Protection against Myocardial Ischemia and Reperfusion:
Role of Peroxynitrite in Late Preconditioning and in Postconditioning

Summary of Ph.D. Thesis

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INTRODUCTION

Therapeutic strategies to protect the ischemic myocardium have been studied extensively. Reperfusion is the definitive treatment for acute coronary syndromes, especially acute myocardial infarction; however, paradoxically, the act of reperfusing the ischemic area can itself induce additional damage and can affect cardiac function.

Ischemic preconditioning (Pre) of myocardium is a well described adaptive response in which brief exposure to ischemia/reperfusion (I/R) before sustained ischemia markedly enhances the ability of the heart to withstand a subsequent ischemic insult. The application of brief repetitive episodes of I/R at the immediate onset of reperfusion, which has been termed "postconditioning" (Post) reduces the extent of reperfusion injury, in some cases equivalent to that observed with Pre.

Pre and Post confer remarkable cardioprotection in a variety of species, including humans. Although several studies have reported that the effectiveness of Pre may be influenced in some disease states such as hypertension, diabetes, heart failure, hyperlipidemia, atherosclerosis, etc. However, very little is known about the effect of Post under pathological conditions. Therefore, in the present thesis, we prepared a hyperlipidemic rat model by feeding 2% cholesterol diet, to examine the effects of hyperlipidemia on the Post-mediated cardioprotection against I/R.

There is still a considerable debate regarding the exact cellular mechanism of endogen cardioprotective phenomenona. Among several other mediators, nitric oxide (NO), oxygen free radicals, and peroxynitrite ($ONOO^-$) play an essential role as key triggers and mediators of Pre and Post. In this thesis we focused on the role of reactive oxygen species, especially $ONOO^-$ in the development of late Pre and Post.

$ONOO^-$ is a powerful oxidant species, which can be formed in vivo by the non-enzymatic reaction of nitric oxide and superoxide ($O_2^{-}\bullet$) anion at an extremely rapid rate, limited only by diffusion. It is widely accepted now that enhanced $ONOO^-$ formation contributes to oxidative and nitrosative stress in a variety of cardiovascular and other pathologies. It has been shown that $ONOO^-$ is produced during the early reperfusion of ischemic hearts and that drugs which inhibit $ONOO^-$ formation or antagonize its toxicity protect the heart from reperfusion injury. In contrast, increasing evidence suggests that physiological levels of $ONOO^-$ may act as a regulator of several physiological functions. Endogenous $ONOO^-$ has been shown to trigger ischemic stress adaptation of the rat myocardium, and to activate stress response pathways.

$ONOO^-$ was reported to activate some full length matrix metalloproteinase (MMP) zymogens. MMPs, a family of zinc-dependent endopeptidases, are implicated in a number of pathological including I/R injury in the heart. Acute release of MMP-2 during reperfusion after ischemia contributes to cardiac mechanical dysfunction. However, their role in late Pre is unknown.

AIMS

Study 1

To investigate the role of NO and peroxynitrite–MMP signalling in the development of late Pre in rats in vivo.

Study 2

A) To study the role of ONOO in rat hearts subjected to Post protocol ex vivo.

B) To examine whether experimental hyperlipidemia induced by cholesterol-enriched diet interferes with the cardioprotective effect of Post.

MATERIALS AND METHODS

Study 1

Animals and experimental groups

Male Wistar rats weighing 300–350 g were used throughout the experiments without any treatment. Animals were preconditioned by 5 consecutive cycles of 4-min ischemia and 4-min reperfusion in vivo. 24 hours after the Pre protocol, 30-min test-ischemia followed by 180-min reperfusion was performed. The control group was subjected to the same procedure without Pre protocol. To measure infarct size after coronary occlusion, hearts were isolated and perfused for 5 min in a Langendorff system to wash out the blood, then the coronary artery was re-occluded and the hearts were perfused with 0.1% Evans-blue. After Evans-blue staining, hearts were frozen, cut into slices and incubated in 1% 2,3,5-triphenyl-tetrazolium chloride for 10 minutes at 37ºC. The slices were scanned and the size of the infarcted area related to the area at risk was determined by computer planimetry.

