Myocardial stress adaptation: role of peroxynitrite and capsaicin-sensitive sensory nerves

Ph.D. Thesis

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INTRODUCTION

Short ischaemic episodes increase tolerance against subsequent severe ischaemia in the heart and in other tissues. This phenomenon is termed ischaemic preconditioning. Preconditioning involve reduction of necrotic tissue mass (infarct size), improvement of cardiac performance and reduction of arrhythmias following ischaemia and reperfusion. There is still a considerable debate regarding the exact cellular mechanism of ischaemic preconditioning. Among several other mediators, nitric oxide (NO), oxygen free radicals as peroxynitrite ($ONOO^-$), and antioxidant enzymes have been suggested to be, and also refuted as key triggers and mediators of preconditioning. In this study we focused on the role of reactive oxygen species, especially $ONOO^-$ in the development of classic preconditioning.

$ONOO^-$ 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 peroxynitrite 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 acute reperfusion of ischaemic 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 ischaemic stress adaptation of the rat myocardium, and to activate stress response pathways.

Chemical preconditioning is a novel and practical strategy of cardioprotection and neuroprotection. The term chemical preconditioning was used first by Riepe and Ludolph for the induction of hypoxic tolerance by using 3-nitropropionic acid

(3-NPA) in the brain. 3-NPA, a plant mycotoxin, is an irreversible inhibitor of succinate dehydrogenase which is an important enzyme of the Szent-Györgyi-Krebs cycle in complex II of the mitochondrial electron transport chain. Subtoxic chemical inhibition of oxidative phosphorylation can induce preconditioning. The action mechanism of 3-NPA strongly relates to the generation of reactive oxygen species, especially $ONOO^-$. We have previously shown that $ONOO^-$ triggers ischaemic preconditioning in the heart and that ischaemic preconditioning in turn attenuates the overproduction of NO, superoxide anion ($O_2^{-}\bullet$), and $ONOO^-$ during a subsequent episode of ischaemia and reperfusion, thereby confers cardioprotection. Although 3-NPA administration is a well-established method to induce chemical preconditioning in the brain, little is known on the effect of 3-NPA induced chemical preconditioning in the myocardium.

In contrast to the adrenergic and cholinergic innervation of the heart, less attention has been paid to the functional significance of the rich sensory innervation of the myocardium and the coronary vascular system. Sensory nerves have strong influence on cardiac function and adaptive responses due to their NO and vasoactive peptide content, such as calcitonin gene-related peptide (CGRP) and substance P. The thin sensory nerve endings act as potential sensor machinery for ischaemia, since ischaemia, hypoxia, lactate, K^+, and low pH were shown to stimulate cardiac sensory nerves in association with a release of their transmitters. Very little is known about the physiological and pathological role of sensory nerves in the regulation of cellular functions in the heart. The mechanism of the development of sensory neuropathy due to the risk factors (e.g. diabetes, etc.) for cardiovascular diseases is also unknown. Capsaicin is a highly selective sensory neurotoxin that leads to a selective functional blockade and/or ablation of a

morphologically well-defined population of primary sensory neurons. By the use of capsaicin, we and others have previously shown that cardiac sensory nerves play a role in cardiac adaptation to ischaemic stress, i.e. ischaemic preconditioning and in the regulation of cardiac NO-cGMP system. Therefore, it seems that capsaicin-sensitive cardiac nerves regulate a series of complex cellular events contributing to physiological and pathological myocardial function. However, the exact role of capsaicin-sensitive sensory nerves and capsaicin receptor (TRPV1) in cellular mechanisms under normal and pathological conditions are largely unknown especially in the heart. The traditional biochemical and pharmacological approaches have been insufficient so far to explore the key cellular events in the heart due to depletion of sensory nerves. Surprisingly, no attempt has been made so far to explore gene expression changes induced by selective chemodenervation of sensory nerves by systemic capsaicin pretreatment in any tissues. We have previously shown that systemic capsaicin pretreatment leads to impaired myocardial relaxation with a concomitant decrease of cardiac NO content, however, its mechanism remained unclear. Decreased basal NO may lead to decreased peroxynitrite ($ONOO^-$) formation and decreased S-nitrosylation of SERCA, the major player in myocardial relaxation. Nitrosylation of Cys349 has been shown to be responsible for the activation of the SERCA by $ONOO^-$. Consequently, it is plausible to speculate that sensory chemodenervation-induced decrease in NO formation may lead to impaired relaxation of the myocardium via decreased $ONOO^-$ formation and S-nitrosylation of SERCA2a.

