CARDIAC STRESS AND STRESS ADAPTATION: ROLES OF PEROXYNITRITE AND CHANGES IN GENE EXPRESSION

Summary of PhD Thesis

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INTRODUCTION

Ischemic heart disease, a major cause of mortality in industrialized societies, is characterized by insufficient blood supply to regions of the myocardium which leads to tissue necrosis (infarction). Ischemic heart disease may develop as a consequence of hypertension, atherosclerosis, hyperlipidemia, and diabetes. Although reperfusion of ischemic myocardium is the definitive treatment to attenuate myocardial injury, reperfusion itself causes additional tissue damage which may lead to further complications. Therefore, development of cardioprotective agents is of great clinical importance.

Preconditioning confers a remarkable cardioprotection in a variety of species including humans. Ischemic preconditioning is a well-described adaptive response in which brief exposure to ischemia markedly enhances the ability of the heart to withstand a subsequent ischemic injury. Preconditioning can be elicited by different sublethal stress signals, such as brief periods of ischemia, hypoxia, rapid electrical pacing, heat stress, or administration of bacterial endotoxin, etc. The cardioprotective effect of preconditioning shows two distinct phases. The early phase ("classic preconditioning") is manifested within minutes after the preconditioning stimulus and has a duration of less than 3 h. The late phase (second window of protection) is characterized by a slower onset (20 h) and a duration of up to 72 h. Both phases of preconditioning involve reduction of necrotic tissue mass (infarct size), improvement of cardiac performance and reduction of arrhythmias following ischemia and reperfusion.

Among several other mediators, NO, oxygen free radicals, and antioxidant enzymes have been suggested to be, and also refuted as key triggers and mediators of preconditioning. There is still a considerable debate regarding the exact cellular mechanism of ischemic preconditioning. Understanding the cellular pathways involved in the ischemic adaptation of the myocardium may lead to the development of "preconditioning mimetic" drugs for patients suffering from ischemic heart disease.

NO is necessary for normal cardiac physiology, but it is potentially toxic in excess concentrations. It is now understood that many of the toxic actions of NO are not directly due to NO itself but are mediated via production of peroxynitrite ($ONOO^-$), the reaction product of NO with $O_2^{\cdot-}$. Although formation of $ONOO^-$ has been shown to contribute to several cardiovascular pathologies including ischemia/reperfusion injury, the possible physiological role of $ONOO^-$ is not known.

The possible downstream targets of $ONOO^-$ which mediate its toxicity are several. Its highly reactive decomposition products at physiological or acidic pH can attack protein, lipids, and DNA. This results in the depletion of antioxidants and most often in the inhibition of several enzymes. $ONOO^-$ has been also shown to trigger apoptosis of cardiac myocytes.

High-cholesterol diet is regarded as an important factor in the development of cardiac diseases since it leads to development of hyperlipidemia, atherosclerosis, and ischemic heart disease. The heart of hyperlipidemic/atherosclerotic patients adapts poorly to oxidative or other kinds of stress, suggesting that the endogenous adaptive mechanisms against myocardial stress are impaired. The focus of research so far has been mainly on the coronary effects of cholesterol, and the possible direct effect of hypercholesterolemia on the heart was neglected. It was found cardiac NO level is significantly decreased in hearts of cholesterol-fed rats; however, the mechanism of reduced NO level in the heart is not known. Reduced vascular NO release in hyperlipidemia has been also shown as a consequence of increased formation of superoxide, which then reacts with NO to form peroxynitrite ($ONOO^-$). Although it is not known if hyperlipidemia leads to increased formation of reactive oxygen species in the heart, it is plausible to speculate that this mechanism is involved in the enhanced breakdown of NO in the myocardium in hyperlipidemia.

AIMS

(i) To examine the effect of preconditioning on $ONOO^-$ formation in the myocardium during ischemia/reperfusion and to identify new cellular pathways involved in cardiac ischemia and ischemic preconditioning

(ii) To identify the mechanism by which cardiac NO content is decreased due to experimental hyperlipidemia.

METHODS

Isolated working rat heart

Male Wistar rats were anesthetized with diethyl ether. The hearts were excised and perfused in Langendorff mode or in a "working" mode. The perfusion was carried out with an oxygenated, normothermic Krebs-Henseleit buffer. Preload and afterload were kept constant

throughout the experiments. Heart rate (HR), left ventricular developed pressure (LVDP), maximum and minimum values of the first derivative of LVDP ($+dP/dt_{max}$ and $-dP/dt_{max}$) and left ventricular end-diastolic pressure (LVEDP) were derived from the left intraventricular pressure curve. Coronary flow (CF) was measured by collecting effluent from the right atrium in a measuring cylinder for a timed period and aortic flow (AF) was measured by a calibrated rotameter. Plasma, coronary effluent and myocardial tissue were sampled for further biochemical measurements.