For investigation of the biochemical parameters and gene expression changes during the development of late Pre, additional experimental groups were generated in separate experiments. A group of animals without any treatment served as 0h control. Four separate groups were preconditioned and 3, 6, 12, or 24 hours after the Pre protocol the hearts were isolated, perfused for 5 min on a Langendorff system to remove blood and frozen in liquid nitrogen. Four separate sham operated control groups undergone the same procedure as the preconditioned groups, but no occlusion was performed with the suture in these groups, then 3, 6, 12 and 24 hours later the hearts were sampled. The frozen hearts were powdered and homogenized in four volumes of ice-cold homogenization buffer with an ultrasonic homogenizer.

Finally, after evaluation of the biochemical parameters in the experimental groups, to verify if late Pre-induced inhibition of MMPs has a causative role in the reduction of infarct size, in separate experiments, 30-min test ischemia followed by 120-min reperfusion was applied after treatment with 1.5 μmol kg^{-1} ilomastat, a non-selective MMP inhibitor, and vehicle, respectively.

Ilomastat and its vehicle were administered 5 min before the coronary occlusion and at the 10th and 25th min of ischemia as well as at the 10th min of reperfusion to maintain its plasma level based on the half life of the molecule.

Measurement of cardiac NO metabolism

To examine the role of NO and NOS in late Pre, NO content of ventricular tissue was measured using electron spin resonance (ESR) spectroscopy after in vivo spin trapping with the NO-specific Fe^{2+}-diethyl-dithiocarbamate. To estimate endogenous enzymatic NO production, Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities in ventricular homogenates were measured by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline.

Measurement of enzymatic O_2^-• production and degradation

In situ detection of O_2^-• anion was performed by confocal laser scanning microscopy using a fluorescent dye dihydroethidium. Activities of xanthine oxidoreductase (XOR) and NADPH oxidase, major sources of O_2^-• in rat hearts were determined from ventricular homogenates. XOR activity was determined by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine. NADPH-stimulated O_2^-• production in freshly prepared ventricular homogenates was assessed by a lucigenin-enhanced chemiluminescence method. Total activity of superoxide dismutase (SOD), a major enzyme converting O_2^-•to H_2O_2, was measured by a spectrophotometric assay using a kit.

Measurement of 3-nitrotyrosine, a marker of $ONOO^-$

To investigate the role of $ONOO^-$, we measured free 3-nitrotyrosine content as a marker of $ONOO^-$ formation by enzyme-linked immunosorbent assay (ELISA) from the myocardium. $ONOO^-$ promotes nitration of phenolic compounds such as tyrosine, the nitration of which leads to the formation of stable product, 3-nitrotyrosine.

Zymographic analysis of MMP activity

Myocardial MMP-2 and MMP-9 activities heart ventricular was homogenized in absence of any protease inhibitor. Proteins were separated on gelatine gel. Following electrophoresis gels were washed with 2.5% Triton X-100 and incubated for 24 at 37°C in incubation buffer. Gels were then stained with 0.05% Coomassie Brilliant Blue (G-250; Sigma-Aldrich) in a mixture of methanol/acetic acid/water and destained in aqueous 4% methanol/8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark-blue background. Zymograms were digitally scanned, and band intensities were quantified.

Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) was performed with gene-specific primers using SybrGreen protocol to assess expression changes of NOS, MMP and TIMP genes. Relative expression ratios were normalized to β-actin. Nontemplate control sample was used for each PCR run to check the genomic DNA contaminations of cDNA template. Analysis of results was done using Pfaffl method. With the use of this calculation method, differences between the amplification efficiencies of reactions could be corrected.

Study 2

Animals and experimental groups

Six-week old male Wistar rats were fed normal or 2% cholesterol-enriched rat chow for 12 weeks. At the conclusion of the experimental diet period, animals were anesthetized with diethyl ether and given 500 U/kg heparin. Hearts were isolated and perfused with Krebs-Henseleit buffer with constant pressure according to Langendorff. Regional ischemia was induced by 30 min coronary occlusion followed by 120 min reperfusion. Post was induced by six or twelve consecutive cycles of 10 s coronary occlusion and 10 s reperfusion immediately at the onset of reperfusion. Electrocardiogram was monitored during the study to measure heart rate and incidence of reperfusion-induced ventricular fibrillation (VF). At the end of the 120 min reperfusion, infarct size was determined by triphenyltetrazolium chloride staining and evaluated by planimetry. Infarct size was expressed as a percentage of area at risk

To analyze, if $ONOO^-$ plays a role in the cardioprotective mechanism of Post, normolipidemic hearts were subjected to 30 min regional ischemia followed by the 6x10 s cycles Post protocol in separate experiments, in the presence or absence of 5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III] (FeTPPS; 20 mg/L). Krebs-Henseleit solution contained FeTPPS only during the last 20 s of the 30-min ischemic period and during Post. At the end of the 120-min reperfusion period, infarct size was measured as described above.