AIMS

Study 1

to investigate whether 3-NPA induces chemical preconditioning in the isolated rat heart and whether alterations

of metabolism of NO, O_2^{-}•, and $ONOO^-$ are involved in the cardioprotective effect of 3-NPA.

Study 2:
to study if the selective sensory chemodenervation of the heart by capsaicin pretreatment leads to a decreased myocardial relaxation by the attenuation of $ONOO^-$ formation, thereby reducing S-nitrosylation of SERCA2a. Furthermore, in the hope of identifying new cellular pathways regulated by capsaicin-sensitive cardiac sensory nerves, here we have used cDNA microarrays of 6400 genes to monitor gene expression pattern of the heart associated with chemodenervation of sensory nerves by systemic capsaicin pretreatment.

MATERIALS AND METHODS

Animals and experimental groups
Study 1.
Male Wistar rats weighing 300–350 g were used throughout the experiments without any treatment. Animals were divided into three groups: In the preconditioned group, served as positive control, after 10 min stabilization period, preconditioning was induced by three intermittent cycles of 5 min no-flow ischaemia, separated by 5 min aerobic perfusion, which was followed by 30 min global ischaemia and 120 min reperfusion. In the control group, solvent for 3-NPA was applied intraperitoneally 3 h before isolation of the hearts. After isolation of the hearts 10 min stabilization period followed by 30 min normal perfusion was applied, then hearts were subjected to 30 min global ischaemia followed by 120 min of reperfusion. In the 3-NPA treated group, animals were pretreated in vivo by a single injection of 3-NPA 3 h before the perfusion protocol. A single subtoxic dose of 3-NPA (20 mg kg^{-1}) was applied intraperitoneally. After isolation of the hearts, 10 min stabilization period, 30 min aerobic perfusion, 30 min

global ischaemia, and 120 min reperfusion were applied as in the non-treated control group.

Study 2.
For selective chemodenervation of primary sensory nerves, rats were treated with solvent or capsaicin subcutaneously in the sequence of 10, 30, and 50 mg kg^{-1} single daily doses in 1 mL kg^{-1} volume for 3 days. To exclude any nonspecific pharmacological effects of capsaicin, isolated heart experiments were commenced seven days after the last injection when depletion of peptide-containing myocardial sensory nerves is already complete. Hearts form capsaicin- and solvent-treated animals were isolated and perfused for measurement of cardiac function and biochemical parameters.

Measurement of cardiac NO metabolism
To examine the role of NO in chemical preconditioning and in the selective sensory chemodenervation, in separate experiments, 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
O_2^{-}• production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence. 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 NAD(P)H 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. NAD(P)H-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 nitrotyrosine, a marker of $ONOO^-$

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

S-nitrosylation of SERCA2a

To assess SERCA2a S-nitrosylation, SR was isolated as described previously by Komuro et al. Isolated SR fractions were resuspended in 40 µl solution containing 150 mM KCl and 1 mM TES to load 20 µg of total protein on 8% polyacrylamide gel. After electrophoresis and blotting, nitrocellulose membranes were incubated with mouse monoclonal anti-SERCA2a antibody or rabbit polyclonal SNO-Cys antibody. Membranes were developed with an enhanced chemiluminescence kit, exposed to X-ray film, scanned, and density of SNO-Cys bands were measured and normalized to the density of SERCA2a bands and SNO-Cys/SERCA2a ratio was expressed in arbitrary units.

RESULTS

Study 1

In control hearts, test ischaemia/reperfusion resulted in a large *infarction and LDH release*. When ischaemic preconditioning was applied before test ischaemia (positive control), both infarct size and LDH release significantly

decreased showing the protective effect of ischaemic preconditioning against acute ischaemia/reperfusion injury. Administration of 3-NPA 3 h before the perfusion protocol showed an infarct size limiting effect similar to that of ischaemic preconditioning. LDH release was significantly decreased by 3-NPA pretreatment when compared to controls, however, LDH release remained significantly higher than that observed in the ischaemic preconditioned group.

