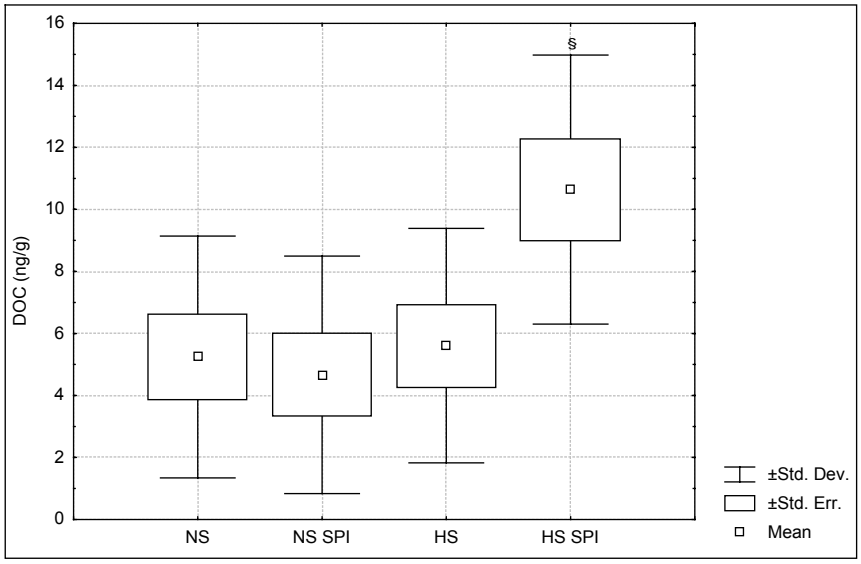


Fig.66a **Deoxycorticosterone** levels in brain of **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. § p<0,001 HS SPI vs. HS.

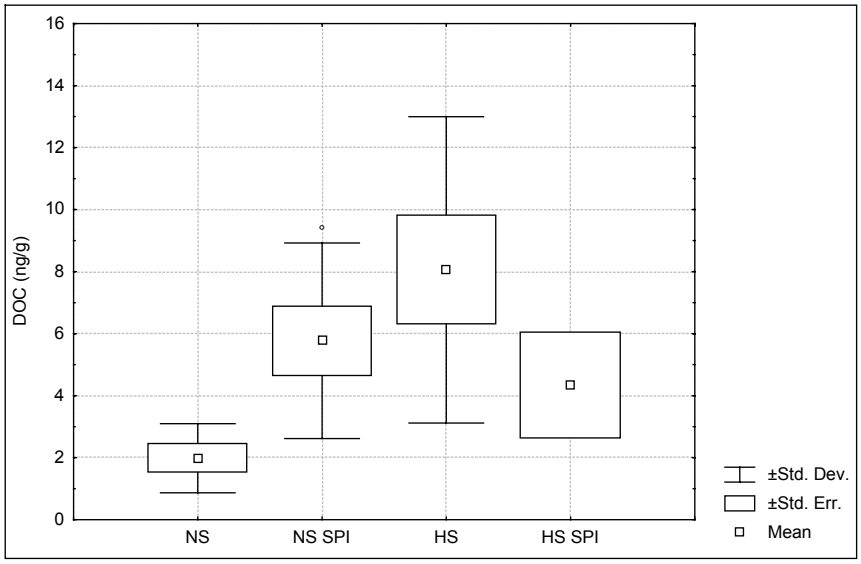


Fig.66b **Deoxycorticosterone** levels in brain of **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=6. ° p<0,05 NS SPI vs. NS.

COMPARISON OF BN AND BNK RATS

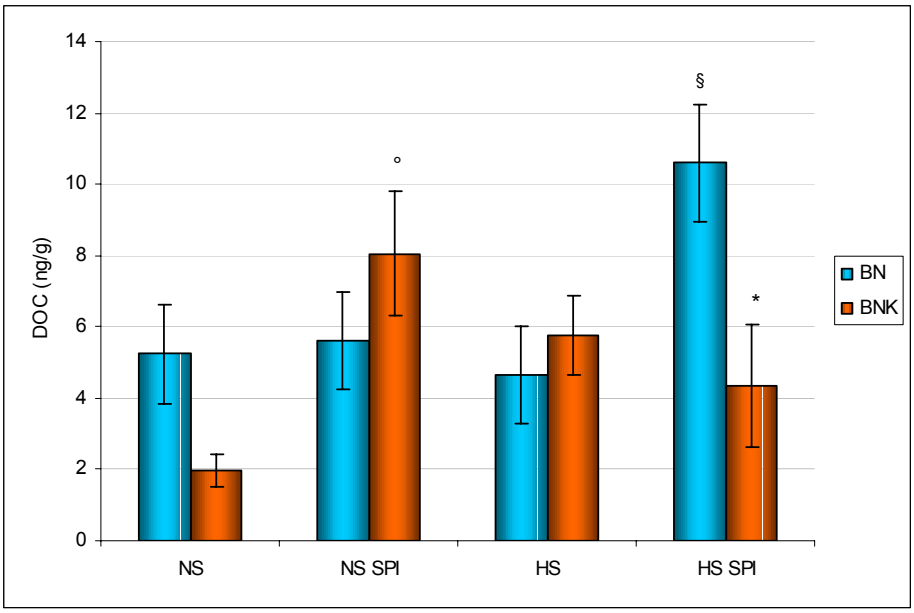


Fig.66c **Deoxycorticosterone** levels in brain of BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=6. *** p<0,05 BNK rats vs. BN rats**; ° p<0,05 NS SPI vs. NS in rats of the same strain; § p<0,05 HS SPI vs. HS in rats of the same strain.

DOC levels in brain tissue were lower in BNK rats than in BN rats (Fig.66c). The same impairment appeared in plasma and urine of BNK rats (Fig.54c and 63c). In BNK rats, spironolactone caused a significant elevation of brain DOC levels. Similarly, following high salt diet the DOC concentration in the brain tended to increase. In BN rats, neither spironolactone nor high salt diet influenced DOC concentration in the brain. In contrast, following both, spironolactone in combination with high salt diet, the DOC levels in brain tissue significantly increased.

4.5.1.2. BRAIN CORTICOSTERONE

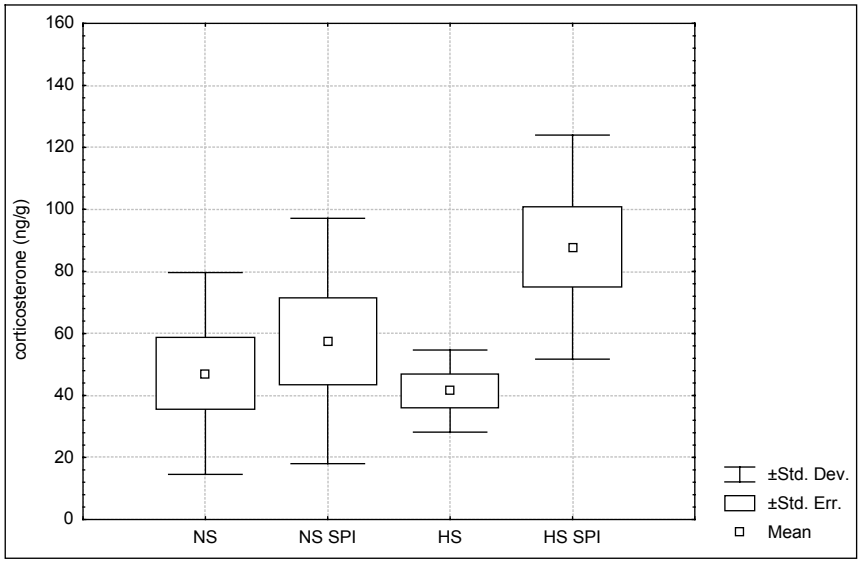


Fig.67a **Corticosterone** levels in brain of **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8.

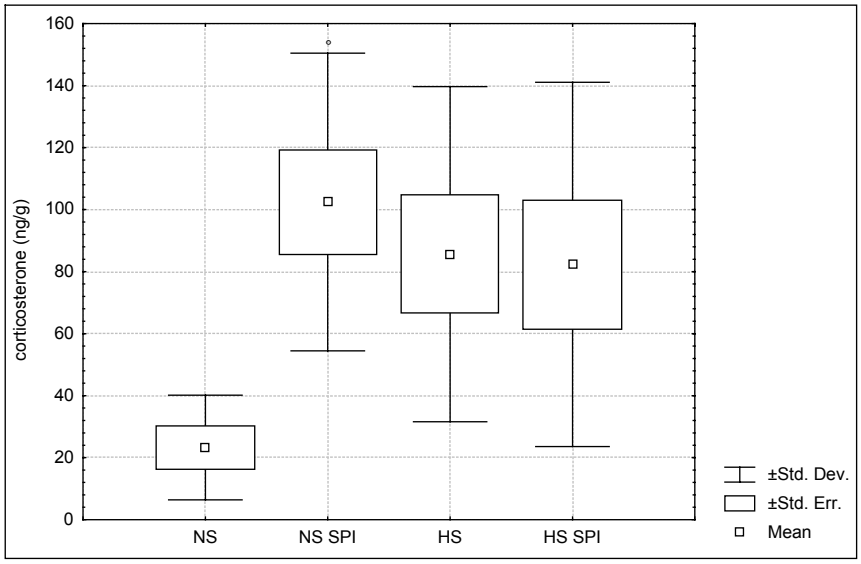


Fig.67b **Corticosterone** levels in brain of **BNK rats** after normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=6. ° p<0,05 NS SPI vs. NS.

COMPARISON OF BN AND BNK RATS

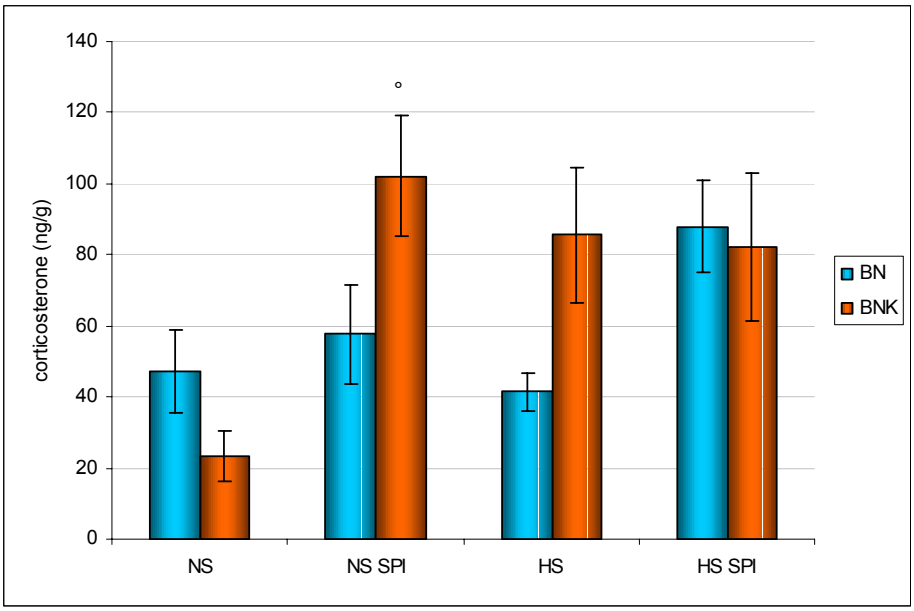


Fig.67c **Corticosterone** levels in brain of BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SEM, n=8 in all groups except of BNK NS, where n=6. ° $p<0,05$ NS SPI vs. NS in rats of the same strain.

Basal corticosterone concentration in the brain was lower in kininogen-deficient BNK rats than in BN control rats (Fig.67c). In BNK rats, spironolactone significantly increased corticosterone levels in the brain. Also following high salt diet, independently of spironolactone, brain corticosterone tended to increase (Fig.67b). In BN rats, neither spironolactone nor high salt diet influenced the corticosterone concentration in the brain. Following high salt diet and spironolactone, brain corticosterone tended to increase (Fig.67a).

4.5.1.3. BRAIN ALDOSTERONE

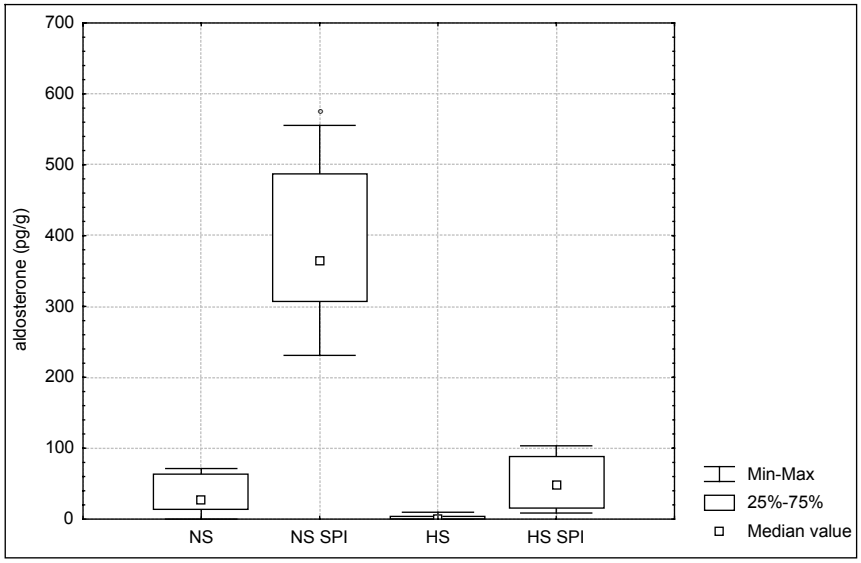


Fig.68a **Aldosterone** levels in brain of **BN rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8. ° p<0,05 NS SPI vs. NS.

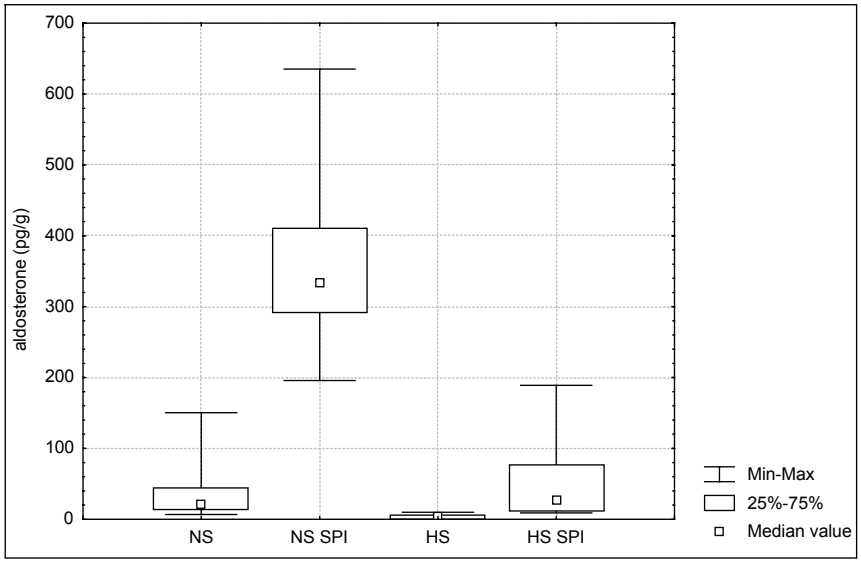


Fig.68b **Aldosterone** levels in brain of **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone, n=8 except of NS, where n=6.

COMPARISON OF BN AND BNK RATS

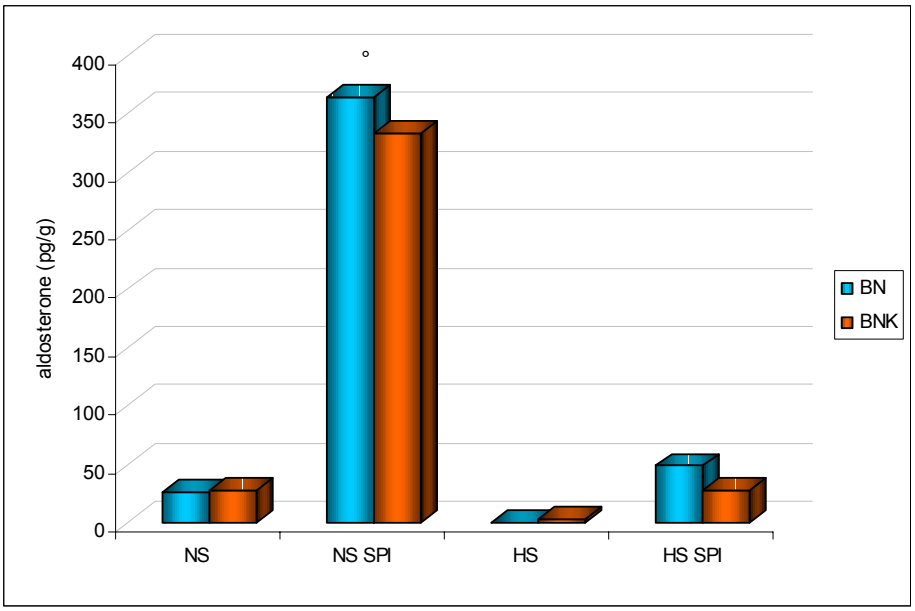


Fig.68c **Aldosterone** levels in brain of BN control rats and kininogen-deficient BNK rats fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the median, n=8 in all groups except of BNK NS, where n=6. ° $p<0,05$ NS SPI vs. NS in rats of the same strain.

In brain tissue, aldosterone levels were identical in BN control rats and kininogen-deficient BNK rats (Fig.68c). In both strains, following treatment with spironolactone, aldosterone concentration in the brain significantly increased, which demonstrated aldosterone's displacement from the MR. High salt diet lowered brain aldosterone concentration almost to the minimum. Additional administration of spironolactone restored aldosterone levels in the brain tissue to basal levels. These results demonstrated that even under conditions of increased Na^+ intake the MR is occupied by residual aldosterone.

SUMMARY OF BRAIN ALDOSTERONE AND ITS PRECURSORS

Measurements of DOC, corticosterone and aldosterone in the brain revealed no significant differences between BN control rats and kininogen-deficient BNK rats. Although between-strain differences appeared, they hardly reached statistical significance because of relative great variance among single values. Generally, results from measurements of brain corticoids were in approximate accordance with the investigation of their urinary levels. Also the effect of salt diet or spironolactone on concentration of these hormones was similar in brain and in urine, with one exception. Following high salt diet, DOC levels significantly increased in urine but remained unaffected in the brain. In BNK rats, treatment with spironolactone caused a significant increase in brain levels of DOC and corticosterone. Similar effect was demonstrated also following high salt diet when aldosterone levels were minimal.

4.5.2. EXPRESSION OF GENES CODING FOR 11ß-HYDROXYLASE AND ALDOSTERONE SYNTHASE IN ADRENAL GLAND

The 11ß-hydroxylase catalyses the conversion of DOC to corticosterone. In parallel, the aldosterone synthase catalyses three consecutive reactions forming aldosterone from DOC. Using the real time RT-PCR the expression of CYP11B1 (gene coding for 11ß-hydroxylase) and CYP11B2 (gene coding for aldosterone synthase) mRNAs in adrenals was quantified in LightCycler®. Graphs display the relative expression ratios of CYP11B1 or CYP11B2, which represents the expression of these genes standardized by a non-regulated housekeeping gene PBGD.

Fig. 117 and 118 show relative expression ratios of CYP11B1 and CYP11B2 mRNA in adrenal gland of BN control rats and kininogen-deficient BNK rats, respectively. Scale x represents the basal relative expression of these genes in the groups of standard diet. Scale y in $\log_2$ emphasizes the down- or up-regulation of these two genes in comparison to basal levels.

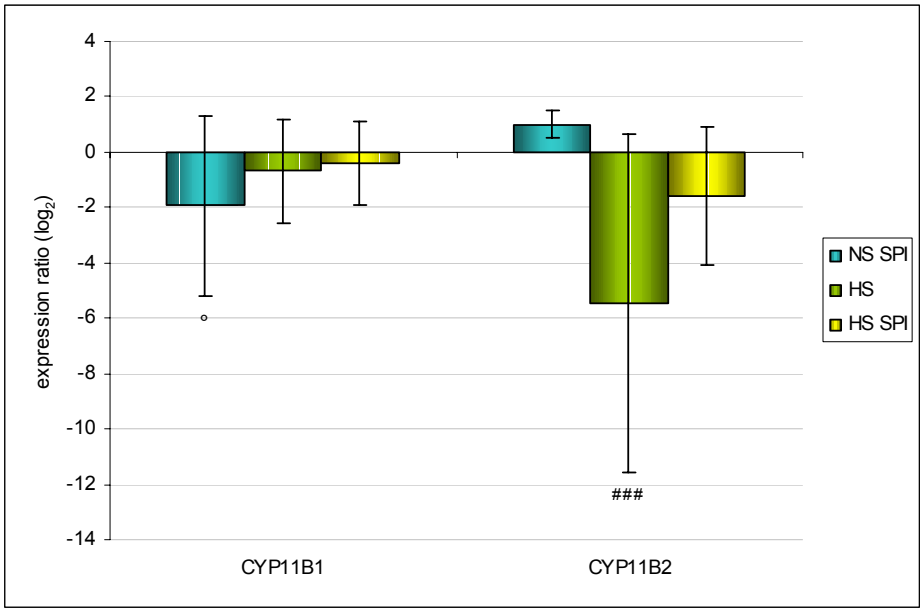


Fig.69a Relative expression ratio of **11ß-hydroxylase** (CYP11B1) and **aldosterone synthase** (CYP11B2) in adrenal gland of **BN control rats** fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SE in reference to standard diet, n=8. ° p<0,05 NS SPI vs. NS; ### p<0,001 HS vs. NS.

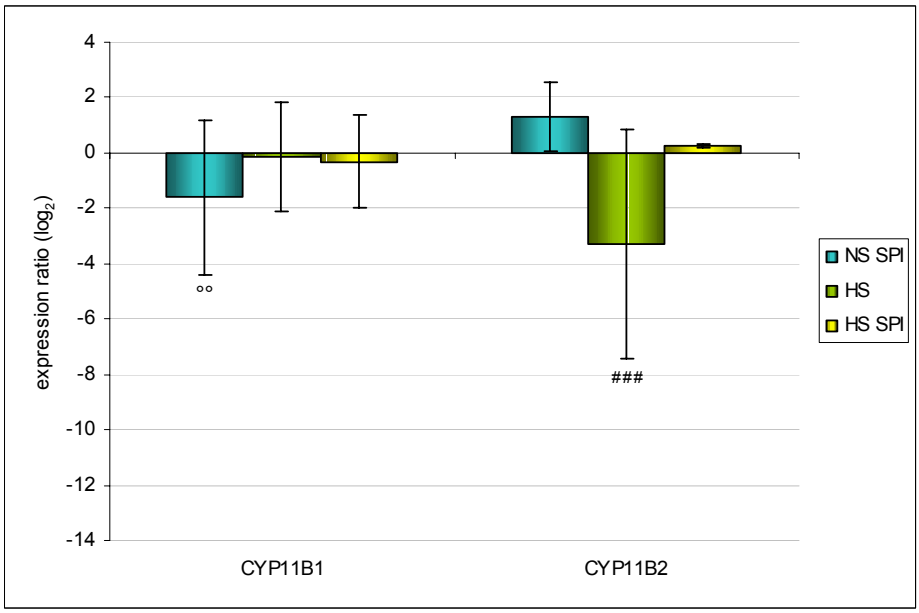


Fig.69b Relative expression ratio of **11ß-hydroxylase** (CYP11B1) and **aldosterone synthase** (CYP11B2) in adrenal gland of kininogen-deficient **BNK rats** fed with normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SE in reference to standard diet, n=8 except of NS, where n=20 and HS, where n=14. °° p<0,01 NS SPI vs. NS; ### p<0,001 HS vs. NS.

In both, BN and BNK rats, spironolactone significantly down-regulated adrenal expression of 11ß-hydroxylase. In contrast, the expression of aldosterone synthase remained unchanged (Fig.69a and 69b). High salt diet did not influence the adrenal expression of 11ß-hydroxylase, but significantly down-regulated aldosterone synthase expression in BN and BNK rats. Combination of high salt diet and spironolactone normalize the expression of these enzymes in adrenals of both strains.

COMPARISON OF BN AND BNK RATS

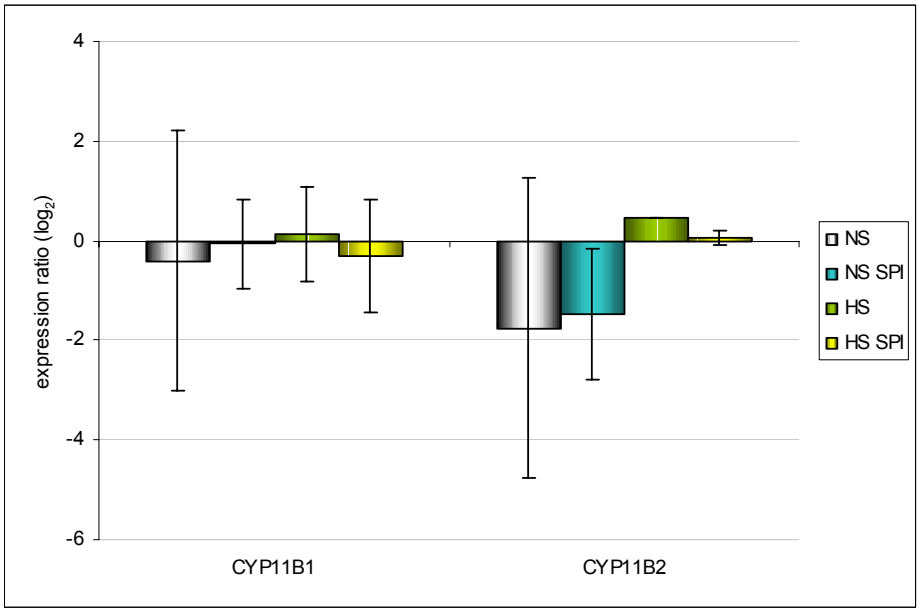


Fig.69c Differences in relative expression ratio of **11ß-hydroxylase** (CYP11B1) and **aldosterone synthase** (CYP11B2) in adrenal gland of kininogen-deficient BNK rats in reference to BN control rats after normal and high salt diet in presence or absence of spironolactone. Values are expressed as the mean ± SE, n=8 in all groups except of BNK NS, where n=20 and BNK HS, where n=14.

Comparison of adrenal expression of 11ß-hydroxylase and aldosterone synthase between BN and BNK rats is shown in Fig.69c. Scale x represents the expression ratios of both steroidogenic enzymes in BN control rats. The bars display the difference in expression in BNK rats in reference to BN rats.

Expression of adrenal 11ß-hydroxylase was identical in BN and BNK rats under all experimental conditions. The expression of aldosterone synthase was found to be lower in BNK rats than in BN rats, especially under standard conditions and following spironolactone.

SUMMARY OF EXPRESSION ANALYSIS OF 11ß-HYDROXYLASE AND ALDOSTERONE SYNTHASE IN ADRENAL GLAND

The expression of genes coding for 11ß-hydroxylase was identical in both rat strains. The basal expression of genes coding for aldosterone synthase tended to be lower in kininogen-deficient BNK rats than in BN control rats. The adrenal expression of 11ß-hydroxylase was significantly down-regulated by spironolactone, which was in contrast to the measurements of urinary corticosterone that increased following treatment with spironolactone. The adrenal expression of aldosterone synthase was significantly down-regulated by high salt diet, which agreed with minimal aldosterone levels in urine. High salt diet in combination with spironolactone restored the expression of both enzymes, CYP11B1 and CYP11B2, to basal levels.

4.6. LANGENDORFF HEART PERFUSION

The Langendorff perfusions were performed with hearts isolated from BN control rats and kininogen-deficient BNK rats fed with standard and high salt diet in presence or absence of spironolactone for 10 days. Basal cardiac parameters and the effects of 30min ischaemia, with or without antecedent IPC, on basic cardiac parameters were characterised in rats of both strains.

Line graphs show changes in heart rate (4.6.1.), left ventricular developed pressure (LVDP) (4.6.2.), left ventricular end-diastolic pressure (LVEDP) (4.6.3.), dp/dt_{max} (4.6.4.), dp/dt_{min} (4.6.5.), coronary flow (4.6.6.), and creatine kinase activity (4.6.7.) during the heart perfusion. Results are presented in two different ways:

I. COMPARISON OF BN AND BNK RATS

This type of presentation emphasizes the between-strain differences in the effects of ischemia and IPC on cardiac function. The results are shown separately for each experimental group:
a) standard diet
b) standard diet & spironolactone
c) high salt diet
d) high sat diet & spironolactone

In each graph, the same four legends occur:

BN CO – control experiment in BN hearts
BN IPC – IPC experiment in BN hearts
BNK CO – control experiment in BNK hearts
BNK IPC – IPC experiment of BNK hearts

II. EFFECT OF SALT DIET AND SPIRONOLACTONE

This type of presentation emphasizes the effect of salt diet or/and spironolactone on cardiac function after ischemia and IPC. The results are shown separately for each strain and perfusion protocol:
a) control experiment in BN hearts
b) IPC experiment in BN hearts
c) control experiment in BNK hearts
d) IPC experiment in BNK hearts

In each graph, the same four legends occur:

NS – group of standard diet (normal salt)
NS SPI – group of standard diet & spironolactone
HS – group of high salt diet
HS SPI – group of high salt diet & spironolactone

4.6.1. HEART RATE

The heart rate (HR) was derived from the left ventricular pressure trace measured by an intraventricular balloon catheter.

COMPARISON OF BN AND BNK RATS

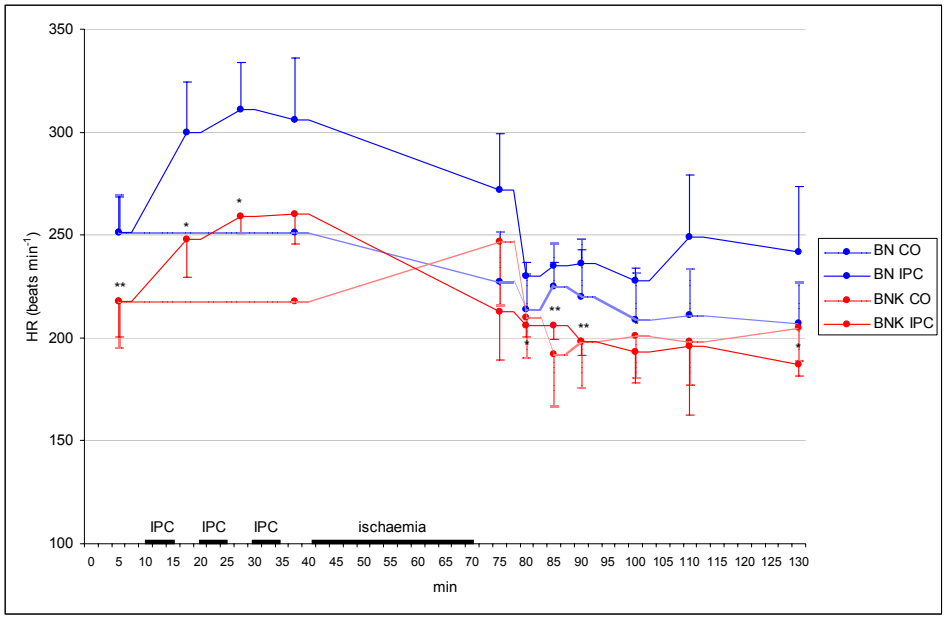


Fig.70a Time course of **heart rate** during perfusion of hearts from BN and BNK rat fed with **normal salt diet**, mean ± SEM, n=4-6. * p<0,05; ** p<0,01 BNK hearts vs. BN hearts.

