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

Ph.D. Thesis

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2008

Contents

1. List of publications 4
2. Summary 5
3. Abbreviations 6
4. Introduction 7
4.1. Classic preconditioning as adaptation mechanism to myocardial oxidative stress 7
4.2. Role of $ONOO^-$ in myocardial physiology and pathophysiology 7
4.3. Chemical preconditioning: role of 3-NPA in $ONOO^-$ generation 9
4.4. Role of capsaicin-sensitive sensory innervation of the myocardium 10
5. Aims 12
6. Materials and methods 13
6.1. Animals and experimental groups 13
6.1.1. Study 1. 13
6.1.2. Study 2. 13
6.2. Isolated heart preparations, measurement of cardiac function 14
6.2.1. Study 1. 14
6.2.2. Study 2. 14
6.3. Measurement of myocardial infarct size and LDH release 15
6.4. CGRP and TRPV1 immunohistochemistry 15
6.5. Measurement of cardiac NO metabolism 16
6.6. Measurement of enzymatic superoxide production and degradation 17
6.7. Measurement of nitrotyrosine, a marker of $ONOO^-$ 18
6.8. RNA preparation, microarrays and probes; scanning and data analysis 19
6.9. Real-time QRT-PCR 20
6.10. S-nitrosylation of SERCA2a 21
6.11. Statistics 21
7. Results 22
7.1 Study 1 22
7.1.1. Infarct size and cardiac LDH release 22
7.1.2. NO content and cardiac NO synthase 23

7.1.3. Cardiac superoxide and XOR, NAD(P)H oxidase, and SOD activity 23
7.1.4. $ONOO^-$ 24
7.2 Study 2.. 25
7.2.1. CGRP and TRPV1 immunohistochemistry.. 25
7.2.2. Effects of sensory chemodenervation by capsaicin on myocardial function. 26
7.2.3. Gene expression changes 27
7.2.4. Effects of sensory chemodenervation on cardiac NO 30
7.2.5. Effects of sensory chemodenervation on cardiac superoxide 32
7.2.6. Effects of sensory chemodenervation on cardiac $ONOO^-$ and S-nitrosylation of SERCA 33
8. Discussion 34
8.1. New findings: 34
8.2. The role of NO and superoxide in 3-NPA induced chemical preconditioning 34
8.3. The role of $ONOO^-$ in 3-NPA-induced chemical preconditioning 35
8.4. Myocardial effects of capsaicin induced sensory chemodenervation 36
8.5.The role of $ONOO^-$ in the regulation of myocardial relaxation 37
8.6. Limitations of the studies. 38
9. Conclusions 39
10. Acknowledgements 40
11. References 41

1. List of publications

List of 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]

2. Summary

It has been widely accepted that enhanced peroxynitrite ($ONOO^-$) formation contributes to oxidative and nitrosative stress in a variety of cardiovascular and other pathologies. However, increasing number of evidences suggest that physiological production of $ONOO^-$ may act as a regulator of various physiologic cellular functions as well as endogenous cardioprotective mechanisms, such as preconditioning. Therefore, our aims were in the present Thesis (*Study 1*) to investigate the role of $ONOO^-$ in the development of chemical preconditioning induced by acute 3-nitropropionic acid (3-NPA) treatment and to examine (*Study 2*) 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 sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a).

We have shown in *Study 1* that 3-NPA pretreatment, similarly to ischaemic preconditioning, markedly reduced infarct size and lactate dehydrogenase (LDH) release in isolated rat hearts subjected to global ischaemia/reperfusion. Cardiac superoxide ($O_2^{-}\bullet$) content and serum nitrotyrosine level were also decreased 3 h after 3-NPA treatment due to decreased activities of xanthine oxidoreductase (XOR) and NADH oxidase, main sources of $O_2^{-}\bullet$ generation in the heart, whereas superoxide dismutase (SOD) activity was not changed. We have also shown that 3-NPA pretreatment increased cardiac nitric oxide (NO) content whereas activities of NO synthase (NOS) were not changed. These results provide evidence for the first time in the rat heart that 3-NPA induces pharmacological preconditioning thereby limiting infarct size and that this effect is associated with increased cardiac NO bioavailability and reduced $ONOO^-$ and $O_2^{-}\bullet$ formation via inhibition of cardiac XO and NADH oxidase activities.

We have found in *Study 2* that systemic sensory chemodenervation by capsaicin pretreatment decreased cardiac NO availability via a decrease in the expression and activity of Ca^{2+}-dependent NOS and an increase in SOD activity thereby leading to decreased basal $ONOO^-$ formation and a reduction of S-nitrosylation of sarcoplasmic reticulum Ca^{2+}-ATPase

2a (SERCA2a), which causes impaired myocardial relaxation characterized by increased left ventricular end-diastolic pressure (LVEDP). This is the first demonstration that capsaicin-sensitive sensory neurons regulate myocardial relaxation via maintaining basal $ONOO^-$ formation and SERCA2a S-nitrosylation.

We conclude that attenuation of pathological increase of $ONOO^-$ formation may be involved in chemically induced preconditioning. Furthermore, basal $ONOO^-$ formation is necessary for physiological regulation of myocardial relaxation.

3. Abbreviations

3-NPA	- 3-nitropropionic acid
AF	- aortic flow
CF	- coronary flow
ELISA	- enzyme linked immunosorbent assay
LDH	- lactate dehydrogenase
LVDP	- left ventricular developed pressure
LVEDP	- left ventricular end diastolic pressure
NO	- nitric oxide
NOS	- nitric oxide synthase
$O_2^{-}\bullet$	- superoxide anion
$ONOO^-$	- peroxynitrite
QRT-PCR	- quantitative real time polymerase chain reaction
SERCA	- sarcoplasmic reticulum Ca^{2+}-ATPase
SOD	- superoxide dismutase
TRPV1	- transient receptor potential vanilloid receptor 1
XOR	- xanthine oxidoreductase

4. Introduction

4.1. Classic preconditioning as adaptation mechanism to myocardial oxidative stress

Short ischaemic episodes increase tolerance against subsequent severe ischaemia in the heart and in other tissues. This phenomenon is termed ischaemic preconditioning (Murry et al., 1986),[see (Ferdinandy et al., 1998 and 2003) for reviews]. Preconditioning confers a remarkable cardioprotection in a variety of species including humans [see for review: (Przyklenk et al., 1998)], although the cardioprotective effectiveness of ischaemic preconditioning might be attenuated in the heart during aging and some disease states such as hyperlipidaemia and diabetes [see for review: (Ferdinandy et al., 2007)]. Preconditioning can be elicited by different sublethal stress signals, such as brief periods of ischaemia, hypoxia, rapid electrical pacing, heat stress, or administration of bacterial endotoxin, etc. Preconditioning involve reduction of necrotic tissue mass (infarct size), improvement of cardiac performance and reduction of arrhythmias following ischaemia and reperfusion [see for reviews: (Baxter et al., 2001; Ferdinandy et al., 1998; Przyklenk et al., 1998)]. Understanding the cellular pathways involved in the ischaemic adaptation of the myocardium may lead to the development of "preconditioning mimetic" drugs for patients suffering from ischaemic heart disease. However, there is still a considerable debate regarding the exact cellular mechanism of ischaemic preconditioning [see for review: (Schulz et al., 2001)]. 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.

4.2. Role of $ONOO^-$ in myocardial physiology and pathophysiology

$ONOO^-$ is a powerful oxidant species, which can be formed in vivo by the non-enzymatic reaction of NO and O_2^{-}• anion at an extremely rapid rate limited only by diffusion (Fig. 1). It is widely accepted now that enhanced $ONOO^-$ formation contributes to oxidative and nitrosative stress in a variety of cardiovascular and other pathologies [see for reviews: (Ferdinandy et al., 2001 and 2003; Denicola et al., 2005)].