Myocardial NO content was significantly increased 3 h after 3-NPA treatment as measured by ESR spectroscopy after in vivo spin trapping of NO compared to the non-treated control group. Endogenous enzymatic sources of NO, Ca^{2+}-dependent and Ca^{2+}-independent NOS activities in the myocardium were not affected by 3-NPA treatment.

Cardiac O_2^{-}• generation was significantly decreased due to 3-NPA treatment as compared to controls. To test possible changes in the enzymatic synthesis of O_2^{-}•, we measured activity of XOR, one of the major enzymatic source of O_2^{-}• in rat hearts. XOR and XO enzyme activities were significantly decreased in the 3-NPA treated group. Other main sources of O_2^{-}• generation in the myocardium are the NADH and NADPH oxidases. NADPH oxidase activity was not influenced by 3-NPA treatment, however, NADH oxidase activity showed significant decrease when compared to controls. Total activity of myocardial SOD was not changed in 3-NPA-treated hearts when compared to controls.

Plasma-free nitrotyrosine (as a marker for systemic $ONOO^{-}$ formation) was decreased approximately two-fold in 3-NPA treated rats as compared to controls.

Study 2

We measured parameters of *myocardial hemodynamics* to assess the effect of capsaicin-induced chemodenervation on

basal cardiac function in isolated heart preparations. LVEDP, the most sensitive marker for myocardial relaxation, was significantly increased after systemic capsaicin treatment, while other functional parameters were not affected as compared to solvent-treated controls.

Among the 6400 rat genes examined by *DNA microarray* in the present study an average of 3324 showed significant intensity and 2.6% (86 genes) showed significantly altered expression: 47 genes exhibited significant up-regulation and 33 were down-regulated 7 days after the end of the systemic capsaicin-treatment protocol.

In the solvent treated group, *basal cardiac NO content* was detected by ESR spectroscopy. In the capsaicin-treated group, the specific signal for NO was markedly reduced to a level near the detection limit. To further explore cardiac NO synthesis, activities and gene expressions of NOS were measured. Ca^{2+}-dependent activity significantly decreased in the capsaicin-pretreated group, while Ca^{2+}-independent activity did not change. The mRNA level of endothelial NOS was significantly down-regulated by capsaicin-treatment, however, the expression of neuronal and inducible NOS was not affected.

To further test the mechanism of reduced cardiac NO due to systemic capsaicin pretreatment we systematically analyzed *myocardial* O_2^{-}• synthesis and $ONOO^-$ formation. Myocardial O_2^{-}• as assessed by dihydroethidium staining was approximately 30% lower in the capsaicin-treated group, however, it was not statistically significant difference. We also measured the activity of XOR and NADPH oxidase enzyme activities, the major enzymatic sources of O_2^{-}• in rat hearts. There was no significant difference in activities of xanthine oxidase, xanthine dehydrogenase and NADPH oxidase. We also tested the total activity of SOD in the heart, which was significantly increased in the capsaicin pretreated group.

We studied whether decreased NO synthesis and increased SOD activity due to chemodenervation changed basal *cardiac $ONOO^-$ formation* in cardiac and extracardiac tissues. Therefore, myocardial and serum free nitrotyrosine concentration was measured. Both serum and myocardial free nitrotyrosine markedly decreased in capsaicin-pretreated group when compared to controls.

To assess, if decreased $ONOO^-$ may decrease the degree of *S-nitrosylation of cardiac SERCA2a*, we assessed S-nitrosylation in cardiac sarcoplasmic reticulum preparations isolated from both group by Western blotting. We have found that sensory chemodenervation significantly decreased S-nitrosylation of SERCA2a in the capsaicin-pretreated group when compared to solvent treated controls.