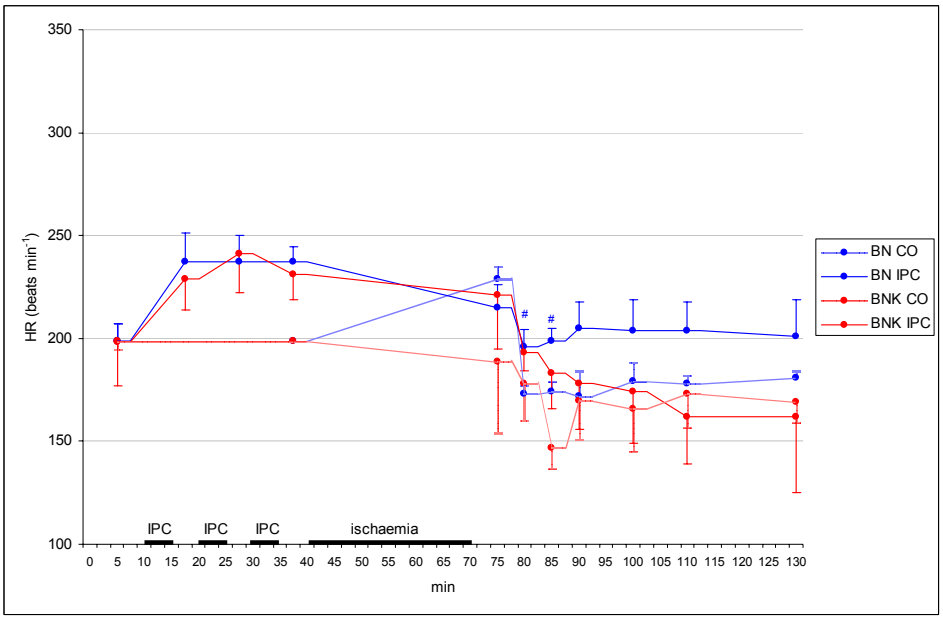


Fig.70b Time course of **heart rate** during perfusion of hearts from BN and BNK rats fed with **normal salt diet** and treated with **spironolactone**, mean ± SEM, n=4. # p<0,05 IPC vs. control experiment.

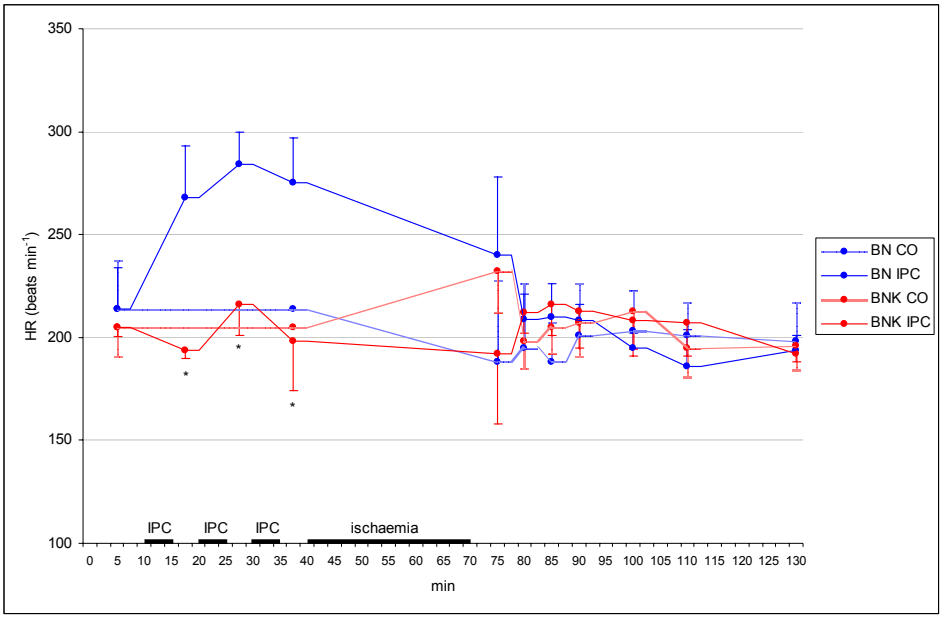


Fig.70c Time course of **heart rate** during perfusion of hearts from BN and BNK rats fed with **high salt diet**, mean ± SEM, n=4. * p<0,05 BNK hearts vs. BN hearts.

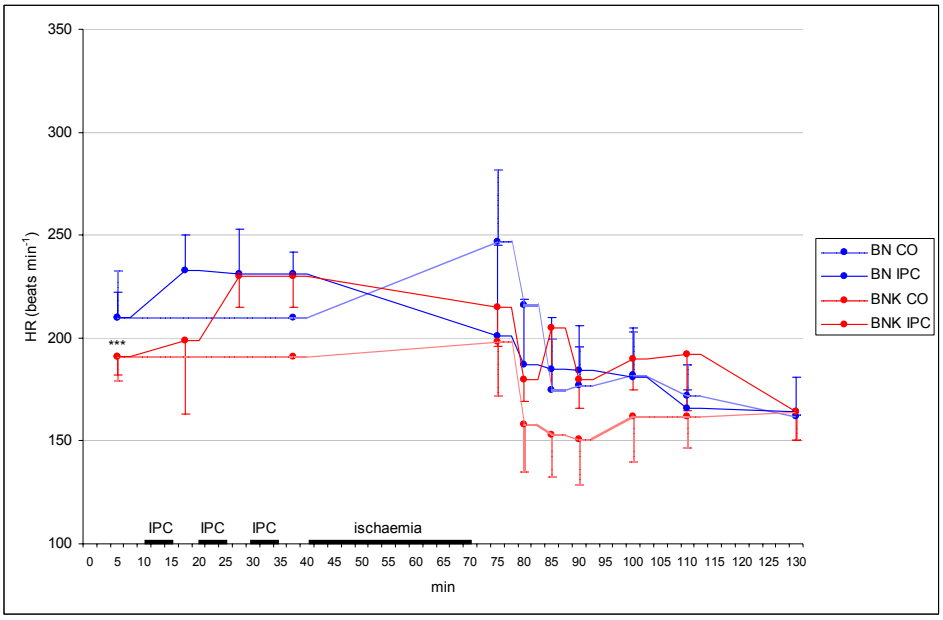


Fig.70d Time course of **heart rate** during perfusion of hearts from BN and BNK rats fed with **high salt diet** and treated with **spironolactone**, mean ± SEM, n=4. *** p<0,001 BNK hearts vs. BN hearts.

Basal heart rate was significantly lower in the hearts of kininogen-deficient BNK rats than in those of BN control rats. In BN hearts, but not in BNK hearts, IPC caused an increase in the post-ischaemic heart rate (Fig.70a). In BN rats, spironolactone

treatment lowered the heart rate whereby diminished the difference in the basal heart rate of BN and BNK hearts. In spironolactone treated BN rats but not BNK rats, IPC caused an increase in post-ischemic heart rate (Fig.70b). In both strains, high salt diet caused a decrease of heart rate. Hearts from BNK rats displayed no change in heart rate during the periods of IPC as it appeared in hearts from BN rats fed with high salt diet. Post-ischaemic heart rate was almost identical in all experimental groups (Fig.70c). Heart rate of BNK rats fed with high salt diet and simultaneously treated with spironolactone was significantly lower than that of BN control rats. Following ischaemia, the heart rate was similar in all experimental groups (Fig.70d).

EFFECT OF SALT DIET AND SPIRONOLACTONE

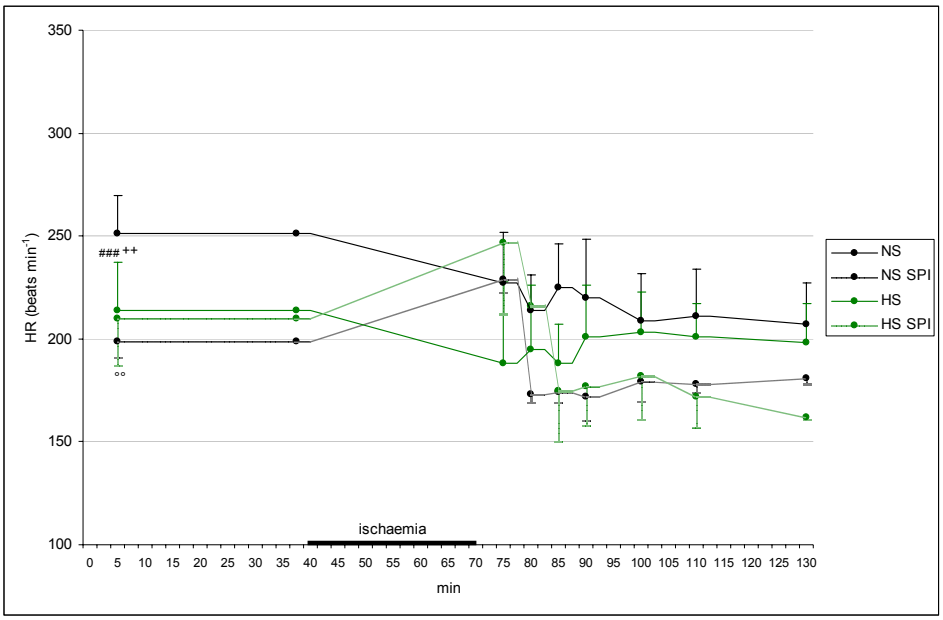


Fig.71a Time course of **heart rate** during **control experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. °° p<0,01 NS SPI vs. NS; ### p<0,001 HS vs. NS; ++ p<0,01 HS SPI vs. NS.

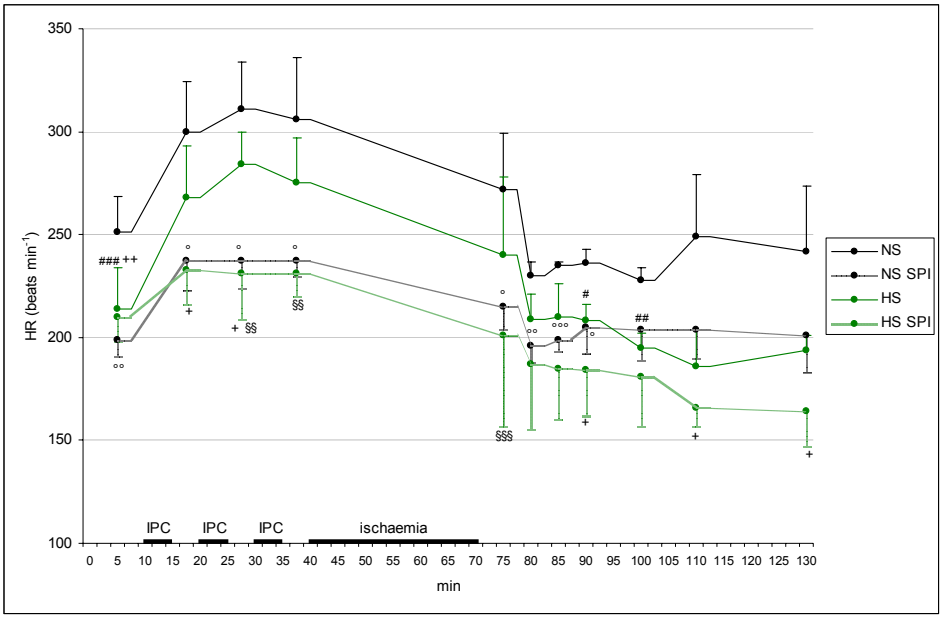


Fig.71b Time course of **heart rate** during **IPC experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05; °° p<0,01; °°° p<0,001 NS SPI vs. NS; # p<0,05; ## p<0,01; ### p<0,001 HS vs. NS; + p<0,05; ++ p < 0,01 HS SPI vs. NS; §§ p<0,01; §§§ p<0,001 HS SPI vs. HS.

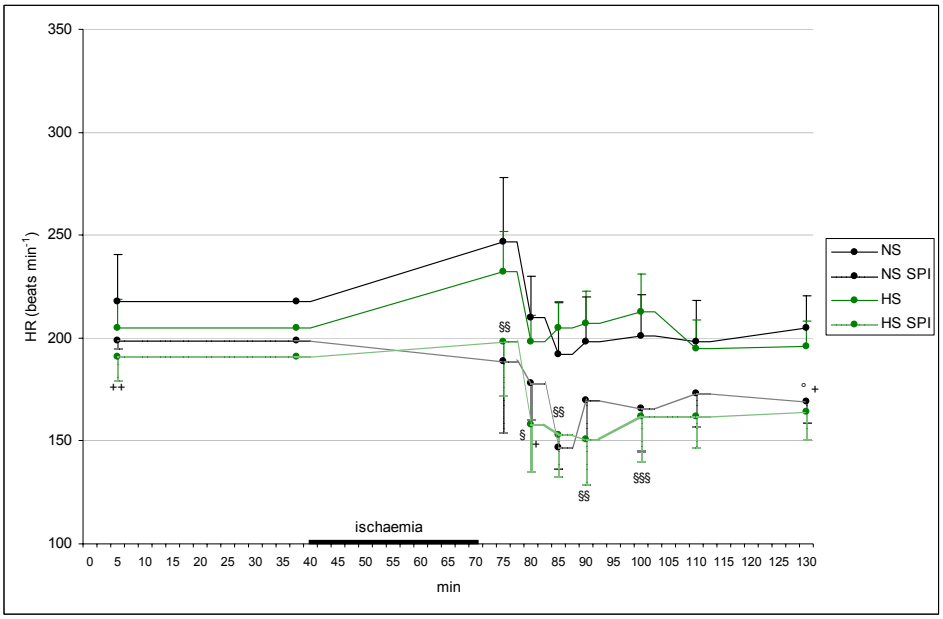


Fig.71c Time course of **heart rate** during **control experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-6. ° p<0,05 NS SPI vs. NS; + p<0,05; ++ p<0,01 HS SPI vs. NS; § p<0,05; §§ p<0,01; §§§ p<0,001 HS SPI vs. HS.

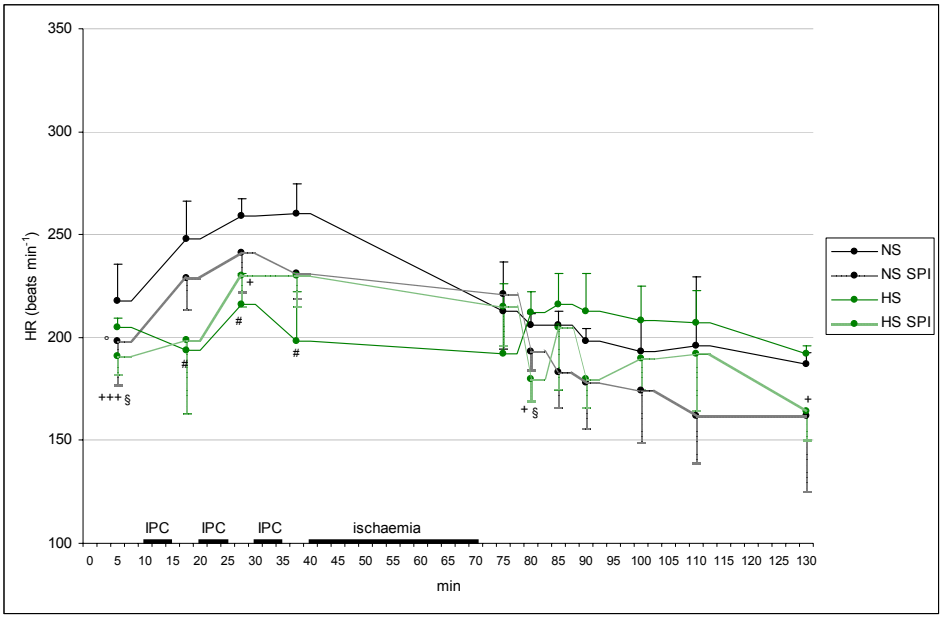


Fig.71d Time course of **heart rate** during **IPC experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-5. ° p<0,05 NS SPI vs. NS; # p<0,05 HS vs. NS; + p<0,05; +++ p<0,001 HS SPI vs. NS; § p<0,05 HS SPI vs. HS.

In BN hearts of both, spironolactone and high salt diet treated rats, the basal heart rate significantly decreased (Fig.71a and 71b). Following 30min ischaemia, the heart rate was similar in all non-preconditioned BN hearts. IPC significantly lowered the post-ischemic heart rate of BN hearts from rats treated with spironolactone when compared with BN hearts from untreated rats. The decrease developed already during the IPC periods. High salt diet caused a moderate decrease in the post-ischaemic heart rate of preconditioned BN hearts in comparison to untreated hearts.

Independently of salt diet, spironolactone significantly decreased the basal heart rate also in hearts of kininogen-deficient BNK rats (Fig.71c and 71d). This reduction in heart rate was more pronounced after 30min ischaemia in non-preconditioned BNK hearts (Fig.71c). IPC did not alter the post-ischaemic heart rate in any group of BNK hearts. Hearts from BNK rats fed with high salt diet displayed significantly reduced heart rate during the IPC periods (Fig.71d)

SUMMARY OF HEART RATE

Taken together, it was confirmed, that KKS plays an important role in the protective mechanism of IPC, as kininogen-deficient BNK rats did not respond to it. Concerning the heart rate, the respond to 30min ischaemia was identical in preconditioned and non-preconditioned hearts of BNK rats fed with standard diet. Neither spironolactone nor high salt diet could recover the effects of IPC on heart rate in BNK hearts.

Aldosterone seems to play an important role in the regulation of heart rate, as hearts from spironolactone treated rats, independently of salt diet, displayed significantly decreased heart rate in both rat strains. In BN hearts, the decline developed already during the IPC periods, which reduced the effect of IPC on heart rate during the reperfusion. These data suggest that aldosterone may increase the heart rate. Hearts from rats treated with high salt diet displayed a decline in heart rate whereby the effect of IPC was abolished, especially in BN hearts. In BNK but not in BN hearts, the heart rate was reduced also during the IPC periods.

4.6.2. LEFT VENTRICULAR DEVELOPED PRESSURE

The isovolumetric measurement of myocardial force was determined by means of an intraventricular balloon catheter. Left ventricular developed pressure (LVDP) is defined as the maximum left ventricular pressure (LVP_{max}) minus LVP_{min} (LVEDP).

COMPARISON OF BN AND BNK RATS

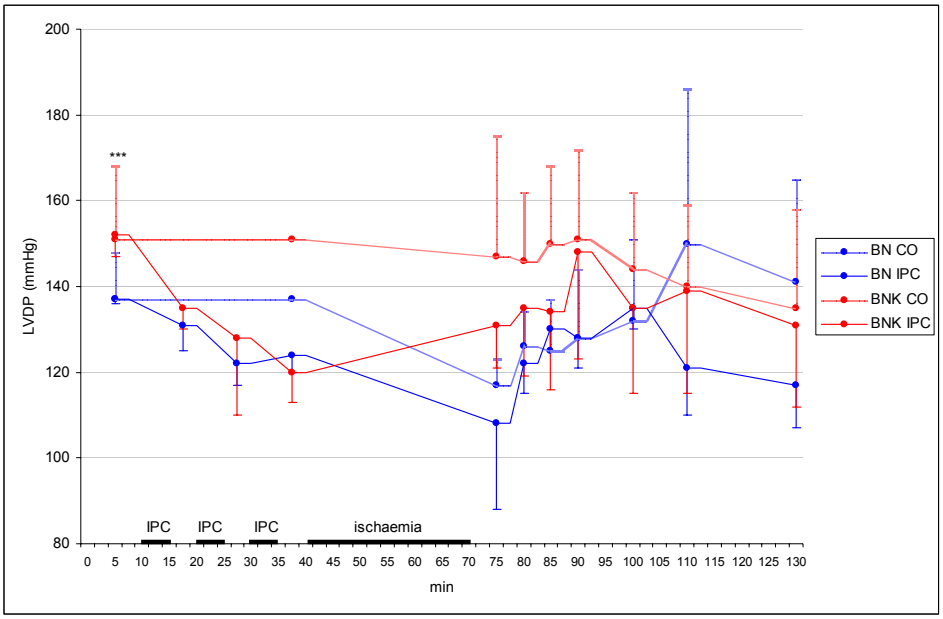


Fig.72a Time course of **left ventricular developed pressure** during perfusion of BN and BNK hearts on **standard diet**, mean ± SEM, n=4-7. *** p<0,001 BNK hearts vs. BN hearts.

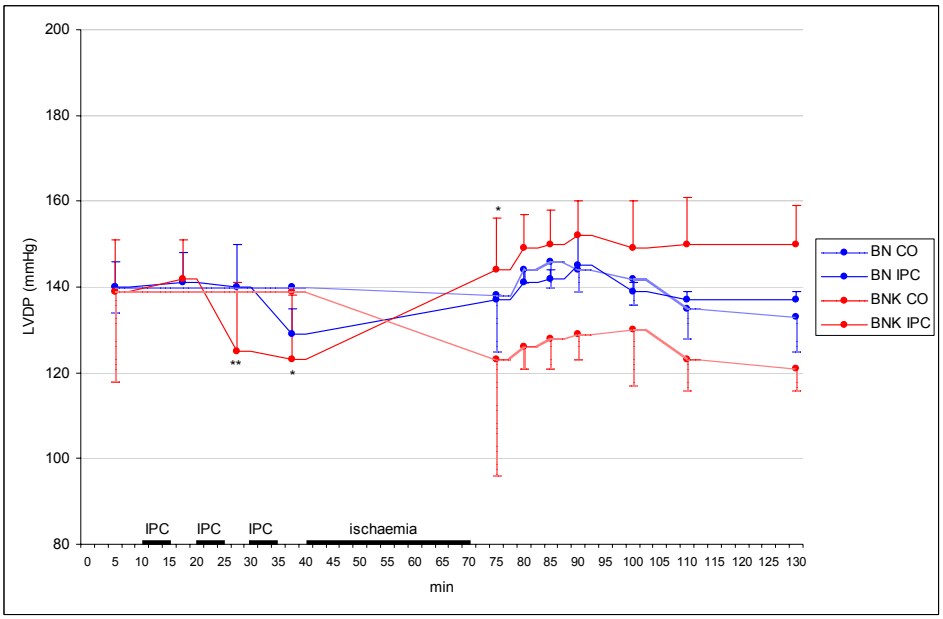


Fig.72b Time course of **left ventricular developed pressure** during perfusion of BN and BNK hearts after **spironolactone upon standard diet**, mean ± SEM, n=4. * p<0,05; ** p<0,01 BNK hearts vs. BN hearts.

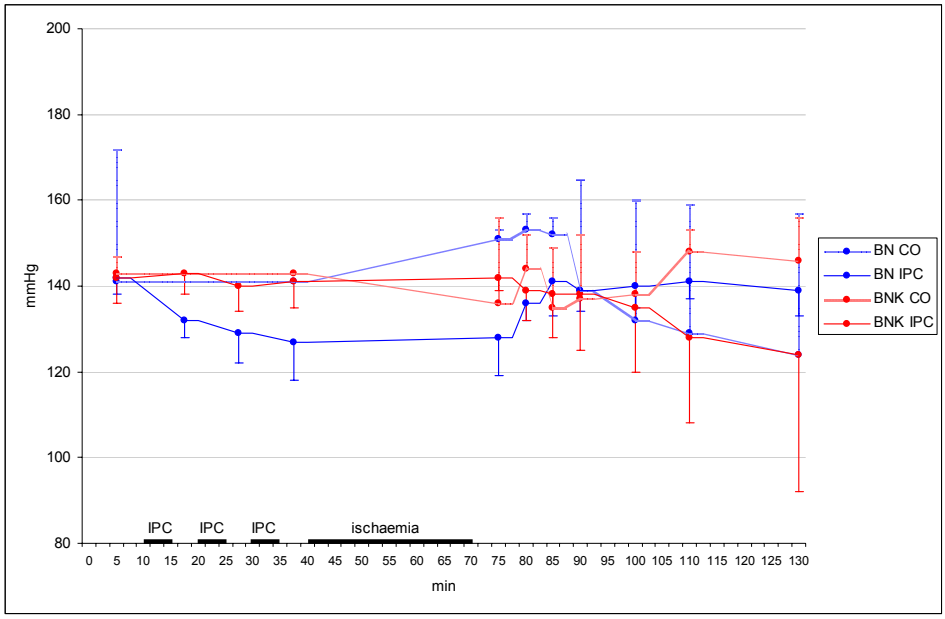


Fig.72c Time course of **left ventricular developed pressure** during perfusion of BN and BNK hearts on **high salt diet**, mean ± SEM, n=4.

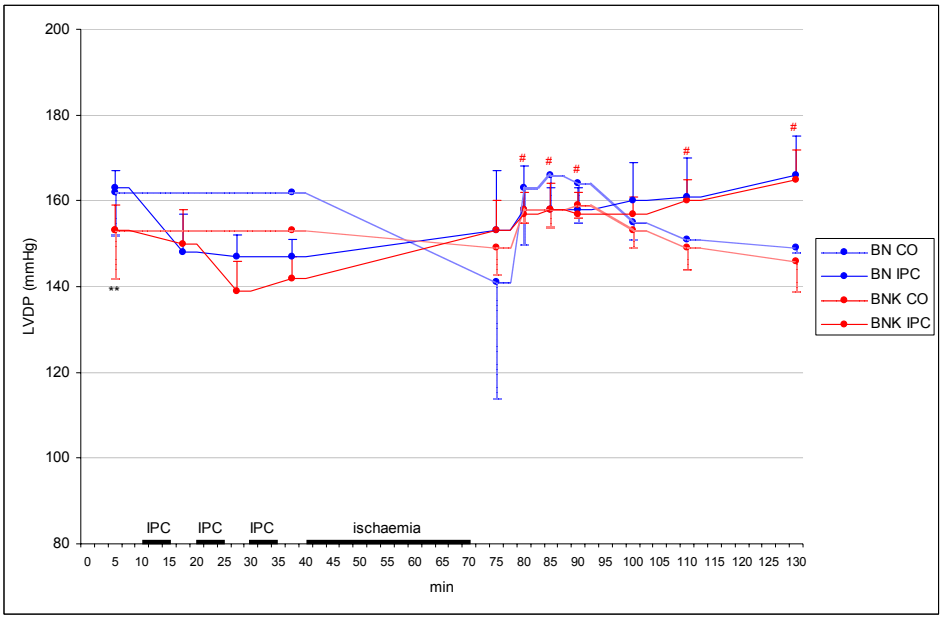


Fig.72d Time course of **left ventricular developed pressure** during perfusion of BN and BNK hearts after **spironolactone upon high salt diet**, mean ± SEM, n=4. ** p<0,01 BNK hearts vs. BN hearts; # p<0,05 IPC vs. control experiment.

Prior to ischaemia, the basal LVDP was significantly higher in BNK hearts than in BN control hearts. The post-ischaemic LVDP was not significantly different among the four groups, although it was slightly higher in BNK hearts than in BN hearts. In both

strains, IPC was accompanied by a reduction of LVDP (Fig.72a). In BNK hearts, spironolactone reduced the basal LVDP to the levels of BN hearts. Spironolactone inhibited the fall of LVDP during the IPC periods in BN hearts but not in BNK hearts. The post-ischaemic LVDP was increased in preconditioned hearts of BNK rats treated with spironolactone but remained unchanged in hearts of spironolactone treated BN rats (Fig.72b). Similarly to spironolactone, the basal LVDP decreased in hearts of BNK rats fed with high salt diet to the levels of BN hearts whereby abolished the differences in post-ischaemic LVDP between preconditioned and non-preconditioned BN and BNK hearts (Fig.72c). Treatment with spironolactone upon high salt diet significantly decreased the basal LVDP in BNK hearts in comparison with BN hearts. In BNK hearts, IPC significantly increased the post-ischaemic LVDP. Similar effect was observed also in BN hearts (Fig.72d).

EFFECT OF SALT DIET AND SPIRONOLACTONE

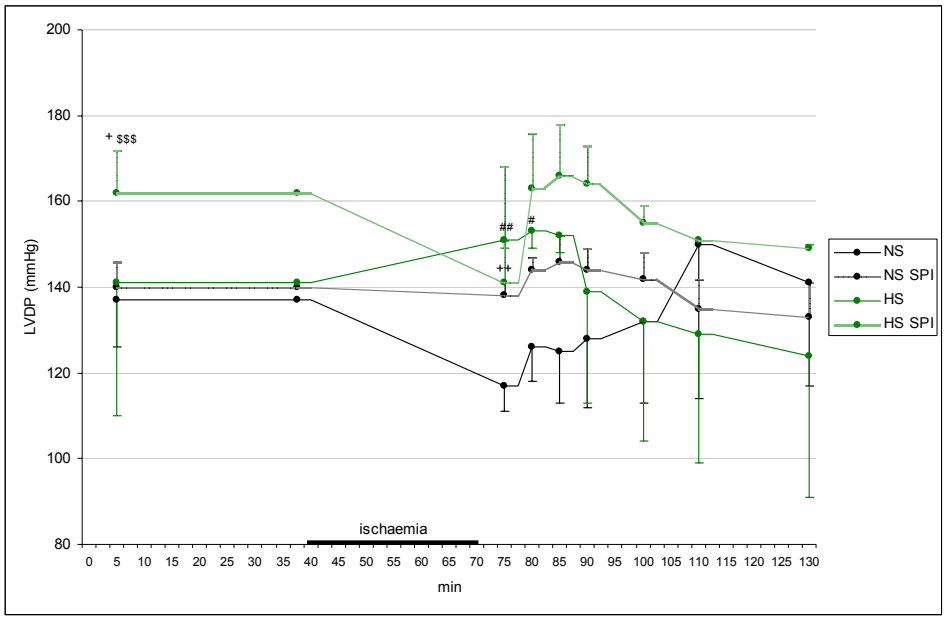


Fig.73a Time course of **left ventricular developed pressure** during **control experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05; °° p<0,01 NS SPI vs. NS; $^{+}$ p<0,05; $^{++}$ p<0,01 HS SPI vs. NS; $^{\$\$\$}$ p<0,001 HS SPI vs. NS SPI.

