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The Mechanism of Ischemic Preconditioning
Adenosine mediates protein kinase C e activity via A_1 and A_3 receptors in isolated guinea pig heart

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The mechanism of cardiac ischemic protection has been highly emphasized, because acute coronary occlusion diseases are the major cause of mortality. However, the cardiologists have been frustrated by the attempts of ß adrenergic receptor blockers, free radical scavengers, Ca^{2+} channel antagonists, anti-inflammatory agents and so on, since none of them enhance the viability of myocardium during ischemia and reperfusion. In 1986, Murry et al. designed a serious of experiments to investigate the ATP depletion during ischemic injury. The protocol included four circles of 5 minutes ischemia separated by 5 minutes reperfusion. They found less ATP depletion during sustained ischemia and less infarction at the end of experiment. This phenomena was named ischemic preconditioning.
It is clear that during ischemia ATP is broken down to ADP and AMP, which in turn produces adenosine. So a large quantity of adenosine is released. But concerning the influence of ischemic preconditioning on adenosine release, it is not clear whether IPC reduces ATP depletion and in turn inhibits adenosine formation and release or enhances it, thereby providing receptor-mediated protection. Using pharmacological study it is confirmed that adenosine-A_1 and A_3 -receptors are involved in the protective effect of IPC and protein kinase C activation is one of the common key steps. Unfortunately, so far there are no direct evidences of PKC activation and correlation with A_1/A_3 receptors. Our present study focused on these questions.

Methods:

A total of 188 young female Dunkin Hartley guinea pigs were used in this study. The body weight was 300±50 g. After anesthesia the heart was isolated and mounted to a Langendorff apparatus and perfused with Krebs-Henseleit buffer at constant pressure of 55 mm Hg. Left ventricular systolic (LVDs) pressure, heart rate (HR), coronary flow (CF), ±dP/dt were monitored and recorded automatically every ten seconds during the whole experiment. Ischemia was produced by stop flow technique, and reperfusion was produced by restore flow and perfusion pressure. Coronary effluent and cardiac tissue were collected at specific time points for further analysis.
The animals were randomly divided into 5 groups and underwent different experimental procedures, which were a non-treated-, CHE-, V1-2 P-, DPCPX-, and a MRS 1220-treated group, respectively. These agents were PKC inhibitor, PKC e translocation inhibitor, A_1 and A_3 receptor antagonists, respectively. Each group was further divided into three groups. Sham hearts were perfused for 60 min without ischemia and drug perfusion for 20 min. They are

described as Sham, CHE-Sham, V1-2 P-Sham, DPCPX-Sham and MRS 1220-Sham. Control hearts (Cont) underwent ischemia and reperfusion for only 30 min each and drug was perfused for 20 min each before ischemia was induced. They are described as Cont, CHE-Cont, V1-2 P-Cont, DPCPX-Cont and MRS 1220-Cont. Ischemic preconditioning hearts (IPC) underwent three circles of 5 min ischemia and reperfusion prior to 30 min ischemia. Each drug was perfused 5 min before IPC and continued to three periods of 5 min reperfusion with a total perfusion time of 20 min. They are termed as IPC, CHE-IPC, V1-2 P-IPC, DPCPX-IPC and MRS 1220-IPC, respectively.

Formetabolite analysis a fully automated HPLC system was used equipped with a dual pump system and a photodiode array detector. The adenine nucleosides were assayed with Nova-Pak C_{18} reversed-phase column. Absorbency of adenosine, inosine and hypoxanthine was monitored at 254 nm. The adenine nucleotides and creatine phosphate were assayed with column Partisil SAX, the absorbencies of which were monitored at 254 and 210 nm, respectively. Peaks of different substances were identified and quantified with appropriate standards by comparison of the respective retention times and spectra.

Frozen myocardial tissue samples were powdered, homogenized and centrifuged to obtain cytosolic and membrane fractions. PKC e activity was analyzed by ELISA.

All data are presented as mean ± SEM. One-way ANOVA combined with Scheffe's post hoc test and paired T-test were used. Pearson's correlation and Multivariate ANOVA also were used in present study. A p value of < 0.05 was considered to be significant.

Results:

Pretreatment with CHE, V1-2 P, DPCPX or MRS 1220 for 20 min, did not induce significant changes of HR, LVPs, CF and ± dP/dt in sham hearts. All hemodynamic data were significantly decreased at the end of 30 min reperfusion in both control and IPC hearts ($p<0.05$ vs. Baseline). There were no differences between control and IPC groups.