For biochemical analysis, hearts from control and cholesterol-fed groups were subjected to 30 min coronary occlusion followed by 5 min reperfusion with or without Post, and used for 3-nitrotyrosine measurements.

Measurement of 3-nitrotyrosine, a marker of $ONOO^-$

Left ventricular tissue was sampled, homogenized, and used for 3-nitrotyrosine measurements by ELISA as mentioned above and by Western Blot analysis. Homogenized heart tissue for Western Blot analysis was centrifuged. Protein concentrations of supernatants were measured by the bicinchoninic acid assay. Equal amounts (20 μg) of proteins were separated by

10% SDS-PAGE and transferred to nitrocellulosemembrane, then the blot was blocked in Tris buffered saline/Tween 20 supplemented with 5% nonfat dry milk overnight. The primary 3-nitrotyrosine antibody was used at the manufacturer-recommended dilution. The membrane was developed with an enhanced chemiluminescence kit, exposed to X-ray film, and scanned. Band density was calculated by integrating the area (pixels x intensity, expressed in arbitrary units). By this method, we detected the degree of nitrosylation of tyrosine side chains of proteins, since low-molecular weight free 3-nitrotyrosine is eliminated in the SDS-PAGE.

RESULTS

Study 1

In control hearts ischemia and reperfusion resulted in a large infarction. When Pre was applied before ischemia, infarct size significantly decreased showing the protective effect of late Pre against acute I/R injury.

Ca^{2+}-dependent *NOS activity* and expression levels of endothelial and neuronal NOS remained unchanged. However, Ca^{2+}-independent NOS activity increased gradually after the Pre protocol and at 12h the elevation reached the level of significance then further increased by 24h. iNOS mRNA content measured at 24h was also increased significantly as compared to the sham operated group, however, gene expression of other NOS isoforms remained unchanged. Despite the increased iNOS expression and activity, NO signal did not change during the development of late Pre.

In cardiac *superoxide metabolism*, we observed a gradual increase of XOR and NADPH oxidase activities in both preconditioned and sham operated groups as compared to the 0h control group, however, there was no difference between Pre and sham operated groups. A significant decrease of myocardial total SOD activity is shown in the Pre group 24h after the Pre protocol, when compared to 0h control and to 24h sham operated group, respectively. We compared myocardial superoxide content in Pre and sham operated groups and found a significant elevation 24h after the Pre protocol, when measured cardiac superoxide by dihydroethidium staining.

$ONOO^-$ generation was attenuated significantly when compared to sham operated group 24h after the Pre. Characteristic MMPs of the myocardium was performed by zymography. Both, MMP-9 and MMP-2 activities have shown a significant reduction 24h after the Pre protocol compared to the sham operated animals. Ischemic late Pre resulted in a decrease in the *gene expression* of MMP-2, however, the amount of MMP-9 mRNA was increased. Gene expression of tissue inhibitor of metalloproteinases TIMP1, TIMP2 and TIMP4 were not changed, however, TIMP-3 mRNA showed a significant decrease in the Pre group, when compared to sham operated group.

To verify if Pre-induced inhibition of MMP activity has a causative role in the reduction of infarct size, Ilomastat, a non-selective MMP inhibitor was used. Ilomastat (1.5 μmol kg^{-1}) given 5 min before ischemia followed by infusion during ischemia and the first 10 min of reperfusion to maintain plasma concentration, decreased infarct size significantly as compared to the vehicle treated animals.

Study 2

Post with 6x10 s cycles significantly decreased infarct size and the incidence of VF in hearts of rats with normolipidemic diet and was lost in hyperlipidemic hearts. To test if a more potent Post stimulus could protect the hyperlipidemic heart, in a separate set of experiments, Post with *12x10 s* cycles of brief ischemia-reperfusion was applied. However, this Post protocol failed to reduce infarct size not only in hyperlipidemic but also in normolipidemic hearts.