DISCUSSION

In the present *study 1* we showed that cardiac NO signal intensity significantly increased in response to the 3-NPA administration, however, activities of endogenous enzymatic sources of NO, Ca^{2+}-dependent and Ca^{2+}-independent NO synthases, were not changed. According to these results, the increase in cardiac NO bioavailability cannot be explained by increased NO synthesis. Nevertheless, it is plausible to speculate that reduced formation of O_2^{-}• played a major role in the increased cardiac NO level found in our present study. Indeed, we have also shown here that chemical preconditioning with 3-NPA significantly decreased O_2^{-}• level, and activities of XOR and NAD(P)H oxidase enzymes, major sources of O_2^{-}• in the rat heart. SOD activity, which is responsible for dismutation of O_2^{-}• was not changed, therefore, it did not influence O_2^{-}• content after 3-NPA administration. These findings are supported by Riepe et al., who reported that chemical preconditioning induced by 3-NPA delayed the

hypoxia-induced increase in NADH oxidase activity in rat hippocampus. In contrast, others have shown that 3-NPA treatment is associated with generation of reactive oxygen species in brain. However, in these studies high concentration or repeated applications of 3-NPA were applied not to induce chemical preconditioning but to examine the neurotoxic effect of 3-NPA to study neurodegenerative disorders. To investigate the role of $ONOO^-$ in 3-NPA-induced chemical preconditioning, we measured 3-nitrotyrosine, a $ONOO^-$ marker concentration in the serum 3 h after 3-NPA treatment, and we found that 3-NPA administration significantly reduced formation of $ONOO^-$. Decreased cardiac XOR and NADH oxidase activities which resulted in a reduced O_2^{-}• production explain the reduced generation of $ONOO^-$. In our previous studies, we have shown that although the first brief cycle of preconditioning ischaemia/reperfusion significantly enhanced $ONOO^-$ formation, after the third cycle of ischaemia/reperfusion $ONOO^-$ formation was significantly reduced. This showed that $ONOO^-$ might act as a trigger of preconditioning but preconditioning in turn decreases increased formation of $ONOO^-$ upon ischaemia/reperfusion and thereby confers cardioprotection. Taken together, acute 3-NPA treatment may induce increased formation of reactive oxygen species, however, 3 h after treatment, a significant reduction in formation of reactive oxygen species including $ONOO^-$ occurs, which results in cardioprotection.

Our *study 2* is the first demonstration that capsaicin-sensitive sensory neurons regulate myocardial relaxation via maintaining basal $ONOO^-$ formation and SERCA2a S-nitrosylation. We have previously shown that sensory chemodenervation leads to impaired myocardial relaxation with a concomitant decrease in cardiac NO content, however, its mechanism remained unclear. In the present study, we confirmed that capsaicin-induced sensory denervation leads to

impaired cardiac relaxation characterized by elevation of LVEDP and to decreased cardiac NO availability. Here we further examined the mechanism of reduced cardiac NO content and showed that it is due to decreased expression of endothelial NOS and therefore decreased enzymatic activity of Ca^{2+}-dependent NOS.

Our further interesting finding is that capsaicin-pretreatment led to altered expression of several genes of neural and non-neural origin, such as TRPV-1 (capsaicin receptor), endothelial NOS, matrix metalloproteinase-13, cytochrom P450, farnesyl-transferase, etc. None of the genes have been previously shown to be involved in the mechanism of the cardiac functional effects of sensory chemodenervation by capsaicin. These results points out that capsaicin-sensitive sensory nerves play a significant role in the regulation of a variety of neuronal and non-neuronal genes in the heart.

To further examine if reduced basal NO content may lead to decreased basal $ONOO^-$ formation, we systematically measured O_2^{-}• formation, as $ONOO^-$ is formed from the rapid non-enzymatic reaction of NO and O_2^{-}•. Activities of major enzymatic sources of O_2^{-}• in the heart, XOR and NADPH oxidase were not changed, however, cardiac activity of SOD, the major enzyme for O_2^{-}• removal was significantly increased by systemic sensory chemodenervation. Myocardial O_2^{-}• content as assessed by dihydroethidium staining was approximately 30% lower in the capsaicin-treated group, however, it did not reach a statistically significant difference. Nevertheless, these findings indicate that at least there is a tendency of decreased basal O_2^{-}• availability in the myocardium when sensory neural function was ablated by capsaicin. To examine if diminished NO and O_2^{-}• availability in the myocardial tissue may lead to decreased formation of $ONOO^-$, we measured serum and cardiac free nitrotyrosine (marker of $ONOO^-$) levels. Myocardial free nitrotyrosine level

was significantly decreased due to capsaicin pre-treatment. This was further supported by decreased nitrotyrosine level in the serum. These results clearly show that due to systemic sensory chemodenervation, there is a decrease in basal cardiac and systemic $ONOO^-$ formation.