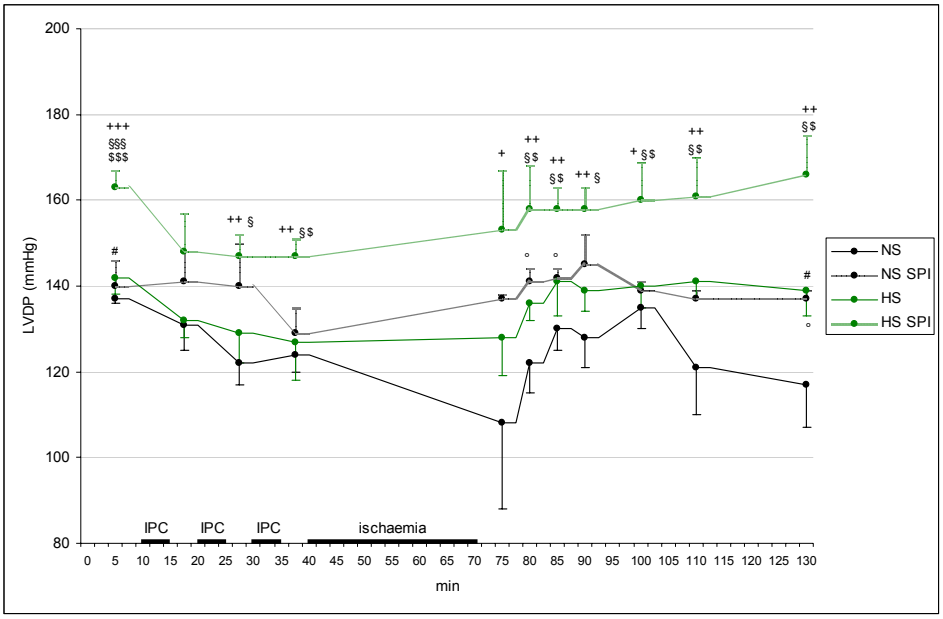


Fig.73b Tim course of **left ventricular developed pressure** during **IPC experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05 NS SPI vs. NS; # p<0,05 HS vs. NS; + p<0,05; ++ p<0,01; +++ p<0,001 HS SPI vs. NS; § p<0,05; §§§ p<0,001 HS SPI vs. HS; $ p<0,05; $$$ p<0,001 HS SPI vs. NS SPI.

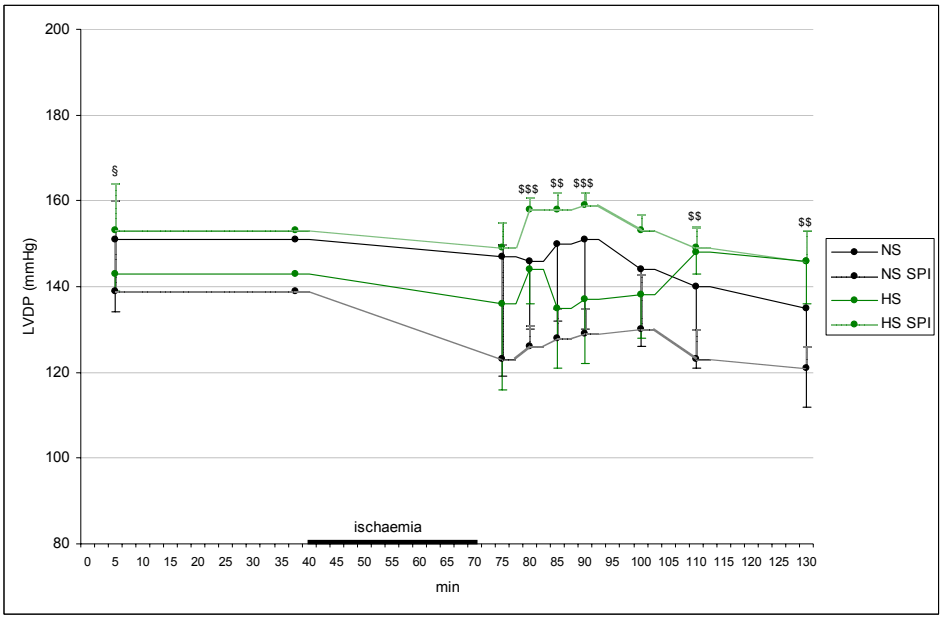


Fig.73c Time course of **left ventricular developed pressure** during **control experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-7. § p<0,05 HS SPI vs. HS; $$ p<0,01; $$$ p<0,001 HS SPI vs. NS SPI.

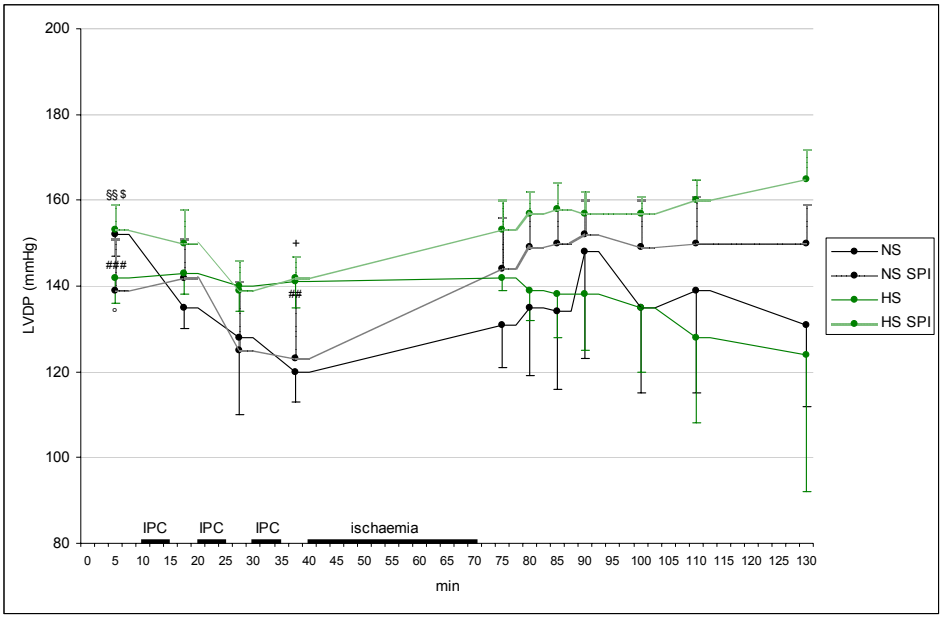


Fig.73d Time course of **left ventricular developed pressure** during **IPC experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05 NS SPI vs. NS; ## p<0,01; ### p<0,001 HS vs. NS; + p<0,05 HS SPI vs. NS; §§ p<0,01 HS SPI vs. HS; $ p<0,05 HS SPI vs. NS SPI.

In hearts of BN rats fed with high salt diet and simultaneously treated with spironolactone the basal as well as post-ischaemic LVDP significantly increased. Spironolactone caused a moderate, not significant increase in LVDP during the reperfusion. In contrast, hearts of rats fed with high salt diet displayed a significant increase in immediate post-ischemic recovery of LVDP (Fig.73a). In BN hearts of rat additionally treated with spironolactone, IPC significantly increased the post-ischaemic LVDP (Fig.73b).

In contrast hearts from BNK rats fed with high salt diet and treated with spironolactone displayed no increase in the basal LVDP. Spironolactone as well as high salt diet caused a decline of the LVDP of BNK rat hearts (Fig.73c). In BNK rats, spironolactone caused an increase in the LVDP following IPC. High salt diet significantly diminished the decrease of LVDP during the IPC periods (Fig.73d).

SUMMARY OF LEFT VENTRICULAR DEVELOPED PRESSURE

These data suggest that aldosterone may alter the LVDP as in both strains, the LVDP was increased by spironolactone, high salt diet as well as combination of both. This increase was less pronounced in kininogen-deficient BNK hearts. In BNK but not BN hearts IPC caused an increase in LVDP. This mechanism was influenced by aldosterone, as spironolactone treatment increased the post-ischaemic LVDP. In non-preconditioned BNK rats, LVDP tended to decrease.

4.6.3. LEFT VENTRICULAR END-DIASTOLIC PRESSURE

The left ventricular end-diastolic pressure (LVEDP) was determined with an intraventricular balloon catheter.

COMPARISON OF BN AND BNK RATS

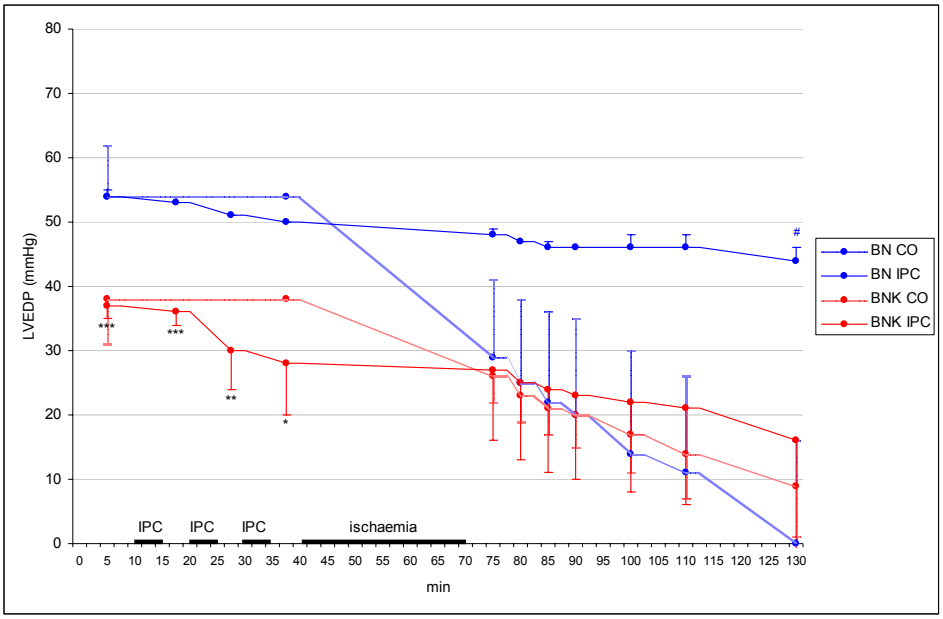


Fig.74a Time course of **left ventricular end-diastolic pressure** during perfusion of BN and BNK hearts on **standard diet**, mean ± SEM, n=4-7. * p<0,05; ** p<0,01; *** p<0,001 BNK hearts vs. BN hearts; # p<0,05 IPC vs. control experiment.

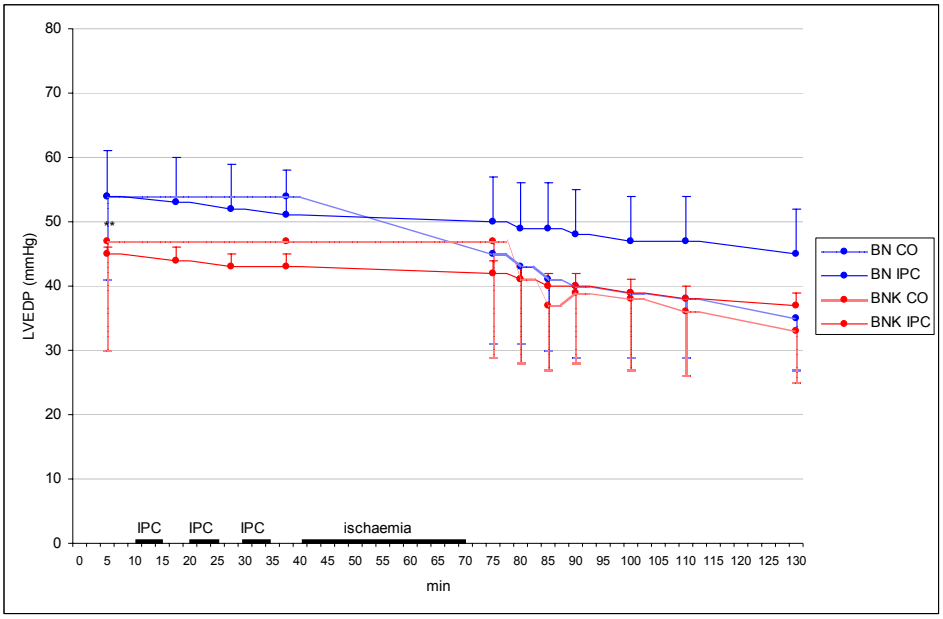


Fig.74b Time course of **left ventricular end-diastolic pressure** during perfusion of BN and BNK hearts after **spironolactone upon standard diet**, mean ± SEM, n=4. ** p<0,01 BNK hearts vs. BN hearts.

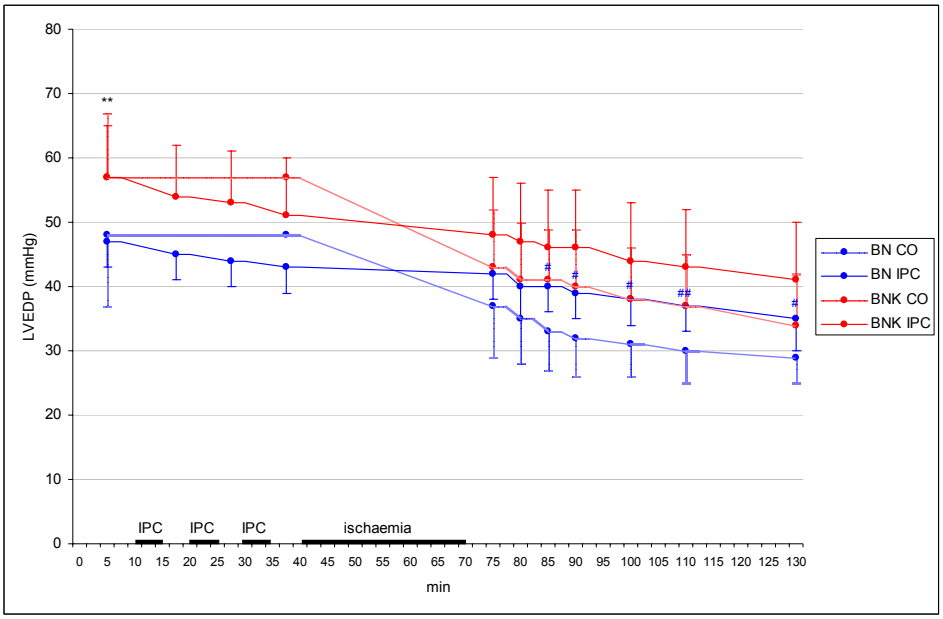


Fig.74c Time course of **left ventricular end-diastolic pressure** during perfusion of BN and BNK hearts on **high salt diet**, mean ± SEM, n=4. ** p<0,01 BNK hearts vs. BN hearts.

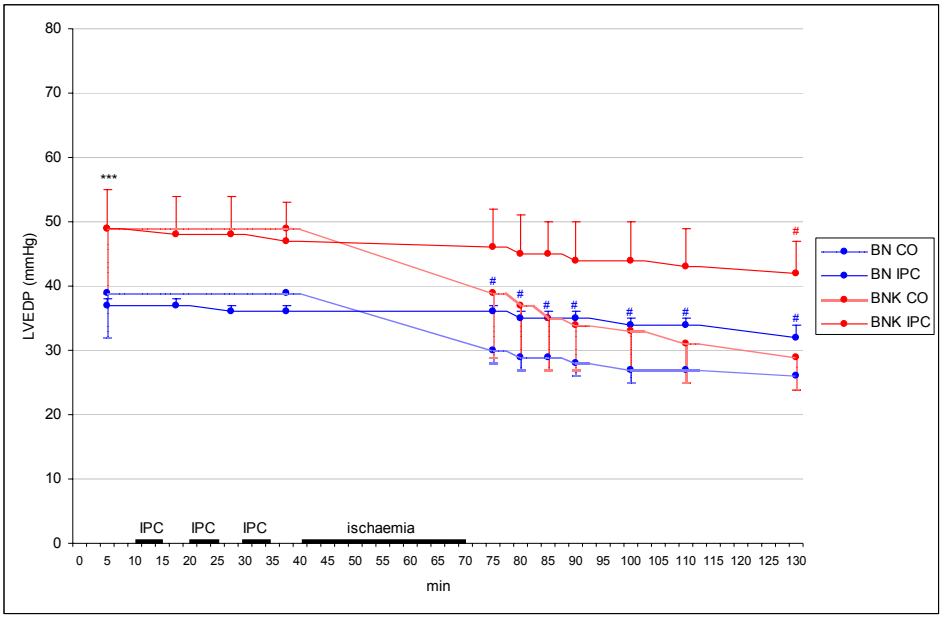


Fig.74d Time course of **left ventricular end-diastolic pressure** during perfusion of BN and BNK hearts after **spironolactone upon high salt diet**, mean ± SEM, n=4. *** p<0,001 BNK hearts vs. BN hearts; # p<0,05 IPC vs. control experiment.

The basal LVEDP was significantly lower in hearts of BNK rats than in those of BN rats. In non-preconditioned hearts of both strains, the LVEDP substantially decreased during the 60min of reperfusion. In both, BN and BNK hearts, IPC increased the post-ischaemic LVEDP (Fig.74a). The basal LVEDP was significantly lower in hearts of BNK rats than in hearts of BN rats treated with spironolactone (Fig.74b). This between-strain difference in LVEDP was smaller than in hearts from untreated rats (Fig.74c). The post-ischaemic LVEDP was almost identical in preconditioned and non-preconditioned hearts of both strains. In hearts of BNK rats fed with high salt diet the basal LVEDP significantly increased. In hearts of BN rats, IPC caused a significant increase in post-ischaemic LVEDP in comparison to non-preconditioned hearts (Fig.74c). In hearts of BNK rats fed with high salt diet and treated with spironolactone the basal LVEDP was significantly higher than in hearts of BN rats. In non-preconditioned hearts of both strains, the post-ischaemic LVEDP continuously decreased. In both strains, IPC recovered the LVEDP during the reperfusion (Fig.74d).

EFFECT OF SALT DIET AND SPIRONOLACTONE

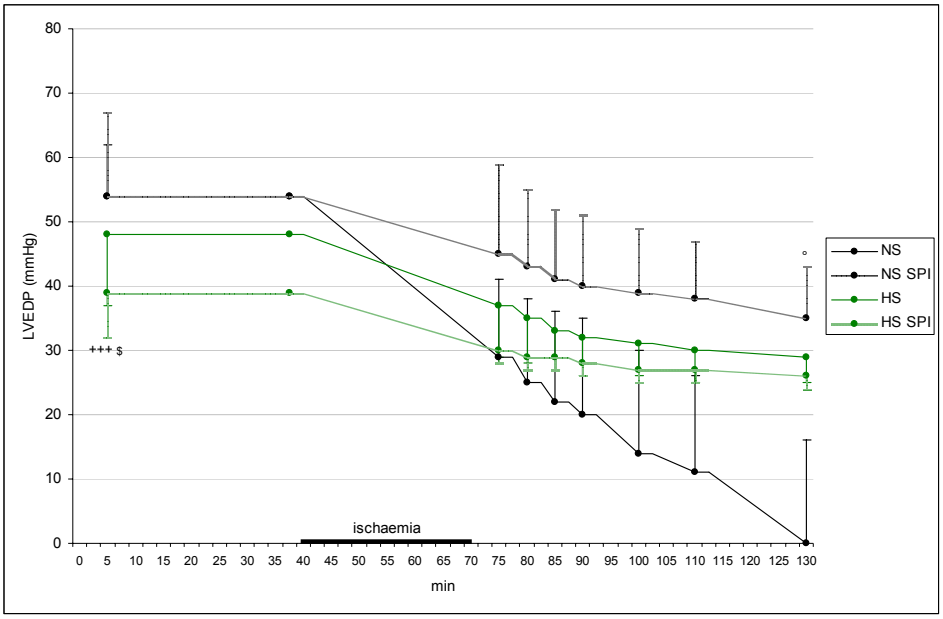


Fig.75a Time course of **left ventricular end-diastolic pressure** during **control experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05 NS SPI vs. NS; $^{+++}$ p<0,001 HS SPI vs. NS; $^{\$}$ p<0,05 HS SPI vs. NS SPI.

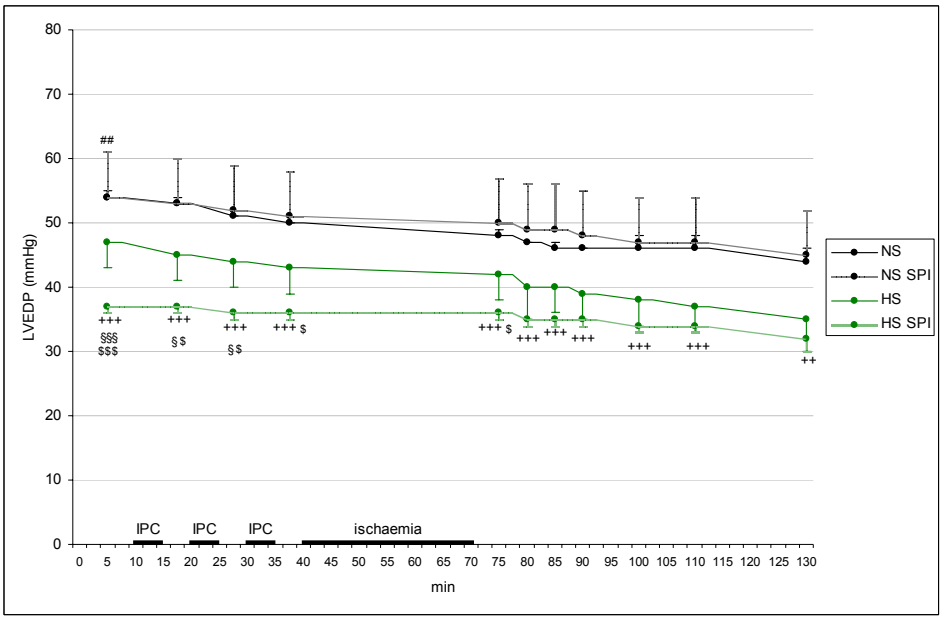


Fig.75b Time course of **left ventricular end-diastolic pressure** during **IPC experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. $^{\#\#}$ p<0,01 HS vs. NS; $^{++}$ p<0,01; $^{+++}$ p<0,001 HS SPI vs. NS; § p<0,05; §§§ p<0,001 HS SPI vs. HS; $^{\$}$ p<0,05; $^{\$\$\$}$ p<0,001 HS SPI vs. NS SPI.

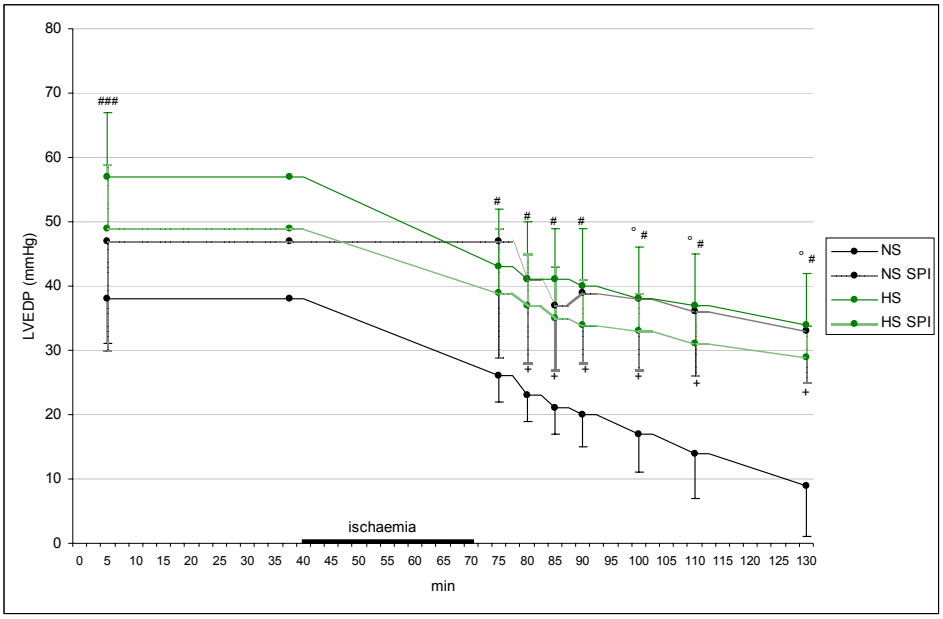


Fig.75c Time course of **left ventricular end-diastolic pressure** during **control experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-7. ° p<0,05 NS SPI vs. NS; # p<0,05; ### p<0,001 HS vs. NS; + p<0,05 HS SPI vs. NS.

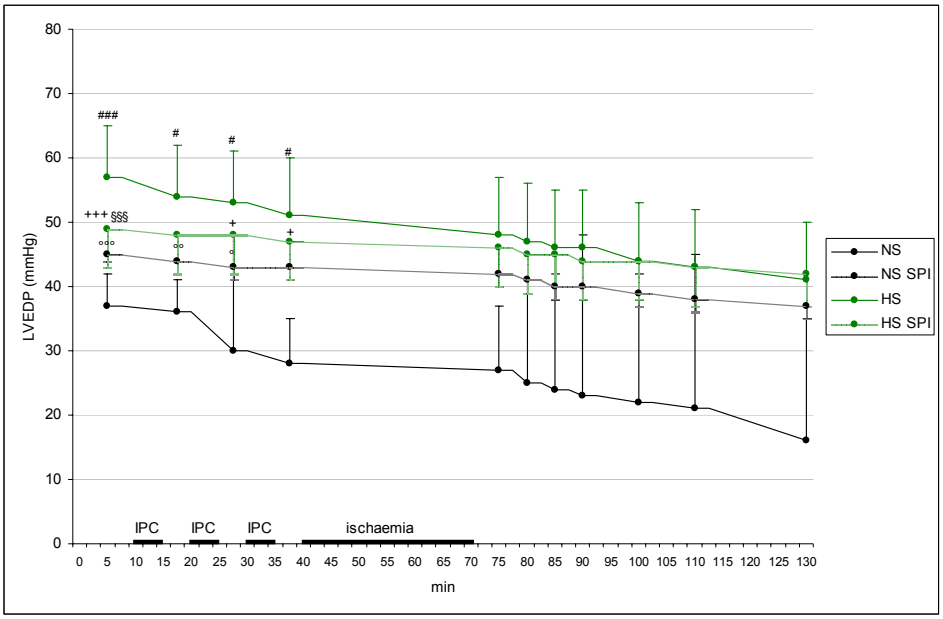


Fig.75d Time course of **left ventricular end-diastolic pressure** during **IPC experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05; °° p<0,01; °°° p<0,001 NS SPI vs. NS; # p<0,05; ### p<0,001 HS vs. NS; + p<0,05; +++ p<0,001 HS SPI vs. NS; §§§ p<0,001 HS SPI vs. HS.

In hearts of BN rats fed with high salt diet and treated with spironolactone the basal LVEDP was significantly increased. In non-preconditioned BN hearts, the post-ischaemic LVEDP was higher in all experimental groups when compared with hearts from untreated rats (Fig.75a). In all hearts of BN rats IPC abolished the decrease of post-ischaemic LVEDP (Fig.75b).

In hearts of BNK rats fed with high salt diet the basal LVEDP was significantly increased. In non-preconditioned BNK hearts, the post-ischaemic LVEDP was significantly higher under all experimental conditions (Fig.75c). Also in preconditioned hearts BNK rats treated with spironolactone the LVEDP was higher during the reperfusion period (Fig.75d).

SUMMARY OF LEFT VENTRICULAR END-DIASTOLIC PRESSURE

The hearts of kininogen-deficient BNK rats displayed significantly higher basal LVEDP than the hearts of BN control rats. Spironolactone increased LVEDP of hearts from BNK rats but not from BN rats. The LVEDP significantly increased in hearts of BNK rats fed with high salt diet. The most pronounced changes occurred in rats fed with high salt diet and simultaneously treated with spironolactone. In hearts of BNK rats, the basal LVEDP significantly increased. In hearts of BN rats, LVEDP significantly decreased. The LVEDP during the reperfusion period was always linked to the initial LVEDP. In non-preconditioned hearts of both strains 30min ischaemia caused a substantial decrease in LVEDP during the 60min of reperfusion. The post-ischaemic decrease of LVEDP was attenuated by high salt diet as well as by spironolactone. Independently of experimental conditions, IPC recovered the post-ischaemic LVEDP.

4.6.4 MAXIMUM CONTRACTION VELOCITY (dp/dt_{max})

Maximum contraction velocity (dp/dt_{max}) was determined by means of an intraventricular balloon.

COMPARISON OF BN AND BNK RATS

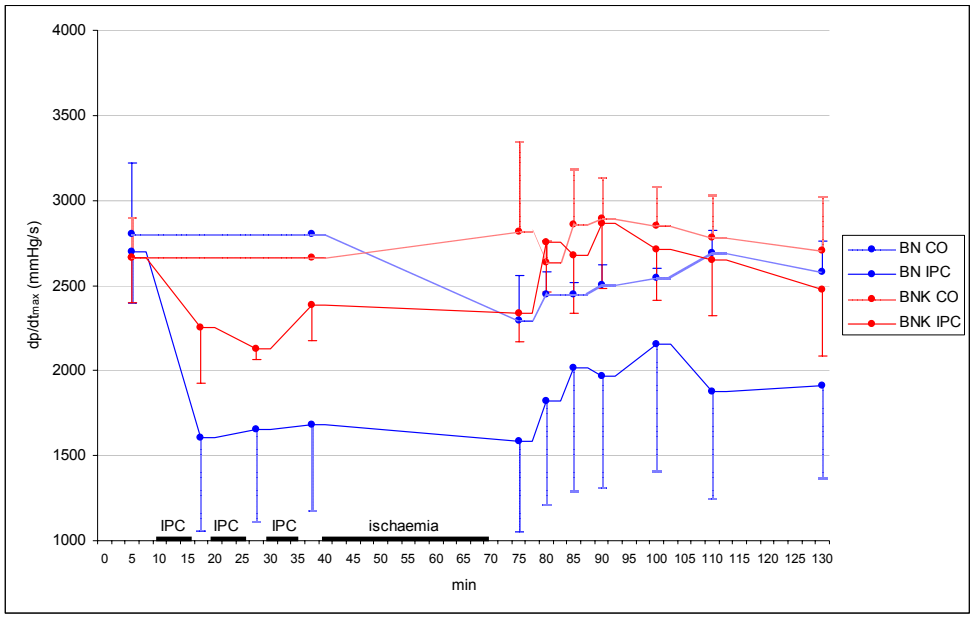


Fig.76a Time course of **maximum contraction velocity** during perfusion of BN and BNK hearts on **standard diet**, mean ± SEM, n=4-7.