Preconditioning studies

To examine the effect of preconditioning and subsequent ischemia/reperfusion on myocardial $ONOO^-$ formation, hearts were subjected to either a test ischemia/reperfusion protocol or a preconditioning protocol followed by a test ischemia/reperfusion.

To identify new cellular pathways involved in cardiac ischemia and ischemic adaptation, we monitored global gene expression changes by DNA microarray analysis of 3200 rat specific genes and by real-time quantitative PCR in rat hearts.

Hyperlipidemic studies

To examine the direct effect of hyperlipidemia on myocardium, rats were fed 2% cholesterol-enriched diet or normal diet for 8 weeks. Separate groups of normal and hyperlipidemic rats were injected twice intraperitoneally with 2x20 μmol/kg FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]), a $ONOO^-$ decomposition catalyst, 24 hours and one hour before isolation of the hearts. Isolated hearts were perfused in a working mode.

Measurement of cardiac NO, superoxide content, $ONOO^-$ formation and LDH release

NO content of ventricular tissue was measured using electron spin resonance spectroscopy after loading the heart with the NO-specific spin trap Fe^{2+}-N-methyl-D-glucosamine-dithiocarbamate (MGD). Superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence.

$ONOO^-$ promotes nitration of phenolic compounds such as tyrosine, the nitration of which leads to the formation of stable products, dityrosine and 3-nitrotyrosine. We measured both dityrosine by spectrofluorometry and free nitrotyrosine by ELISA in the effluent as markers of cardiac $ONOO^-$ formation and plasma nitrotyrosine as a marker of systemic $ONOO^-$ generation.

Cardiac lactate dehydrogenase (LDH) release was assessed from coronary effluent by spectrophotometer.

RESULTS AND DISCUSSION

$ONOO^-$ and preconditioning

In nonpreconditioned control hearts, test ischemia/reperfusion resulted in a marked decrease in aortic flow (AF) and a considerable increase in left ventricular end-diastolic pressure (LVEDP) and LDH release. When test ischemia was preceded by preconditioning elicited by three brief periods of no-flow ischemia, postischemic AF increased and LVEDP and LDH release decreased showing the protective effect of classic preconditioning against acute ischemia/reperfusion injury.

In the nonpreconditioned control group, test ischemia/reperfusion markedly increased cardiac nitrotyrosine formation. Preceding preconditioning significantly attenuated nitrotyrosine formation upon test ischemia/reperfusion, however, the first brief cycle of preconditioning ischemia/reperfusion markedly enhanced nitrotyrosine formation, which was attenuated after the third cycle of no-flow ischemia. This may show that $ONOO^-$ formed during ischemia/reperfusion might act as a trigger for preconditioning, but preconditioning in turn decreases formation of $ONOO^-$ upon subsequent cycles of ischemia/reperfusion.

These results strongly suggest that formation of $ONOO^-$ is an important oxidative stimulus to trigger cellular adaptive mechanisms.

Gene expression alteration due to ischemia/reperfusion, and preconditioning

The exact molecular mechanisms of ischemic preconditioning is very complex. We found that there are a number of genes the expression of which is significantly altered in the rat heart due to ischemia/reperfusion and preconditioning.

Several genes exhibited changes in their expression in response to ischemia/reperfusion, i.e. heat-shock proteins, ubiquinone oxidoreductase, ubiquinone binding protein, collagen, tubulin, and atrial natriuretic factor. We have detected extensive changes in heat shock proteins: a chaperonin (subunit ε) and two heat shock proteins (86 and 105 kDa) were upregulated due to cardiac ischemia/reperfusion. Ischemia/reperfusion repressed several genes including some mitochondrial genes and aconitase, a major enzyme of the citrate cycle.

Changes in the expression of some genes by preconditioning followed by ischemia/reperfusion were similar to those changed by ischemia/reperfusion alone (overexpressed: a secretory membrane protein, an amino acid transporter, an anion exchanger, a ribosomal RNA and a chaperonin gene; repressed: β-tubulin). Because in the case of preconditioning the control sample was ischemia/reperfusion alone, therefore, the expression of these genes changed more dramatically when compared to nonishemic controls. This suggests that these genes might have significant roles in ischemic adaptation of the heart during a single ischemia without preconditioning as well.