Although earlier $ONOO^-$ has been thought to be a purely toxic reactive nitrogen species, recently, it has been shown that basal $ONOO^-$ production plays an important role as a regulator of several cellular mechanisms via protein nitrosylation of some enzymes. Nitrosylation of Cys349 has been shown to be responsible for the activation of the SERCA2a by $ONOO^-$ thereby maintaining the normal physiological function of SERCA2a in myocardial relaxation. Here we have found that decreased basal $ONOO^-$ formation due to sensory chemodenervation resulted in a significant decrease in S-nitrosylation of SERCA2a and impaired relaxation of the heart characterized by increased LVEDP, the most sensitive hemodynamic marker for impairment of myocardial relaxation.

NEW OBSERVATIONS:

1. 3-NPA induces pharmacological preconditioning thereby limiting infarct size
2. This effect is associated with increased cardiac NO bioavailability and reduced $ONOO^-$ and $O_2^{-}\bullet$ formation via inhibition of cardiac XOR and NADH oxidase activities.
3. Capsaicin-sensitive sensory nerves may play a significant role in the regulation of the expression of a variety of neuronal and non-neuronal genes in the heart and possibly in other tissues as well.
4. Sensory chemodenervation decreases cardiac NO availability via decreased expression of Ca^{2+}-dependent NOS and increases SOD activity thereby leading to decreased basal $ONOO^-$ formation and a reduction of S-nitrosylation of

SERCA2a, which causes impaired myocardial relaxation characterized by increased LVEDP.

5. We here demonstrate for the first time in the literature that capsaicin-sensitive sensory neurons regulate myocardial relaxation via maintaining basal $ONOO^-$ formation and SERCA2a S-nitrosylation.

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PUBLICATIONS

Full papers directly related to the subject of the Thesis:

I. Turan N, Csonka C, Csont T, Giricz Z, Fodor G, **Bencsik P**, Gyöngyösi M, Cakici I, Ferdinandy P. (2006) The role of peroxynitrite in chemical preconditioning with 3-nitropropionic acid in rat hearts.
Cardiovasc Res. **70**:384-90. **IF: 5.826**

II. Zvara A, **Bencsik P**, Fodor G, Csont T, Hackler L Jr, Dux M, Fürst S, Jancsó G, Puskás LG, Ferdinandy P. (2006) Capsaicin-sensitive sensory neurons regulate myocardial function and gene expression pattern of rat hearts: a DNA microarray study.
FASEB J. **20**:160-2. **IF: 6.721**

III. **Bencsik P**, Kupai K, Giricz Z, Görbe A, Huliák I, Fürst S, Dux L, Csont T, Jancsó G, Ferdinandy P. (2008) Cardiac capsaicin-sensitive sensory nerves regulate myocardial relaxation via S-nitrosylation of SERCA: role of peroxynitrite.
Br J Pharmacol. **153**:488-96. **IF: 3.825.**

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

IV. Giricz Z, Lalu MM, Csonka C, **Bencsik P**, Schulz R, Ferdinandy P. (2006) Hyperlipidemia attenuates the infarct size-limiting effect of ischemic preconditioning: role of matrix metalloproteinase-2 inhibition.
J Pharmacol Exp Ther. **316**:154-61. **IF: 3.956**

V. Csont T, Bereczki E, **Bencsik P**, Fodor G, Görbe A, Zvara A, Csonka C, Puskás LG, Sántha M, Ferdinandy P. (2007) Hypercholesterolemia increases myocardial oxidative and nitrosative stress thereby leading to cardiac dysfunction in apoB-100 transgenic mice.
Cardiovasc Res. **76**:100-9. **IF: 5.826**