MYOCARDIAL STRESS ADAPTATION: ROLE OF HYPERLIPIDEMIA

Ph.D. Thesis

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1. List of Publications

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

I. **Kocsis GF**, Csont T, Varga-Orvos Z, Puskás LG, Murlasits Z, Ferdinandy P. Expression of genes related to oxidative/nitrosative stress in mouse hearts: effect of preconditioning and cholesterol diet. Medical Sci Mon. 2010; 16: BR32-9. [IF: 1.514]

II. Csont T, Bereczki E, Bencsik P, **Fodor G**, Görbe A, Zvara A, Csonka C, Puskás LG, Sántha M, Ferdinandy P. Hypercholesterolemia increases myocardial oxidative and nitrosative stress thereby leading to cardiac dysfunction in apoB-100 transgenic mice. Cardiovasc Res. 2007; 76: 100-9. [IF: 6.127]

III. **Kocsis GF**, Pipis J, Fekete V, Kovacs-Simon A, Odendaal L, Molnar E, Giricz Z, Janaky T, van Rooyen J, Csont T, Ferdinandy P. Lovastatin interferes with the infarct size-limiting effect of ischemic preconditioning and postconditioning in rat hearts. Am J Physiol Heart Circ Physiol. 2008; 294: H2406–H2409. [IF: 3.724]

IV. Faragó N, **Kocsis GF**, Fehér LZ, Csont T, Hackler L Jr, Varga-Orvos Z, Csonka C, Kelemen JZ, Ferdinandy P, Puskás LG. Gene and protein expression changes in response to normoxic perfusion in mouse hearts. J Pharmacol Toxicol Methods. 2008; 57: 145-54. [IF: -]

Cumulative IF of papers directly related to the thesis**: 11.365**

1. 2. List of full papers directly not related to the subject of the Thesis:

I. Csonka C, Kupai K, **Kocsis GF**, Novák G, Fekete V, Bencsik P, Csont T, Ferdinandy P. Measurement of myocardial infarct size in preclinical studies. J Pharmacol Toxicol Methods. 2010; (doi:10.1016/j.vascn.2010.02.014). [IF: -]

II. Lakkisto P, Csonka C, **Fodor G**, Bencsik P, Voipio-Pulkki LM, Ferdinandy P, Pulkki K. The heme oxygenase inducer hemin protects against cardiac dysfunction and ventricular fibrillation in ischaemic/reperfused rat hearts: role of connexin 43. Scand J Clin Lab Invest. 2009; 69:209-18. [IF: 1.235]

III. Turan N, Csonka C, Csont T, Giricz Z, **Fodor G**, Bencsik P, Gyöngyösi M, Cakici I, Ferdinandy P. The role of peroxynitrite in chemical preconditioning with 3-nitropropionic acid in rat hearts. Cardiovasc Res. 2006; 70: 384-90. [IF: 6.127]

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

V. Szekeres M, **Fodor G**, Fazekas A, Radnai M, Turzó K, Dékány I. Formation of octacalcium phosphate by heterogeneous nucleation on titania surface. COLLOID POLYM SCI. 2005; 283: 587-592. [IF: 1.249]

VI. Szekeres M, **Fodor G**, Radnai M, Turzó K, Dékány I, Fazekas A. Formation of crystalline calcium phosphate coating on the titanium dioxide layer of dental implants' surface. Fogorv Sz. 2002; 95: 209-14.