The rest of the genes (metallothionein, coagulation factor VII, cystein proteinase inhibitor, peroxisome proliferator activator receptor γ and myosin light chain kinase genes) were specifically and differentially expressed in response to preconditioning and were not altered after a single ischemia/reperfusion. We have found here that 2'-5' oligoadenylate synthase gene, chaperonin subunit ε and natriuretic peptide precursor type B also exhibited up-regulation due to preconditioning. A more dramatic induction was detected in the expression of a cGMP phosphodiesterase (PDE9A1). Alterations in cGMP levels in the heart have been previously shown in response to preconditioning. Peroxisome proliferator activator receptor γ (PPARγ) exhibited one of the most pronounced repression due to preconditioning. PPARγ has been shown to be involved in several cardiovascular pathologies including atherosclerosis and ischemic heart disease. Degradation of myocardial structural proteins in myocardial infarction has been shown to be reduced by a cystein proteinase inhibitor. In our present study a repression has been detected. It seems that the activity of cystein proteases are favoured in preconditioning. Another gene related to protein degradation was also repressed: protease 28 subunit had also repression; this protease subunit has a regulatory function in proteasome for small protein substrate degradation and has implications for oxidative stress.

$ONOO^-$ and cholesterol-enriched diet

Cholesterol-enriched diet resulted in a decrease of cardiac NO level, an increase of cardiac formation of superoxide and their reaction product $ONOO^-$. Dityrosine in the perfusate, a marker of cardiac $ONOO^-$ formation, and plasma nitrotyrosine, a marker for systemic $ONOO^-$ formation, were both elevated in hyperlipidemic rats. LVEDP was significantly elevated in cholesterol-fed rats as compared to controls. To further test if hyperlipidemia-induced elevation of LVEDP was due to enhanced $ONOO^-$ formation, hyperlipidemic and normal rats were treated with FeTPPS, a $ONOO^-$ decomposition catalyst.

In the hyperlipidemic group, LVEDP was recovered to control values after FeTPPS treatment, however, FeTPPS did not change LVEDP in the normal group.

These findings strongly suggest that sustained exposure to dietary cholesterol leads to an increase in cardiac $ONOO^-$ formation which can be in associated with the deterioration of cardiac function and the endogenous adaptive mechanisms.

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PUBLICATIONS

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

I. Csonka C, Csont T, **Ónody A**, Ferdinandy P. Preconditioning decreases ischemia/reperfusion-induced peroxynitrite formation. *Biochem Biophys Res Commun.* 285(5):1217-1219 (2001). [IF: 2.935]

II. **Ónody A**, Zvara A, Hackler L Jr, Vigh L, Ferdinandy P, Puskas LG. Effect of classic preconditioning on the gene expression pattern of rat hearts: a DNA microarray study. *FEBS Lett.* 536(1-3):35-40 (2003). [IF: 3.912]

III. **Ónody A**, Csonka C, Giricz Z, Ferdinandy P. Hyperlipidemia induced by a cholesterol-rich diet leads to enhanced peroxynitrite formation in rat hearts. *Cardiovasc Res* 58(3):663-70 (2003). [IF: 4.692]

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

IV. Giricz Z, Csonka C, **Ónody A**, Csont T, Ferdinandy P. Role of cholesterol-enriched diet and the mevalonate pathway in cardiac nitric oxide synthesis. *Basic Res Cardiol* 98(5):304-10 (2003). [IF:1.994]

V. Csonka C, **Ónody A**, Csont T, Ferdinandy P. Defibrillatory action of glibenclamide is independent from ATP-sensitive K^+-channels and free radicals. *J Cardiovasc Pharmacol.* 41(6):916-922 (2003). [IF:1.602]

VI. D'Souza SP, Yellon DM, Martin C, Schulz R, Heusch G, **Ónody A**, Ferdinandy P, Baxter GF. B-type natriuretic peptide limits infarct size in rat isolated hearts via K-ATP channel opening. *Am J Physiol Heart Circ Physiol* 284(5):H1592-H1600 (2003). [IF:3.369]

VII. Csont T, CsonkA C, **Ónody A**, Gorbe A, Dux L, Schulz R, Baxter GF, Ferdinandy P. Nitrate tolerance does not increase production of peroxynitrite in the heart. *Am J Physiol Heart Circ Physiol.* 283(1):H69-H76 (2002). [IF:3.369]

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