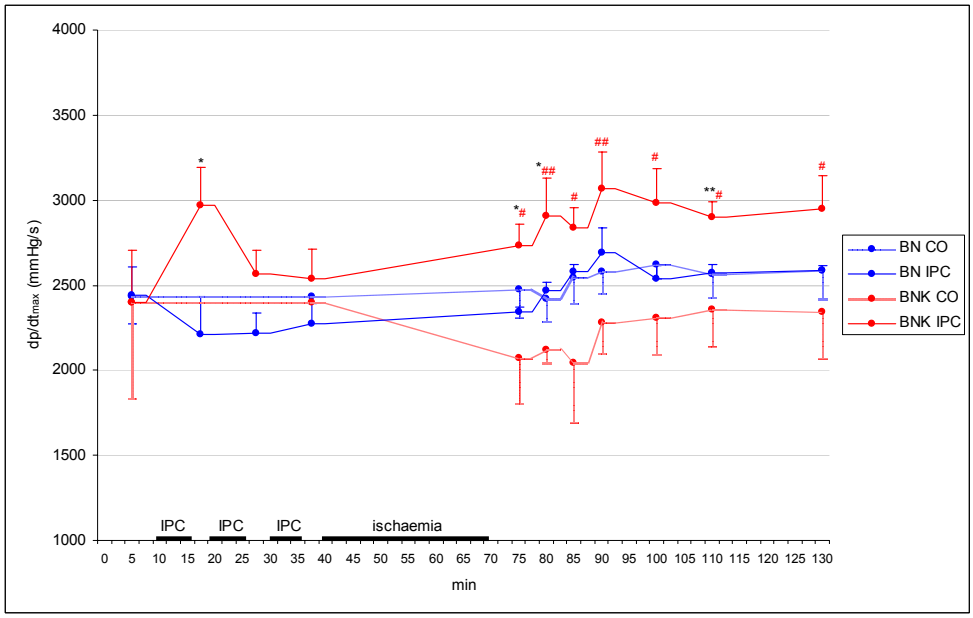


Fig.76b Time course of **maximum contraction velocity** during perfusion of BN and BNK hearts after **spironolactone upon standard diet**, mean ± SEM, n=4. * p<0,05; ** p<0,01 BNK hearts vs. BN hearts; # p<0,05; ## p<0,01 IPC vs. control experiment.

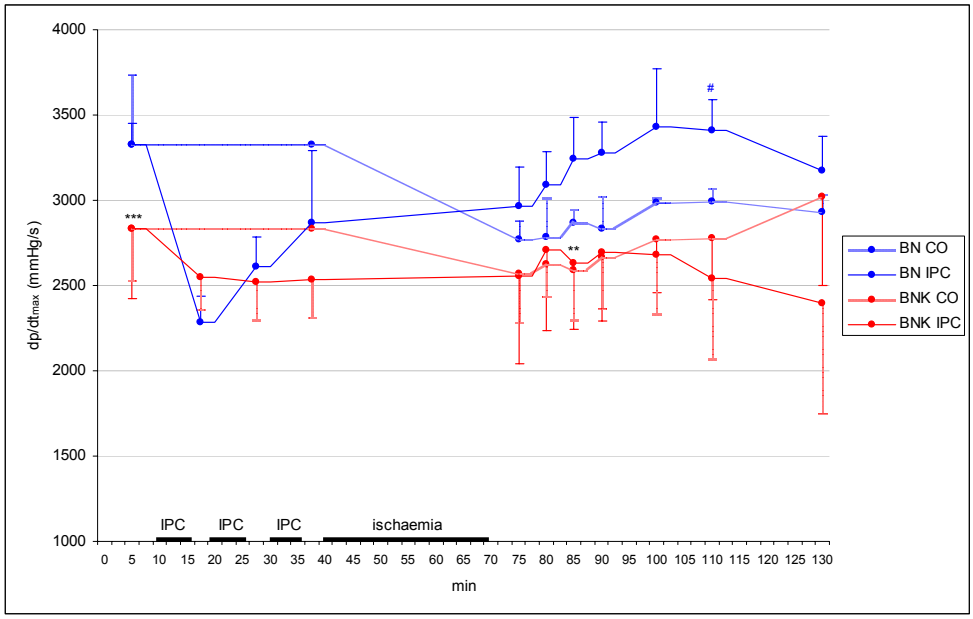


Fig.76c Time course of **maximum contraction velocity** during perfusion of BN and BNK hearts on **high salt diet**, mean ± SEM, n=4. ** p<0,01; *** p<0,001 BNK hearts vs. BN hearts; # p<0,05 IPC vs. control experiment.

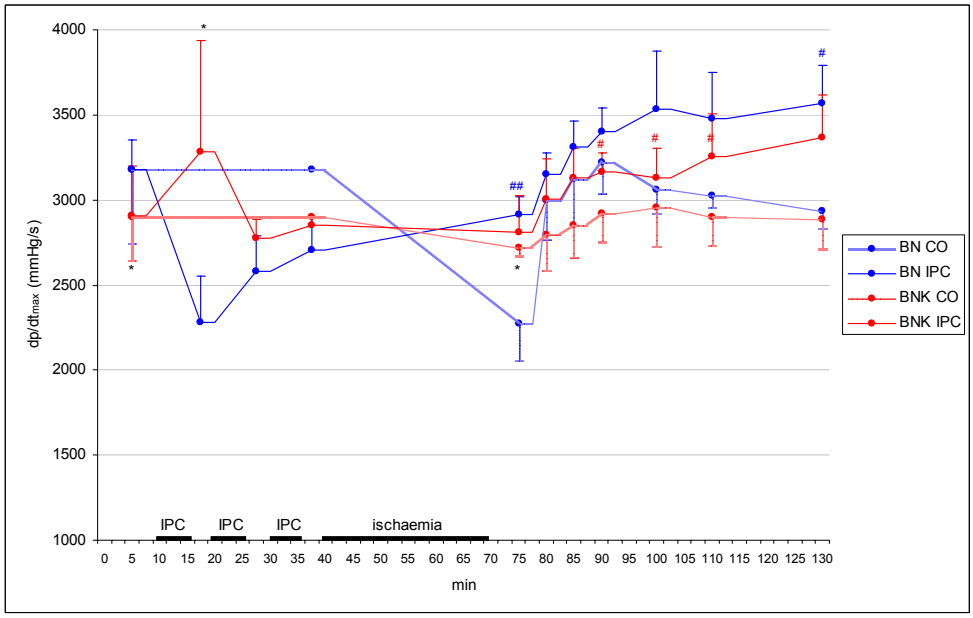


Fig.76d Time course of **maximum contraction velocity** during perfusion of BN and BNK hearts after **spironolactone upon high salt diet**, mean ± SEM, n=4. * p<0,05; ** p<0,01 BNK hearts vs. BN hearts; # p<0,05; ## p<0,01 IPC vs. control experiment.

Following ischaemia the contractility rapidly decreased already during the IPC periods. In hearts of BN rats, the negative inotropic effect of ischaemia was more

pronounced and the contractility remained reduced also during the reperfusion. In BNK hearts 30min ischaemia did not influence the post-ischaemic contractility (Fig.76a). In hearts of BN rats spironolactone inhibited negative inotropic effect of ischaemia that appeared in untreated rats. In hearts of BNK rats treated with spironolactone IPC had significant positive inotropic effect during IPC periods as well as during reperfusion (Fig.76b). In hearts of BN rats fed with high salt diet the basal contractility significantly increased. During IPC periods, the contractility substantially decreased. IPC mediated positive inotropic effects on post-ischaemic contractility of hearts from BN rats fed with high salt diet (Fig.76c). In hearts of BN rats fed with high salt diet and simultaneously treated with spironolactone the basal contractility significantly increased. In both strains IPC mediated positive inotropic effect during reperfusion (Fig.76d).

EFFECT OF SALT DIET AND SPIRONOLACTONE

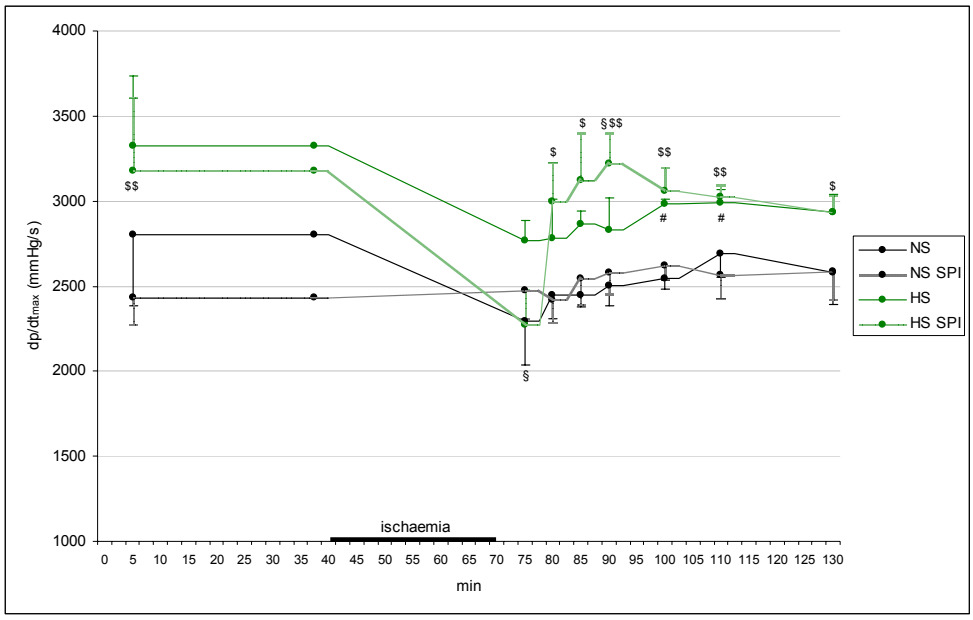


Fig.77a Time course of **maximum contraction velocity** during **control experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. # p<0,05 HS vs. NS; § p<0,05 HS SPI vs. HS; $ p<0,05; $$ p<0,01 HS SPI vs. NS SPI.

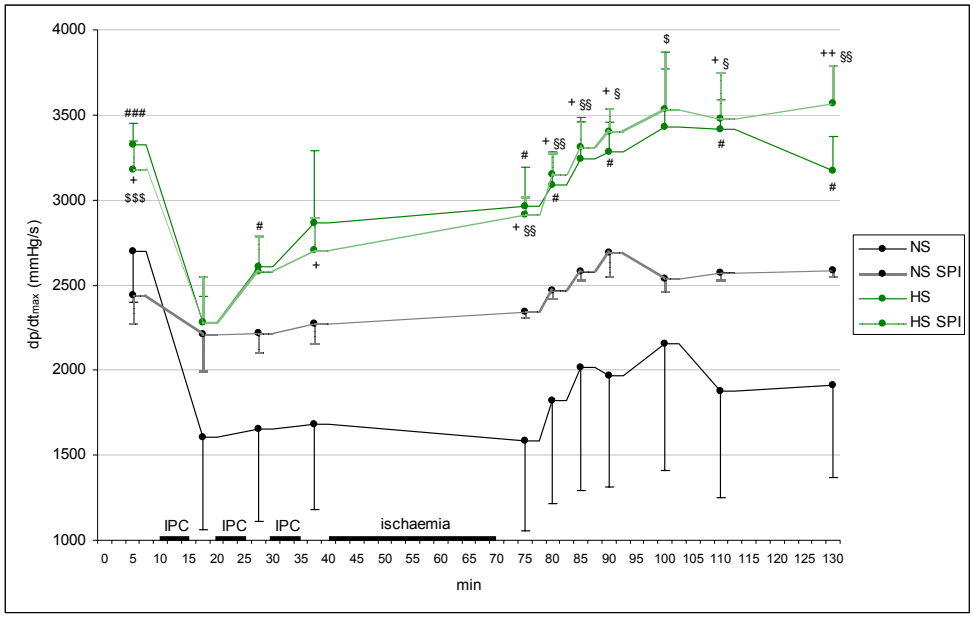


Fig.77b Time course of **maximum contraction velocity** during **IPC experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. $^{\#}$ p<0,05; $^{\#\#\#}$ p<0,001 HS vs. NS; $^{+}$ p<0,05; $^{++}$ p < 0,01 HS SPI vs. NS; § p<0,05; §§ p<0,01 HS SPI vs. HS; $^{\$}$ p<0,05; $^{\$\$\$}$ p<0,001 HS SPI vs. NS SPI.

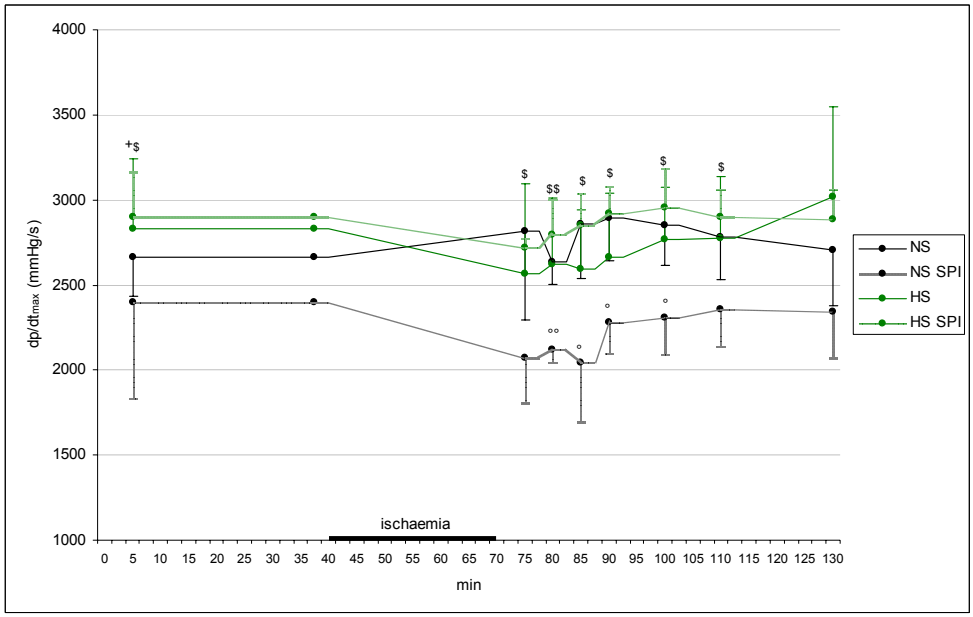


Fig.77c Time course of **maximum contraction velocity** during **control experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-7. ° p<0,05; °° p<0,01 NS SPI vs. NS; $^{+}$ p<0,05 HS SPI vs. NS; $^{\$}$ p<0,05; $^{\$\$}$ p<0,01 HS SPI vs. NS SPI.

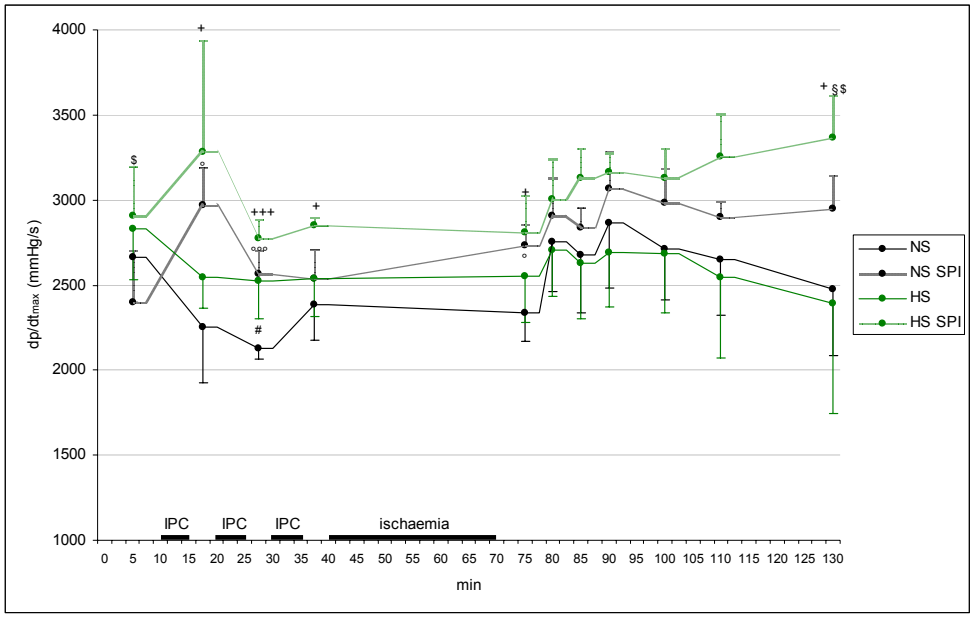


Fig.77d Time course of **maximum contraction velocity** during **IPC experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-6. ° p<0,05 °°° p<0,001 NS SPI vs. NS; # p<0,05 HS vs. NS; + p<0,05; +++ p<0,001 HS SPI vs. NS; § p<0,05 HS SPI vs. HS; $ p<0,05 HS SPI vs. NS SPI.

Although treatment with spironolactone caused negative inotropic effects on the myocardium of BN rats, the contractility remained unaffected by ischaemia (Fig.77a). High salt diet increased contractility that remained higher also during reperfusion if compared with untreated hearts. High salt diet significantly increased the post-ischaemic contractility in preconditioned hearts of BN rats (Fig.77b). Treatment with both, high salt diet and spironolactone, mediated positive inotropic effects that significantly increased contractility also after 30min ischemia, particularly in comparison with hearts of rats treated with spironolactone.

In hearts of kininogen-deficient BNK rats, 30min ischaemia did not influence contractility (Fig.77c). High salt diet mediated a significant increase in the basal contractility of hearts of BNK rats. On the contrary, spironolactone significantly reduced contractility, especially after 30min ischaemia. In pre-conditioned hearts of BNK rats, none of the experimental conditions influenced contractility during the reperfusion (Fig.77d).

SUMMARY OF MAXIMUM CONTRACTION VELOCITY

The basal contractility was similar in hearts of untreated BN and BNK rats. IPC had negative inotropic effect in hearts of BN rats but not in those of BNK rats. Contractility remained unaffected by ischaemia in hearts of BN and BNK rats treated with spironolactone. High salt diet mediated positive inotropic effect, especially in preconditioned hearts of BN rats.

4.6.5 MAXIMUM RELAXATION VELOCITY (dp/dt_{min})

Maximum relaxation velocity (dp/dt_{min}) was determined by means of an intraventricular balloon.

COMPARISON OF BN AND BNK RATS

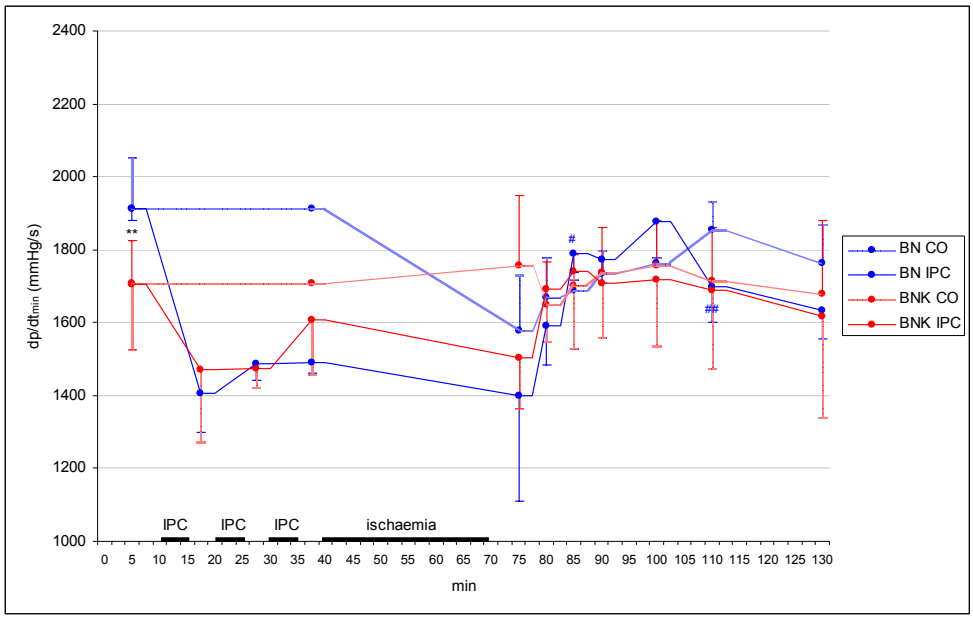


Fig.78a Time course of **maximum relaxation velocity** during perfusion of BN and BNK hearts on **standard diet**, mean ± SEM, n=4-7. * p<0,05 BNK hearts vs. BN hearts; # p<0,05; ## p<0,01 IPC vs. control experiment.

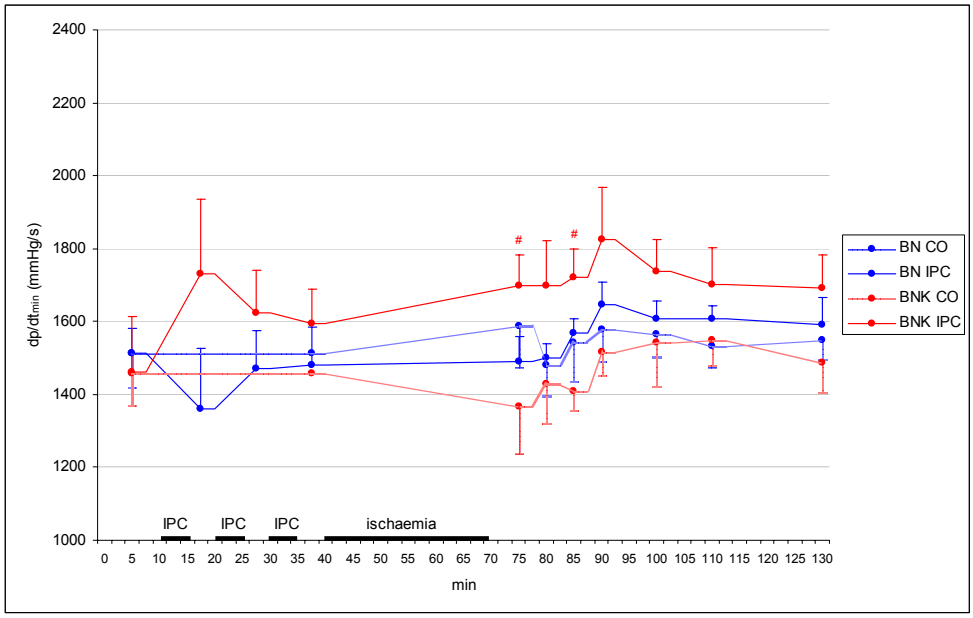


Fig.78b Time course of **maximum relaxation velocity** during the perfusion of BN and BNK hearts after **spironolactone upon standard diet**, mean ± SEM, n=4. # p<0,05 IPC vs. control experiment.

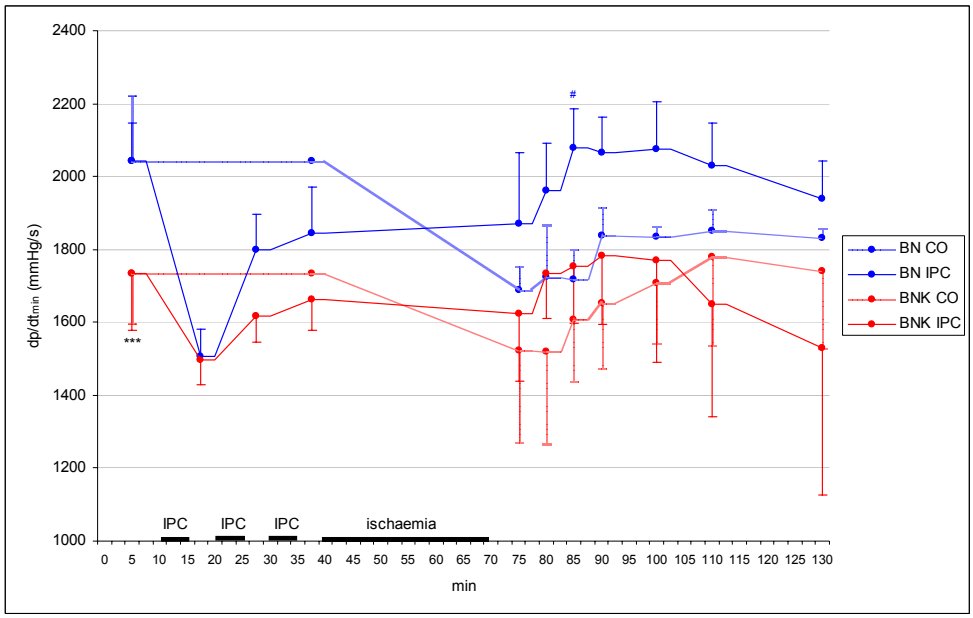


Fig.78c Time course of **maximum relaxation velocity** during perfusion of BN and BNK hearts on **high salt diet**, mean ± SEM, n=4. *** p<0,001 BNK hearts vs. BN hearts; # p<0,05 IPC vs. control experiment.

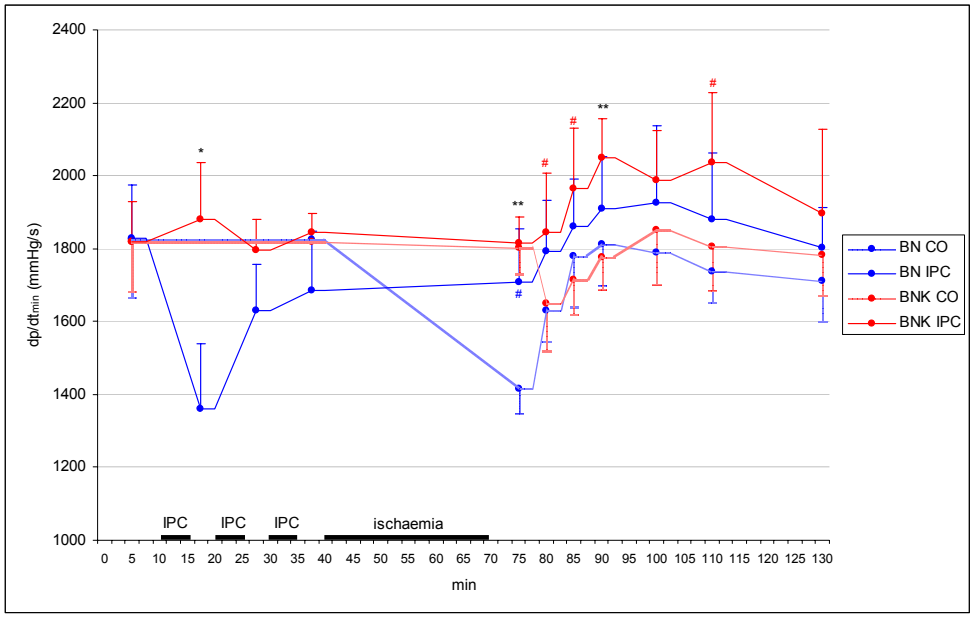


Fig.78d Time course of **maximum relaxation velocity** during the perfusion of BN and BNK hearts after **spironolactone upon high salt diet**, mean ± SEM, n=4. * p<0,05; ** p<0,01 BNK hearts vs. BN hearts; # p<0,05 IPC vs. control experiment.

Under standard conditions the hearts of BNK rats displayed a higher rate of relaxation than those of BN rats. In both strains, IPC mediated negative lusitropic (affecting rate of relaxation) effects (Fig.78a). In both strains, spironolactone decreased the basal rate of relaxation. In hearts of BN rats, spironolactone abolished differences in the post-ischaemic maximum relaxation velocity between preconditioned and non-preconditioned hearts. In hearts of BNK rats, IPC mediated positive lusitropic effect, already during the IPC periods (Fig.78b). In hearts of BN rats fed with high salt diet the basal relaxation velocity significantly increased. IPC was able to recover the rate of relaxation after ischaemia, even if the dp/dt_{min} markedly decreased during the IPC (Fig.78c). The basal rate of ventricular relaxation was identical in rats of both strains fed with high salt diet and treated with spironolactone. The maximum relaxation velocity significantly decreased during the IPC periods in BN hearts but not in BNK hearts. IPC mediated positive lusitropic effect in hearts of both strains (Fig.78d).

EFFECT OF SALT DIET AND SPIRONOLACTONE

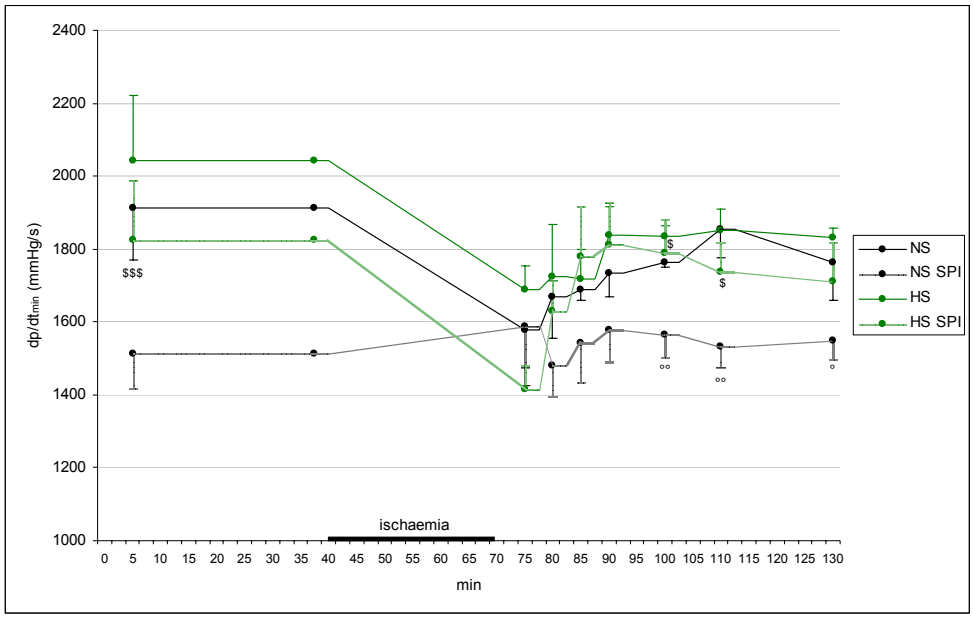


Fig.79a Time course of **maximum relaxation velocity** during **control experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05 °° p<0,01 NS SPI vs. NS; $^{\$}$ p<0,05; $^{\$\$\$}$ p<0,001 HS SPI vs. NS SPI.

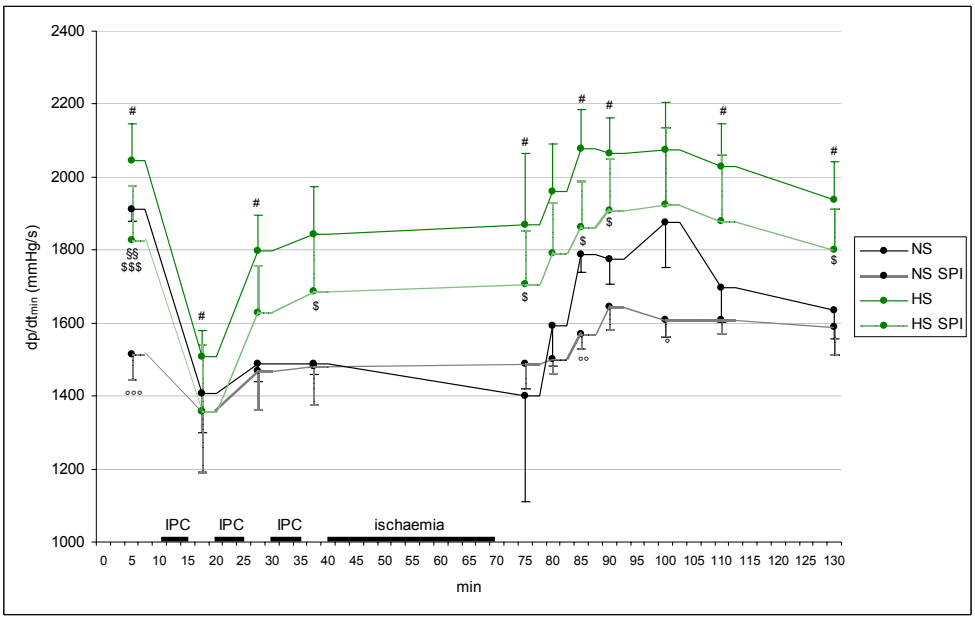


Fig.79b Time course of **maximum relaxation velocity** during **IPC experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05; °° p<0,01; °°° p<0,001 NS SPI vs. NS; $^{\#}$ p<0,05 HS vs. NS; §§ p<0,01 HS SPI vs. HS; $^{\$}$ p<0,05; $^{\$\$\$}$ p<0,001 HS SPI vs. NS SPI.