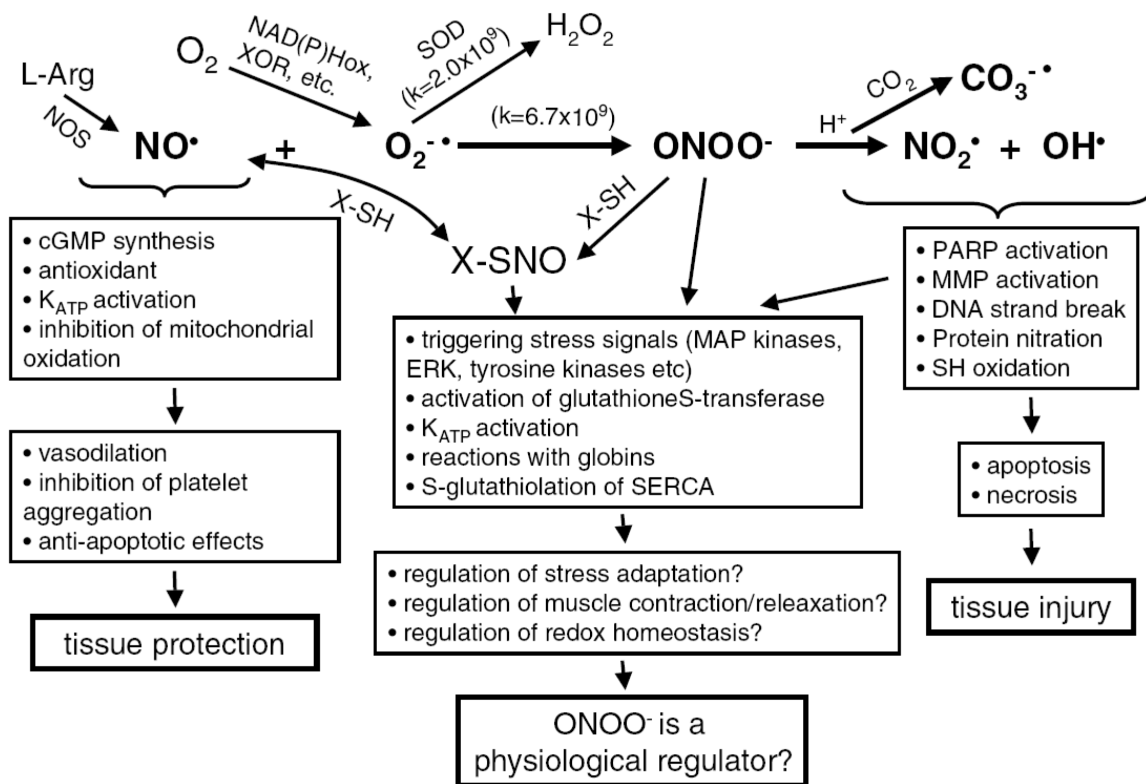

Figure 1. Cellular mechanisms of the actions of NO, superoxide (O_2^{-}•), and $ONOO^-$. NO is an important cardioprotective molecule via its vasodilator, antioxidant, antiplatelet, and antineutrophil actions and it is essential for normal cellular function. However, excess NO could be detrimental if it combines with O_2^{-}• to form $ONOO^-$ which rapidly decomposes to highly reactive oxidant species leading to tissue injury. There is a critical balance between cellular concentrations of NO, O_2^{-}•, and superoxide dismutase (SOD) which physiologically favor NO production but in pathological conditions such as, for example, ischaemia and reperfusion result in $ONOO^-$ formation. $ONOO^-$ might be converted to NO donors if it combines with SH-group containing molecules (X-SH) to form S-nitroso compounds (X-SNO) including S-nitrosoglutathione. S-nitrosylation and S-glutathiolation are proposed mechanisms by which $ONOO^-$ regulates protein functions. Increasing evidence suggests that physiological levels of $ONOO^-$ act as regulator of several physiological functions. MMP, matrix metalloproteinase; NOS, NO synthase; PARP, poly-ADP ribose polymerase; XOR, xanthine oxidoreductase; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; KATP, ATP sensitive potassium channel. (Original figure in Ferdinandy, *Br J Pharmacol,* 2006)

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 (Cheung et al., 2000; Yasmin et al., 1997). In contrast, increasing evidence suggests that physiological levels of $ONOO^-$ may act as a regulator of

several physiological functions (Ferdinandy et al., 2001 and 2003; Herold et al., 2005; Ji et al., 2006). However, still very little is known about the physiological roles of endogenous peroxynitrite formation, possibly due to the number of technical limitations of detecting low, physiological levels of $ONOO^-$ in biological systems (Daiber et al.; 2003; Tarpey et al., 2001). Increasing evidence suggests that $ONOO^-$ may act as a regulator of various physiologic cellular functions. Endogenous $ONOO^-$ has been shown to trigger ischaemic stress adaptation of the rat myocardium [(Altug et al., 2000; Csonka et al., 2001); see for review (Ferdinandy et al., 2003)], and to activate stress response pathways [see for review (Klotz et al., 2002)].

4.3. Chemical preconditioning: role of 3-NPA in $ONOO^-$ generation

Chemical preconditioning is a novel and practical strategy of cardioprotection and neuroprotection (Horiguchi et al., 2003; Ockaili et al., 2001; Riepe et al., 1997b). The term chemical preconditioning was used first by Riepe and Ludolph (Riepe et al., 1997b) for the induction of hypoxic tolerance by using 3-nitropropionic acid (3-NPA) in the brain. Other studies also suggested that the application of 3-NPA in a single dose up to 20 mg/kg is a suitable strategy to induce chemical preconditioning with an early onset and long duration in the brain (Aketa et al., 2000; Riepe et al., 1997b), [see (Rejdak et al., 2001) for review]. 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 (Alston et al., 1977; Hassel et al., 1995). Subtoxic chemical inhibition of oxidative phosphorylation can induce preconditioning (Nandagopal et al., 2001). It has to be noted, however, that 3-NPA given systematically in sufficiently low dose over a period of weeks results in neuronal death predominantly in striatum, therefore, 3-NPA is widely used in animal models of neurodegenerative disorders such as Hungtinton's disease [see (Borlongan et al., 1997) for review]. The action mechanism of 3-NPA strongly relates to the generation of reactive oxygen species (Schulz et al., 1996; Teunissen et al., 2002; Wang et al., 2001). $O_2^{-}•$ combines with nitric oxide at a reaction rate that is only limited by diffusion to form $ONOO^-$, a highly reactive molecule with cytotoxic effects [see (Ferdinandy et al. 2003; Schulz et al., 1997) for review)] described that after systemic 3-NPA treatment in rats an increased production of $ONOO^-$ was observed in brain. We have previously shown that $ONOO^-$ triggers ischaemic preconditioning in the heart and that ischaemic preconditioning in turn attenuates

the overproduction of NO, $O_2^{-}\bullet$, and $ONOO^-$ during a subsequent episode of ischaemia and reperfusion, thereby confers cardioprotection [(Csonka et al., 1999 and 2001), see (Ferdinandy et al., 2003) for review]. 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. Only one study (Ockaili et al., 2001) showed that 3-NPA is able to induce chemical preconditioning in rabbit heart.

4.4. Role of capsaicin-sensitive sensory innervation of 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 (Franco-Cereceda 1988; Holzer 1991; Ren et al., 1995; Sosunov et al., 1996). 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 [see (Franco-Cereceda 1988; Nagy et al., 2004) for reviews].

Sensory neuropathy develops in the presence of various risk factors for cardiovascular diseases, such as diabetes, dyslipidemia, and obesity, however, it should be noted that other non-cardiovascular diseases are also associated with sensory neuropathy, such as trauma, HIV infection, pain disorders, etc (Abrams et al., 2007; Facer et al., 2007; Herman et al., 2007; Hughes et al., 2004; Kassem et al., 2005; Polydefkis et al., 2004). Very little is known about the physiological and pathological role of sensory nerves in the regulation of cellular functions in the heart and other organs. The mechanism of the development of sensory neuropathy due to the risk factors 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 (Jancso et al., 1977; Jancso et al., 1968). Hence, capsaicin has become one of the most important probes for investigations of sensory neural pathology and pharmacology [see (Franco-Cereceda 1988; Holzer 1991; Jancso 1992) for reviews]. However, it has been appreciated only in the last decade that the selective action of capsaicin and related

compounds on a subset of sensory nerve fibers is mediated at least in part by an agonist activity on a ligand-gated ion channel called capsaicin receptor or the transient receptor potential vanilloid receptor 1 (TRPV1; Van Der Stelt et al., 2004).

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 (Ferdinandy et al., 1997; Li et al., 1996; Wang et al., 2005; Zhong et al., 2007), in the regulation of cardiac NO-cGMP system (Csont et al., 2003) and in the mechanism of doxorubicin-induced heart failure (Katona et al., 2004). Therefore, it seems that capsaicin-sensitive cardiac nerves regulate a series of complex cellular events contributing to physiological and pathological myocardial function. Capsaicin has become one of the most important probes for investigations of sensory neural pathology and pharmacology (Szallasi et al., 2007). 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 (Holzer 2006; Wang et al., 2005).

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. A few years ago, Szallasi and Fowler envisioned in their review (Szallasi et al., 2002) that the application of gene chip technologies will address the global profile of vanilloid-induced changes in gene expression and their contribution to cellular events. 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 (Csont et al., 2003; Ferdinandy et al., 1997). Decreased basal NO formation may lead to decreased $ONOO^-$ formation (Ferdinandy et al., 2003; Ferdinandy 2006) and decreased S-nitrosylation of sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a), the major player in myocardial relaxation. Nitrosylation of Cys349 has been shown to be responsible for the activation of the SERCA2a by $ONOO^-$ (Adachi et al., 2004; Viner et al., 1999). 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.

5. Aims

Therefore, the aims of our present studies were:

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.

6. Materials and methods

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

6.1. Animals and experimental groups

6.1.1. Study 1.

Male Wistar rats weighing 300–350 g housed in a room maintained at 12 h light–dark cycles and a constant temperature of 22±2 ºC 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. 3-NPA (Sigma, St Louis, MO) was dissolved in distilled water at 1 mg mL^{-1} (pH 7.4, adjusted with NaOH). A single subtoxic dose of 3-NPA [20 mg kg^{-1}, (Riepe et al., 1997b)] was applied intraperitoneally as described previously (Aketa et al., 2000). 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.

6.1.2. Study 2.

Male Wistar rats weighing 300-350 g housed in a room maintained at 12-h light–dark cycles and a constant temperature of 22±2 ºC were used throughout the experiments. For selective chemodenervation of primary sensory nerves, rats were treated with solvent or

capsaicin (1% w v^{-1}, Fluka, Buchs, Switzerland, dissolved in physiological saline containing 6% v v^{-1} ethanol and 8% v v^{-1} Tween 80) subcutaneously in the sequence of 10, 30, and 50 mg kg^{-1} single daily doses in 1 mL kg^{-1} volume for 3 days as described (Ferdinandy et al., 1997). All injections of capsaicin and its solvent were given under general anesthesia using diethyl-ether. 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 (Csont et al., 2003; Ferdinandy et al., 1997). Hearts form capsaicin- and solvent-treated animals were isolated and perfused for measurement of cardiac function and biochemical parameters. Similarily to our previous studies (Csont et al., 2003; Ferdinandy et al., 1997) systemic capsaicin treatment in the present series of experiments resulted in an elimination of CGRP immunoreactivity from the heart tissue as assessed by immunohistochemistry (see below) and a lack of CGRP release from the heart as assessed by radioimmunoassay as described (Csont et al., 2003; Ferdinandy et al., 1997; Katona et al., 2004).

6.2. Isolated heart preparations, measurement of cardiac function

6.2.1. Study 1.

To isolate the hearts, rats were anesthetized with diethyl ether. After i.v. administration of heparin (500 IU kg^{-1}), the chests were opened and the hearts were rapidly excised and mounted on a non-recirculating Langendorff perfusion apparatus perfused at 37 ºC with Krebs–Henseleit bicarbonate buffer containing (in mmol L^{-1}) NaCl 118.4, KCl 4.1, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.17, $MgCl_2$ 1.46 and glucose 11.1; gassed with 95% O_2 and 5% CO_2, pH 7.4 (Csonka et al., 1999; Ferdinandy et al., 2000).