Total IF: 26.697

2. List of abbreviations

AAR	**A**rea **a**t **r**isk
Akt	Protein kinase B
ApoB-100	**Apo**lipoprotein type **B100**
FeTPPS	(5,10,15,20-**t**etrakis-[4-**s**ulfonato**p**henyl] **p**orphyrinato-**iron**[III])
IS	**I**nfarct **s**ize
IPost	**I**schemic **Post**conditioning
IPC	**I**schemic **Pre**conditioning
I/R	**I**schemia/**r**eperfusion
HDL	**H**igh **d**ensity **l**ipoprotein
HMG-CoA	**H**idroxy-**m**ethyl-**g**lutaryl-**Co**enzyme **A**
LDL	**L**ow **d**ensity **l**ipoprotein
MMP	**M**atrix **m**etallo**p**rotease
n-, e-, iNOS	**n**euronal-, **e**ndothelial-, **i**nducible-**N**itric **o**xide **s**ynthase (isoenzymes)
Phox 4	Nicotinamide adenine dinucleotide **ph**osphate (NADPH) **ox**idase subunit 4
p42/p44, MAPK, ERK1/2	**M**itogen **a**ctivated **p**rotein **k**inase
qRT-PCR	**q**uantitative **R**eal-**T**ime **P**olymerase **C**hain **R**eaction
SOD	**S**uper**o**xide **d**ismutase
TIMP	**T**issue **i**nhibitor of **m**etallo**p**roteases
VLDL	**V**ery **l**ow **d**ensity **l**ipoprotein
WT	**W**ild**t**ype
XOR	**X**antine **o**xido**r**eductase

3. Summary

In developed societies cholesterol-enriched and fatty diet together with the lack of exercise and accumulating environmental stress leads to the development of atherosclerosis and consequent coronary heart disease and often lead to a lethal ischemic episode. Short periods of ischemia applied before or immediately after a sustained period of ischemia are very important adaptive mechanisms of the healthy heart that protect the heart against the consequences of a lethal ischemia. These phenomena, termed ischemic preconditioning (IPC) and ischemic postconditioning (IPost), have been demonstrated to be abolished by hyperlipidemia. In this thesis our aim was to collect further knowledge to help the understanding of the role of hyperlipidemia in myocardial stress adaptation.

Using DNA microarray analysis our group has previously shown that hyperlipidemia alters cardiac gene expression profile in the rat. Similarly, in a separate study, IPC was demonstrated to modify I/R-induced gene expression pattern. In our first study we looked at the effects of cholesterol-enriched diet on I/R- and IPC-induced cardiac gene expression pattern in the mouse heart focusing on genes involved in NO and free radical signaling and in the mevalonate pathway. We found differential alterations in cholesterol-fed mice on I/R-, or IPC-induced gene expression response. These results suggest that alterations in cardiac gene expression pattern in response to cholesterol-enriched diet may contribute to the loss of IPC effect in hyperlipidemia.

Our group has previously shown that hyperlipidemia-induced oxidative/nitrosative stress leads to moderate contractile dysfunction. Therefore, in an apolipoproteinB-100 (apoB-100) transgenic mouse model we have investigated the mechanism of hyperlipidemia-induced oxidative/nitrosative stress and tested if high serum cholesterol or high serum triglyceride leads to the deterioration of myocardial function. We have found that hypercholesterolemia but not hypertriglyceridemia is responsible for the hyperlipidemia-induced, peroxynitrite-mediated cardiac dysfunction in human apoB-100 transgenic mice and that increased cardiac NADPH oxidase activity is a major source of increased oxidative stress in hypercholesterolemia.

In the treatment of hyperlipidemia and in the prevention of atherosclerosis-induced ischemic heart disease cholesterol lowering statins are widely prescribed drugs. In previous studies of our group we have shown that cholesterol-enriched diet blunts IPC

but it can be recaptured with farnesol, an intermedier in endogenous cholesterol biosynthesis. Since statins inhibit this pathway, we hypothesized that statins have some rude effects on endogenous cardiac adaptation. We have shown that acute lovastatin treatment abolished the infarct size-limiting effect of IPC and that chronic lovastatin treatment attenuated the protective effect of IPost. We have also demonstrated that both chronic and acute lovastatin treatment decreased myocardial coenzyme Q9 level and attenuated phosphorylation of Akt. However, phosphorylation of p42 MAPK/ERK was increased only by acute lovastatin treatment and remained unaffected by chronic lovastatin treatment. The interaction of statins with cardiac adaptive mechanisms warrants attention in some clinical situations.