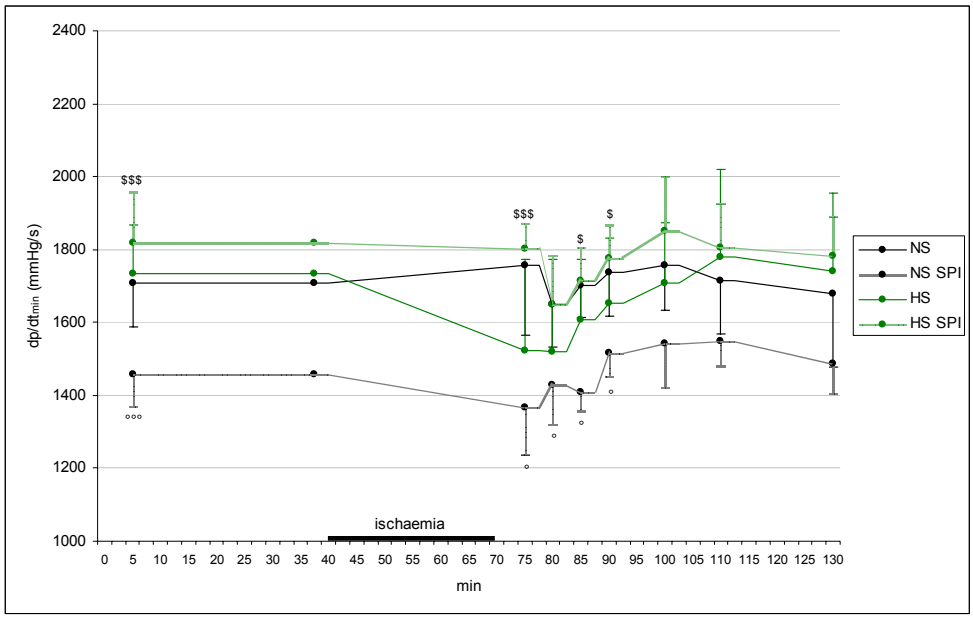


Fig.79c Time course of **maximum relaxation velocity** during **control experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-7. ° p<0,05; °°° p<0,001 NS SPI vs. NS; $ p<0,05; $$$ p<0,001 HS SPI vs. NS SPI.

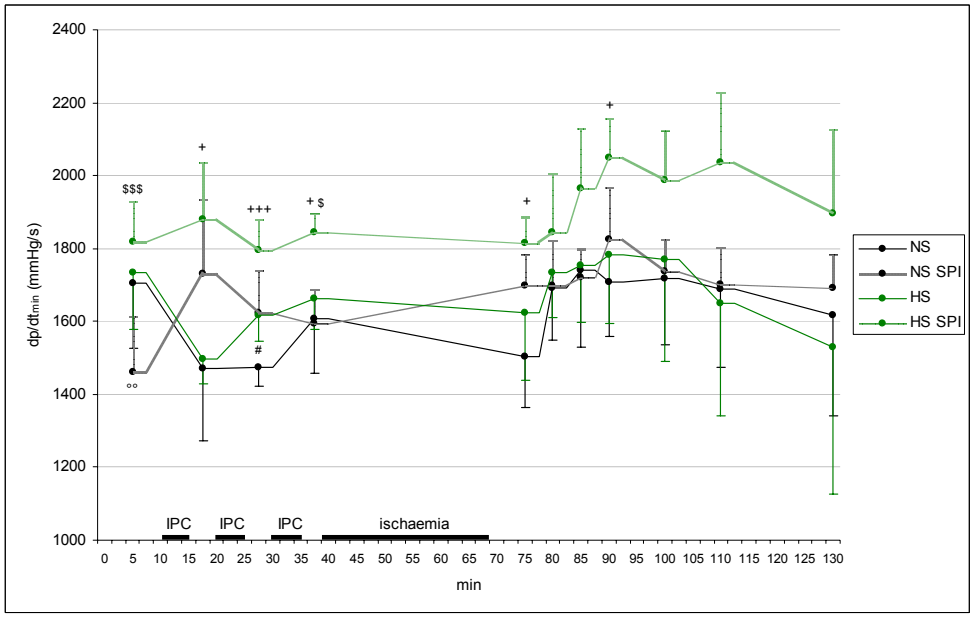


Fig.79d Time course of **maximum relaxation velocity** during **IPC experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-6. °° p<0,01 NS SPI vs. NS; # p<0,05 HS vs. NS; + p<0,05; +++ p<0,001 HS SPI vs. NS; $ p<0,05; $$$ p<0,001 HS SPI vs. NS SPI.

In hearts of BN rats fed with high salt diet the basal rate of ventricular relaxation increased. In hearts of rats treated with spironolactone the maximum relaxation velocity was significantly reduced during the whole perfusion period but it was not reduced after ischaemia as in other experimental groups of BN hearts (Fig.79a). Also in the preconditioned hearts of BN rats treated with spironolactone the most negative lusitropic effects in comparison with untreated rats was observed. In general, IPC mediated positive lusitropic effects in all BN hearts. High salt diet mediated significant increase in dp/dt_{min} during the whole perfusion (Fig.79b). In BNK hearts, treatment with spironolactone significantly reduced both, the pre-ischaemic and post-ischaemic rate of ventricular relaxation (Fig.79c). In BNK rats fed with high salt diet and simultaneously treated with spironolactone a positive lusitropic effects was observed. These effects were more pronounced following IPC (Fig.79d).

SUMMARY OF MAXIMUM RELAXATION VELOCITY

In hearts form kininogen-deficient BNK rats, the basal relaxation velocity was significantly reduced. Hearts from BN rats but BNK rats fed with high salt diet displayed an increase in the rate of relaxation. Spironolactone mediated the most negative lusitropic effects in both strains. Treatment with spironolactone and high salt diet mediated negative lusitropic effect in hearts of BN rats and positive lusitropic effect in hearts of BNK rats. In hearts of untreated rats, IPC did not alter the post-ischaemic rate of ventricular relaxation. In general already the first short ischaemic stimulus caused an intense negative lusitropic effect. The decrease was more pronounced in hearts of BN rats and was reversed by spironolactone in hearts of BNK rats. Positive lusitropic effects during reperfusion were observed in preconditioned hearts of BN rats but not in those of BNK rats fed with high salt diet. Additional treatment with spironolactone mediated significant positive lusitropic effects in preconditioned hearts of BNK rats but not in those of BN rats.

4.6.6. CORONARY FLOW

Coronary flow (CF) was determined by means of the perfusion controller. The values reflected the action of the peristaltic pump that was adjusted to the constant perfusion pressure of 65mmHg.

COMPARISON OF BN AND BNK RATS

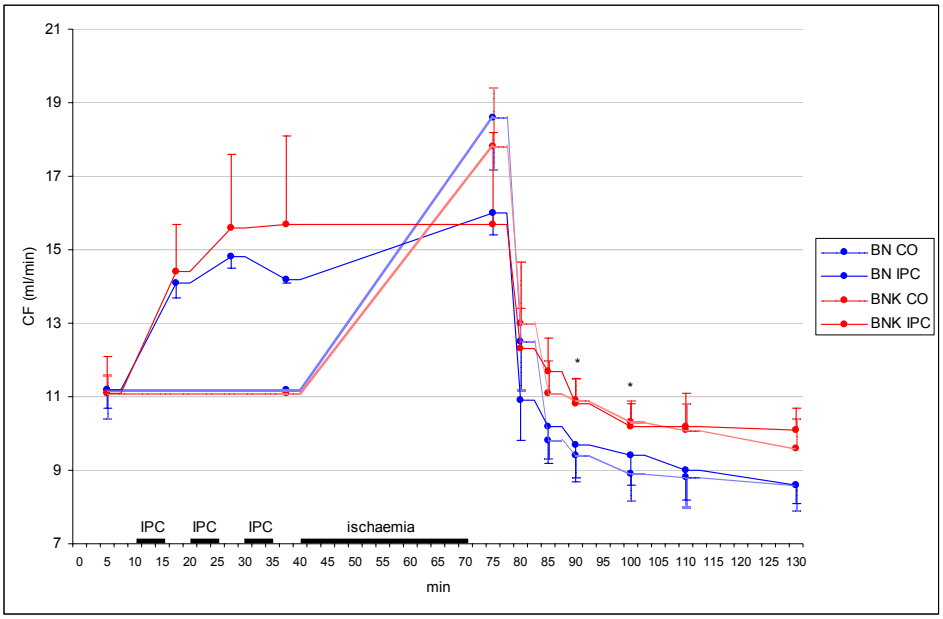


Fig.80a Time course of **coronary flow** during perfusion of BN and BNK hearts on **standard diet**, mean ± SEM, n=4-6. * p<0,01 BNK hearts vs. BN hearts.

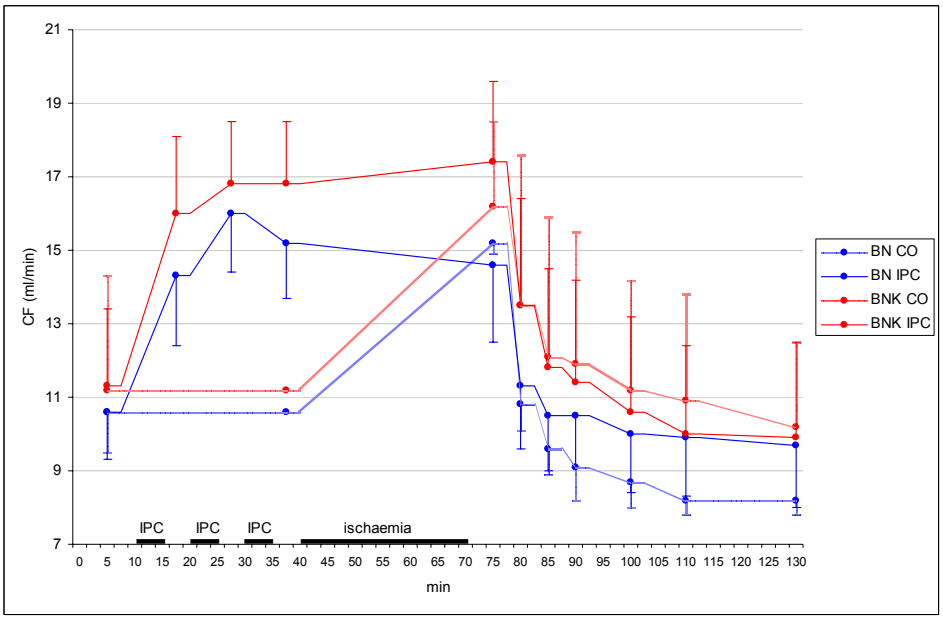


Fig.80b Time course of **coronary flow** during perfusion of BN and BNK hearts after **spironolactone upon standard diet**, mean ± SEM, n=4.

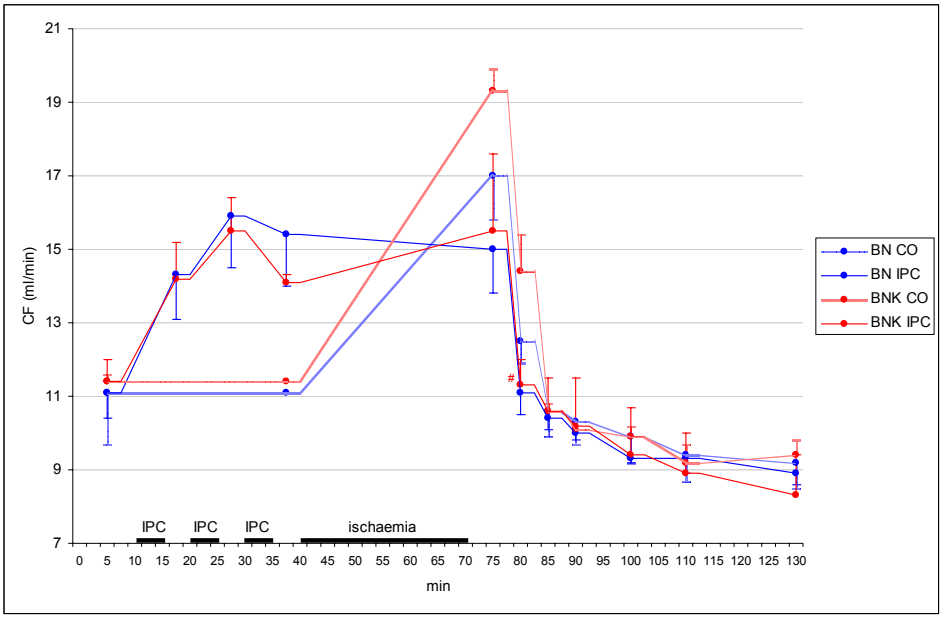


Fig.80c Time course of **coronary flow** during perfusion of BN and BNK hearts on **high salt diet**, mean ± SEM, n=4. # p<0,05 IPC vs. control experiment.

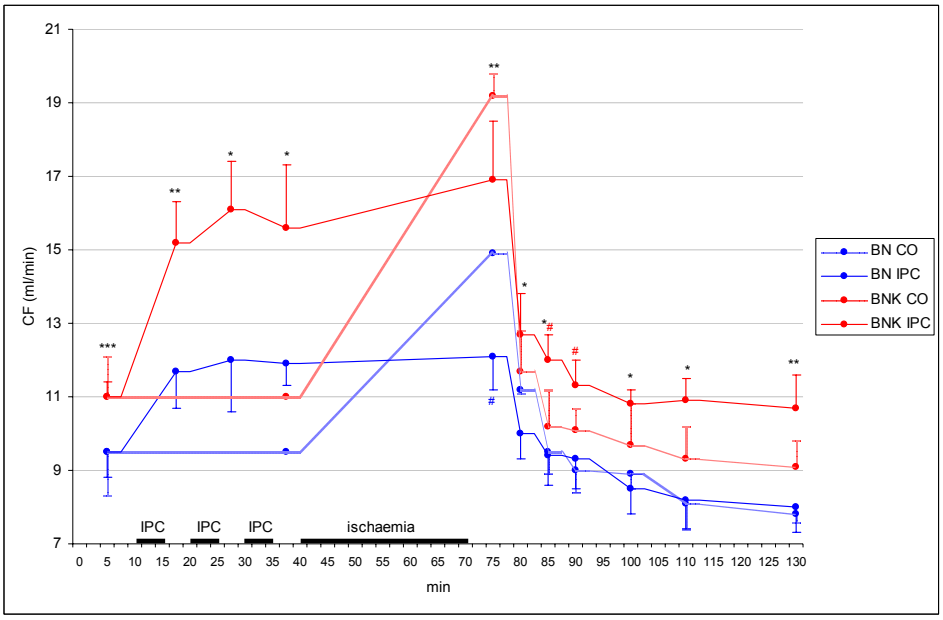


Fig.80d Time course of **coronary flow** during perfusion of BN and BNK hearts after **spironolactone upon high salt diet**, mean ± SEM, n=4.

In both strains the initial coronary flow was identical. The post-ischaemic coronary flow was significantly higher in hearts of BNK rats than in those of BN rats. IPC did not alter the coronary flow in any group (Fig.80a). In hearts of BNK rats, treatment with spironolactone slightly increased basal and post-ischaemic coronary flow. IPC

mediated a moderate increase in the post-ischaemic coronary flow in hearts of BN rats but not in those of BNK rats (Fig.80b).

High salt diet abolished all differences in the coronary flow between the strains and between preconditioned and non-preconditioned hearts (Fig.80c). In hearts of BNK rats fed with high salt diet and simultaneously treated with spironolactone the basal coronary flow significantly increased. Moreover, coronary flow was significantly higher already during IPC periods and remained higher during the reperfusion when compared with hearts of BN rats. Following IPC, coronary flow increased in hearts of BNK rats but not in those of BN rats (Fig.80d).

EFFECT OF SALT DIET AND SPIRONOLACTONE

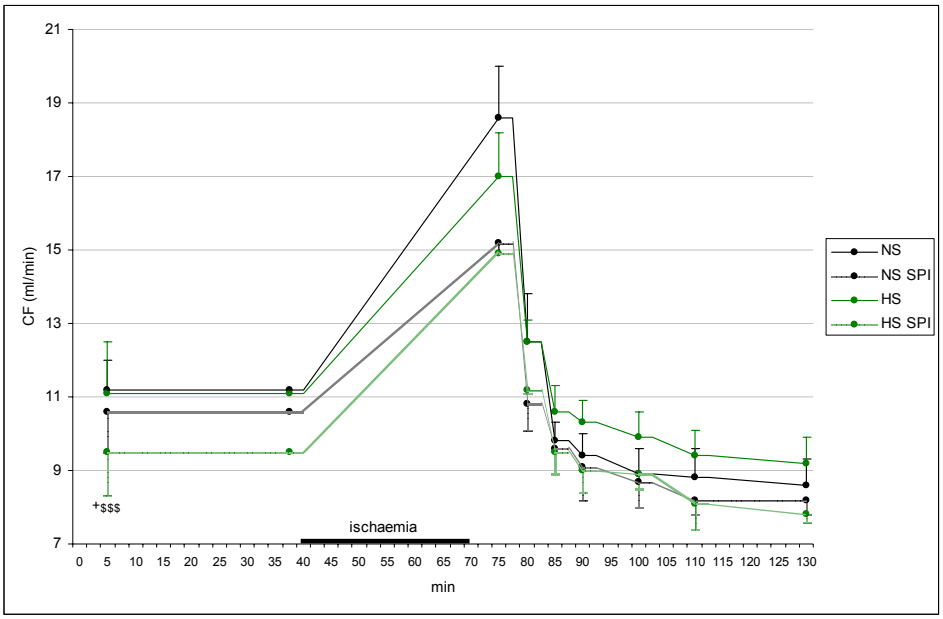


Fig.81a Time course of **coronary flow** during **control experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. $^{+}$ p<0,05 HS SPI vs. NS; $^{\$\$\$}$ p<0,001 HS SPI vs. NS SPI.

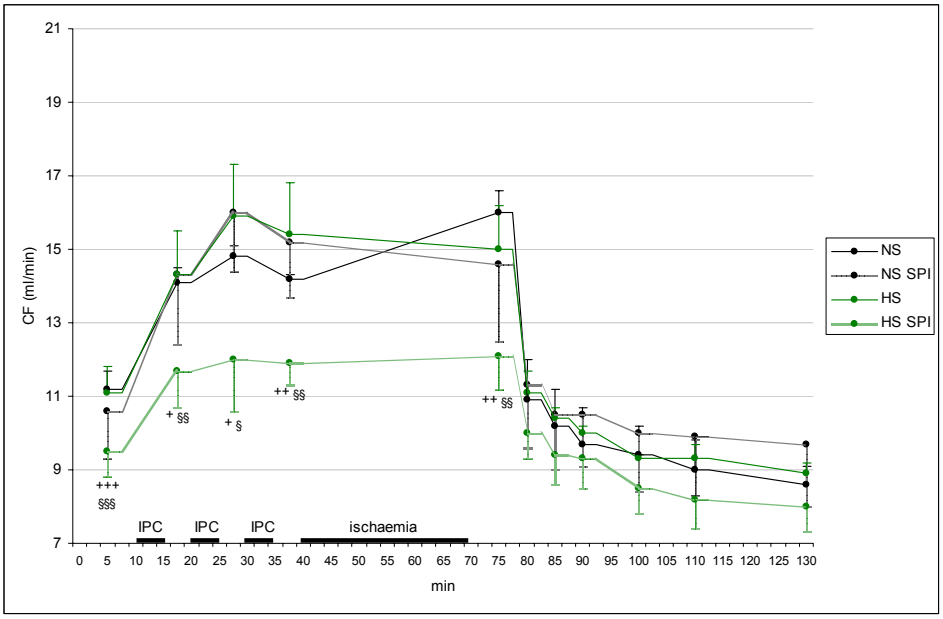


Fig.81b Time course of **coronary flow** during **IPC experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. $^{+}$ p<0,05; $^{++}$ p<0,01; $^{+++}$ p<0,001 HS SPI vs. NS; § p<0,05; §§ p<0,01; §§§ p< 0,001 HS SPI vs. HS.

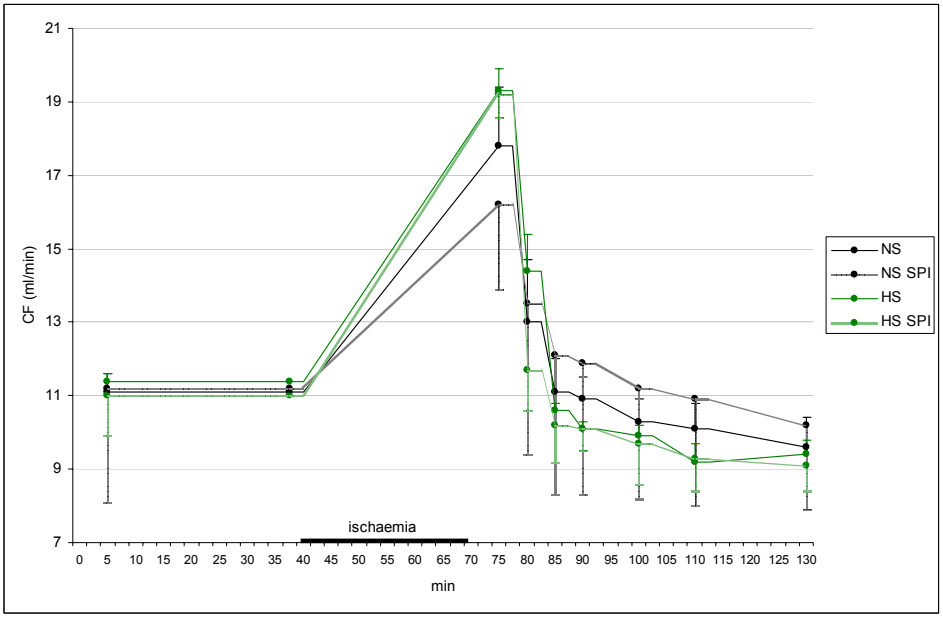


Fig.81c Time course of **coronary flow** during **control experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-6.

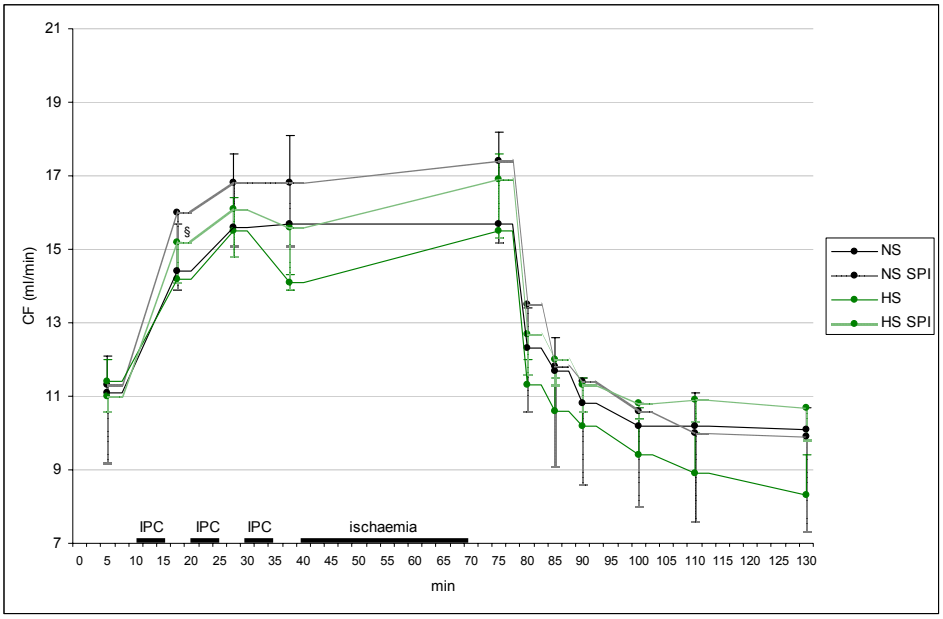


Fig.81d Time course of **coronary flow** during **IPC experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4-5. § p<0,05 HS SPI vs. HS.

In hearts of BN rats, treatment with spironolactone significantly decreased the initial coronary flow (Fig.81a). This effect was more pronounce in rats fed with high salt diet. IPC mediated further reduction of the post-ischaemic coronary flow (Fig.81b). In hearts of BNK rats, any of the experimental conditions influenced the basal coronary flow (Fig. 81c). High salt diet slightly decreased the post-ischaemic coronary flow in preconditioned BNK hearts (Fig.81d).

SUMMARY OF CORONARY FLOW

The basal coronary flow was identical in hearts of BN and BNK rats. In hearts of BNK rats any experimental condition altered the coronary flow. In hearts of BN rats treatment with spironolactone slightly reduced coronary flow. Spironolactone in combination with high salt diet significantly decreased coronary flow. The post-ischaemic coronary flow markedly increased during the first minutes of reperfusion but within 15min decreased to initial pre-ischaemic levels. Hearts of untreated rats of both strains showed no response to IPC. In hearts of BN rats fed with high salt diet and additionally treated with spironolactone IPC significantly reduced the post-ischaemic coronary flow.

4.6.7. CREATINE KINASE ACTIVITY IN CORONARY EFFLUENT

The measurement of the creatine kinase (CK) activity was performed in order to evaluate cardiac damage caused by ischaemia. Creatine kinase was measured in the coronary effluent that was collected during the perfusion. The activity of creatine kinase was determined spectrophotometrically at 340 nm after an enzymatic reaction.

COMPARISON OF BN AND BNK RATS

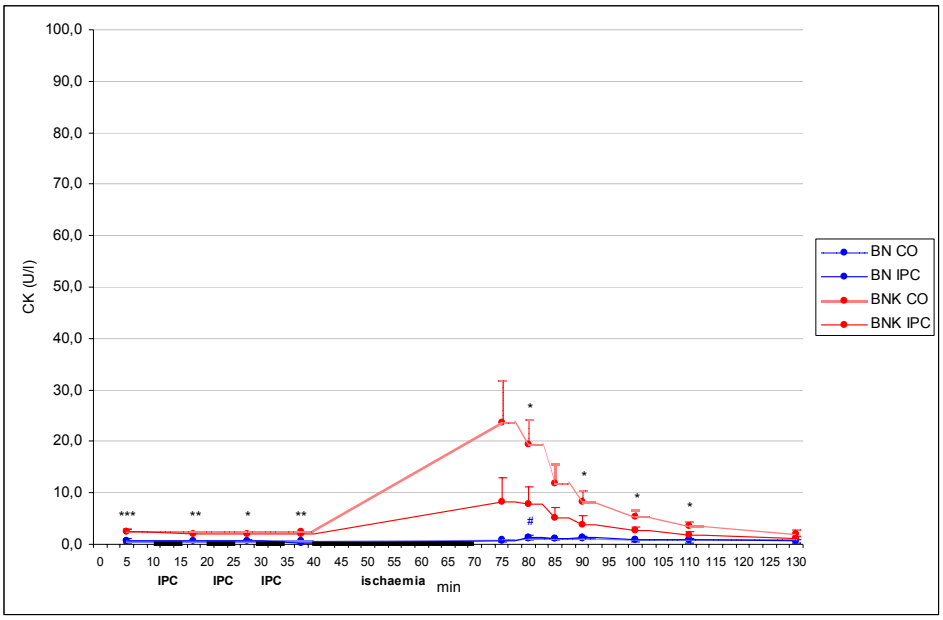


Fig.82a Time course of **creatine kinase activity** during perfusion of BN and BNK hearts on **standard diet**, mean ± SEM, n=4. * p<0,01; ** p<0,01; *** p<0,001 BNK hearts vs. BN hearts; # p<0,05 IPC vs. control experiment.

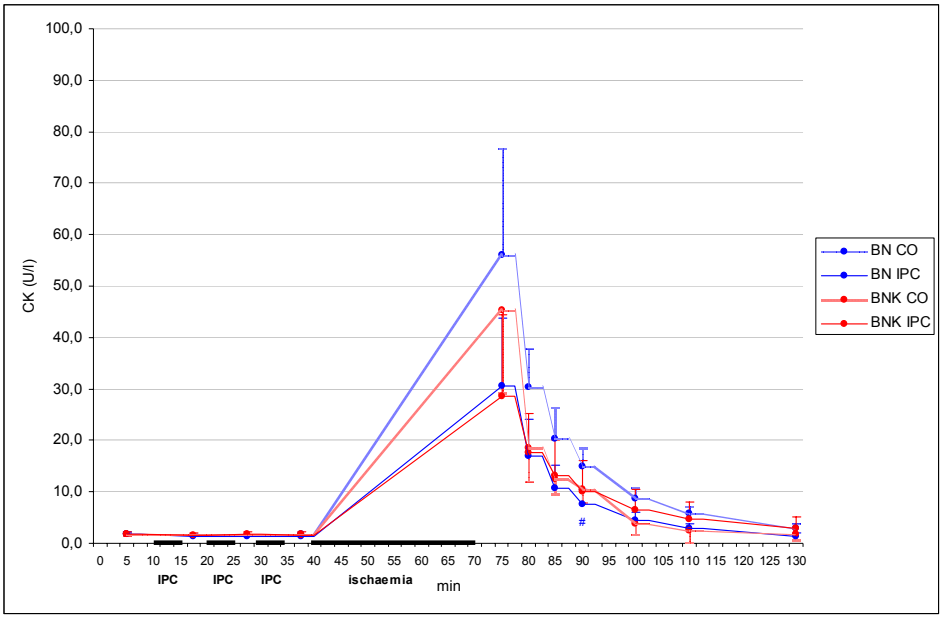


Fig.82b Time course of **creatine kinase activity** during perfusion of BN and BNK hearts after **spironolactone upon high salt diet**, mean ± SEM, n=4. # p<0,05 IPC vs. control experiment.