In all groups ATP depletion was significantly lower at the end of 30 min ischemia than baseline and associated with an accumulation of AMP. In Cont, CHE-Cont, V1-2 P-Cont, DPCPX-Cont and MRS 1220-Cont, ATP levels were decreased at the end of 30 min ischemia and 30 min reperfusion ($p<0.05$ vs. Baseline). ATP levels of IPC were decreased at the end of 30 min ischemia ($p<0.05$ vs. Baseline), but at the end of 30 min reperfusion ATP recovered to pre-ischemic levels ($p>0.05$ vs. Baseline). The ATP restoration effect of IPC during reperfusion was completely blocked by CHE, V1-2 P, DPCPX or MRS 1220 pretreatment. Creatine phosphate was decreased at the end of 30 min ischemia in all control and IPC groups ($p<0.05$ vs. Baseline). Creatine phosphate increased to baseline level at the end of reperfusion.

The total release of adenosine during the first five minutes of reperfusion after long ischemia showed no differences between Cont and IPC hearts. Surprisingly, adenosine release was increased in CHE IPC ($p<0.05$ vs. Cont CHE or IPC). Pretreatment with DPCPX and MRS 1220 did not significantly increase adenosine release at these points. The total release of inosine and hypoxanthine during first five minutes were no difference in all groups. Adenosine release during three intervals of ischemic preconditioning was increased by pretreatment with either CHE, V1-2 P, DPCPX or MRS 1220. MRS 1220 attenuated the release of hypoxanthine of first period of IPC, and no differences in inosine and hypoxanthine release were obtained during the IPC-related reperfusion periods.

PKC e activity was measured in membrane and cytosolic fractions. In the present study the activity of membrane fraction was undetectable in all hearts. At the end of three circles of IPC, PKC e activity in the cytosolic fraction was increased by two times. At the end of 30 min ischemia, the activity was decreased both in Cont and IPC hearts ($p<0.05$ vs. Baseline). CHE pretreatment inhibited the PKC e activation effect of ischemic preconditioning at the end of IPC. V1-2 P pretreatment increased the PKC e activity by 2.5 times at the end of IPC. With

either DPCPX or MRS 1220 pretreatment, PKC e activation effect of ischemic preconditioning was completely inhibited at the end of IPC.

Discussion and conclusion:

In present study the isolated guinea pig heart was used, which is characterized by the following advantages:

- IPC was produced successfully in isolated guinea pig heart by three circles of 5-min ischemia.
- Adenosine formation and release have extensively been studied under physiologic and ischemic conditions.
- Adenosine A_1 and A_3 receptors have been confirmed to exist in guinea pig myocardium and involve in IPC.
- In pharmacological studies, several PKC isoforms have been found including e isoform. E. g., acute mechanical stretch of left ventricle induces translocation of PKC e isoform and mechanical stretch shares same mechanism as ischemic preconditioning, although the direct evidence is missing.

Recovery of contractile function during reperfusion is more dependent on the species and the protocols of IPC, the data of guinea pig heart is still missing. ATP depletion is the predominate source for adenosine formation during ischemia. It is not clear whether IPC decrease adenosine formation and release as an index of ATP depletion or increase adenosine formation and release to provide receptor-mediated protection. The direct evidences of PKC e activation and translocation during IPC and mediated via adenosine receptors are still missing. Our present study was designed to answer these questions.

The following conclusions can be made from the present study:

- Ischemic preconditioning does not attenuate cardiac dysfunction during reperfusion after long ischemia in isolated guinea pig heart. The dysfunction recovery is more species dependent. Attenuated myocardial dysfunction is not a prerequisite for infarct size-limitation effect.
- Pretreatment with adenosine A_1 receptor antagonist DPCPX delays the cardiac function recovery during early reperfusion after long term ischemia. But A_3 receptor antagonists and PKC inhibitors have no effect on the functional recovery.
- Although three circles of IPC reduce the levels of ATP by about 30%, it improves ATP preservation at the end of reperfusion. The protection effect is not strong enough to protect ATP depletion at the end of a 30 min lasting ischemia.
- The protection effect of ATP depletion is abolished by pretreatment with either adenosine A_1 or A_3 receptor antagonists, PKC inhibitor or PKC e translocation inhibitor.
- IPC has a salutary effect on CrP during sustained ischemia, but has no effect during reperfusion. The effect is independent to PKC activation and adenosine A_1 and A_3 receptors occupation.
- Although ATP depletion is attenuated by IPC, adenosine release is not enhanced. More interestingly, pretreatment with either CHE, V1-2 P, DPCPX or MRS 1220 can block the protective effect on energy metabolism by the heart.
- Ischemic preconditioning increased cytosolic PKC e activity at the end of three circles 5-min ischemia. By pretreatment with PKC e translocation inhibitor, V1-2 P, cytosolic PKC e activity even higher than IPC alone. Taking together, we assume that IPC activates PKC e and the activated PKC e isoform translocate from cytosolic fraction to membrane.
- PKC e activation and translocation effect of IPC is abolished by adenosine A_1 and/or A_3 receptor antagonists pretreatment.

- Adenosine is a key metabolite in the control of the activation of protein kinase C, solely under conditions of IPC.
- The action of adenosine is specifically mediated by adenosine receptors, in particular A_1- and A_3-receptors.
- Adenosine does not control PKC translocation from the cytosolic to the membarne compartment.