4. Introduction

4. 1. Ischemia/reperfusion and cardiac adaptation

4. 1. 1. Ischemia/reperfusion injury

In the last few decades hyperlipidemia and especially hypercholesterolemia became a very frequent clinical sign of inappropriate lifestyle including “western type” diet, lack of exercise, stressful environment, etc. in developed countries [1]. Hyperlipidemia serves as a risk for the development of atherosclerosis. The complete thrombotic occlusion of a coronary artery at the site of a ruptured atherosclerotic plaque, heralds the onset of an acute myocardial ischemia, a major manifestation of coronary heart disease, the most prevalent health problem in the world, and a major cause of morbidity and mortality. Myocardial ischemia is defined as an imbalance between fractional uptake of oxygen and the rate of cellular oxidation in the heart [2]. Ischemia/reperfusion (I/R) cascade further include various events such as reperfusion arrhythmias, depressed contractile function, micro-vascular damage, and decreased coronary flow. Both myocardial stunning and infarction are seen [3]. During ischemic conditions many biochemical changes happen including various cellular events like altered membrane potential, altered ion distribution, cellular swelling and cytoskeleton disorganization [2]. Ischemia due to anaerobic metabolism leads to catabolism of adenine nucleotides, which results in depletion of adenosine triphosphate (ATP) and accumulation of hypoxanthine within cells [2]. While timely reperfusion of acute ischemic myocardium is essential for myocardial salvage, reperfusion results in a unique form of myocardial damage termed reperfusion injury. With the prolongation of ischemia or restoration of the coronary flow, alterations in ions and overall Ca^{2+} homeostasis occur, together with an oxidative/nitrosative stress mediated by oxygen free radicals (reactive oxygen species) and reactive nitrogen species, which are not adequately counteracted by the cellular antioxidant defenses. In this process oxidative/nitrosative stress mediated DNA damage occurs leading to the activation of the nuclear enzyme poly(ADP-ribose) polymerase, which initiates an important pathway of cell dysfunction and tissue injury during myocardial infarction [4]. Over the last two decades, it has become increasingly clear that the oxidative/nitrosative stress serves as a critical central mechanism of post-ischemic injury [3].

4. 1. 2. Ischemic preconditioning and it's molecular mechanisms

The heart is known to possess a remarkable ability to adapt to ischemia/reperfusion (I/R) stress. The phenomenon in which brief periods of ischemia protect the heart against subsequent lethal I/R injury termed ischemic preconditioning (IPC) [5, 2]. The protective effects of IPC are seen as a reduction of a myocardial infarct size [5], decrease in the incidence of ventricular arrhythmias and in improvement of functional recovery during reperfusion [6]. This powerful cardioprotective effect appears to be a ubiquitous endogenous response having been reproduced in all species tested including humans and a variety of organs other than the heart including the kidney, liver, and brain [7]. The brief episodes of I/R which constitute the IPC stimulus elicit two distinct windows of cardioprotection: the first window of protection or classical IPC manifests immediately and wanes after 2–3 h and is replaced by a second window of protection which appears 12–24 h later and lasts 2–3 days [8]. The mechanistic pathways underlying these endogenous cardioprotective phenomena are not entirely clear, complex in nature and have conventionally been divided into trigger factors, mediators and effectors [9]. IPC recruit signaling pathways comprising cell surface receptors to numerous autacoids (such as adenosine, bradykinin and opioids), signaling kinases (phosphatidylinositol-3-kinase-Akt-eNOS, mitogen activated protein kinase (ERK1/2, p42/p44 MAPK), protein kinase C, and protein kinase G) and mitochondrial components (the mitochondrial ATP sensitive potassium channels, the mitochondrial permeability transition pore, protein kinase C, signaling reactive oxygen species) prior to the index myocardial ischemic episode. The oxidant formation is generated through a series of interacting pathways in cardiac myocytes and endothelial cells. Nitric oxide (NO) production and NO levels are also greatly increased in ischemic and post-ischemic myocardium, and this occurs through NO synthase (NOS)-dependent NO formation and NOS-independent nitrite reduction. Recently, it has been shown that the pathways of oxygen radical and NO generation interact and modulate each other. Under conditions of oxidant stress, NOS may switch from NO to oxygen radical generation. Under ischemic conditions, xanthine oxidase may reduce nitrite to generate NO. NO and peroxynitrite may inhibit pathways of oxygen radical generation, and, in turn, oxidants can inhibit NO synthesis from NOS. IPC markedly decreases NO and oxidant generation, and this appears to be an important mechanism contributing to IPC-induced myocardial protection [10-12, 15],