6.2.2. Study 2.

To exclude any nonspecific pharmacological effects of capsaicin, isolated heart experiments were commenced seven days after the end of capsaicin treatment, when depletion of peptide containing myocardial sensory nerves and elimination of capsaicin is complete (Csont et al., 2003; Ferdinandy et al., 1997). Hearts were excised after anesthesia with diethylether and prepared for working heart perfused at 37°C with Krebs-Henseleit bicarbonate buffer containing (in mmol L^{-1}) 118 NaCl, 4.3 KCl, 2.4 $CaCl_2$, 25 $NaHCO_3$, 1.2

KH_2PO_4, 1.2 $MgSO_4$, and 11.1 glucose, gassed with 95% O_2 - 5% CO_2 (Csonka et al., 1999; Ferdinandy et al., 2000). After a 5 min initial normoxic, normothermic Langendorff perfusion to normalize sinus rhythm, the perfusion was switched to working perfusion for 10-min to measure cardiac mechanical functional parameters, such as heart rate, coronary flow, aortic flow, cardiac output, left ventricular developed pressure and its first derivatives ($\pm dP\ dt_{max}^{-1}$), and left ventricular end-diastolic pressure (LVEDP) were monitored as described (Csonka et al., 1999; Csont et al., 1999; Ferdinandy et al., 2000). Ventricular tissue samples for biochemical measurements were taken in separate experiments after the initial 5 min perfusion to eliminate blood before tissue sampling.

6.3. Measurement of myocardial infarct size and LDH release

To measure infarct size in *study 1*, at the end of reperfusion hearts from all groups were perfused with 1% triphenyltetrazolium-chloride (Sigma) dissolved in phosphate buffer and incubated for 5 min at 37ºC. Hearts were then sectioned (2 mm) and fixed in 4% formaldehyde. Infarct size was determined from images of the sections by planimetry.

Coronary effluents were collected for 5 min before and after global ischaemia in all groups. LDH levels were measured immediately after collection of the effluent and evaluated spectrophotometrically at 340 nm wavelength using a kit (Diagnosticum Rt, Budapest, Hungary).

6.4. CGRP and TRPV1 immunohistochemistry

For immunohistochemical studies in *study 2*, rats treated with capsaicin (n=3) or its solvent (n=3) were perfused via the left heart ventricle with 4% formaldehyde in phosphate buffer (0.1 M, pH 7.4) one week after the completion of the treatment. The hearts, and the 8th cervical and 1-3rd thoracic dorsal root ganglia innervating the heart (Benson et al., 1999) were removed and after a post-fixation period of 3 hours they were placed in a buffer solution and stored at 4°C until sectioning. Transverse sections through the ventricles and sections from the dorsal root ganglia were cut at a thickness of 20 mm and were processed for immunohistochemical staining with the indirect immunofluorescence technique by using a rabbit and guinea pig polyclonal antisera raised against CGRP (Sigma Chemicals, St. Louis,

MO, USA, 1:500) and TRPV1 (Neuromics Antibodies, Bloomington, MN, USA, 1:1000). Goat anti-rabbit and donkey anti-guinea pig IgGs labelled with Cy3 (carboxymethylindocyanin, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA, 1:500) were used as secondary antibodies. The specimens were viewed under a Leitz DMLB fluorescence microscope equipped with an appropriate filter combination and photographed with a digital camera.

6.5. 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 (DETC) as described (Csonka et al., 1999). The spin-trap for NO was prepared freshly before each experiment. 200 mg kg^{-1} DETC, 50 mg kg^{-1} $FeSO_4$, and 200 mg kg^{-1} sodium citrate were slowly administered to the non-treated control and treated animals intraperitoneally under diethyl ether anesthesia, respectively. DETC was dissolved in distilled water and injected separately from $FeSO_4$ and sodium-citrate in 0.5 ml volume to avoid precipitation of Fe^{2+}-$(DETC)_2$. $FeSO_4$ and sodium citrate were dissolved in distilled water (pH 7.4). Five minutes after DETC, $FeSO_4$, and citrate treatment, hearts were isolated and perfused in Langerdorff mode for 1 min to eliminate blood. Tissue samples from the apex of the heart (approximately 150 mg) were placed into quartz ESR tubes and frozen in liquid nitrogen. ESR spectra of NO–$Fe^{2+}(MGD)_2$ adducts were recorded with a Bruker ECS106 spectrometer (Rheinstetten, Germany; ESR parameters: X band, 100 kHz modulation frequency, 160 K temperature, 10 mW microwave power, 2.85 G modulation amplitude, 3356 G central field) and analyzed for NO signal intensity as described (Csonka et al., 1999).

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 as previously described (Ferdinandy et al., 2000). Powdered frozen ventricular tissue was placed in four volumes of ice-cold homogenization buffer (composition given in (Schulz et al., 1992) and homogenized with an Ultra-Turrex disperser using three strokes of 20 s duration each. The homogenate was centrifuged (1,000 g for 10 min) at 4°C and the supernatant was kept on ice for immediate assay of enzyme activities.

Samples were incubated for 25 min at 37°C in the presence or absence of EGTA (1 mM) or EGTA plus N-monomethyl-L-arginine (1 mM) to determine the level of Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities, respectively. Protein concentration was measured from the supernatant using a Lowry–Folin method. NO synthase activities were expressed in pmol $minute^{-1}$ $mg_{protein}^{-1}$.

6.6. Measurement of enzymatic superoxide production and degradation

Superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence in separate experiments as described (Onody et al., 2003a). Hearts from control and treated animals were isolated and perfused with Krebs–Henseleit solution to eliminate blood. Approximately 100 mg of the apex of the right ventricle was placed in 1 ml air-equilibrated Krebs–Henseleit solution containing 10 mmol L^{-1} HEPES (pH 7.4) and 5 μmol L^{-1} lucigenin. Chemiluminescence was measured at room temperature in a liquid scintillation counter using a single active photomultiplier positioned in out-of-coincidence mode in the presence or absence of the O_2^{-}• scavenger nitroblue tetrazolium (NBT, 200 μmol L^{-1}). NBT-inhibitable chemiluminescence was considered an index of myocardial O_2^{-}• generation. It should be noted that NBT, like other O_2^{-}• scavengers, is not entirely specific for O_2^{-}•.

In situ detection of O_2^{-}• anion was performed by confocal laser scanning microscopy using a fluorescent dye dihydroethidium (Sigma) as described (Csont et al., 2007). Dihydroethidium is freely permeable to cell membranes and fluorescences red when oxidized by O_2^{-}•. Frozen native heart sections (30 μm) were placed on glass slides covered with PBS buffer (pH 7.4) and collected at +4°C. Then slides were submerged in 10^{-6} mol L^{-1} dihydroethidium (Sigma) in PBS buffer (pH 7.4) and incubated at 37°C for 30 minutes in a dark humidified container. Fluorescence in heart sections was detected by a confocal microscope (Olympus™ FV1000) using a 530 nm long-pass filter for excitation. Images of the hearts that were treated with saline (negative control) were measured first. After the basal settings of the confocal microscope were adjusted, images of the hearts were collected digitally. Eight images were taken randomly of each slides and fluorescence intensity was analysed by ImageJ 129 program.

Activities of xanthine oxidoreductase (XOR xanthine oxidase and xanthine dehydrogenase) and NADPH oxidase, major sources of O_2^{-}• in rat hearts were determined from ventricular homogenates (protein concentration: 8-10 μg μL^{-1}). Ventricular homogenates were prepared as for the measurement of NO synthase activity. XOR activity was determined by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total xanthine oxidoreductase activity) and absence (xanthine oxidase activity) of the electron acceptor methylene blue, as described (Beckman et al., 1989).

NAD(P)H-stimulated O_2^{-}• production in freshly prepared ventricular homogenates was assessed by a lucigenin-enhanced chemiluminescence method. To estimate background level of luminescence 30 μL of the ventricular homogenate was added to 1 mL Krebs–Henseleit buffer (room temperature) containing 10 mmol L^{-1} HEPES–NaOH (pH 7.4) and 5 μmol L^{-1} lucigenin (Sigma). Chemiluminescence was measured with a liquid scintillation counter (Packard, Meriden, CT) every 15 s for 5 min and the last 6 readings were averaged. To measure NAD(P)H stimulated O_2^{-}• generation, either 100 μmol L^{-1} NADH or 100 μmol L^{-1} NADPH (Sigma) was added to the tube following background measurements and changes in luminescence were recorded. The background luminescence was subtracted from the readings with NADH or NADPH. Values were standardized to the amount of protein present and expressed as counts $minute^{-1}$ $mg_{protein}^{-1}$.

Total activity of SOD, a major enzyme converting O_2^{-}• to H_2O_2, was measured by a spectrophotometric assay using a kit (Randox Laboratories Ltd, Crumlin UK). Approximately 100 mg ventricular tissue was homogenized in 10 volumes of ice-cold phosphate buffer (0.01 M, pH 7.0). Total SOD activity in homogenates was determined by the inhibition of formazan dye formation due to O_2^{-}• generated by xanthine and XO.

6.7. 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, 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. Briefly, 220 μL serum samples were added to 4x volume of ethanol at 4°C, vortexed and centrifuged at 3000 g for 10 min. Supernatant was evaporated under nitrogen and redissolved in 105 μL of ultra-

pure water. Samples were then incubated with nitrotyrosine acetylcholinesterase tracer in precoated (mouse antirabbit IgG) microplates followed by development with Ellman's reagent as we described previously (Onody et al., 2003a). Reagents were provided by Cayman Chemicals (Ann Arbor, MI, USA) and the detailed protocol to conduct the ELISA measurement is available at the website of Cayman Chemicals. Serum nitrotyrosine concentration is expressed as nmol L^{-1}.

6.8. RNA preparation, microarrays and probes; scanning and data analysis

Total RNA was purified from each group (25-25 mg tissue from each heart) with RNeasy Fibrous Tissue Mini Kit from Qiagen (Maryland, USA) according to the manufacturer's instructions as described (Onody et al., 2003b). The quantities and qualities of RNA from each sample were assessed by gel electrophoresis as well as spectrophotometry (NanoDrop spectrophotometer, NanoDrop, USA). Two RNA pools were prepared from each group (n=4, randomly selected from each group) and used in replica experiments. Total RNA was used for microarray analysis as well as for reverse transcription quantitative PCR.