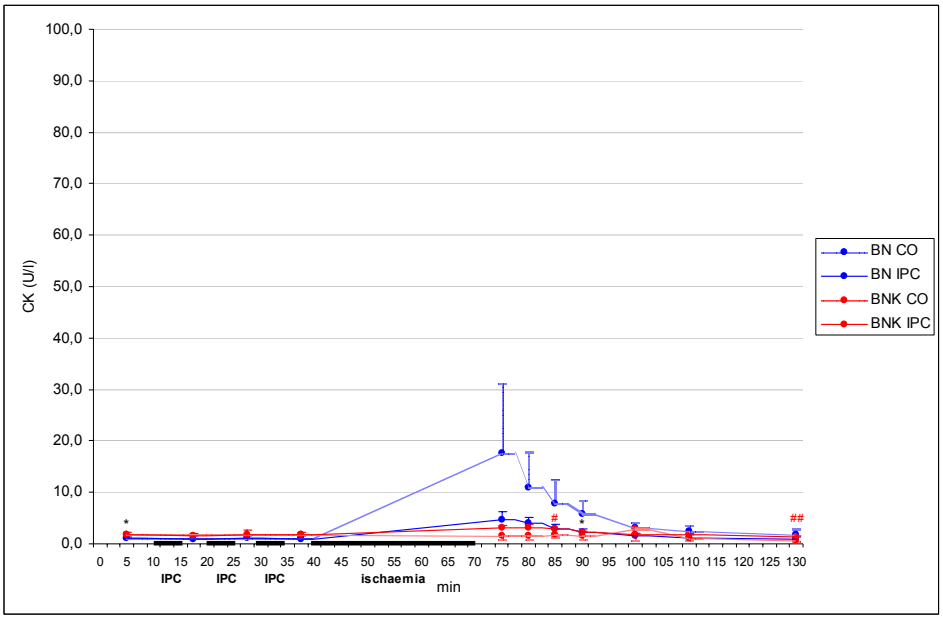


Fig.82c Time course of **creatine kinase activity** during perfusion of BN and BNK hearts on **high salt diet**, mean ± SEM, n=4. * p<0,01 BNK hearts vs. BN hearts; # p<0,05; ## p<0,01 IPC vs. control experiment.

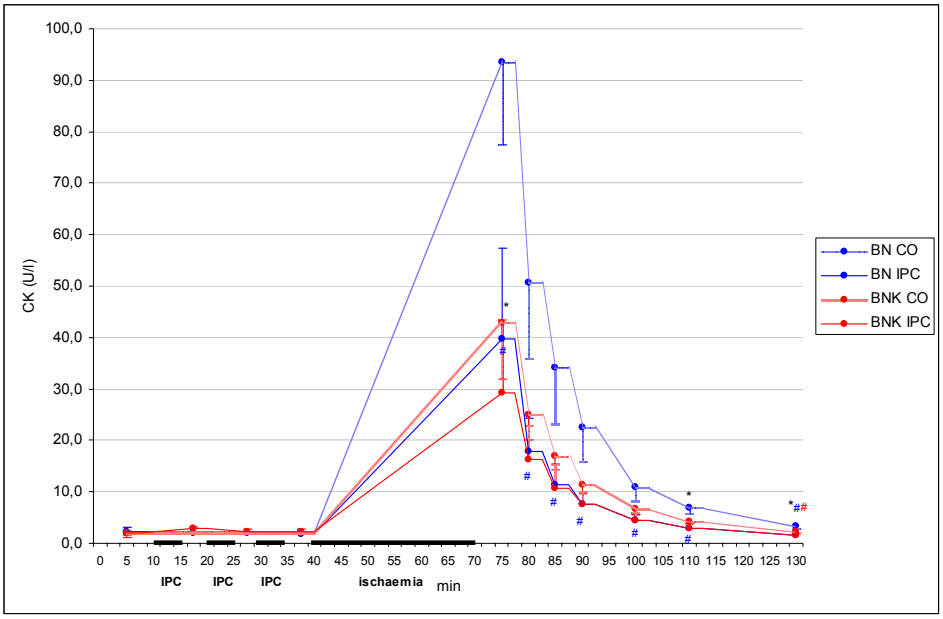


Fig.82d Time course of **creatine kinase activity** during perfusion of BN and BNK hearts after **spironolactone upon high sat diet**, mean ± SEM, n=4. * p<0,05 BNK hearts vs. BN hearts; # p<0,05 IPC vs. control experiment.

Initial creatine kinase activity was slightly higher in coronary effluent of hearts of BNK rats showing that these animals are more sensitive to ischaemia or cardiac damage caused during the surgical procedure. In hearts of BNK rats ischaemia significantly increased the creatine kinase activity and IPC was even more detrimental, according to the creatine kinase activity. In untreated hearts of BN rats ischaemia caused a small increase in the creatine kinase activity (Fig.82a). In hearts of both strains treatment with spironolactone was linked with a marked increase in creatine kinase activity. In hearts of spironolactone treated BN and BNK rats IPC mediated protective effects on myocardium. Nevertheless, this effect may not be relevant as the levels of creatine kinase activity were significantly higher than in hearts of untreated rats (Fig.82b). In hearts of BN rats fed with high salt diet an increase in post-ischaemic creatine kinase activity was found. In hearts of BNK rats high salt diet mediated positive myocardial effects as the creatine kinase activity was markedly reduced in both, preconditioned and non-preconditioned hearts (Fig.82c). In hearts of both strains fed with high salt diet and treated with spironolactone a substantial increase in the post-ischaemic creatine kinase activity was observed. Although IPC showed to be protective and decreased the creatine kinase activity in these experimental groups, it was irrelevant at these creatine kinase activity levels (Fig.82d).

EFFECT OF SALT DIET AND SPIRONOLACTONE

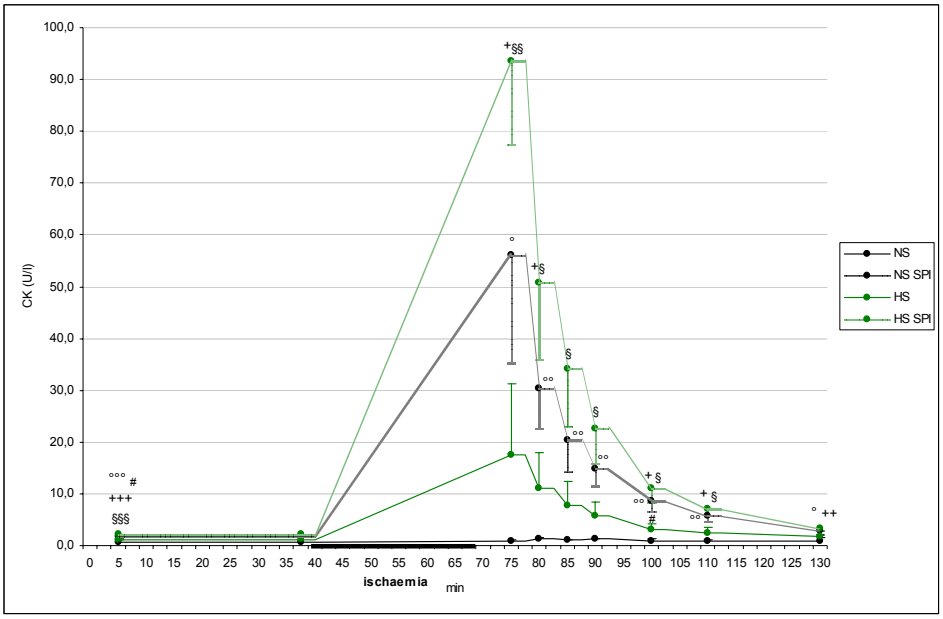


Fig.83a Time course of **creatine kinase activity** during **control experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05; °° p<0,01; °°° p<0,001 NS SPI vs. NS; # p<0,05 HS vs. NS; + p<0,05; ++ p<0,01; +++ p<0,001 HS SPI vs. NS; § p<0,05; §§ p<0,01; §§§ p< 0,001 HS SPI vs. HS.

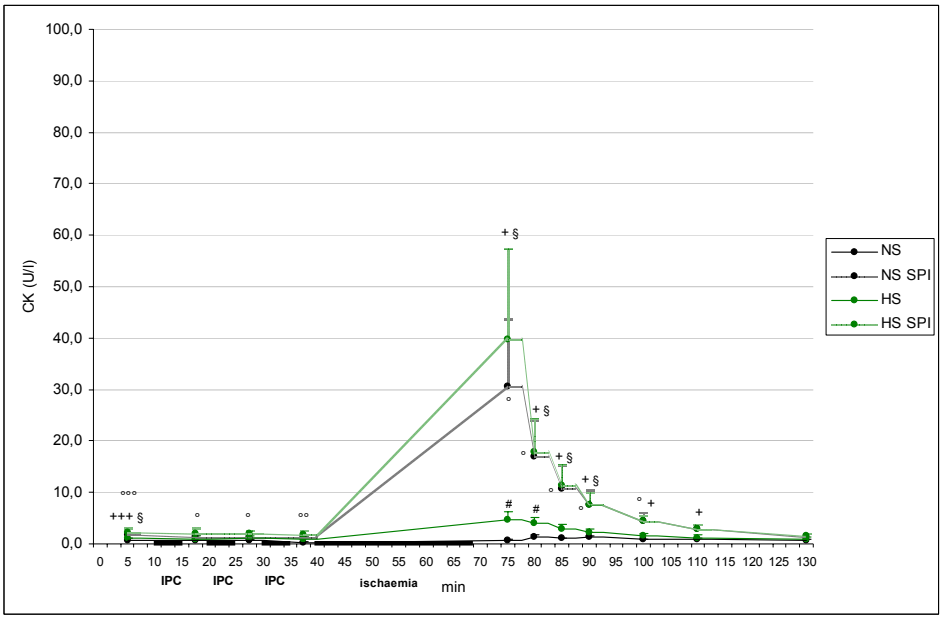


Fig.83b Time course of **creatine kinase activity** during **IPC experiment** of **BN hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05; °° p<0,01; °°° p<0,001 NS SPI vs. NS; # p<0,05 HS vs. NS; + p<0,05; +++ p<0,001 HS SPI vs. NS; § p<0,05 HS SPI vs. HS.

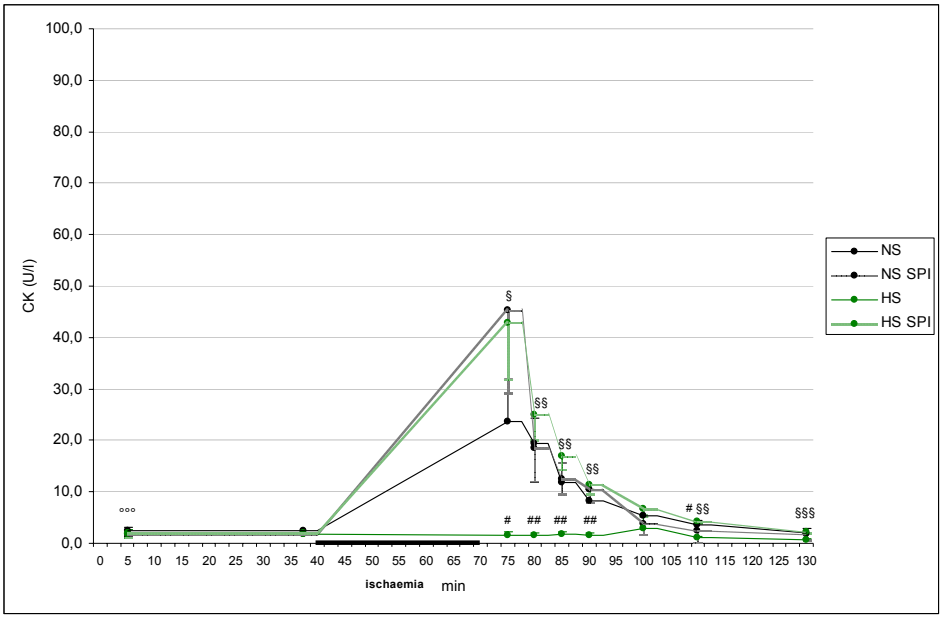


Fig.83c Time course of **creatine kinase activity** during **control experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. °°° p<0,001 NS SPI vs. NS; # p<0,05; ## p<0,01 HS vs. NS; § p<0,05; §§ p<0,01; §§§ p< 0,001 HS SPI vs. HS.

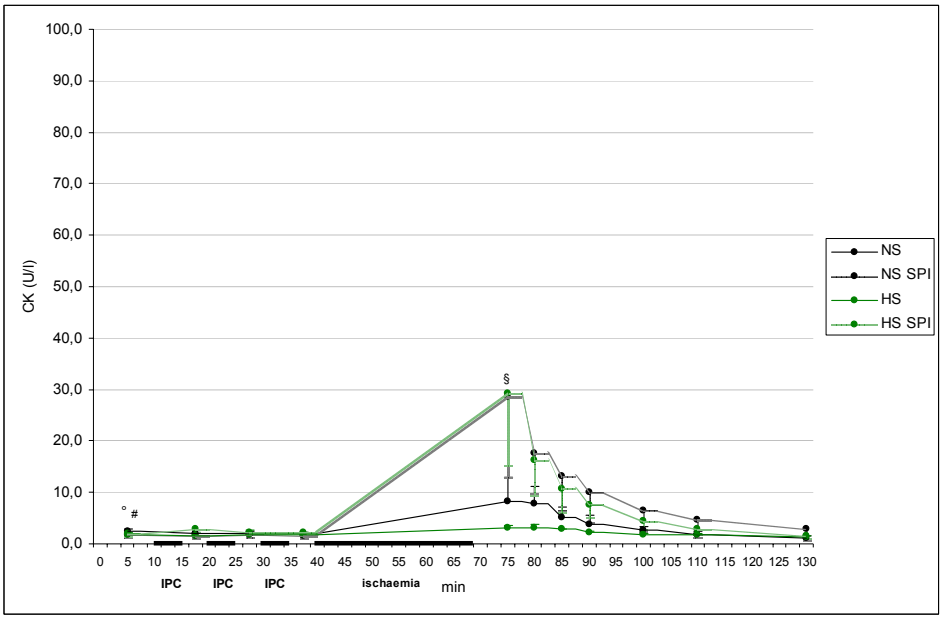


Fig.83d Time course of **creatine kinase activity** during **IPC experiment** of **BNK hearts** after normal and high salt diet in presence or absence of spironolactone, mean ± SEM, n=4. ° p<0,05 NS SPI vs. NS; # p<0,05 HS vs. NS; § p<0,05 HS SPI vs. HS.

In hearts of BN rats fed with high salt diet and treated with spironolactone the initial creatine kinase activity significantly increased. The post-ischaemic creatine kinase activity significantly increased in all experimental groups when compared with hearts of untreated BN rats (Fig.83a). Although IPC reduced creatine kinase activity in each experimental group, it was still too high to be judged as protective (Fig.83b).

Also in hearts of BNK rats spironolactone significantly increased the creatine kinase activity. High salt diet mediated even protective effects, as the creatine kinase activity was significantly reduced if compared with hearts of untreated rats (Fig.83c). IPC modestly reduced the post-ischaemic creatine kinase activity in hearts of all treated BNK rats (Fig.83d).

SUMMARY OF CREATINE KINASE ACTIVITY

The hearts of kininogen-deficient BNK rats displayed higher initial creatine kinase activity than the hearts of BN rats. The hearts of untreated BN rats were significantly less damaged than the hearts of BNK rats. Furthermore, hearts of untreated BNK rats were even more injured after IPC by additional ischaemic stimuli. In hearts of BNK rats fed with high salt diet the creatine kinase displayed lower levels. The most striking results revealed that treatment with spironolactone might be deleterious to the myocardium. It may suggest that aldosterone exerts cardioprotective actions following ischaemia.

5. DISCUSSION

The kallikrein-kinin system is generating the vasodilating peptides bradykinin and kallidin by the proteolytic enzymes plasma and tissue kallikreins, respectively, from precursor proteins HMW and LMW kininogens. The importance of the KKS has been discussed in the development of salt sensitive hypertension. Furthermore, the KKS has been shown to act cardioprotective, especially during ischaemic preconditioning. The development of mutant BNK rats, which have a congenital deficiency in plasma kininogens and a lower level of plasma prekallikrein, has enabled investigation of the role of KKS in these issues.

Although BNK rats have already been extensively studied, we have revised and completed previous results, especially after having detected KLP (KAL equivalent) in rats (Hilgenfeldt et al., 2005). The KAL/KLP specific antiserum developed in our laboratory (Hilgenfeldt et al., 1995) provided a good tool to analyse the levels of BK and KLP independently, not only in kininogen-deficient BNK rats but also in wild type BN rats. Furthermore, the fact that the deleterious effects of Na^+ accumulation induced either by higher Na^+ intake or aldosterone following Ang II infusion were entirely abolished by spironolactone (Majima and Katori, 1995), has inspired us to investigate the precise role of aldosterone in BNK rats.

EXPERIMENTAL DESIGN

The following experiments were designed in order to compare the kininogen-deficient BNK rats with the BN control rats. The primary experimental group (NS) of both rat strains remained without any treatment. The rats were given standard diet (0,5% NaCl) and served for general characterization and comparison of both strains. In the second experimental group (NS SPI) both rat strains were treated with spironolactone (20mg/day) for 10 days. This experiment should describe basic effects of spironolactone in both strains under standard conditions. In the third experimental set the BN and BNK rats (HS) were fed with a high salt diet (5% NaCl) for 10 days. These experiments should investigate the handling and actions of Na^+ in both strains under conditions of increased salt intake. In the fourth experimental group (HS SPI) both rat strains were given high salt diet (5% NaCl) and

simultaneously treated with spironolactone (20mg/day) for 10 days. This combination should preferentially characterize local effects of spironolactone under conditions of high Na^+ intake when aldosterone levels are significantly diminished.

Besides general characterization of experimental animals, we have focused on the characterization of the KKS, ET-1 and corticoid hormones in plasma, urine and relevant tissues. All these hormonal systems were found to play important role in cardiovascular diseases. Additionally, functional cardiac parameters were assessed in isolated rat hearts. Subsequently, the effects of ischaemic preconditioning in reperfused heart following 30min of global ischaemia were investigated.

5.1. GENERAL DESCRIPTION OF BN AND BNK RATS

It is generally accepted that under normal conditions the kininogen-deficient BNK rats show no apparent syndromes. Similarly, we found only moderate differences between BN and BNK rats. Due to different suppliers of both rat strains the body weight of BNK rats was slightly higher than that of BN control rats. The weight difference was also reflected in a higher heart weight and left kidney weight of BNK rats. The BNK rats excreted slightly more urine than BN rats, which originated in a higher water intake. Both rat strains gained with a standard diet on average 12g of body weight in 10 days. The basal mean blood pressure and heart rate, measured by tail cuff method, were slightly higher in BNK rats.

Obviously, the analysis of the components of the plasma KKS revealed significant differences between the wild type BN rats and mutant BNK rats. Oh-ishi & colleagues (1982) described complete congenital deficiency in HMW and LMW kininogen in BNK rats. Consequently, negligible levels of kinins were observed in their plasma and urine. A point mutation of Ala^{163} to Thr in the common part of the gene coding for both kininogens was found to be responsible for a defective secretion of HMW and LMW kininogens from hepatocytes of BNK rats (Hayashi et al., 1993). On the contrary to other investigators (Majima et al., 1991) we were able to detect both, HMW and LMW kininogens also in BNK rats, possibly due to the very careful handling of the samples and our very specific RIA. Of course, in BNK rats both plasma kininogens were substantially reduced. The levels of plasma HMW kininogen

were 35-fold lower so as the levels of LMW kininogen were 10-fold lower in BNK rats than in BN rats.

Kininogens were determined indirectly. BK was measured after the kininogens were digested with trypsin. The digestion conditions were optimised to avoid production of T-kinin from T-kininogen (Majima et al., 1991). Trypsin is specific for the cleavage of peptide bonds after Lys and Arg. Similarly to HMW kininogen, Arg is the BK preceding amino acid in the LMW kininogen sequence. Therefore the method should be equally sensitive to both kininogens. However, the LMW deficiency was less pronounced than that of HMW kininogen. Unfortunately, there is not great knowledge about the localisation and function of LMW kininogen in plasma. Lower levels of LMW kininogen in plasma may be attributed either to higher tissue kallikrein activity or to higher occurrence of LMW kininogen in a bound form.

The measurements of plasma kallikrein activity revealed a 2-fold reduction in BNK rats in comparison to BN rats. This impairment originated in the kininogen deficiency. The HMW kininogen acts as a carrier protein for plasma prekallikrein (Mandle et al., 1976). Lower HMW kininogen levels attenuated the binding of plasma prekallikrein and uncomplexed plasma prekallikrein was unstable and accounted for the reduced concentration of plasma kallikrein. In comparison to the HMW kininogen deficiency the range of plasma kallikrein activity reduction in BNK rats was markedly smaller. We may suggest that under normal conditions not all HMW molecules transport a molecule of prekallikrein. The low levels of HMW kininogen in BNK rats were definitely not sufficient to bind all prekallikrein molecules. Plasma kallikrein was found to have a high affinity to HMW kininogen. Similarly, the LMW kininogen is the primary substrate for tissue kallikrein. Unfortunately, as no specific substrate for tissue kallikrein in plasma is available, we were unable to analyse its activity.

The deficiency in kininogens and plasma kallikrein led to a significant reduction of kinin levels in plasma of BNK rats. Plasma BK, a product of the plasma kallikrein cleavage of the HMW kininogen, reached in BNK rats only 35% of BK in BN control rats. This result is rather consistent with the plasma kallikrein activity that was reduced in a similar order than with the amount of the HMW kininogen. It

demonstrates that not the amount of the substrate but the activity of plasma kallikrein is essential for the kinin generation.

One of the reasons why we were interested in the revision of the formal data, not only of BNK rats, was the discovery of kallidin-like-peptide (KLP) (Hilgenfeldt et al., 2005). Originally, it was believed that rats are able to generate only BK. The rat sequence of LMW kininogen displays little differences, especially in the kinin domain. It contains an Arg residue instead of a Lys in the human molecule in front of the BK sequence (Fig.6). By means of our highly specific antiserum for the KAL molecule, we could easily detect KLP in rat plasma and urine. Lys and Arg, exchanged in the amino-terminus, are both basic amino acids similar in their properties, which provided a cross-reaction of KAL-antiserum with KLP of about 80%.

However, the isolation of KLP turned out to be extremely difficult. Due to Arg, the basic properties of KLP in comparison to KAL are more pronounced. This fact accounted for the complications during the sample handling and measurements. The urine and plasma samples had to be treated very carefully. Under usual conditions in pH range from neutral to acidic KLP is charged and easily and quickly disappears by retention to the material walls. Furthermore, the half time of kinins in biological materials was described to be very short. Therefore the urine samples were collected on ice bath to reduce the enzymatic degradation. Similarly, plasma was collected into pre-chilled tubes. After the collection they were aliquoted to avoid repeated thawing and freezing. The samples were immediately frozen and were kept always on ice during analysis. Furthermore, the samples were in contact only with siliconized material or with special low retention tubes that helped to reduce the adsorption to the walls. Both, this very special handling and specific antisera that were able to distinguish between BK and KAL (KLP) (Hilgenfeldt et al., 1995) were essential for the detection of KLP in rat.

It is assumed that KLP is cleaved from LMW kininogen by tissue kallikrein. Unfortunately, as mentioned above, we have no information about the activity of tissue kallikrein in plasma. These data would help to understand the origin of plasma KLP. In BN control rats the KLP levels were approximately 10-fold higher than those of BK. Although we have not determined or specifically inhibited the enzymatic

degradation of kinins we believe that due to the careful sample handling the data reflect the physiological important levels of both kinins. According to previously reported amounts of HMW kininogen, plasma kallikrein and BK we suggest that the activity of tissue kallikrein responsible for the generation of KLP may be significantly higher than that of plasma kallikrein. Due to the lack of specificity of the most kinin assays the distinct physiological roles of KAL and BK were not described until now. On the basis of our results it seems likely that for most physiological functions KLP is more important than BK. In BNK rats the plasma KLP levels reached 65% of the levels of BN control rats. The tissue kallikrein activity should not be affected by the kininogen-deficiency, which may explain the smaller difference in the amount of KLP between both strains. If the hypothesis were true that KLP is the major physiologically important kinin it would help to explain no apparent syndromes in BNK rats under normal conditions.

Measurements of the components of KKS in urine showed mostly no significant differences between BN and BNK rats. In contrast to other investigators, we were able to detect urinary kininogen as well as kinins in urine of BNK rats. Kininogens and kinins found in urine are supposed to be of renal origin. Kinins filtered from the glomeruli are probably destroyed immediately in the proximal tubules where the kininases, e.g. NEP and ACE are located. The collecting tubules are equipped with full repertoire of the tissue KKS, which is distinct from plasma KKS (Majima and Katori, 1995). The LMW kininogen is synthesised in the principal cells of the collecting duct and is believed to be the main source of renal kinins. Similarly, tissue kallikrein was found in adjacent connecting tubule cells. The close proximity of these cells suggests that kinins are generated within the lumen of the initial segment of collecting duct (Figueroa et al., 1988).

It seems probable that the defect in secretion of kininogens is relevant only for the hepatic cells and therefore the kininogen-deficiency is present only in plasma. As typical secretory proteins kininogens undergo glycosylation prior to secretion from hepatic cells (Bhoola et al., 1992). Whether the mutation to Thr^{163} in the kininogen molecule of BNK rats provides a new site for glycosylation or it impairs original glycosylation hereby it may influence the kininogen secretion. The glycosylation depends on the enzymatic equipment of each cell. It is generally accepted that

glycosylation is not necessary for the secretion of proteins in different tissues. In case the renal LMW kininogen is secreted without a glycosylation this might be a possible explanation for the normal kininogen levels in urine of BNK rats although the kininogen molecule is mutated. The measurement of urinary LMW kininogen revealed comparable levels in both strains. Similarly, no major differences were found in the activity of urinary kallikrein. In BNK rats the kallikrein activity was slightly higher which correlated with the moderate increase in urine volume. Also Majima & colleagues (1993) found such a correlation. Their interpretation suggested that an increase in urinary kallikrein activity was accompanied with an increase in urine volume. Anyway the regulation of urine volume is a very complex mechanism and we believe it cannot be so strongly influenced by only one component. On the contrary, it seems likely that the urinary kallikrein activity depends on the urine volume. *In vitro* experiments revealed that K^+ is one of the few regulators that release renal kallikrein (Lauar et al., 1982). Our previous data showed a slightly lower urinary excretion of K^+ in BNK rats than in BN rats, which suggest an increased extracellular K^+ levels in BNK rats and may be linked with the higher urinary kallikrein activity.

Consequently, no significant differences were found in BK urinary excretion of both strains. Interestingly, urinary KLP was detectable in BNK rats but not in BN rats. It is generally accepted that KAL/KLP is the representative kinin of tissue KKS. Because any component of the plasma KKS is present in the urine we suggest that urinary BK is a metabolite of KAL/KLP generated by urinary aminopeptidases. Unfortunately, there are very little experimental data about the differences between BK and KAL. This was in part caused by the availability of only unspecific commercial assays for kinins. BK and KAL are usually detected together but the data are often presented as BK. Furthermore, theoretically KAL could be investigated only in human or mice because it was believed that rats generate only BK. Consequently, almost no data about rat urinary aminopeptidases exist that could be responsible for the cleavage of N-terminal Arg. We may only suggest that BNK rats have different renal enzymatic equipment, rather the KLP degrading than the KLP generating system.

Supplementary investigations revealed no between-strain differences in the expression of the renal B_2 receptor (data not shown) that is responsible for the most physiological actions of kinins. B_2 receptor was shown to be constitutively expressed

and highly conserved (Bachvarov et al., 2001). KAL and BK exert equal affinity to the B_2 receptor. We suggest that rat KLP have similar properties in binding to the B_2 receptor, but precise data are not available yet.

The KKS system counterbalances the effects of the RAAS. The RAAS is known to be of great importance in many physiological and pathological situations, e.g. regulating blood pressure and salt homeostasis. In fact, it was awaited that the KKS is of similar importance. In recent years the scientific progress provided the possibility to work with knockout mice lacking the B_1, B_2 or both receptors. Surprisingly, the phenotype of these animals did not notably differ from that of wild type animals (Bader, 2003). At present there is a general believe that the KKS is not involved in the regulation of blood pressure under normal conditions but is activated in pathological states, e.g. at an excess of salt intake and inflammation. Anyway this finding has left some doubts about the role and importance of kinins.

Kinins were shown to inhibit Na^+ reabsorption and accelerate its urinary excretion whereby antagonising the renal effects of aldosterone. Therefore we focused on the investigation of aldosterone and its precursor molecules in kininogen-deficient BNK rats. Deoxycorticosterone (DOC) is the precursor molecule for the synthesis of corticosterone and aldosterone. The conversion of corticosterone, which is the main glucocorticoid in rat, is catalysed by 11ß-hydroxylase, encoded by CYP11B1 gene. Aldosterone is synthesised in three sequential steps by aldosterone synthase, encoded by CYP11B2 gene. Both enzymes are members of the cytochrome P450 family and in rat are to 88% homologous. Recently, local steroid synthesis was described in the brain tissue, which is believed to be relevant in hypertensive states. Still controversial remains the existence of aldosterone synthase and aldosterone generation in the cardiac tissue.

We have analysed the concentration of DOC, corticosterone and aldosterone in plasma, urine and brain tissue. These data revealed only moderate differences between BN and BNK rats under standard conditions. The DOC levels were slightly reduced in plasma, urine and brain of BNK rats. Basal corticosterone concentration was almost identical in all three sample types. Corticosterone represents the last molecule in the glucocorticoid synthesis. Its concentration is about 100 and 1000-fold

higher as that of DOC and aldosterone, respectively. Therefore a moderate alteration of its concentration would not be reflected in the final values. Majima et al. (1993, 1994) showed in experiments with BNK rats that they are very sensitive to the actions of aldosterone antagonist spironolactone. Therefore we speculated whether BNK rats operate with higher aldosterone levels. On the contrary, the BNK rats displayed rather a modest reduction of urinary aldosterone in comparison with BN rats. None of the measurements in plasma, urine and brain revealed significant differences in aldosterone concentration between the BN and BNK rats.

These results were based on measurement of the free fraction of these hormones. We investigated also the expression of genes coding for the 11ß-hydroxylase and aldosterone synthase in the adrenal gland. The most suitable method for the expression analysis is the detection and quantification of mRNA carrying the information about respective proteins. After the isolation the mRNA was reverse transcribed and a specific part of cDNA coding for the protein was quantified with aid of specific primers in a real-time PCR performed in LightCycler®. As already mentioned, the sequences of genes coding for these two enzymes are highly homologous. To ensure specificity, the primers used in the PCR were chosen according to the publication of Gomez-Sanchez (2004). According to Romero (2005) the primers were controlled for the specificity for each of the genes separately. The method of relative quantification is based on comparing expressions of a target gene and a housekeeping gene that is expressed in standard amounts under respective experimental conditions. Until recently, commonly used housekeeping genes were GAPDH or ß-actin. Experiences revealed that both of them are strongly regulated in common experiments. Furthermore they belong to a group of pseudogenes that are strongly present in genomic DNA, which can falsely influence the quantification. A list of new potential housekeeping genes was suggested by Roche, e.g. 18S, PBGD. It was shown that any gene can be up or down-regulated. Therefore it is necessary to prove the suitability of each housekeeping gene in every experiment. We used PBGD as the housekeeping gene, especially because the expression of this gene was in the same order of magnitude like those of 11ß-hydroxylase and aldosterone synthase. The stable expression of PBGD was tested in all experimental groups and neither spironolactone nor high salt diet altered its expression. Similarly to corticosterone levels, the expression of 11ß-hydroxylase, which is responsible for corticosterone

generation, was almost identical in both strains. Expression analysis of genes coding for aldosterone synthase revealed a moderate reduction in BNK rats. This result was consistent with the measurements of aldosterone that displayed a moderate reduction of aldosterone levels of BNK rats.