however, small amount of NO and oxidant generation is necessary for IPC to elicit its trigger mechanisms [6, 16]. The MAPK family members such as 42/44 kDa ERK 1/2 have been reported to act as additional mediators of cardioprotection conferred by IPC [17, 18]. Several of the above-mentioned mediators finally induce myocardial protection by opening mitochondrial ATP sensitive potassium channels [13, 19], blocking mitochondrial permeability transition pore [20], upregulating heat shock proteins [21], activating antioxidant defense system [22], improving myocardial energy balance [23], inhibiting both the activation of caspase-3 and cleavage of nuclear enzyme poly(ADP-ribose) polymerase, general markers of apoptosis [24], and inhibiting the release of proapoptotic substances [25], which are collectively known as end-effectors in IPC-induced cardioprotection (Fig. 1.). The potent means of mimicking IPC-mediated myocardial protection by pharmacological agents is referred to as pharmacological preconditioning [26]. Because both classical IPC and second window of protection require an intervention which can be implemented before the onset of the index myocardial ischemia, their clinical application has been largely restricted to specific scenarios such as cardiac surgery, in which the ischemic insult can be anticipated. However, the recent discovery of ischemic postconditioning has provided an intervention which can be applied after the onset of the index myocardial ischemia at the time of reperfusion, facilitating its application to patients presenting with an acute myocardial infarction [9].

4. 1. 3. Ischemic postconditioning and it's molecular mechanisms

Studies published in the mid-1980s first established that ischemic myocardial injury could be reduced, if the myocardial reperfusion process was modified to a staged or gradual form of myocardial reperfusion [27, 28]. Zhao et al. have reported first that brief episodes of coronary occlusion and reperfusion at the onset of reperfusion after sustained ischemic insult conferred cardioprotection against I/R injury by means of limiting infarct size. This phenomenon is known as ischemic postconditioning (IPost) [29]. However, the term IPost was first described by Na et al. who reported that ventricular premature beat-driven intermittent restoration of coronary blood flow reduced the incidence of reperfusion-induced ventricular fibrillation in a cat model of regional ischemia [30].