6400 rat gene-specific, amino-modified oligonucleotides were purchased (Sigma-Operon) and were resuspended in 50% dimethyl-sulfoxide/water at a final concentration of 300 fmol L^{-1}. Oligonucleotides were arrayed from 384 well plates onto PXM oligonucleotide slides (Full Moon Biosystems, Sunnyvale, CA, USA) by using a MicroGrid Total Array System (BioRobotics, Cambridge, UK) spotter with 16 pins in a 4×4 format. The diameter of each spot was approximately 200 μm. After printing, slides were incubated in a humid chamber for 14h at 42ºC. Prior to hybridization, the slides were processed as described previously (Puskas et al., 2002a. and 2002b).

For probe preparation, 2 μg of total RNA was reverse transcribed using poly-dT and random primed Genisphere Expression Array 900MPX Detection system. All the other probe preparation steps were done according the manufacturer's instructions (Genisphere, Hatfield, USA). Each array was scanned under a green laser (543 nm for Cy3 labeling) or a red laser (633 nm for Cy5 labeling) using a ScanArray Lite (GSI Lumonics, Billerica, MA) scanning confocal fluorescent scanner with 10 μm resolution (Laser power: 85% for Cy5 and 90% for Cy3, Gain: 80% for Cy5 and 75% for Cy3; Onody et al., 2003b). Scanned output files were analyzed using the GenePix Pro 5.0 software (Axon Instruments Inc., Foster City, CA). For

each channel the median values of feature and local background pixel intensities were determined (Onody et al., 2003b; Palotas et al., 2004). The background corrected expression data was filtered for flagged spots and weak signal. Technical replicates on the same array were averaged. Normalization was performed using the print-tip LOWESS method (Yang et al., 2002). Next we used the one-sample t-test in order to determine the genes to be regarded as regulated in response to treatment. Logarithm was taken from each expression ratio to fulfill the t-test's requirement for a normal distribution. Genes for which the mean of log-ratios across the biological replicates was equal to zero at a significance level $\alpha=0.05$ are considered to have an unchanged expression. On the other hand, genes having a p-value smaller than α and the average-fold change (increase or decrease) of the four data points were at least 1.9-times were considered as regulated genes.

6.9. Real-time QRT-PCR

Real-time quantitative reverse transcription-PCR (QRT-PCR) was performed on a RotorGene 2000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers and SybrGreen protocol to confirm expression changes of 10 selected genes observed by microarrays (see for original article). An additional 11 genes strongly related to sensory neural signaling but not available on the DNA-chip were also investigated by QRT-PCR (see for original article). 2 µg of total RNA from each sample was reverse transcribed in the presence of random primers in a total volume of 20 µL. After dilution with 20 µL of water, 1 µL of the diluted reaction mix was used as template in QRT-PCR. The 20 µL reaction volume contained 0.2 mM of dNTP, 1× PCR reaction buffer (ABGene, Epsom, UK), 6 mM of each primer, 4 mM of $MgCl_2$, 1× SYBR Green I (Molecular Probes, Eugene, Oregon) at final concentration, and 0.5 units of thermostart Taq DNA polymerase (ABGene). The amplification was carried out with the following cycling parameters: 600 sec heat start at 95°C, 45 cycles of denaturation at 95°C for 25 sec, annealing at 60°C for 25 sec and fluorescence detection at 72°C for 15 sec. Relative expression ratios were normalized to β-actin. Non-template 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 (Pfaffl 2001). Using this calculation method differences between the amplification efficiencies of reactions could be corrected.

6.10. S-nitrosylation of SERCA2a

To assess SERCA S-nitrosylation, sarcoplasmic reticulum (SR) was isolated as described previously (Komuro et al., 1989; Nakanishi et al., 1984). All procedures were performed at 4°C. The left ventricles (approximately 1.5 g) were minced with scissors and homogenized three times with a polytron tissue processor for 5 s in 4x volume of the isolation solution containing 10 mM Na_2HCO_3 (pH 7.1 with 0.1 N HCl). The homogenate was centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 14,000 g for 20 min three times to remove mitochondria. The supernatant from the third spin was centrifuged at 45,000 g for 30 min. The pellet was suspended in a solution containing 0.6 M KCl and 10 mM N-tris(hydroxymethyl) methyl-2-aminomethane-sulfonic acid (TES; pH 7.1) using a glass-Teflon homogenizer. The suspension was centrifuged at 45,000 g for 30 min. The resulting pellet was suspended in solution containing 150 mM KCl and 1 mM TES (pH 7.1) and used as SR fractions.

Isolated SR fractions were resuspended in 40 µl solution containing 150 mM KCl and 1 mM TES (pH 7.1) 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 (ab2861, Abcam plc. Cambridge; UK and NISC11-A, Alpha Diagnostics Intl. Inc., San Antonio, TX; USA, respectively) for 1.5 h. Polyclonal rabbit anti-mouse IgG and polyclonal goat anti-rabbit IgG secondary antibodies (DakoCytomation Denmark A/S, Glostrup, Denmark) were used for incubation at room temperature for 1 h, as appropriate. Membranes were developed with an enhanced chemiluminescence kit (ECL Plus; GE Healthcare, Little Chalfont, Buckinghamshire, UK), 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.

6.11. Statistics

Data were expressed as mean±S.E.M. or S.D. Analysis were done by one-way ANOVA followed by Tukey's test or student t-test as appropriate; $p < 0.05$ was accepted as statistically significant difference.

7. Results

7.1 Study 1.

7.1.1. Infarct size and cardiac LDH release

In control hearts, test ischaemia/reperfusion resulted in a large infarction (Fig. 2/A) and LDH release (Fig. 2/B). 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.

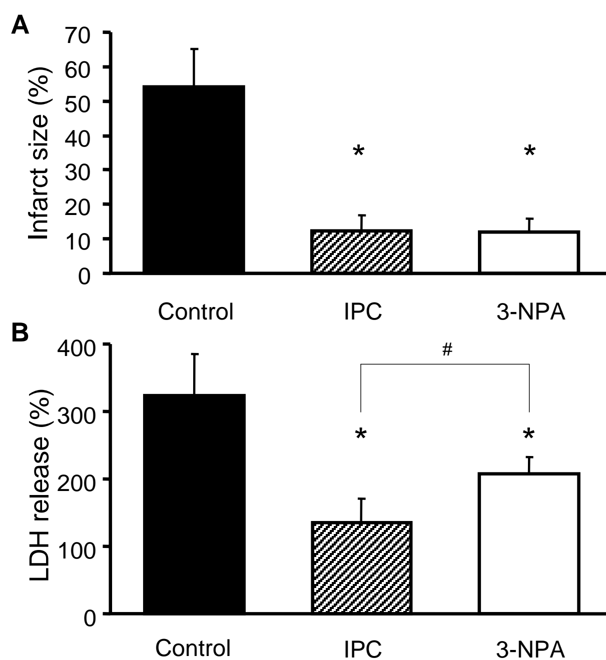

Figure 2. Infarct size (Panel A) and lactate dehydrogenase (LDH) release (Panel B) in ischaemic preconditioned, control, and 3-nitropropionic acid (20 mg/kg, i.p.; 3-NPA) treated groups after 30 min ischaemia followed by 120 min reperfusion. Values are mean±S.E.M., *p <0.05 compared to control groups; #p <0.05 between preconditioned and 3-NPA groups (n =7 in each groups).

7.1.2. NO content and cardiac NO synthase

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 (Fig. 3/A). To test enzymatic synthesis of NO in the heart, we also measured cardiac activities of NOS. Endogenous enzymatic sources of NO, Ca^{2+}-dependent and Ca^{2+}-independent NOS activities in the myocardium were not affected by 3-NPA treatment (Fig. 3/B).

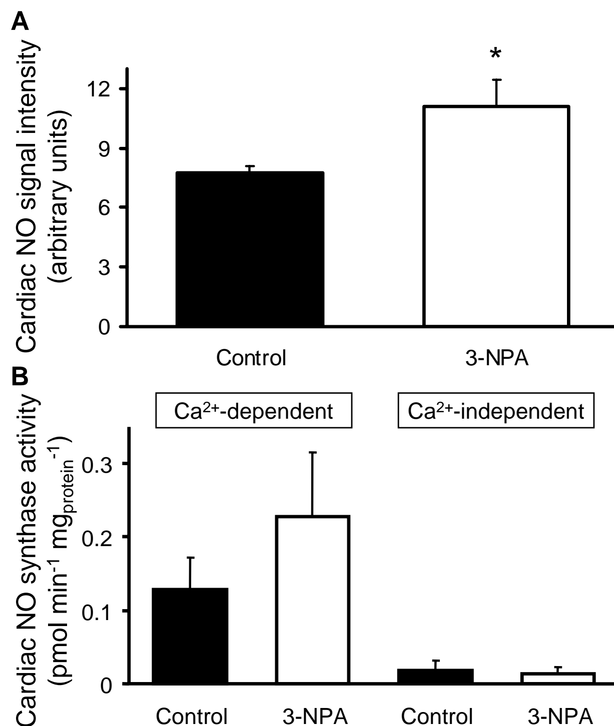

Figure 3. Cardiac nitric oxide (NO) content measured in vivo from the myocardium using electron spin resonance spectroscopy after in vivo spin trapping (Panel A, n =4 in both groups) and NO synthase activity (Panel B, n =7 in both groups) measured from isolated heart tissue after 10 min normoxic perfusion in control and 3-nitropropionic acid treated (3-NPA) groups 3 h after 3-NPA injection. Values are mean±S.E.M., *p <0.05.

7.1.3. Cardiac superoxide and XOR, NAD(P)H oxidase, and SOD activity

To test if 3-NPA treatment influences cardiac O_2^{-}• generation, we performed a lucigenin-enhanced chemiluminescence assay in freshly minced cardiac tissue. Cardiac O_2^{-}• generation was significantly decreased due to 3-NPA treatment as compared to controls (Fig. 4/A). 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 activity was significantly decreased in the 3-NPA treated group (Fig. 4/B). Other main sources of O_2^{-}• generation in the myocardium are the NADH and NADPH oxidases. NADPH oxidase activity

was minimal in the heart tissue and was not influenced by 3-NPA treatment (data not shown), however, NADH oxidase activity showed significant decrease when compared to controls (Fig. 4/C). We also assayed total activity of SOD in the myocardium, the enzyme responsible for detoxification of O_2^{-}•. SOD activity was not changed in 3-NPA-treated hearts when compared to controls (Fig. 4/D).