Post significantly increased peroxynitrite-induced 3-*nitrotyrosine* formation in normolipidemic hearts, but not in hyperlipidemic conditions at the 5th min of reperfusion.

To test if the early increase in nitrosative stress signal observed in normolipidemic animals is a necessary trigger for the development of cardioprotection by Post, in separate experiments, Post was induced in the presence of a *$ONOO^-$ decomposition catalyst FeTPPS* in normolipidemic animals. Although FeTPPS did not affect infarct size significantly in the nonpostconditioned group, it abolished the infarct size-limiting effect of Post.

DISCUSSION

In the present *study 1* we showed that infarct size significantly decreased in response to the late Pre. We have confirmed that the cardioprotective effect of ischemic late Pre was accompanied by the induction of iNOS as described previously by several studies. In our present study, surprisingly, iNOS activation did not lead to increased NO level in the myocardium as measured by electron spin resonance spectroscopy 24 h following the Pre stimulus. These findings show that the role of iNOS in late Pre may be not related to increased NO production. This is further supported by the finding that 1400W, a selective iNOS inhibitor, did not decrease cardiac NO content at late Pre in our present study.

Many of the physiologic actions of NO are mediated through $ONOO^-$, the reaction product of NO and superoxide. Therefore, we examined myocardial levels of superoxide and $ONOO^-$ as well as the expression and activity of the enzymes involved in superoxide production and its elimination. We have found here a markedly increased superoxide level in the myocardium 24 h after Pre as compared to the non-preconditioned group. Neither XOR activity nor NADPH oxidase activities were changed, however, SOD activity was decreased 24 h after the Pre stimulus as compared to the non-preconditionined controls. These results show that the source of increased myocardial superoxide can be a decreased activity of SOD. Moreover, it is well known that iNOS is able to produce superoxide in certain circumstances e.g. absence of tetrahydrobiopterin and/or L-arginine and altered ratio of reactive oxygen and nitrogen species. Therefore, since NO production was not increased in the present study, increased iNOS activity could be the source of increased superoxide level during late Pre. We have previously shown that $ONOO^-$ may trigger classic ischemic Pre and subsequent cycles of ischemia/reperfusion result in a gradual decrease in $ONOO^-$ formation. In *Study 1* we measured cardiac 3-nitrotyrosine levels as a marker of cardiac $ONOO^-$ generation and showed for the first time that late Pre significantly diminishes myocardial $ONOO^-$ formation.

It is known that $ONOO^-$ at low concentrations contributes to the beneficial effects of Pre on ischemia in isolated rat hearts. However, no attempt has been made so far to measure cardiac $ONOO^-$ during late Pre, although $ONOO^-$ was suspected to be a trigger of late Pre in conscious rabbits. In *Study 1* we measured cardiac 3-nitrotyrosine levels as a marker of cardiac $ONOO^-$ generation and showed that late Pre diminishes myocardial $ONOO^-$ significantly.

It has been described that $ONOO^-$ activates MMPs, which may play a role in a variety of cardiovascular pathologies including I/R injury. Moreover, acute treatment with MMP-inhibitors is able to attenuate I/R injury and decrease myocardial infarct size and acute heart failure induced by pro-inflammatory cytokines in rats. To study if late Pre induced MMP inhibition has a causative role in its infarct size reduction, in separate experiments, rats were treated with a non-selective

MMP inhibitor ilomastat during coronary occlusion and reperfusion. We have shown that pharmacological inhibition of MMP also leads to the reduction of infarct size in the rat infarction model *in vivo*. To study if increased TIMPs inhibit MMPs, we measured gene expression of 4 isoforms of TIMPs. As gene expression of TIMP1, TIMP2, and TIMP4 were not changed and that of TIMP3 was decreased, MMP inhibition by altered TIMP levels is unlikely, nevertheless, the role of TIMPs in late Pre cannot be excluded. Therefore, it is plausible to speculate that inhibition of MMP activity is due to a decreased activation of MMPs by Pre-induced decrease in peroxynitrite. These results show that late Pre-induced inhibition of peroxynitrite–MMP signalling is involved in the cardioprotective effect of late Pre, and shows that pharmacological inhibition of MMPs is able to reduce infarct size *in vivo*.

The role of reactive oxygen species (ROS), including $ONOO^-$ in cardioprotection is still not clear. Furthermore, little is known about the balance between the detrimental and protective effects of $ONOO^-$.