Such a well-established method was necessary to be able to investigate the expression of aldosterone synthase in the cardiac tissue. Cardiac expression of aldosterone synthase was described to be extremely low in case that it at all exists. Similarly to Ye et al. (2005), we attempted to detect expression of the CYP11B2 gene using rat ventricles and atria of BN and BNK rats. On the contrary to the one-step RT-PCR performed by Ye et al. (2005), our two-step approach could increase the sensitivity of RT-PCR. Nevertheless, even that method failed to perform consistent and reproducible data. In our previous experiments with isolated rat hearts we could observe that the longer the heart was perfused the less aldosterone was found in the cardiac tissue. Consequently, we were able to detect eluted aldosterone in the coronary effluent in the beginning of the perfusion but later on its detection failed. We can agree that, however the CYP11B2 gene is not expressed or it exists at such a low levels, any physiological significance of local aldosterone synthesis in the cardiac tissue is extremely unlikely.

The principal effector of the cellular response to mineralocorticoids is the mineralocorticoid receptor (MR). Although the MR primarily acts as a transcription factor recent evidence suggests that it may also mediate non-genomic activation of second messenger pathways. In addition there is a growing body of evidence that some actions of aldosterone may involve a receptor other than the MR (Fuller and Young, 2005). In 2004 new discoveries about a mutation of the MR in BN rats were published by Marissal-Arvy et al.. They found a Tyr to Cys substitution (Y73C) in the N-terminal part of the BN MR, a change from GGT to GGC in codon 221 leading to no amino acid substitution and an Asp to Gly (N487G) exchange. *In vitro* studies further revealed that the Y73C substitution induces greater transactivation of the MR by aldosterone and surprisingly by progesterone as well. Thus progesterone could substitute for aldosterone after adrenalectomy in BN rats. *In vivo* they found increased progesterone levels in BN rats that partially compensated for aldosterone in adrenalectomy. It was necessary to confirm or disconfirm such a MR mutation in

BN rats used in our experiments. Furthermore, we were interested whether the BNK rats share this mutation. Very recent data really confirmed respective mutations in the MR gene sequence of BN rats. Furthermore, we found an additional fourth mutation of Ala to Gly (A771G). In BNK rats the gene sequence coding for the MR shared the same mutations like that of BN rats but they displayed two other mutations in codon 632 and 636 leading to Val to Leu and His to Leu exchange respectively (Engel, 2006). The precise consequences of these mutations have to be investigated in binding studies. Theoretically, we may expect different MR properties in either strain.

Generally, the classical MR shows the same high affinity for aldosterone and corticosterone. As the circulating levels of glucocorticoids are at least 100-fold higher than those of aldosterone Mihailidou and Funder (2005) tried to answer the question of how aldosterone can access MR in mineralocorticoid target tissues. The MR function is in part regulated at a prereceptor level. At least in epithelial tissues the MR is coexpressed with the enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD2), which in rat metabolises corticosterone to 11-dehydrocorticosterone. The enzyme reduces intracellular glucocorticoid levels from about 100-fold those of aldosterone to about 10-fold. These data suggest that even in epithelial tissues where it is protected by 11ß-HSD2, the MR is occupied but not activated by glucocorticoids. When the enzyme is inhibited or deficient the glucocorticoid-MR complex is activated, possibly caused by decreased levels of NADH, which change the intracellular redox state and possibly activates other corepressors. Similarly, in tissues in which the MR is expressed in the absence of 11ß-HSD2 differential effects of corticosterone and aldosterone may be the result of interactions with ligand-specific coactivators. These observations indicate a degree of plasticity of the receptor allowing the conformation to be in part dictated by the ligand (Fuller and Young, 2005).

Additionally to the KKS and corticoid hormones we investigated plasma and urinary levels of ET-1 in both rat strains. There are several known relationships between aldosterone and ET-1. ET-1 is a potent vasoconstrictor generated especially in endothelial cells. Furthermore, it is also produced by the heart, kidney and CNS. The synthesis and release of this peptide can be stimulated by many factors including Ang II and mineralocorticoids (Lüscher et al., 1993). ET-1 stimulates release of

aldosterone. Furthermore, ET-1 stimulates endothelial ACE activity whereby stimulating kinin degradation. The overall cardiovascular effect of endogenous ET-1 depends on the balance between ET_A and ET_B mediated effects. Activation of vascular smooth muscle ET_A receptors causes vasoconstriction and tends to elevate blood pressure. Activation of endothelial and renal ET_B receptors promotes vasodilation and natriuresis and tends to decrease blood pressure. Locally produced ET-1 plays an important role in modulation of renal excretion of Na^+ and water. ET-1 blocks reabsorption of Na^+ by inhibiting the tubular Na^+/K^+-ATPase activity in the proximal tubule and collecting duct and blocks the reabsorption of water in the collecting duct by inhibiting the effects of ADH on tubular osmotic permeability (Haynes and Webb, 1998). Plasma ET-1 levels are in picomolar range, lower than those required to evoke vasoconstriction. These concentrations may stem from the efficient clearance of this potential harmful peptide, mainly by ET_B receptors localized within the lung, liver, and kidney as well as by NEP present in the renal, pulmonary, and vascular tissues (Abassi et al., 2001). We found that kininogen-deficient BNK rats have significantly higher plasma ET-1 levels than the BN rats. The excretion of urinary ET-1 was also increased but only moderately. Whether this between-strain difference originates in the misbalance of ET_A and ET_B receptors in BNK rats call for detailed investigation.

All these findings opened us a new insight into the regulation of salt homeostasis in the kininogen-deficient BNK rats. Until now especially the role of the renal KKS was emphasized. The knowledge about the mutation in the MR and higher ET-1 levels in BNK demanded a careful revision of previous results.

The kininogen-deficient BNK rats were extensively used in experiments investigating the mechanism of myocardial ischaemic preconditioning. Ischaemic preconditioning (IPC) was found to be the strongest protective mechanism against ischaemic injury. The understanding of these mechanisms would provide an access to a new spectrum of therapeutics treating myocardial infarction and cerebral stroke. After a series of experiments three most important candidates triggering the protection were found. Besides adenosine and the mediators from neural origin, e.g. noradrenaline and opioids, BK was described to be involved. Therefore the kininogen-deficient BNK rats should be a suitable and convenient experimental model for the investigation of the

role of kinins in IPC. The widespread usage of spironolactone as antihypertensive and its positive cardiovascular effects exceeding its diuretic potential suggested new roles of aldosterone in the cardiovascular system. On the basis of our recent knowledge about the MR in BN and BNK rats, we were interested in the cardiac consequences. Nevertheless, also increased levels of ET-1 in BNK rats may influence cardiac physiology. ET-1 has potent positive chronotropic and inotropic effects. It is a potent constrictor of coronary vessels, causing myocardial ischaemia and ventricular arrhythmias. The Langendorff model of isolated perfused heart served for the determination of basic cardiac parameters. Furthermore we have investigated the effects of 30min global ischaemia with or without IPC in hearts of both strains.

In experiments with BNK rats Liu et al. (2000) showed that under normal conditions endogenous kinins play only a minor role in maintaining basal blood pressure and cardiac homeostasis. Basal cardiac hemodynamics and function as well as histology were not different from rats with intact KKS. A local KKS exists in the heart, which enables it to synthesise and release kinins (Nolly et al., 1994). Several studies showed that kinins are rapidly released from the heart during acute myocardial ischaemia. Recently, we could show that both BK and KLP are released during IPC from the rat heart and that the cardioprotective effect could be blocked by administration of a specific antiserum against KAL suggesting that KLP is the cardioprotective kinin in the rat heart (Liu et al., 2005). *In vivo* models of myocardial infarction and heart failure revealed no differences in myocardial ischaemia-reperfusion injury between kininogen-deficient BNK rats and rats with intact KKS (Liu et al., 2000) However, in response to stimuli such as ischaemic preconditioning or administration of ACE inhibitors, the cardioprotective effect was almost abolished in BNK rats (Linz et al., 1996). These findings showed that increased release or decreased degradation of kinins might have profound cardioprotective significance. We have not explicitly investigated the cardiac KKS but according to our findings about the renal KKS it seems likely that the cardiac KKS is also not affected by the kininogen-deficiency in contrast to the plasma KKS and therefore comparable in both strains. *In vivo* the plasma KKS naturally contributes to the cardiac effects of kinins as the heart operates with a very high blood circulation. However, in *in vitro* Langendorff experiments of isolated hearts the plasma is removed and the heart is

perfused with a perfusion medium supplying only necessary ions and substrates. Accordingly, from this point of view we could determine rather the long-termed consequences of the plasma kininogen-deficiency on the myocardium.

The basal heart rate of BNK rats was significantly lower than that of BN rats. On the contrary, the heart rate measured *in vivo* during the blood pressure measurement displayed a moderate elevation in BNK rats. As previously mentioned ET-1 exerts chronotropic effects and stimulates the activity of the sympathetic nervous system. Therefore the higher plasma ET-1 levels in BNK rats may be also responsible for the increased heart rate, besides for the higher blood pressure. *In vivo* the heart rate generated in the sinus node is controlled by the parasympathetic and sympathetic activity of the nervous system. In isolated hearts the innervation is abolished after the removing of the pericardium. Therefore the fall of the heart rate in BNK rats following the isolation of the heart may suggest a higher reactivity of BNK rats to sympathetic stimuli. Another explanation would be a higher sensitivity of BNK rat hearts to the ischaemic damage caused even during the short time of the heart isolation.

Immediately after the first 5min stimulus of IPC the heart tried to supplement the missing substrates and the heart rate reflectively increased. After these short ischaemic insults the heart rate always returned to basal levels. Following 30min global ischaemia the heart rate immediately increased and after a stabilisation period remained slightly lower than the basal values. In non-preconditioned hearts the post-ischaemic heart rate was similar in both strains. IPC showed to be protective in BN but not in BNK rat hearts. Indeed, the hearts of BN rats that underwent IPC displayed higher post-ischaemic heart rate than the non-preconditioned hearts, which was not found in BNK rats. In this context, higher heart rate could be considered as protective because it assures delivery of oxygen and substrates to the myocardium and particularly confirms the functionality of the sinus or AV nodes and also that of the cardiac conduction system. Nevertheless, it should be mentioned that higher heart rate, which also increases oxygen consumption, might be too high to allow sufficient substrate and oxygen distribution in post-ischaemic circumstances.

The contractile function of the hearts was evaluated by dp/dt_{max} and dp/dt_{min} which represent the capacity of the left ventricle to contract during systole and its ability to

relax during diastole. Moreover, the left ventricular developed pressure (LVDP) and left ventricular end-diastolic pressure (LVEDP) were assessed. The BNK rat hearts were characterised by a higher LVDP and a lower LVEDP. Anyway the contractility was identical in both strains. The rate of the myocardial relaxation was significantly lower in BNK rats, which may have been partially responsible for the higher blood pressure in intact BNK rats. ATP depletion caused by ischaemia led to a decrease in the myocardial contractile function. After the onset of ischaemia the contractility rapidly decreased. In BNK rat hearts the dp/dt_{max} decreased to a lesser extent than in BN rat hearts and remained reduced also during the reperfusion period. Similarly, the LVDP was slightly higher in BNK rat hearts, probably due to the differences in the initial values. Ischaemia impaired also the rate of myocardial relaxation and the LVEDP. IPC turned out to be rather harmful as the contractility even more decreased in preconditioned BN rat hearts.

The perfusions were performed at constant perfusion pressure, which is advantageous in experiments investigating the effects of ischaemia. The coronary arteries were allowed to autoregulate during the reperfusion according to the momentary demand of the ischaemic heart. The lacking oxygen and substrates were then compensated by an increase in the coronary flow. The basal coronary flow was identical in hearts of both rat strains. Immediately after the 30min ischaemia the coronary flow naturally increased as the heart tried to deliver more substrates. The later post-ischaemic coronary flow was higher in BNK rat hearts than in BN rat hearts, which actually described greater relaxation of the coronary arteries in BNK rats. We may only speculate about the molecular basis of this finding. This result is rather contradictory as kinins are substances with vasodilating properties. As mentioned above we believe that the BNK rats are comparably equipped with the cardiac KKS as the BN rats. It is known that ET-1 may also mediate vasodilation through ET_B receptors on endothelial cells via generation of NO. Based on the findings that BNK rats displayed higher ET-1 levels this might be a possible explanation. Even though a prompt reperfusion is required to save the ischaemic myocardium, paradoxically, increased coronary flow may cause reperfusion injury, which may in part explain the worse response of BNK rat hearts to ischaemia. It is known that the ischaemic tissue releases mediators stimulating processes leading to tissue damage and necrosis. During the reperfusion these mediators are distributed

even to the healthy tissue and possibly initiate pathological processes as well. IPC did not alter the post-ischaemic coronary flow in any of the strains. In accordance, Baxter and Ebrahim (2002) reported that the cardiac response to IPC is not attributed to changes in coronary flow.

The myocardial damage caused by ischaemia was assessed by the measurement of creatine kinase (CK) activity in the coronary effluent. CK is an intracellular enzyme that is secreted after tissue damage. It is routinely used for the diagnosis of myocardial infarction. The CK activity was significantly higher in the effluent of BNK rat hearts than in that of BN rat hearts. It suggests that the BNK rat hearts were indeed more sensitive to ischaemia and were more injured already during the isolation of the heart, which may correlate with the increased post-ischaemic coronary flow of BNK rats. BN rat hearts displayed only small ischaemic damage that was slightly improved by IPC. On the contrary, preconditioned BNK rat hearts displayed even larger ischaemic damage than the non-preconditioned ones. The release of creatine kinase from the tissue is depending on the cellular integrity. Therefore, it seems probable that the BN and BNK rat hearts may also differ in their microstructure.

These results are in accordance with previous reports on BNK rats showing diminished response to myocardial IPC of these rats. So far, these effects were explained by the lack of functional KKS in BNK rats. We suggest that the cardiac KKS is similar in both strains. The question is to which extent the deficient plasma KKS influenced the cardiac tissue. We could show that there may be other mediators that could contribute to the differences between BN and BNK rats, not only in mediating the effect of IPC.

5.2. EFFECTS OF SPIRONOLACTONE IN BN AND BNK RATS

Previous experiments showed that the kininogen-deficient BNK rats display increased sensitivity to the actions of spironolactone (Majima et al., 1993 and 1994). Spironolactone is a specific antagonist of aldosterone acting primarily through competitive binding to the MR. This mechanism of action could be confirmed by the measurements of corticoid hormones. Although recent investigations suggest rather

wide occupancy of the MR by glucocorticoids we found that aldosterone levels in plasma, urine and brain tissue substantially increased following spironolactone treatment. The antiserum used in aldosterone assay displayed no crossreactivity with spironolactone. Therefore these data demonstrate that aldosterone was replaced from its binding sites at the MR, which increased the free fraction of aldosterone that was then assessed. In case of MR occupancy by glucocorticoids, aldosterone would not increase in this order of magnitude.

We found that following spironolactone plasma and urinary aldosterone was significantly higher in BNK rats in comparison to BN rats. Under standard conditions the expression of aldosterone synthase and aldosterone levels were rather lower in BNK than in BN rats. In accordance with the mutation analysis of the renal MR in BN and BNK rats it opens the possibility of a different sensitivity of the MR to aldosterone. The physiological consequence of these additional mutations has to be determined in binding studies. Furthermore, additional expression analysis revealed an overexpression of the MR in kidneys of BNK rats (Engel, 2006). These findings may explain not only the higher levels of aldosterone in BNK rats following spironolactone but also the differences between BN and BNK rats that could not be accounted for the kininogen-deficiency.

Spironolactone caused a modest increase in the expression of aldosterone synthase in adrenal tissue. In BNK rats, similarly to standard conditions, the expression was slightly lower. Spironolactone also known as K^+ sparing diuretics increases extracellular K^+ levels. Production of aldosterone is acutely sensitive to very small changes in extracellular K^+ concentrations. Increased extracellular K^+ concentration causes a membrane depolarisation leading to the opening of voltage depending Ca^+ channels and a rapid rise in Ca^{2+} concentration. This leads to the activation of calmodulin and Ca^{2+}/calmodulin-dependent protein kinases, which phosphorylate transcription factors that stimulate CYP11B2 gene transcription (Spat & Hunyady, 2004).

As the BNK rats overexpress the MR it seems likely that the additional mutations of the receptor alter the signalling cascade, possibly by changing the affinity to various steroids, which leads to different effects of spironolactone in these rats.

Spironolactone caused also a significant increase in DOC levels in urine, plasma and brain tissue of BNK rats. In BN rats the levels of DOC were not altered. DOC is a potential ligand on the MR. According to the mutation and overexpression of the MR in BNK rats, these results suggest that the excess of MR was occupied, besides aldosterone, also by DOC. Spironolactone significantly down-regulated the 11ß-hydroxylase to the same extent in both strains. By contrast, in both strains we found increased levels of corticosterone following spironolactone treatment. In BNK rats corticosterone levels in urine, plasma and brain tissue were significantly higher than those of BN rats. Spironolactone can also act as a weak glucocorticoid receptor (GR) antagonist and therefore may be responsible for the increased corticosterone levels in BN rats. Nevertheless, although we have not investigated the GR in these rats the higher corticosterone levels of BNK rats may also refer to the higher number of MR in this strain. In fact, corticosterone was found to exert higher affinity to MR than to GR (Funder, 2005). The consequences of this additional binding of DOC and corticosterone to MR in BNK rats, caused by mutation and overexpression of the MR, will have to be analysed in more detail. The mutations of the MR found in BNK rats may influence the signalling cascade as well. Under normal conditions glucocorticoids act as MR antagonists but when the protective enzyme 11ß-HSD2 is blocked or deficient corticosterone becomes a MR agonist. The same antagonist-to-agonist change is seen when intracellular redox state is altered by generation of e.g. reactive oxygen species, which may be enhanced during ischaemia (Funder, 2005).

We may hypothesise that the overexpression of the MR in BNK rats is present also in the brain tissue. Under normal conditions both, brain DOC and corticosterone were significantly lower in BNK rats. On the contrary, following spironolactone administration their levels were significantly higher than those in BN rats. In comparison to GR that is widely distributed throughout the CNS in neurons and glial cells there are fewer MR and these are localized predominantly in the hippocampus, the septum and the granular cells of the cerebellum. Generally, MR in the CNS do not colocalize with 11ß-HSD2 and the majority of brain MR is occupied by glucocorticoids.

Brain MR may play a key role in a number of homeostatic mechanisms including blood pressure regulation, regulation of sympathetic tone, thirst and salt appetite,

learning and memory and hypothalamic/pituitary adrenal axis regulation. Nevertheless, it is often difficult to distinguish between glucocorticoid and mineralocorticoid effects on the MR (Connell and Davies, 2005). Steroid biosynthesis in the CNS is a well documented phenomenon. All of the key genes involved in the corticosteroid biosynthesis are expressed at fairly high levels in specific CNS regions where it correlates with areas with high MR expression. This is consistent with an autocrine or paracrine model of corticosteroids actions in the brain. Although there is a strong evidence to support the production of aldosterone of CNS it is much more difficult to establish whether locally produced aldosterone exerts any significant physiological or pathophysiological effects. Aldosterone production within the brain may suggest regulation of blood pressure (Gomez-Sanchez et al., 1990). It is necessary to mention that as steroids are lipophilic and able to cross the blood-brain barrier it is not surprising that most of the corticoid hormones present in the normal brain are derived from the circulation (Gomez-Sanchez et al., 2005).

During the 10 days of spironolactone treatment the body weight decreased even below the initial body weight at the beginning of the experiment. It was accompanied also by the loss of kidney weight. It is likely that the loss in body weight was caused by the diuretic activity of spironolactone. The diuretic effect of spironolactone was stronger in BNK rats than in BN rats, which might be caused by the mutation and overexpression of the MR in BNK rats. Consequently, the body weight loss was more pronounced in BNK rats. Even though spironolactone is successfully used in the therapy of hypertension in humans the mean blood pressure after the 10 days of spironolactone treatment displayed a very moderate increase in both rat strains. Under normal conditions the BNK rats displayed higher heart rate then the BN rats. The expression of the MR in the cardiac tissue of BNK rats has to be first analysed but according to the data we may propose that the number of MR in the myocardium will be increased. Similarly, artificial overexpression of the human MR in mice produced a significant increase in heart rate but no change in systolic blood pressure (Ouvrard-Pascaud et al., 2005). Consequently, spironolactone decreased the elevated heart rate of BNK rats. On the contrary in BN rats spironolactone caused an increase of the heart rate.

In general, spironolactone influenced the plasma KKS only moderately. In comparison to BN rats all of the components of plasma KKS were significantly lower in BNK rats. Following spironolactone treatment plasma HMW and LMW kininogens slightly increased in both strains, which suggests that aldosterone may inhibit the synthesis or release of kininogens. In both, BN and BNK rats plasma kallikrein activity remained unaltered. Consequently, the kinin levels in plasma of BN rats stayed constant. In BNK rats plasma BK slightly increased but plasma KLP was significantly reduced. As the plasma kallikrein activity was unchanged we may suggest that spironolactone influenced the degradation of BK in BNK rats. Aldosterone was found to up-regulate the expression of ACE mRNA in rat endothelial cells (Sugiyama et al., 2005). ACE is known to have higher affinity for BK than for Ang I, resulting in more favourable kinetics for BK degradation than for Ang II generation (Zisman, 1998). Therefore we may suggest that spironolactone decreased the activity of ACE, which was more pronounced in BNK rats due to the MR properties and overexpression in BNK rats. Unfortunately, as we have no information about the activity of tissue kallikrein in plasma we cannot interpret the decreased KLP levels in BNK rats.

The effects of spironolactone on the renal KKS were more pronounced than those on plasma KKS. Indeed, kidney is the main target tissue of aldosterone. Treatment with spironolactone caused an increase in urinary LMW kininogen, apparently significant in BNK rats. Spironolactone increased also urinary kallikrein activity, which again correlated with urine volume and was significantly more pronounced in BNK rats. As spironolactone increases extracellular K^+ these results are in accordance to the above mentioned mechanism of renal kallikrein release by K^+ ions. Independently of increased urine volume the levels of BK and KLP in urine increased, which may be a consequence of increased urinary kininogen. In both strains spironolactone caused a significant increase in urinary KLP, which was more pronounced in BNK rats. Similarly, the excretion of urinary BK increased, which was found significant in BNK rats. In comparison to BN rats, the BNK rats excreted more KLP and less BK. Similarly to plasma, these findings suggest that spironolactone influenced the degradation of kinins. It is believed that the kininases in urine are different from those in plasma which may explain the opposite effects. It is likely that spironolactone inhibited the genomic actions of aldosterone mediated by the MR. These findings

suggest that aldosterone down-regulates the transcription of renal LMW kininogen. Furthermore, aldosterone indirectly inhibits the release of renal kallikrein by the decrease of extracellular K^+. These data also suggest that KLP is the main kinin in urine responsible for the diuretic and natriuretic actions and that urinary BK is a metabolite of KLP.

Spironolactone caused a significant increase in urinary ET-1 in BNK rats, which suggests that aldosterone decreases the urinary ET-1 excretion in BNK rats. In BN rats urinary ET-1 excretion remained unchanged. These data are in contrast to findings of Lüscher et al. (1993) who found that the synthesis and release of ET-1 is stimulated by aldosterone. Also patients with primary aldosteronism have increased circulating ET-1 levels that contribute to the pathophysiology of hypertension (Letizia et al., 1996). It seems likely that the mutation in the MR of BNK rats may influence different signalling mechanism. We could show that the excess of the MR in BNK rats is also occupied by DOC and corticosterone. And these ligands possibly mediate rather antagonistic effects, at least in the renal tissue. In this context, plasma ET-1 levels following spironolactone have to be analysed.

It is now well accepted that aldosterone has physiological and pathophysiological effects in non-epithelial tissues including the heart. The MR are present in cardiomyocytes and blood vessel wall (VSMC and endothelial cells). Cardiomyocytes normally express very low levels of 11ß-HSD2. The receptor is effectively occupied by endogenous glucocorticoids, which have opposing effects to aldosterone. In contrast, VSMCs express the MR and show 11ß-HSD2 activity. The inhibiting of 11ß-HSD2 activity allows physiological levels of glucocorticoids to activate MR, which produce similar responses to aldosterone. In addition to the steroid receptor modulation of transcription there is an increasing evidence of rapid non-genomic effects that involve activation of second messenger pathways. They have recently been reviewed in extension. However, whether aldosterone acts via the classical MR to mediate these effects or via a novel receptor, as is the case for progesterone and estrogen, remains controversial. The non-genomic actions of aldosterone are varied and mediated by a multitude of second messenger systems depending on the cell type involved (Fuller and Young, 2005).

Similarly to the heart rate measured *in vivo* in BNK rats following spironolactone in the Langendorff experiment the basal heart rate decreased in both strains. These findings may be explained by inhibiting the non-genomic actions of aldosterone, possibly mediated by the MR. Aldosterone was found to increase IP_3, DAG and subsequent Ca^{2+} concentration. These stimulate then the translocation of PKC from the cytosol to the membrane. PKC inhibits the rapid activation of the Na^+/H^+ antiporter by aldosterone, which increases the intracellular pH. Furthermore, aldosterone also increases cAMP levels, which activate PKA. This seemingly opposing action of aldosterone on smooth muscle second-messenger system normally associated with both contraction and relaxation may be a mechanism by which the cells are primed for rapid contraction/relaxation cycles (Connell and Davies, 2005). The heartbeats of atria were eliminated and remained only in the ventricles. It appeared that spironolactone caused AV blockade. The RALES study provided convincing evidence that patients with severe heart failure treated additionally to conventional therapy with spironolactone showed a 30% reduction in mortality. Much of this benefit was due to a reduction in sudden cardiac death, suggesting that aldosterone blockade reduced the incidence of cardiac rhythm disturbances (Connell and Davies, 2005). Heart rate remained reduced also during the whole perfusion and did not display such a variation following ischaemia as in the untreated hearts. In BN rats IPC remained protective. The protective effect of IPC on heart rate was not altered by spironolactone. Therefore it seems likely that the MR does not play a role in the mechanism of IPC by regulating the heart rate. In BNK rat hearts IPC remained without an effect.

Aldosterone administration in perfused hearts was shown to increase cardiac myocytes contractile force (Barbato et al., 2004). Spironolactone treatment caused a reduction of the LVDP in BNK rat hearts. The higher basal LVDP and contractility in BNK rat hearts was reduced by spironolactone to the levels of BN rat hearts. These findings assume an overexpression of the cardiac MR in BNK rats, which remains to be determined. Interestingly, the LVDP and contractility of spironolactone treated BN rat hearts remained unaffected by ischaemia. Also the effect of IPC in BN rat hearts was abolished. On the contrary, in spironolactone pre-treated BNK rat hearts IPC increased the post-ischaemic LVDP and contractility. Final effectors of the non-genomic aldosterone actions include K^+ channels. K^+ channels play an important role

in the protective mechanism of IPC. These finding would suggest that aldosterone may be involved in the mechanism of IPC by its non-genomic actions mediated by the MR. Similarly, spironolactone pre-treatment led to and increase in the basal LVEDP in BNK rat hearts. Post-ischaemic LVEDP increased in hearts of both strains. The effects of IPC remained unchanged. Spironolactone reduced also the rate of myocardial relaxation in both strains.

Spironolactone did not alter the coronary flow in any strain. Except of that the basal coronary flow was slightly decreased in spironolactone treated BN rat hearts, it remained unaltered during the perfusion vs. untreated ones. In spironolactone treated BNK rat hearts the post-ischaemic coronary flow was slightly higher than that of BN rat hearts. These finding are consistent with those of Fujita & colleagues (2005). They found that aldosterone administration rapidly decreased coronary flow in ischaemic as well as in non-ischaemic heart *in vivo*. Moreover, aldosterone further worsens the contractile and metabolic functions, which was blunted by PKC inhibitor but not MR antagonist.

All these data suggest that aldosterone non-genomically induces vasoconstriction via PKC-dependent pathways possibly through membrane receptors, which leads to the worsening of the cardiac contractile and metabolic function in the ischaemic heart. There are some reports that eNOS is a PKC substrate and PKC-mediated phosphorylation inhibits eNOS activity. Because NO is a widely known vasodilative agent decreased NO activity could attenuate the vascular tone leading to a decrease in coronary flow (Fleming et al., 2001). Aldosterone can also suppress iNOS and NO in cardiomyocytes in a post-transcriptional manner. The decrease in NO synthesis may also account for the known cardiovascular effects of aldosterone (Chun et al., 2003). Elevated levels of aldosterone may worsen myocardial ischaemia via non-genomic as well as genomic pathways in the ischaemic heart. This knowledge may explain above mentioned higher sensitivity of BNK rat hearts to ischaemia as seen in the pronounced reduction of the heart rate and an increase of CK activity already following the isolation of the heart.

The most surprising and striking results were found after measuring the activity of the CK activity in the coronary effluent of hearts treated with spironolactone. The CK

activity significantly increased already prior to ischaemia. No similar result was found describing that spironolactone could be harmful to the myocardium. There might be a relation, linked with the MR properties, to the increased coronary flow following spironolactone, which may worsen the reperfusion injury. Nevertheless, the precise mechanism remains unclear and should be further investigated. It may possibly show that treatment with spironolactone could be rather deleterious in patients that will suffer from myocardial infarction.

Unfortunately, the interpretation of the Langendorff experiments is based only on suggestions and hypothesis. Additional experiments with a second series of rats that underwent identical treatment are already in progress. The atria and ventricles of these rats were collected for subsequent expression analysis. Relevant components of tested hormonal systems will have to be investigated to get a basic insight into the molecular basis of these effects.

5.3. EFFECTS OF HIGH SALT DIET IN BN AND BNK RATS

The kininogen-deficient BNK rats were often used in experiments with high salt diet because they easily develop hypertension. Majima et al. (1993) demonstrated that the BNK rats showed an increase in blood pressure after receiving a diet containing 2% of NaCl whereas under the same conditions the blood pressure of wild type BN rats remained unaffected. The authors suggested that the absence of renal KKS is responsible for the insufficient excretion of an excess of Na^+. Consequently, Na^+ accumulation leads to hypertension (Fig.1). They achieved similar effects in BNK rats with non-pressor doses of Ang II. Although these effects were completely abolished by spironolactone and therefore mediated by aldosterone, they emphasized the role of renal KKS in the development of the hypertension. Nevertheless, under standard conditions, we found no substantial differences in the renal KKS between the BN and BNK rats. Moreover, on the basis of the discovery of MR mutation and overexpression in BNK rats our data suggest that the role of the renal KKS was overestimated and falsely interpreted.