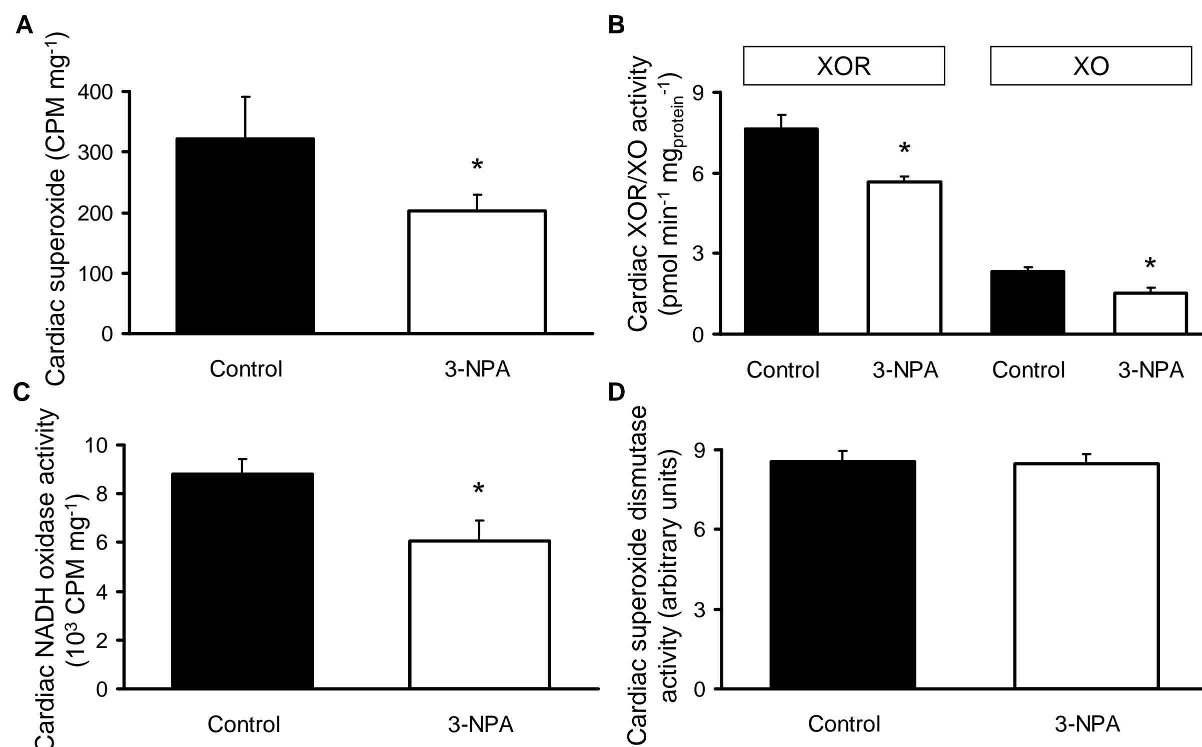

Figure 4. Cardiac O_2^{-}• content (Panel A), cardiac xanthine oxidoreductase (XOR) and xanthine oxidase (XO, Panel B), cardiac NADH oxidase (Panel C), and cardiac superoxide dismutase (SOD, Panel D) activities measured from isolated heart tissue after 10 min normoxic perfusion in control and 3-nitropropionic acid treated (3-NPA) groups. Values are mean±S.E.M., *p <0.05 (n =6 – 7 in each groups).

7.1.4. $ONOO^-$

To study the role of $ONOO^-$ in 3-NPA-induced cardioprotection serum-free nitrotyrosine concentration was measured in non-treated control and 3-NPA treated animals as a marker for systemic $ONOO^-$ formation. Plasma-free nitro-tyrosine was decreased approximately two-fold in 3-NPA treated rats as compared to controls (Fig. 5).

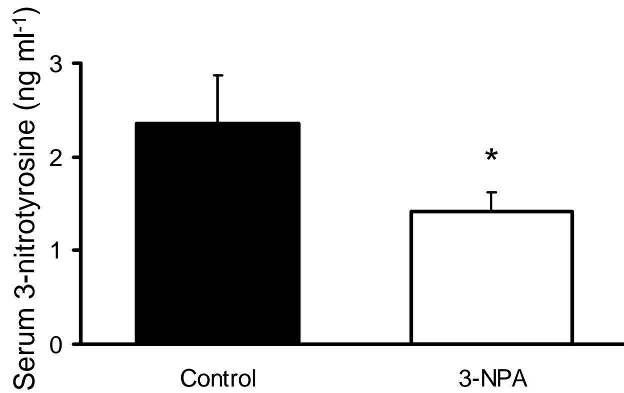

Figure 5. Serum free nitrotyrosine concentration as an indicator of peroxynitrite formation in control and 3-nitropropionic acid treated (3-NPA) groups 3 h after 3-NPA administration. Values are mean±S.E.M., *p <0.05 (n =7– 8).

7.2 Study 2.

7.2.1. CGRP and TRPV1 immunohistochemistry

As it is shown in Fig. 6/a, TRPV1-immunohistochemistry revealed many small to medium-sized and few small neurons showing moderate and very intense staining, respectively, in the C8-Th3 dorsal root ganglia, which provide the sensory innervation of the heart (Benson et al., 1999). TRPV1-immunoreactive neurons could not be demonstrated after capsaicin treatment (Fig. 6/b). However, in agreement with previous findings (Dvorakova et al., 2001; Zahner et al., 2003), we could not detect TRPV1-immunoreactive nerves in the heart control or capsaicin-treated groups using conventional immunohistochemical techniques. CGRP is the major sensory neuropeptide localized in afferent nerves, therefore, CGRP-immunohistochemistry is a reliable tool for the demonstration of the peptidergic sensory innervation of most somatic and visceral organs, including the heart (Franco-Cereceda et al., 1987; Kruger et al., 1989). Accordingly, CGRP-immunohistochemistry demonstrated many immunoreactive axons in the heart ventricles of control rats (Fig. 6/c). In agreement with previous studies, capsaicin treatment resulted in a practically complete depletion of CGRP-containing nerves from the ventricular myocardium indicating a substantial sensory denervation of the heart (Fig. 6/d) (Ferdinandy et al., 1997; Franco-Cereceda et al., 1987; Katona et al., 2004; Rankin et al., 1990).

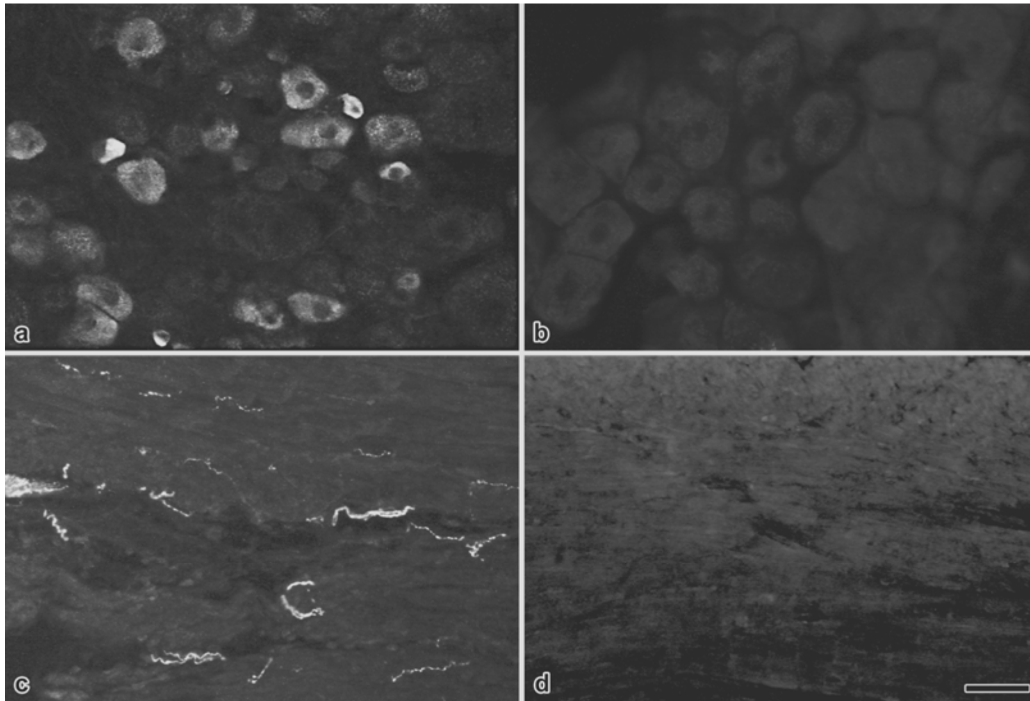

Figure 6. TRPV1-immunohistochemistry reveals many small and medium-sized immunoreactive neurons in the 2nd thoracic dorsal root ganglion of a control rat (a). TRPV1-immunoreactive spinal ganglion cells cannot be detected in the capsaicin-pretreated rat (b). CGRP-immunoreactive nerve fibres in the left ventricle of the heart of a control rat (c). Capsaicin treatment resulted in a complete depletion of CGRP-immunoreactive nerves from the heart (d). Scale bar in panel (d) indicates 50 μm and applies for all microphotographs.

7.2.2. Effects of sensory chemodenervation by capsaicin on myocardial function.

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 when hearts were isolated after systemic capsaicin treatment, while other functional parameters were not affected as compared to solvent-treated controls (Fig. 7. and Table 1.).

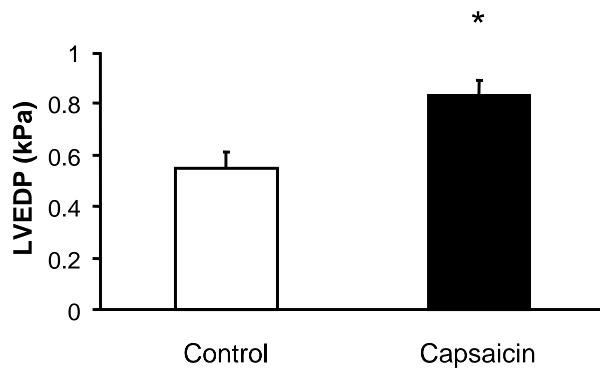

Figure 7. Effect of sensory chemodenervation by systemic capsaicin pretreatment on left ventricular end-diastolic pressure (LVEDP) in isolated rat hearts. Values are means ± S.E.M. (n = 6 in each group; *p < 0.01 vs. control.