Our present results show that Post increases formation of cardiac 3-nitrotyrosine, a marker for peroxynitrite-induced nitrosative stress, at early reperfusion; however, the increased $ONOO^-$ formation was not observed in hyperlipidemic hearts subjected to Post. In hyperlipidemic animals, Post was ineffective and the early increase in $ONOO^-$ after Post was not present, we conclude that the lack of a nitrosative trigger signal may be involved in the loss of Post in hyperlipidemia.

Because $ONOO^-$ has been previously shown to be involved in the trigger mechanism of the cardioprotective effect of Pre it is plausible that $ONOO^-$ may also be involved in the triggering mechanism of Post. To test this hypothesis, in normal animals, Post was performed in the presence of a $ONOO^-$ decomposition catalyst FeTPPS at a dose that significantly reduced $ONOO^-$-induced myocardial effects in different models. We have found that the infarct size-limiting effect of Post was abolished in the presence of FeTPPS, which shows for the first time that increased nitrosative stress at early reperfusion after Post is necessary to trigger its cardioprotective effect. This is another important example of the physiological regulatory role of mild nitrosative stress. Moreover, recent studies suggest that some ROS at low concentrations could protect ischemic hearts. ROS scavengers N-acetyl-L-cysteine or mercaptopropionyl glycine given at the beginning of reperfusion abolished Post induced protection. Nossuli et al. showed that, in a feline model of coronary occlusion/reperfusion, intraventricular infusion of authentic $ONOO^-$ (1 μmol/L) 10 min before reperfusion was associated with a reduction in infarct size. Furthermore, it has been shown that $ONOO^-$ regulates mitogen-activated protein kinases, which are also involved in the mechanism of Post.

NEW OBSERVATIONS:

Study 1

- Late Pre increased the expression and activity of iNOS but this change did not lead to increased cardiac NO formation
- Late Pre decreased $ONOO^-$ formation, but cardiac superoxide was increased.
- Pharmacological inhibition of MMP activity was able to reduce infarct size in a late Pre model of rats with coronary occlusion in vivo

Study 2

- The infarct size-limiting effect of Post was lost in hearts of hyperlipidemic rats
- Cardiac 3-nitrotyrosine content was increased during early reperfusion after Post, which was not seen in hyperlipidemic hearts
- Post in the presence of the $ONOO^-$ decomposition catalyst FeTPPS failed to reduce infarct size in normal hearts

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PUBLICATIONS

Full papers directly related to the subject of the Thesis:

I. Role of iNOS and peroxynitrite-matrix metalloproteinase-2 signaling in myocardial late preconditioning in rats. Bencsik P, **Kupai K**, Giricz Z, Görbe A, Pipis J, Murlasits Z, Kocsis GF, Varga-Orvos Z, Puskás LG, Csonka C, Csont T, Ferdinandy P.Am J Physiol Heart Circ Physiol. 2010 Aug;299(2):H512-8.

IF.: 3.712

II. Cholesterol diet-induced hyperlipidemia impairs the cardioprotective effect of postconditioning: role of peroxynitrite. **Kupai K**, Csonka C, Fekete V, Odendaal L, van Rooyen J, Marais de W, Csont T, Ferdinandy P.Am J Physiol Heart Circ Physiol. 2009 Nov;297(5):H1729-35.

IF.: 3.712

III. Matrix metalloproteinase activity assays: Importance of zymography. **Kupai K**, Szucs G, Cseh S, Hajdu I, Csonka C, Csont T, Ferdinandy P. J Pharmacol Toxicol Methods. 2010 Mar-Apr;61(2):205-9.

IF.: 2.22 (Scopus)

List of full papers indirectly related to the subject of the Thesis:

I. Matrix metalloproteinase inhibitors: a critical appraisal of design principles and proposed therapeutic utility. Dormán G, Cseh S, Hajdú I, Barna L, Kónya D, **Kupai K**, Kovács L, Ferdinandy P. Drugs. 2010 May 28;70(8):949-64

IF.: 4.732

II. Measurement of myocardial infarct size in preclinical studies. Csonka C, **Kupai K,** Kocsis GF, Novák G, Fekete V, Bencsik P, Csont T, Ferdinandy P. J Pharmacol Toxicol Methods. 2010 Mar-Apr;61(2):163-70.

IF.: 2.22 (Scopus)