In details, Majima & colleagues (1993) could show that increases in the dietary salt content from 3-8% caused rapid and significant increases in the blood pressure in

BNK rats. In contrast, in BN rats the raise of blood pressure was mild and gradual. Nevertheless, the final value of blood pressure was identical in both strains. It was suggested that the KKS plays a role in modulating the initiation and rate of the blood pressure increase. Based on the knowledge of the MR properties we suggest that these effects were rather caused by the enhanced mineralocorticoid activity in BNK rats. Indeed, they found for aldosterone typical significant elevations of the Na^+ concentration in the serum, erythrocytes and cerebrospinal fluid of BNK rats. Accumulation of Na^+ in the cells increased the pressor sensitivity of the arterioles against noradrenaline and Ang II. Furthermore, the accumulation of Na^+ in cerebrospinal fluid caused increases in the sympathetic discharge from the CNS.

In accordance to Majima et al. (1993), we have also detected a significant increase in blood pressure following 10 days of high salt diet (5% NaCl). Despite the kininogen-deficiency in BNK rats blood pressure of both strains did not differ. We suggest that the elevated blood pressure may have been in part caused by fluid retention resulting in total volume expansion. In both strains high salt diet naturally increased thirst, consequently water intake and urine volume to the same extent. As we measured blood pressure as late as after 10 days of high salt diet we cannot speculate whether the raise was more rapid in BNK rats. A number of studies explored the mechanisms explaining the dietary Na^+-blood pressure relationship. Besides previously mentioned modulation of vasoreactivity and sympathetic nervous system the role of NO and its precursors was highlighted among which the RAAS, KKS and ET system might be of great importance (Jones, 2004). Nevertheless, the increase in blood pressure was found to be of minor importance regarding the multiple deleterious effects of high salt diet.

Increased salt intake reduced the heart rate of BNK rats. On the contrary, in BN rats the heart rate tended to increase. As described in detail in following section aldosterone levels were substantially reduced under increased Na^+ intake. The absence of aldosterone on the MR is comparable with the action of spironolactone that inhibits the binding of aldosterone to MR. These findings are in accordance with the previous hypothesis that aldosterone may be responsible for the higher heart rate in kininogen-deficient BNK rats. Likewise, Majima et al. (1994) demonstrated that the

increase in heart rate of BNK rats following an infusion of a non-pressor dose of Ang II was completely abolished by spironolactone.

Measurements of aldosterone levels confirmed the classical effect of a high salt diet. Increased levels of Na^+ reduce the release of renin and consequently Ang, which is the main stimulator of aldosterone synthesis. We found that high salt diet significantly down-regulated the expression of aldosterone synthase, which resulted in minimal aldosterone levels and hereby facilitated the excretion of an excess of Na^+. In BN rats aldosterone was almost undetectable. Even though in BNK rats the levels of aldosterone were also minimal they were still higher than those of BN rats. In urine this difference was more pronounced confirming the key role of kidney in handling increased levels of Na^+. These results correlate with the expression of aldosterone synthase that was slightly higher in BNK rats. This finding is rather contradictory to higher Na^+ levels of BNK rats that should lead to lower Ang. We have not determined whether the BNK rats display defective sensitivity of RAS to Na^+ leading to higher Ang levels. Nevertheless, the stimulation of aldosterone synthase expression is dependent on increased intracellular Ca^{2+}, which may be caused also by other mediators. As mentioned previously ET was found to stimulate aldosterone secretion. In this context we showed that the BNK rats showed significantly higher ET-1 levels. These data suggest that even under condition of high Na^+ intake the BNK rats operate with higher aldosterone levels, which may be further intensified by the possibly higher number of MR and account for the elevated intracellular Na^+ levels in BNK rats. This mechanism is of major importance because it describes the reasons for the deleterious effects of high salt diet in BNK rats.

Volpe et al. (1993) described comparable findings in stroke-prone spontaneously hypertensive rats (SHRSP). In male SHRSP a high salt intake was associated with malignant development of hypertension, severe vascular injury and premature death by stroke, which was not fully accounted for the higher blood pressure. They found that during high salt diet plasma aldosterone levels remained significantly higher in SHRSP than in SHR rats, which was associated with reduced Na^+ excretion. The blunted aldosterone suppression was dissociated from the levels of circulating renin since its activity showed comparable suppression in both strains. The unfavourable vascular consequences of high salt intake could be prevented by ACE inhibitor,

which would suggest involvement of the KKS in preventing these deleterious effects. Recent knowledge about the broad spectrum of aldosterone effects in the cardiovascular system may help to define molecular mechanisms leading to these effects. Presumably, the presence of both, aldosterone and increased intracellular Na^+ may open the possibility for investigating of new signalling schemes of aldosterone that may be specifically inhibited by kinins.

High salt diet significantly increased DOC levels in urine and plasma in BNK but not in BN rats. We found also increased corticosterone in urine but not in plasma of BNK rats. In BN rats both plasma and urinary corticosterone levels increased. These findings are rather confusing because the expression of 11ß-hydroxylase remained unaffected by high salt diet in both strains. Therefore we may only suggest that these differences originated in the different expression and affinity of the MR in BN and BNK rats, which remains to be investigated in details. As described in following chapter 5.4., additional administration of spironolactone should help to determine at least the binding of these ligands to the MR.

Following high salt diet the BN rats achieved comparable body weight like the control rats on standard diet. In contrast, the BNK rats lost few grams of body weight. This loss of body weight in BNK rats was not caused by reduced food intake. On the contrary, in BNK rats the food and consequently salt intake was slightly higher which resulted in slightly higher diuresis. It seems likely that this between-strain difference originated in the overexpression of the MR in the brain tissue of BNK rats (see chapter 5.2.). Brain MR and especially the amygdala MR may play a key role in the control of thirst and salt appetite, respectively. As the brain MR is not coexpressed with the 11ßHSD2 it is believed to be predominantly occupied by glucocorticoids (Connell and Davies, 2005). Indeed, additionally we found significantly higher DOC and corticosterone levels in the brain tissue of BNK rats. Nevertheless, the precise effects, agonistic or antagonistic, of these MR ligands are difficult to determine.

As kinins were shown to exert natriuretic effects the higher urine volume and body weight loss in BNK rats may correlate with the significantly higher KLP levels in these rats. In comparison to standard conditions high salt diet slightly increased urinary KLP levels in BNK rats, which was possibly caused by significantly higher LMW

kininogen levels in urine and increased urinary kallikrein activity (correlating with the urine volume).

Similarly to standard conditions, in BN rats urinary KLP was almost undetectable while urinary LMW kininogen and renal kallikrein activity significantly increased. The precise mechanism of an increase in the urinary excretion of LMW kininogen following high salt diet is unclear. To the best of our knowledge there are no studies on the selective release of kininogen from any renal cells. In both strains high salt diet significantly increased urinary excretion of BK. Unfortunately, we can only speculate about the origin of BK. We have already suggested that the BNK rats might have different renal enzymatic system than the BN rats. These data show that high salt diet did not alter the renal degradation of kinins. Although the kinin levels increased, which correlated with increased kininogen and kallikrein, the proportion between KLP and BK remained unchanged. Furthermore, preliminary data revealed unchanged expression of the renal B_2 receptor following high salt diet in both strains.

The experiments with high salt diet revealed no significant changes and between-strains differences in the plasma KKS, except of significantly reduced plasma KLP in BN rats. Kinins are known to have a half-life of about 30s and act in autocrine-paracrine manner. It is not defined to what extent the measured levels reflect actual and local concentrations of kinins. We did not follow enzyme concentration neither that of generating KLP (tissue kallikrein in plasma) nor that of degrading KLP. Therefore we may only speculate about these effects of high salt diet in BN rats.

High salt diet significantly increased urinary excretion of ET-1. In BNK rats the levels of ET-1 in urine were significantly higher than those of BN rats. Similarly to kinins, clearance studies revealed that urinary ET-1 is of renal origin. ET-1 is generated in tubular epithelial cells of renal medulla, mainly in the inner collecting duct, where it acts in autocrine/paracrine fashion. It is involved in tubular handling of Na^+ and water through ET_B receptor subtype (Fig.2). Furthermore, activation of ET_B receptors stimulates the release of NO, which plays a crucial role in the regulation of renal hemodynamics and excretory functions (Abassi et al., 2001). The increase of ET-1 is necessary to handle the excretion of an excess of Na^+. The higher ET-1 levels of BNK rats might have compensated for the increased Na^+ retention caused by the

enhanced mineralocorticoid action. Independently of its indirect effects on blood pressure by modulating renal functions ET exerts direct effects on the vasculature through ET_A receptors. As both strains developed the same increase in blood pressure following high salt diet we suggest that the renal ET-1 did not directly influence the blood pressure. Whether there is a correlation of blood pressure and plasma ET-1 in BN and BNK rats remained to be investigated. Nevertheless, high salt diet was shown to increase plasma levels of ET-1 (Sasser et al., 2002). The role of ET in vascular homeostasis is rather complex because ET receptors have both pressor and depressor effects *in vivo*. The activation of ET_B receptor in endothelial cells is known to reduce the vascular reactivity and thereby counterbalance ET-induced enhancement of the mechanisms of vascular smooth muscle contraction. A possible role of ET in salt-sensitive hypertension was investigated by Smith & colleagues (2003). They demonstrated that low-dose infusion of ET in rats fed with high salt diet increased vascular reactivity that involved Ca^{2+} entry from extracellular space. The enhanced Ca^{2+} influx, particularly during high salt diet, suggested activation of other mechanisms possibly involving PKC (a downstream molecule of ET receptors). These could be ET induced up-regulation of Ca^{2+} channels in VSMC and an increase in the myofilament force sensitivity to Ca^{2+}.

Dietary Na^+ was shown to mediate blood pressure-independent effects leading to cardiac hypertrophy and perivascular fibrosis. Structural changes may be related to Na^+ itself and/or to the subsequent hormonal changes associated with the diet. The vascular and cardiac structure could be normalized with e.g. ET_A antagonist or spironolactone (Schiffrin et al., 1999; Jones, 2004). Partovian et al. (1998) demonstrated that administration of B_2 antagonist in SHR rats fed with high salt diet mediated synergistic effect and enhanced extracellular matrix (elastin and collagen) accumulation. Accordingly, we found that heart weight tended to increase following 10 days of high salt diet. Precisely, the BN rats displayed significant increase in their heart weight. This finding correlate with the significantly reduced plasma KLP levels in BN rats. Even though 10 days of high salt diet might be too short to allow progression of greater structural changes the absence of KLP on the cardiac B_2 receptor might in part be responsible for the increased heart weight of BN rats. As in BNK rats the heart weight did not increase and they showed high plasma levels of

KLP this hypothesis could be potentially proven by administration of B_2 antagonist to these rats fed with high salt diet.

The consequences of high salt diet for cardiac functions were investigated in Langendorff experiments with isolated rat hearts. High salt diet caused a decrease in the basal heart rate of isolated hearts. In BNK rats the reduction of heart rate was moderate, which was comparable with values measured *in vivo*. These data confirm the role of aldosterone in the regulation of heart rate in BNK rats as discussed above. In contrast, the heart rate of isolated BN rat hearts significantly decreased. In accordance with the increased heart weight of BN rats the reduction of the heart rate may be associated with structural changes of the cardiac tissue. Interstitial fibrosis leads to physiologic and morphologic disorganisation of the ventricle, which includes impairment of the electrical synchronization (De Mello, 2004). In BNK rat hearts high salt diet abolished the reflective increase of heart rate during IPC. High salt diet diminished differences between BN and BNK rat hearts especially in the post-ischaemic heart rate and hereby abolished the post-ischaemic effect of IPC in BN rats.

In BN rat hearts excess of dietary Na^+ increased contractility that was accompanied by an increase in LVDP. These effects might have been a consequence of a decreased heart rate. Except the increased intracellular Na^+ concentration alterations in microscopic cardiac anatomy, e.g. cardiomyocyte hypertrophy may alter the contractile process. Moreover, these findings are in accordance with increased vascular reactivity mediated by ET-1 through increased sensitivity to Ca^{2+} (see chapter 5.1.). Both parameters significantly increased following ischaemia, especially in preconditioned hearts, showing that high salt diet impaired the ability of the ischaemic myocardium to recover. Additionally, the rate of relaxation, characterized by dp/dt_{min}, especially in preconditioned BN rat hearts significantly increased. Interestingly, the contractility and LVDP of BNK rat hearts remained unchanged following increased Na^+ intake. Both parameters were not altered by ischaemia and remained constant during the whole experiment. As described in detail in above chapter 5.2. aldosterone was found to worsen the contractile and metabolic function in ischaemic heart through genomic and non-genomic pathways. The contractility and rate of relaxation of BNK rat hearts were found to be significantly lower than

those of BN rat hearts. In accordance, in BNK rat hearts the basal and post-ischaemic LVEDP was significantly higher than in BN rat hearts. On the basis of these adverse findings we may conclude that the increase in blood pressure does not originate in changed cardiac parameters because in both strains the increase in blood pressure was identical.

In accordance, high salt diet diminished the moderate between-strain difference in coronary flow, which may be in accordance with the hypothesis concerning the role of aldosterone in the regulation of coronary flow. High salt diet increased the creatine kinase activity in coronary effluent of BN rats. Likewise to untreated hearts, creatine kinase activity was significantly reduced in preconditioned BN rat hearts vs. the non-preconditioned ones, which lose on significance due to too high levels in comparison to untreated group. On the contrary, coronary effluent of post-ischaemic BNK rat hearts displayed significantly lower creatine kinase activity than that of untreated hearts. Furthermore, in BNK rats the creatine kinase activity was lower than that of BN rat hearts.

It is evident that high salt diet caused severe changes predominantly in BN rat hearts whereby also abolished the effects of IPC in these rats. These data highlight especially the cardioprotective role of endogenous KLP under conditions of an excess of dietary Na^+. It seems likely that kinins through B_2 receptors are able to counterbalance the unfavourable effects of high salt diet. In BNK rats most of the measured cardiac parameters remained unchanged following high salt diet. The ability of BNK rat hearts to exert a reaction to ischaemia was abolished whereby also the response to IPC was disabled. According to the CK activity these data suggest that high salt diet made the BNK rat heart resistant against the deleterious effects of ischaemia. It is plausible that these findings are attributable to the absence of aldosterone under condition of an excess of dietary Na^+ and confirms the dominant role of the MR in BNK rats.

5.4. EFFECTS OF SPIRONOLACTONE IN BN AND BNK RATS FED WITH HIGH SALT DIET

The rational of this set of experiments seems to be at first rather incomprehensive and unsubstantiated as under conditions of a high salt intake aldosterone is down-

regulated. Therefore there seems to be no need to antagonise its effects. Nevertheless, as we found higher aldosterone levels in BNK rats in comparison to BN rats following high salt diet the blockade of its effects on the MR gained on significance. Moreover, even though the potential of spironolactone to antagonise aldosterone effects is significantly limited under conditions of high salt diet spironolactone by binding to the MR replace also other important ligands of this receptor. As described in chapter 5.2., the mutation and overexpression of the MR in BNK rats allowed significant binding of DOC and corticosterone. Even though the BN rats are similarly characterised by a mutation of the MR, which was found to be distant to that of BNK rats, under standard conditions they do not share additional binding of DOC and corticosterone. Nevertheless, based on the knowledge about the affinity of the MR to various ligands under conditions of very low levels of aldosterone it is more than virtual that the MR is occupied by other corticoid hormones whose levels are not diminished under conditions of an excess of Na^+. Furthermore, recent studies have raised the questions concerning the kind of effects (agonistic or antagonistic) mediated by these ligands and what are the critical conditions switching the mechanisms.

We found that even under conditions of high salt diet aldosterone mediate important effects via the MR. Although plasma aldosterone levels remained substantially reduced we found that spironolactone replaced residual aldosterone from the renal and brain MR. Administration of spironolactone under high salt diet increased expression of aldosterone synthase to comparable levels as under standard conditions. We believe that aldosterone levels slightly increased as a consequence of both, increased synthesis and displacement from the MR. These findings describe a negative feedback mechanism between the MR and aldosterone synthase activity. Besides stimulation of ACTH, the precise molecular mechanism possibly involves K^+ ions altered by spironolactone. Even though high salt diet significantly reduced the expression of aldosterone synthase it is evident that residual aldosterone levels are necessary to exert essential aldosterone effects.

The BN rats displayed slightly higher aldosterone levels in urine and brain than the BNK rats. It would be too simple if these results would correspond with our previous findings under standard dietary conditions. The MR of BN rats was predominantly

occupied by aldosterone whereby in BNK rats the MR was supposed to be simultaneously occupied also by other corticoids. Accordingly, these hormones, whose levels are significantly higher under increased dietary Na^+ than those of aldosterone, would hinder aldosterone binding. Nevertheless, measurements of DOC and corticosterone levels, potential ligands of the MR, following spironolactone administration under high salt diet revealed in part confusing, exact opposite findings.

In BN rats, neither high salt diet nor additional administration of spironolactone altered the DOC levels in plasma and urine. This finding might demonstrate that DOC did not bind to the renal MR of BN rats. In BNK rats high salt diet did not alter the DOC levels in plasma but increased its urinary excretion. In contrast to findings under standard conditions following spironolactone both, plasma and urinary DOC levels even decreased, which definitely excludes binding of DOC to the MR and suggest additional mechanisms lowering DOC levels. In the brain of BN rats DOC levels significantly increased following spironolactone showing that under conditions of increased dietary Na^+ the brain MR are occupied by DOC, naturally by a significantly higher rate than by aldosterone. On the contrary, administration of spironolactone did not change the levels of DOC in the brain tissue of BNK rats suggesting no binding of DOC to brain MR. This finding is opposite to what was found under standard conditions.

We showed that in BNK rats but not in BN rats under standard conditions even the corticosterone binds to the renal and brain MR, additionally to GR. Similarly to DOC, levels of corticosterone displayed opposite effects. In BN rats high salt diet increased plasma corticosterone levels that were lowered by spironolactone. Simultaneously, the urinary excretion of corticosterone increased. In the brain tissue corticosterone levels increased after displacement from receptors (MR or GR) by spironolactone. In BNK rats plasma levels of corticosterone slightly decreased but remained unchanged in urine and brain tissue.

Similarly to high salt diet, additional administration of spironolactone did not alter the basal expression rate of 11ß-hydroxylase. If spironolactone was administered under standard dietary conditions the expression of 11ß-hydroxylase decreased. These results are rather contradictory to the negative feedback mechanism whereby the

steroid hormones inhibit their own secretion. One would expect rather an increase of 11ß-hydroxylase expression following spironolactone treatment. However, in case of 11ß-hydroxylase, which is predominantly controlled by ACTH, it seems likely that even in case of glucocorticoids, this mechanism depends on the actual salt status. This effect may be caused or accompanied by changes in the MR expression or MR properties. Besides for the changes in the amount of MR under unchanged rate of synthesis the lower plasma and urinary levels of DOC and corticosterone following spironolactone under high salt diet raised a question, whether spironolactone, especially in BNK rats, could have stimulated the degradation of these corticoids. DOC, as a primary substrate for both aldosterone synthase and 11ß-hydroxylase, is degraded into corticosterone or aldosterone. Nevertheless, the expression of both enzymes was not altered and the levels of respective products were not increased. Corticosterone, especially in epithelial tissues, is degraded by 11ß-HSD2 that is predominantly known to assure aldosterone specificity. Mutations of this enzyme have important pathological implications, e.g. causing syndrome of apparent mineralocorticoid excess leading to hypertension (Connell and Davies, 2005). Unfortunately, there is not a distinct knowledge about the regulation of activity and expression of this enzyme. Nevertheless, the tissue levels of corticosterone did not decrease and it seems improbable that this mechanism could have significantly influenced plasma corticosterone concentration.

Although in the brain a local steroid synthesis was described the amount of the 11ß-hydroxylase and aldosterone synthase mRNA in the brain was found to be exceedingly small and concentrated only in distinct regions. Therefore it is believed that the circulation is one of the most likely sources of brain steroids (Gomez-Sanchez et al., 2005). Nevertheless, our data suggest that the brain tissue regulates the concentrations of these hormones, as the brain levels did not reflect those of plasma. The access of mineralocorticoids to central MR has important implications. An increased activation produces hypertension through an increase in the release of ADH and central sympathetic drive to the kidneys, heart and vascular smooth muscle (Gomez-Sanchez, 1997).

The higher aldosterone and DOC levels in brain of BN rats were linked with a more pronounced reduction of blood pressure than in BNK rats. In both strains the

combination of high salt diet and spironolactone tended to normalize the increased, in case of BN rats, or decreased heart rate, in case of BNK rats, which may again highlight the role of MR in the regulation of cardiovascular functions. Spironolactone could not exert its diuretic effect under conditions of high salt diet. The urine volume remained comparably high as following high salt diet alone. It seems likely that under high salt diet the regulation of diuresis is not controlled by the MR. Furthermore it confirms that the blood pressure lowering effect of aldosterone is not related to its diuretic action.

The higher blood pressure in BNK rats was linked with an increased heart weight. This effect could not originate in higher body weight. Spironolactone decreased salt appetite and consequently reduced water intake and body weight. All these effects were significantly more pronounced in BNK rats and may confirm the overexpression of the central MR in BNK rats, as suggested in chapter 5.2. These in part contradictory data may be related to the mutations of the MR in both rat strains that are supposed to cause an altered affinity of the receptor to different steroid ligands, which will be investigated in details in further studies.

We showed that spironolactone increased the secretion of plasma kininogens and suggested that aldosterone may up-regulate the expression or stimulate the secretion of both HMW and LMW kininogens. This effect of spironolactone was intensified by high salt diet, especially in BNK rats, although the levels of kininogens were substantially lower than those of BN rats. Like in other experimental groups plasma kallikrein activity was not influenced by any of these conditions. The higher kininogen levels were linked with an increase of plasma BK concentration. In BNK rats BK reached almost the levels found in BN rats. Increased dietary Na^+ inhibits the activity of the RAS, whereby inhibits also the activity of ACE that is one of the most important plasma kininases. According to our data we hypothesize that the BNK rats overexpress the MR, which may be responsible for the increased effect of spironolactone. In this context a more pronounced inhibition of ACE may account for the significant increase in plasma BK. In BN rats spironolactone inhibited the effect of high salt diet and increased plasma KLP levels. This effect may have contributed to the higher BK levels. Unfortunately, as already noted we have no information about the activities of respective generating and degrading enzymes. In BNK rats an

opposite effect was observed, which may confirm the role of aldosterone in regulating the KLP levels in plasma via the MR.

Simultaneous administration of spironolactone during high salt diet exerted synergistic effects on the regulation of the renal KKS activity. The urinary LMW kininogen excretion further increased, which was more pronounced in BN rats and correlated with higher levels of aldosterone. These findings confirmed the property of aldosterone to down-regulate the expression of kininogens as mentioned above. Following spironolactone under high salt diet urinary kallikrein activity moderately increased in BN rats but decreased in BNK rats. It is likely that this effect is linked with the ability of spironolactone to increase extracellular K^+ ions and is possibly related to the amount of binding sites for spironolactone in the kidney. Increased amount of substrate and enhanced enzymatic activity may have caused a significant increase in urinary kinin levels in BN rats. In BNK rats spironolactone reversed the effects of high salt diet as it has also been shown for other parameters. Urinary KLP was almost undetectable whereas BK levels remained comparable to those under high salt diet. We have suggested that the BNK rats are characterized by different enzymatic equipment. It seems likely that spironolactone stimulated the degradation of KLP in plasma and urine. These data point to a possible key role of the MR in BNK rats. It is likely that not only the overexpression but also different affinity of the MR to various ligands is leading to diverse effects and may be responsible for these findings. Paradoxically, this kininogen-deficient rat strain has enabled us to investigate the direct relationship between the aldosterone and kinins. Besides for ACE, which simultaneously regulates the levels of Ang II and kinins, aldosterone fulfils another comparable connective link between the RAAS and KKS that gain on importance especially in mediating local tissue effects.

Likewise to the KKS, the combination of spironolactone and high salt diet mediated synergistic effects on the urinary excretion of ET-1. These results are contradictory to generally accepted mechanism of stimulation of ET-1 secretion by aldosterone. On the contrary, from a functional point of view ET-1 mediates natriuretic effects and therefore the synergism of high salt diet and spironolactone does not seem to be meaningless. In accordance with the hypothesis that higher urinary ET-1 of BNK rats correlates with increased intracellular levels of Na^+ the BNK rats excreted

significantly more ET-1 than the BN rats, which may again correlate with the overexpression of the renal MR in BNK rats. Similarly, as we found that spironolactone mediated an attenuation of the negative feedback mechanism between the MR and ACTH it seems plausible that spironolactone did not always exert antagonistic effect on the MR.

In BNK rats fed with high salt diet spironolactone caused a significant increase in the heart weight. Similar results were found in BN rats following high salt diet and were attributed to the lower plasma KLP levels. Additional administration of spironolactone increased the levels of KLP and consequently normalized the heart weight. Likewise, the higher heart weight of BNK rats correlated with reduced KLP levels following administration of spironolactone. These experimental conditions were comparable to conditions that would be obtained by administration of B_2 receptor antagonist as previously suggested. The absence of KLP in plasma turned out to be responsible for the deleterious effects of Na^+ leading to cardiac hypertrophy. Protective effect of endogenous kinins in the development of cardiac hypertrophy was demonstrated in studies using ACE inhibitors. The antihypertrophic effect of ACE inhibitors or chronic administration of BK was abolished by coadministration of a B_2 receptors antagonist (Schoelkens, 1996). This finding highlights the cardioprotective role of KLP under conditions of increased dietary Na^+. The velocity of the increase of heart weight following high salt diet was remarkable. It remains unclear whether this effect is specifically mediated by aldosterone. Under standard condition the BN and BNK rats do not differ in their heart weights although the BNK rats are supposed to overexpress the MR. Gu et al. (1998) showed that Na^+ might have a direct effect to induce cellular hypertrophy in both heart and vasculature, which was only enhanced by aldosterone via increasing intracellular Na^+ levels.

The cardiovascular effects of MR blockade following high salt diet were evaluated in final Langendorff experiments. These experiments also revealed the primary role of aldosterone in controlling the heart rate. In both strains we found that spironolactone reduced heart rate possibly by inhibiting the rapid non-genomic effects of aldosterone. Recent data suggest that also these rapid effects may be mediated via the MR (Connell and Davies, 2005). The precise mechanism of this reduction was already described in details in chapter 5.2.. Aldosterone was suggested to be

involved in the response to cardiac ischaemia. Especially it was proposed that aldosterone may impair cardiac functions. Nevertheless, aldosterone may play a role in mediating the effects of IPC via the same mechanism. We found that IPC did not alter the post-ischaemic heart rate in any of the strain whereby the effect of IPC on heart rate was abolished.

Following spironolactone treatment under high salt diet the LVDP increased in both strains to significantly higher levels than in other experimental groups. Moreover, the differences between both strains in LVDP were abolished. The higher LVDP was linked with a significant increase in contractility, especially in BNK rat hearts, which was comparable with the higher contractility of hearts from BN rats fed with high salt diet. BNK rat hearts displayed significantly higher LVEDP than the BN rat hearts, which was affected neither by ischaemia nor by IPC. This finding may in part explain the higher blood pressure in BNK rats. Similarly, even though the rate of ventricular relaxation was almost identical in both strains it was significantly increased in BNK rats but rather decreased in BN rats if compared with the high salt diet group.

The most significant changes in coronary flow were found following spironolactone treatment and high dietary Na^+. The coronary flow moderately increased in BNK rats but significantly decreased in BN rats. It was reduced especially during the IPC periods, showing the important role of aldosterone in mediating the response to ischaemia.

All these data suggest different actions of spironolactone in BN and BNK rat hearts. Unfortunately, we have no data about the amount of the MR, which would characterize the cardiac tissue in more detail and help to understand these effects. Nevertheless, these findings confirm the important role of aldosterone in cardiac tissue that may be influenced by the actual salt status either directly or through other mediators.

Strikingly, this experimental group displayed the most deleterious effects on the myocardium as concluded from the CK activity in the coronary effluent. The CK activity was substantially higher following spironolactone treatment under high salt diet than in other groups. In BNK rat hearts the activity was similar to that found

following spironolactone alone. In BN rat hearts this effect was more pronounced already prior to ischaemia. These findings confirmed the deleterious effect of spironolactone during ischaemia, described in section 5.2., that was further enhanced by high salt diet. These data may suggest that aldosterone is an important regulator of cardiac functions also during and following ischaemia. The presence of cardiac aldosterone should not always be estimated as detrimental. From this point of view treatment with spironolactone may be unfavourable in some patients. Even though in present there is no doubt about the beneficial effects of treatment with spironolactone if we understand the precise mechanism of our findings it might contribute to even better therapeutical impact of spironolactone.

6. SUMMARY

BNK rats are characterized by plasma kininogen deficiency that is reflected in lower levels of plasma kallikrein activity and consequently in lower plasma BK and KLP levels. In contrast to other investigators, with a specific assay system we found that the renal KKS of BNK rats was almost identical to that of wild type BN rats. Plasma and urinary KLP, rat kinin discovered and characterized in our laboratory, was found to be the major kinin responsible for most physiological effects mediated by the B_2 receptor in both rat strains. We found that the deleterious effects of salt diet in BNK rats are in fact attributable to enhanced mineralocorticoid action. Originally they were believed to be caused by the kininogen deficiency. The renal mineralocorticoid receptor (MR) displayed several mutations that might be responsible for altered affinity and responses toward aldosterone and other ligands, e.g. DOC and corticosterone. Furthermore, it seems likely that these effects are enhanced by the overexpression of the MR in BNK rats in kidney as well as in the brain and possibly other tissues. These findings are important for the salt sensitive hypertension because the central MR was found to control salt appetite. Moreover, the BNK rats have increased levels of plasma and urinary ET-1, which may contribute to the deleterious effects of Na^+ during high salt diet.

Investigation of ischaemic preconditioning (IPC) in isolated hearts revealed no significant findings. Plasma components contribute to a great extent to the acute protective effects of IPC *in vivo*. *In vitro* experiments without any direct pharmacological treatment cannot describe the molecular mechanism responsible for the protective effects of IPC in all details. Nevertheless, long-lasting effects like attenuation of plasma kinins or antagonism of the MR seemed to be of importance for the regulation of cardiac function. The absence of plasma KLP during high salt diet was found to be responsible for the enhanced deleterious effects of Na^+ leading to cardiac hypertrophy. In BNK rat hearts aldosterone was found to be responsible for the higher heart rate and increased contractility besides for the impairment of coronary flow. Aldosterone is also involved in the acute response to ischaemia and consequently in response to IPC. In case of an enhanced mineralocorticoid action like in BNK rats aldosterone may account for the increased sensitivity to ischaemia whereby attenuating the protective effect of IPC.

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