	HR (bpm)	**CF** (mL min^{-1})	**AF** (mL min^{-1})	**LVDP** (kPa)	**+dP dt$_{max}$$^{-1}$** (kPa s^{-1})	**-dP dt$_{max}$$^{-1}$** (kPa s^{-1})
Control	278±6	22.0±1.0	44.3±2.9	18.2±0.6	904±63	472±24
Capsaicin	270±11	22.7±0.7	46.7±1.1	18.7±0.4	903±58	458±32

Table 1. Effect of systemic capsaicin pretreatment on cardiac functional parameters in isolated rat hearts (HR: heart rate; CF: coronary flow; AF: aortic flow; LVDP: left ventricular developed pressure). Values are means ± S.E.M. (n = 6 in each group).

7.2.3. Gene expression changes

Among the 6400 rat genes examined by DNA microarray in the present study an average of 3324 showed significant intensity (see Materials and methods for statistical calculations) and 2.6% (86 genes) showed significantly altered expression: 47 genes exhibited significant up-regulation (Table 2/A) and 33 were down-regulated (Table 2/B) 7 days after the end of the systemic capsaicin-treatment protocol. Genes with altered expression were from various functional clusters, such as neural function, signal transduction, protein processing, cell adhesion, gene regulation, defense response, metabolism including lipid metabolism, transport processes, etc. The gene expression changes ranged from -3.9 to +4.8-fold.

In order to confirm the differential expression of genes revealed by microarray analysis of rat hearts after capsaicin treatment, we have selected 10 genes differentially expressed in hearts due to capsaicin-pretreatment for real-time QRT-PCR analysis. The differential expression of all the 10 selected genes was confirmed by the QRT-PCR data. In case of vanilloid receptor subtype 1, apolipoprotein B, voltage-dependent calcium channel

gamma-3 subunit, farnesyl transferase, farensyl diphosphate synthase, and matrix metalloproteinase-13, the QRT-PCR showed very similar values to microarray data. However, a higher degree of change was observed in the repression of leukotriene B4 receptor 2 and cytochrome P450 subfamily 2A polypeptide 1 genes, and a lower degree of change was observed in the up-regulation of GABA receptor rho-3 subunit and kinesin-related protein 6 genes by QRT-PCR when compared to DNA microarray data (data not shown).

The effect of sensory chemodenervation by capsaicin on expression of an additional 11 genes strongly related to sensory neural signaling but were not available on the DNA-chip was also investigated by QRT-PCR. The expression of neurokinin B was significantly down-regulated by capsaicin-pretreatment, however, mRNAs of alpha calcitonin gene-related peptide, beta calcitonin gene-related peptide, and substance P were not found in control or capsaicin-pretreated groups (data not shown). Neurokinin receptor 2 mRNA was down-regulated due to capsaicin-pretreatment, but the expression of neurokinin receptor 1 was not changed significantly (data not shown). No mRNA for neurokinin receptor 3 was found in the control or capsaicin-treated groups (data not shown).

Function	*Gene Product*	*Acc. no.*	*Microarray (average-fold ± SD)*	
Neural function	vanilloid receptor subtype 1	AF029310	2.16	0.54
	vanilloid receptor-rel. osmotically act. chan.	AF263521	2.19	0.62
	transient receptor potential prot. 5	AF061876	2.06	0.55
	GABA receptor rho-3 subunit	D50671	2.63	0.82
	neural visinin-like Ca^{2+}-binding prot. 3	NM_017356	2.67	0.69
	synaptotagmin VII	U20106	2.23	0.72
	potassium channel Q4	AF249748	2.05	0.32
	phosphohippolin	AF142439	2.04	0.48
Cell adhesion	integrin alpha v subunit	S58528	2.14	0.35
	claudin-1	AF195500	3.07	1.02
Gene regulation	Jun D proto-oncogene	NM_138875	3.30	0.89
	histone deacetylase 2	AF321130	3.93	1.57
	RNA polymerase II	AB017711	2.07	0.06
	arix1 homeodomain protein	U25967	4.48	0.11
	HNF-3/fork-head homolog-7	L13207	2.12	0.56
	TRAF-interacting zinc finger prot. FLN29	AF329825	3.60	1.80
	zinc finger protein 12	U78123	2.53	0.41
	HES-related repressor protein 1	AY059382	2.05	0.24
Signal transduction	cAMP-regulated phosphoprotein	AJ005982	2.07	0.52
	SH3 domain-cont. adapter protein	AF131867	2.02	0.16
	calcium-binding prot.. vitamin D-dep.	NM_012521	2.07	0.47
	munc13-1	U24070	2.09	0.61

	putative pheromone receptor VN6	U36898	2.61	0.80
	PAPIN	AF169411	3.39	1.33
Protein processing	protein phosphatase T	X77237	2.80	0.80
	procollagen C-proteinase enhancer prot.	NM_019237	2.23	0.06
	metalloendopeptidase	M61142	2.28	0.58
	mast cell protease 1 precursor	U67915	3.66	1.69
Defense response	NK receptor KILR-1	AF082533	4.15	1.99
	37K chain of CD8 antigen	X04310	2.04	0.24
	Membrane cofactor pr. /comp. activation/	NM_019190	2.08	0.50
Lipid metabolism	apolipoprotein B	M14952	2.49	0.21
	leptin, murine homolog	NM_013076	3.13	0.76
	farnesyl transferase	NM_012847	1.98	0.34
	farensyl diphosphate synthase	NM_031840	1.97	0.20
Others	voltage-dep. calcium channel γ subunit-like	NM_139095	2.03	0.23
	MEGF4	AB011530	2.25	0.24
	clone DRNBWC04	BG671664	2.06	0.78
	high mobility group pr. 2 /chr. organization/	NM_017187	2.12	0.51
	cytochrome c oxidase VIa /energy pathway/	X12553	2.34	0.43
	kinesin-rel. pr. KRP6 /microtubule process/	AF035955	4.80	2.46
	olp4	X80671	2.63	0.75
	tropoelastin	J04035	2.01	0.39
	parathyroid hormone regulated sequence	X95087	2.13	0.43
	clone E5130-3, estrogen induced gene	S74342	2.94	0.72
	clone E572, estrogen induced gene	S74340	3.27	1.14
	nectin-1	AF091111	2.00	0.13

Table 2/A. Fourty-seven up-regulated genes in rat hearts due to chemodenervation by systemic capsaicin pretreatment.

Function	***Gene Product***	***Acc. no.***	***Microarray (average-fold ± SD)***	
Neural function	5-hydroxytryptamine 3 receptor B	AF155044	-1.95	0.34
Gene regulation	Su(var)3-9 homolog	AA858468	-2.01	0.24
	DNA polymerase beta	NM_017141	-2.41	0.25
	histone TH2B	M18045	-2.33	0.18
	NEDH islet/duodenum homeobox-1 TF	U04833	-2.17	0.47
	zinc finger protein 6	U78134	-1.98	0.45
	cDNA /RNA processing/	CA507392	-2.06	0.22
	cDNA /Pol II transcription initiation/	BQ200557	-2.82	0.16
Transport processes	voltage-dependent calcium channel gamma-3	NM_080691	-2.11	0.32
	C7-1 protein	AF035387	-2.12	0.10
	solute carrier family 16, member 10	NM_138831	-2.93	0.21
Metabolism	cytochrome P450, subfamily 2A, polypeptide 1	NM_012693	-2.03	0.21
	aldehyde dehydrogenase	J03637	-2.10	0.39
	acetyl-CoA carboxylase	AB004329	-1.97	0.36

	type II iodothyronine deiodinase	AB011068	-2.09	0.25
	cDNA /protein biosynthesis/	BQ205308	-2.50	0.28
	cDNA /oxidative phosphorylation/	BI300403	-2.00	0.36
Protein processing	convertase PC5	L14933	-1.97	0.46
	cDNA /glycoprotein catabolism/	CA509514	-2.13	0.36
Signal transduction	MIS type II receptor	U42427	-1.96	0.32
	interleukin 11 receptor, alpha chain 1	NM_139116	-1.98	0.23
	leukotriene B4 receptor 2	NM_053640	-2.70	0.33
	G protein-coupled receptor I5E	NM_138978	-2.16	0.37
	G protein coupled receptor	D38450	-1.94	0.30
Others	hyaluronan binding prot.	Z28366	-1.94	0.50
	matrix metalloprotease 13	XM_343345	-2.54	0.43
	osteoclast inhibitory lectin	NM_130402	-1.95	0.47
	growth & transformation-dependent cDNA	M17412	-3.88	0.52
	SH3-domain binding protein 5 (BTK-assoc)	NM_054011	-2.70	0.41
	WNT7A-like protein	AF481946	-1.98	0.33
	prostatic 22-kD glycoprot. /embryogenesis/	M58169	-2.10	0.29
	Interleukin 7	NM_013110	-2.56	0.22
	cyclin-dependent kinase 4	L11007	-1.94	0.13

Table 2/B. Thirty-three down-regulated genes in rat hearts due to chemodenervation by systemic capsaicin pretreatment.

7.2.4. Effects of sensory chemodenervation on cardiac NO

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 (Fig. 8/A).

To further explore cardiac NO synthesis, activities of NO synthases were measured. Ca^{2+}-dependent activity significantly decreased in the capsaicin-pretreated group, while Ca^{2+}-independent activity did not change (Fig. 8/B).

We also examined the gene expression pattern of the 3 different isoforms of NO synthase in the heart by real-time quantitative PCR. The mRNA level of endothelial nitric oxide synthase (eNOS; NOS-3) was significantly down-regulated by capsaicin-treatment, however, the expression of neuronal nitric oxide synthase (nNOS; NOS-1) and inducible nitric oxide synthase (iNOS; NOS-2), was not affected (Table 3.).

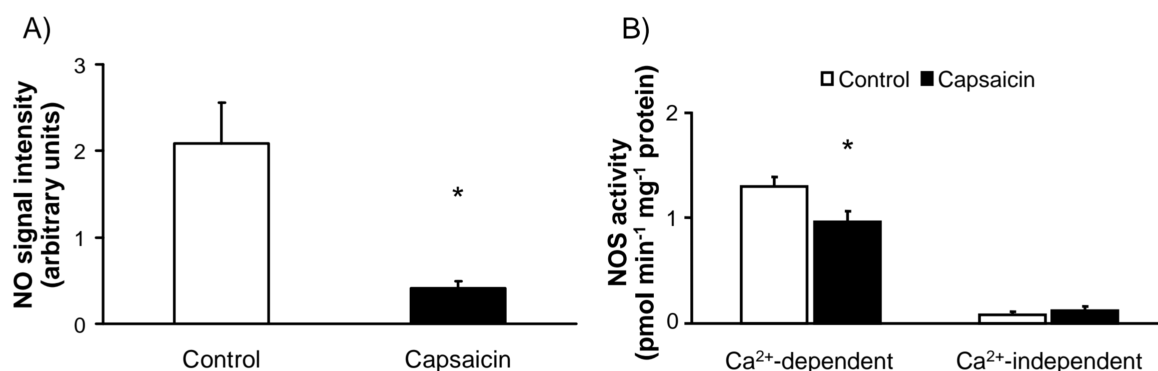

Figure 8. Panel A: NO content of left ventricular tissue samples in solvent and capsaicin pretreated groups as measured by electron spin resonance spectroscopy (Values are means ± S.E.M.; n = 6 in each group, *p < 0.01 vs control, ESR parameters: X band, 100-kHz modulation frequency, 160 K, 10-mW microwave power, 2.85-G modulation amplitude, 340-G sweep width, and 3350-G central field). Panel B: Myocardial activities of NO synthases (NOS). Values are means ± S.E.M. (n = 6 in each group, *p < 0.01 vs control).

Gene product	Access No.	Real time PCR	Forward primer	Reverse primer
endothelial NOS (NOS 3)	AF085195	-1.96 (0.19)*	TGGCAACAGCGACAATTTGA	CACCCGAAGACCAGAACCAT
inducible NOS (NOS 2)	NM012611	-0.27 (0.12)	CGGCTGCCCGGAAAA	TCGTCGGCCAGCTCTTTCT
neuronal NOS (NOS 1)	U67309	-0.68 (0.10)	CAAACCGAGGCAATCTTCGT	CCCGGCCAGCGTAGCT

Table 3. Effect of chemodenervation by systemic capsaicin pretreatment on gene expression pattern of nitric oxide synthase (NOS) isoforms measured by real-time QPCR in rat hearts. Values are mean fold changes ± S.D. (*p < 0.05) shows significant difference as compared to Control.

7.2.5. Effects of sensory chemodenervation on cardiac superoxide

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 (Fig. 9/A). We also measured the activity of xanthine oxidoreductase (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 (Fig. 9/B) and NADPH oxidase (Fig. 9/C). We also tested the total activity of SOD in the heart, the major enzyme responsible for endogenous detoxification of O_2^{-}•. SOD activity significantly increased in the capsaicin pretreated group (Fig. 9/D).

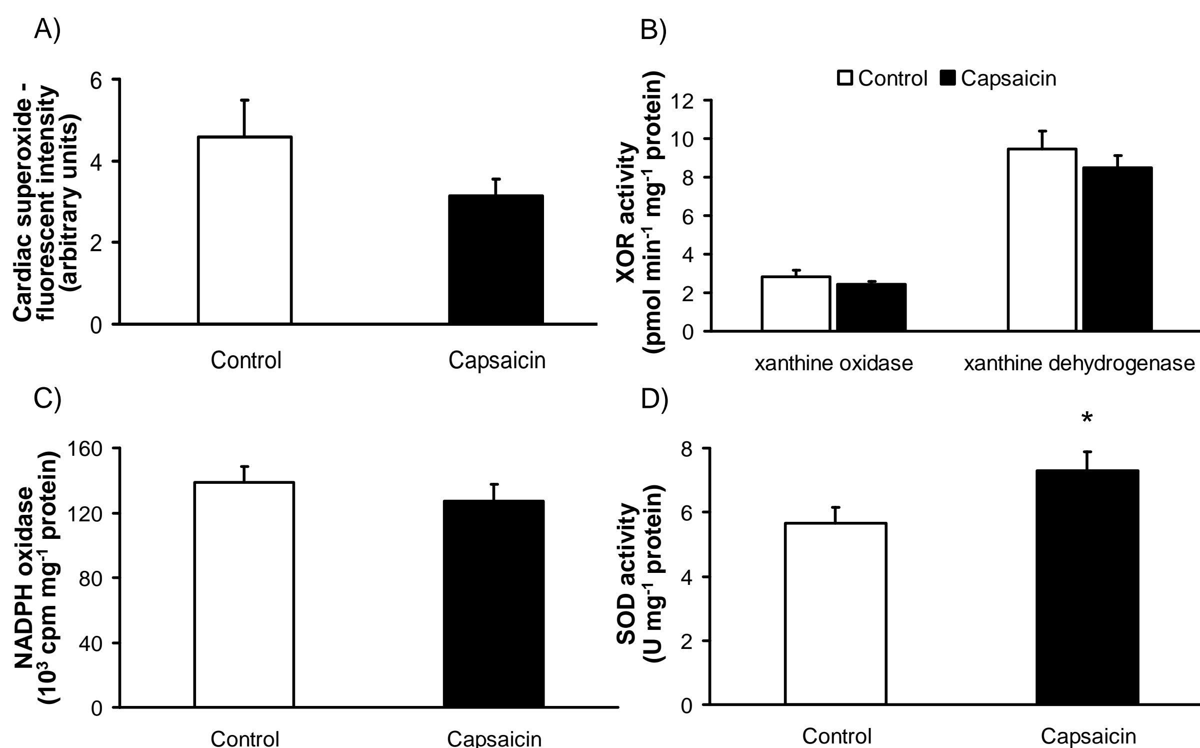

Figure 9. Effect of sensory chemodenervation by systemic capsaicin pretreatment on cardiac superoxide (Panel A), cardiac activities of xanthine oxidoreductase (XOR, Panel B:), NADPH oxidase (Panel C), and superoxide dismutase (SOD, Panel D). Values are mean ± S.E.M. (n=11 in each group; *$p<0.05$ vs control).

7.2.6. Effects of sensory chemodenervation on cardiac $ONOO^-$ and S-nitrosylation of SERCA

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 (as a marker for systemic $ONOO^-$ formation) concentration was measured. Both serum and myocardial free nitrotyrosine markedly decreased in capsaicin-pretreated groups when compared to controls (Fig. 10/A, B).

To assess, if decreased $ONOO^-$ may decrease the degree of S-nitrosylation of cardiac SERCA (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 (Fig. 10/C).

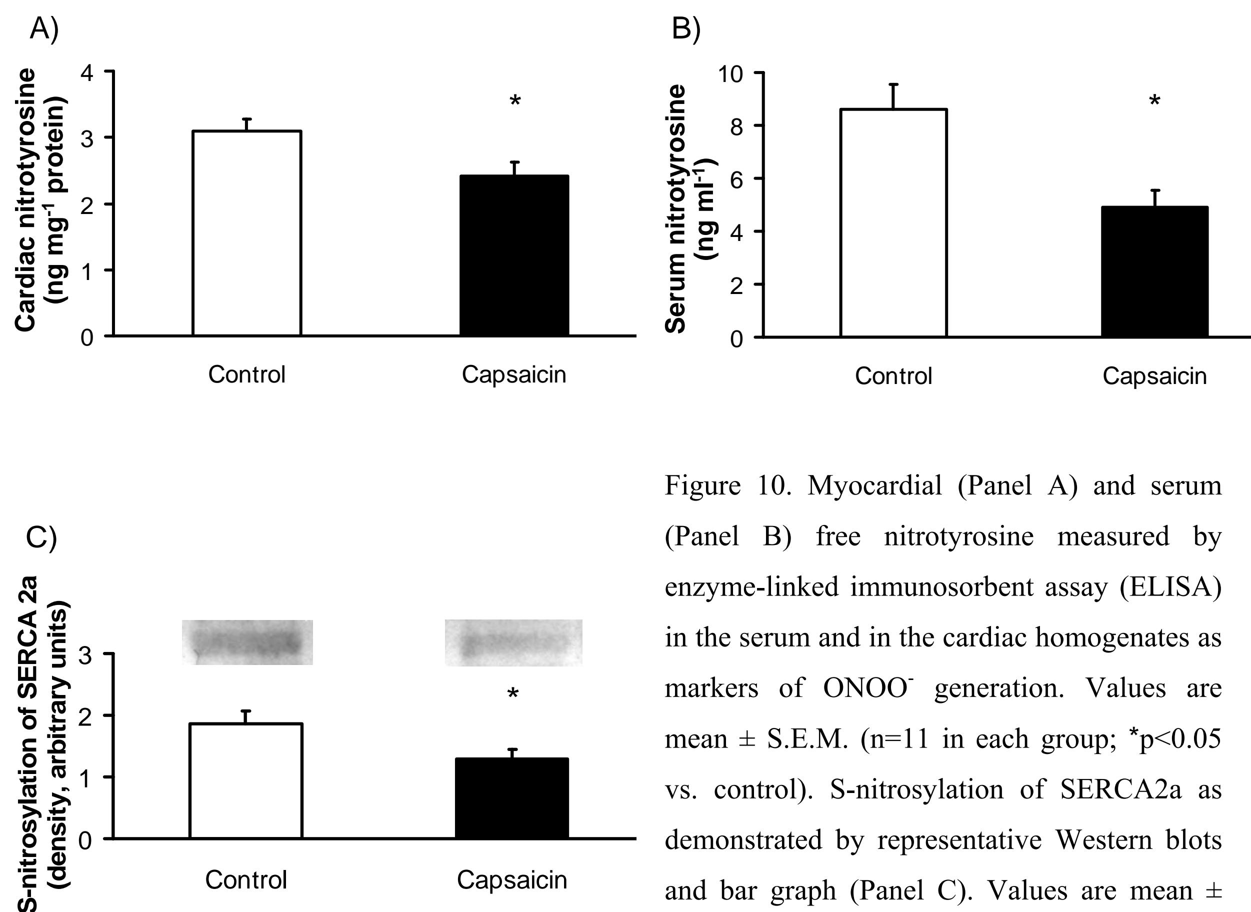

Figure 10. Myocardial (Panel A) and serum (Panel B) free nitrotyrosine measured by enzyme-linked immunosorbent assay (ELISA) in the serum and in the cardiac homogenates as markers of $ONOO^-$ generation. Values are mean ± S.E.M. (n=11 in each group; *p<0.05 vs. control). S-nitrosylation of SERCA2a as demonstrated by representative Western blots and bar graph (Panel C). Values are mean ± S.E.M. (n=9 in each group; *p<0.05 vs control.

8. Discussion

8.1. New findings:

Study 1:

Our results provide evidence for the first time in the rat heart that

(i) 3-NPA induces pharmacological preconditioning thereby limiting infarct size and that

(ii) 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.

Study 2:

(iii) 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.

(iv) sensory chemodenervation decreases cardiac NO availability via decreased expression and activity 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.

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

8.2. The role of NO and superoxide in 3-NPA induced chemical preconditioning

We have shown in *study 1* that 3-NPA pretreatment, similarly to ischaemic preconditioning, markedly reduced infarct size and LDH release in isolated rat hearts subjected to global ischaemia/reperfusion. Cardiac $O_2^{-}\bullet$ content and serum nitrotyrosine level were also decreased 3 h after 3-NPA treatment due to decreased activities of XOR and NADH oxidase, main sources of $O_2^{-}\bullet$ generation in the heart, whereas SOD activity was not changed. We have also shown that 3-NPA pretreatment increased cardiac NO content whereas activities of NOS were not changed. These results provide evidence for the first time in the rat

heart that 3-NPA induces pharmacological preconditioning thereby limiting infarct size and that this effect is associated with increased cardiac NO bioavailability and reduced $ONOO^-$ and $O_2^-\bullet$ formation via inhibition of cardiac XO and NADH oxidase activities.

In the present study 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. In agreement with our results, von Arnim et al. (von Arnim et al., 2001) described that mRNA of neuronal, endothelial, and inducible NO synthases stayed at the control level 12, 24, 72 h after preconditioning with 3-NPA (20 mg kg^{-1}) in mice hippocampal slices. 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 $O_2^-\bullet$ 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 $O_2^-\bullet$ level, and activities of XOR and NAD(P)H oxidase enzymes, major sources of $O_2^-\bullet$ in the rat heart. SOD activity, which is responsible for dismutation of $O_2^-\bullet$ was not changed, therefore, it did not influence $O_2^-\bullet$ content after 3-NPA administration. These findings are supported by Riepe et al. (Riepe et al., 1996 and 1997a), 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 (Schulz et al., 1996; Teunissen et al., 2002; Wang et al., 2001). 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.

8.3. The role of $ONOO^-$ in 3-NPA-induced chemical preconditioning

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 $O_2^-\bullet$ production explain the reduced generation of $ONOO^-$. Some groups showed that at the repeated (Teunissen et al., 2002) or high doses (Beal et al., 1995) of 3-NPA, nitrotyrosine generation could be induced in spheroid cultures from rats or in mouse brain. In this study we

measured $ONOO^-$ after 20 mg kg^{-1} single dose of 3-NPA, 3 h after its intraperitoneal injection, and found a significant decrease in serum nitrotyrosine. In our previous studies (Csonka et al., 2001), 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.

8.4. Myocardial effects of capsaicin induced sensory chemodenervation

We have found in *study 2* that systemic 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. This is the first demonstration that capsaicin-sensitive sensory neurons regulate myocardial relaxation via maintaining basal $ONOO^-$ formation and SERCA S-nitrosylation.

Focusing mostly on the well-known adrenergic and cholinergic effector innervation of the heart, cardiovascular researchers generally neglect the rich sensory innervation of the myocardium and the coronary vascular system, which may have strong influence on cardiac physiology and pathology due to its NO, CGRP, substance P, and other neurokinin content (Franco-Cereceda 1988; Sosunov et al., 1995 and 1996; Szallasi et al., 1999). For example, CGRP affects atrial contractility and relaxation (Huang et al., 1999), and induces a prolongation of the action potential (Franco-Cereceda et al., 1988). Furthermore, it has become well established, that sensory neuropathy develops in the presence of major cardiovascular risk factors, such as diabetes (Facer et al., 2007; Polydefkis et al., 2004), dyslipidemia (Hughes et al., 2004; Kassem et al., 2005), and obesity (Herman et al., 2007). Nevertheless, still very little is known about the physiological and pathological role of sensory nerves in the regulation of the cardiovascular system. Since the original brake through observation by (Jancso et al., 1977), in the late seventies capsaicin has become one of the

most important probes for investigations of sensory neural pathology as selective sensory chemodenervation by capsaicin and its analogs enabled the development of animal models for sensory neuropathy (Jancso et al., 1968; Khan et al., 2002; Szolcsanyi 2004).

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 (Csont et al., 2003; Ferdinandy et al., 1997). 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 vanilloid receptor-1 (capsaicin receptor), transient receptor potential protein, GABA receptor rho-3 subunit, 5-hydroxytryptamine 3 receptor B, neurokinin receptor 2, endothelial nitric oxide synthase, matrix metalloproteinase-13, cytochrom P450, farnesyl-transferase, ApoB, leptin, 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 and possibly in other tissues as well.

8.5. The role of $ONOO^-$ in the regulation of myocardial relaxation

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^{-}• (Ferdinandy et al., 2001 and 2003). 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 $O_2^{-}\bullet$ availability in the myocardial tissue may lead to decreased formation of $ONOO^-$, we measured markers of $ONOO^-$-induced nitration, i.e. cardiac nitrotyrosine level. Myocardial free nitrotyrosine level was significantly decreased due to capsaicin pre-treatment. This was further supported by decreased nitrotyrosine level in the serum, a marker for systemic $ONOO^-$ formation. 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 (Ferdinandy et al., 2001 and 2003), 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 (Ferdinandy 2006; Pacher et al., 2007). Nitrosylation of Cys349 has been shown to be responsible for the activation of the SERCA by $ONOO^-$ thereby maintaining the normal physiological function of SERCA in myocardial relaxation (Adachi et al., 2004; Viner et al., 1999). 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. Nevertheless, the role of other molecular targets of $ONOO^-$ could be also involved in regulation of myocardial relaxation (Viappiani et al., 2006). Recently, a total of 48 putative proteins containing nitrotyrosine were identified in whole heart homogenates by a proteomic approach (Kanski et al., 2005). Through S-nitrosylation, $ONOO^-$ can activate matrix metalloproteinases (MMP-9, MMP-2) and its resultant proteolysis of novel intracellular targets, including troponin I and myosin light chain 1, might also take part in the regulation of myocardial contractile function (Chow et al., 2007).

8.6. Limitations of the studies.

In the *study 1* cardiac NO bioavailability cannot be explained by increased NO synthesis. It should be noted that a possible increase in NO synthase activity in the heart in vivo cannot be excluded due to the limitations of the in vitro NO synthase activity assay used in our present study.

Our present results do not clarify the exact cellular mechanisms of 3-NPA-induced chemical preconditioning. Besides $ONOO^-$, ATP-sensitive potassium channels and altered mitochondrial function may play a role in the chemical preconditioning induced by 3-NPA

(Horiguchi et al., 2003; Nakagawa et al., 2002 and 2003). Furthermore, the mechanism by which 3-NPA leads to decreased activities of XOR and NADH oxidase needs further studies.

Due to its very short half life at physiological pH, endogenous formation of $ONOO^-$ cannot be directly detected in biological systems. Therefore, to estimate basal $ONOO^-$ formation, we measured the most widely accepted marker of $ONOO^-$, free nitrotyrosine, probably underestimating local $ONOO^-$ formation (Ferdinandy 2006).

Furthermore, our present study does not show a direct link between S-nitrosylation of SERCA2a and relaxation, as there is no validated method to replace physiological level of $ONOO^-$ in the intact heart (Ferdinandy 2006).

Finally, we have measured all biochemical and cardiac functional parameters 7 days after the last administration of capsaicin, when depletion of peptide containing myocardial sensory nerves as well as elimination of capsaicin were complete (Csont et al., 2003; Ferdinandy et al., 1997). However, any non-specific systemic effect of capsaicin other than depletion of sensory nerves cannot be excluded (Szallasi et al., 1999).

9. Conclusions

In conclusion, our results clearly show that chemical preconditioning with 3-NPA markedly reduces infarct size via a mechanism that may involve increased bioavailability of NO and decreased $ONOO^-$ formation due to decreased cardiac formation of O_2^-• by 3-NPA-induced inhibition of XOR and NADH oxidase activities.

Furthermore, we provided here the first evidence that 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 regulate myocardial relaxation via maintaining basal cardiac NO, O_2^-• and $ONOO^-$ formation and thereby the physiological level of SERCA2a S-nitrosylation. The possible clinical relevance of our results are substantial as it may emphasize the need for protection of sensory nerves in the presence of various risk factors for cardiovascular diseases, such as diabetes, dyslipidemia, and obesity associated with the development of sensory neuropathy.

Finally, we conclude that attenuation of pathological increase of $ONOO^-$ formation may be involved in chemically induced preconditioning. Furthermore, basal $ONOO^-$ formation seems to regulate myocardial relaxation.

10. Acknowledgements

This work was supported by grants from the Hungarian Ministry of Health (ETT 597/2006, ETT 515/2003), the Hungarian Scientific Research Fund (OTKA F 046810, T046417), and the National Office for Research and Technology (RET OMFB-00067/2005, Asboth-2005, Jedlik NKFP-A1-2006-0029).

I greatly acknowledge to Professor László Dux for providing possibility to work at the Biochemistry department.

I greatly indebted to my tutor, Professor Péter Ferdinandy for his scientific guidance encouragement and support he gave me.

I am especially thankful to Professor Gábor Jancsó, Dr. Tamás Csont and Dr. Csaba Csonka for their useful help and support all the time during my work.

I am grateful to Dr. Ágnes Zvara and Dr. László Puskás for the performance of the DNA-microarray and RT-PCR experiments.

I thank Judit Pipis for the skillful assistance, and Nilufer Turan, Ildikó Huliák, Krisztina Kupai, Dr. Anikó Görbe and Dr. Zoltán Giricz for the collaboration and for the good fellowship.

I am thankful for my wife, Dr. Ágnes Bach, for her assistance and love.

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