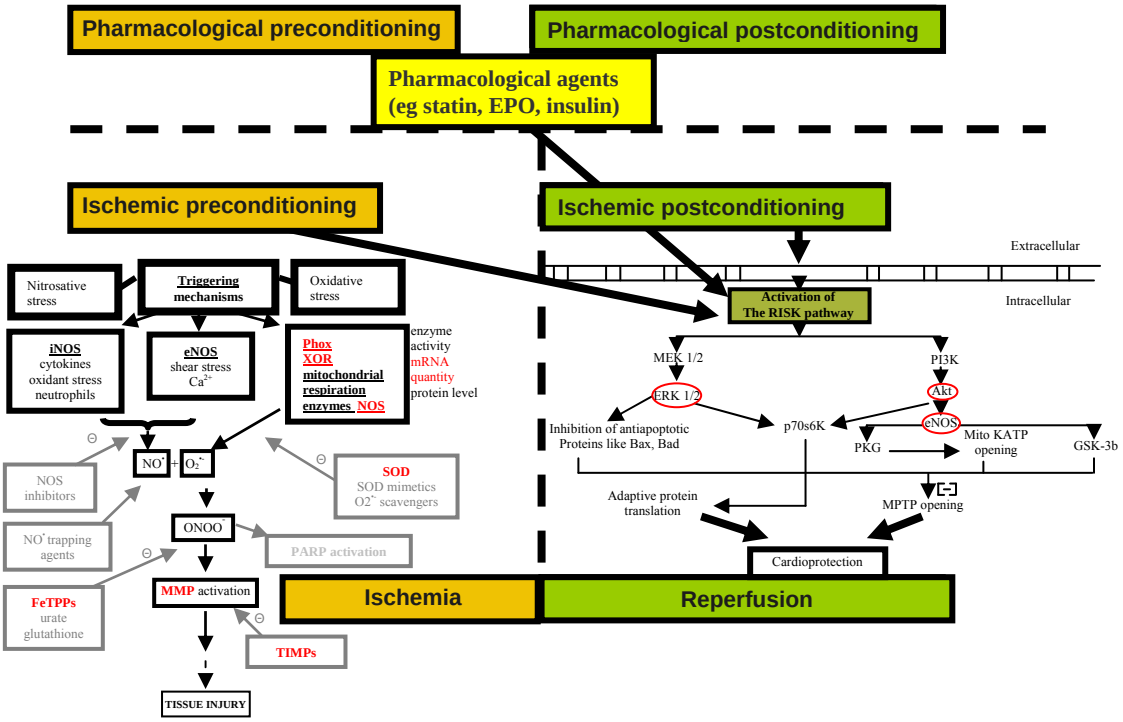

Figure 1. : Proposed key cellular and molecular events in IPC and IPost

(RISK pathway: reperfusion induced salvage kinase pathway, PI3K: phosphatidylinositol-3-kinase, GSK-3b: glycogen synthase-3 beta, MEK: mitogen activated protein kinase kinase)

The mechanisms involved in IPost-mediated cardioprotection have not been fully understood. Several mechanisms have been proposed in IPost-induced myocardial protection against I/R injury (Fig. 1.). IPost has been noted to attenuate the generation of reactive oxygen species, mitochondrial calcium overload and inflammation and improved endothelial function [31-32]. Moreover, IPost has been shown to activate prosurvival kinases such as phosphatidylinositol-3-kinase and Akt in conjunction with reperfusion-induced salvage kinase pathway (Fig. 1.). Reperfusion-induced salvage kinase pathway includes various signaling systems like phosphatidylinositol-3-kinases/Akt, glycogen synthase-3 beta and mitogen activated protein kinase kinase1/2-ERK1/2, which are activated at the time of myocardial reperfusion in both IPC and IPost-mediated cardioprotection [32-33]. Mitochondrial ROS play a paradoxical role in IPost hearts in which ROS is generated during reperfusion of ischemic myocardium and IPost has been experimentally proved to attenuate ROS generation at the time of myocardial reperfusion [34]. Consequent studies have reported the abrogation of reactive oxygen species and reactive nitrogen species like peroxynitrite [35] during myocardial IPost-induced cardioprotection. During the

reperfusion phase of cycles of I/R, the reintroduction of oxygen leads to generation of small amount of ROS that is found to trigger various signaling pathways leading to activation of survival kinases that finally inhibit mitochondrial permeability transition pore opening in order to confer IPost-mediated cardioprotection and this notorious involvement of ROS in mediating cardioprotection is referred as redox signaling [36]. IPost opens mitochondrial ATP sensitive potassium channels, activates eNOS and thus releases (NO) [2]. ATP sensitive potassium channels are potentially act as end-effectors in IPost-mediated cardioprotection [2] (Fig. 1.).

4. 2. Endogenous adaptation influenced by risk factors

Coronary heart disease develops as a consequence of a number of systemic diseases, including systemic arterial hypertension and related left ventricular hypertrophy, hyperlipidemia and atherosclerosis, diabetes and insulin resistance, heart failure, as well as aging. Accumulating evidence shows that both forms of endogenous cardiac adaptations can be modified by certain risk factors [37, 38].

4. 2. 1. The effect of high-cholesterol diet on ischemic preconditioning

Atherosclerotic cardiovascular disease, the major cause of death in Western society, results from complex interactions among multiple genetic and environmental factors. Our group and others have demonstrated that hyperlipidemia induced by a cholesterol-enriched diet attenuates the cardioprotective effect of IPC in rat hearts [39-41]. More importantly, the loss of IPC was also shown in hyperlipidemic/hypercholesterolemic patients [42-43]. Moreover, pacing-induced PC, NO donor-induced late PC and IPost were also impaired in the myocardium of rabbits on cholesterol-enriched diet [44-46]. Hyperlipidemia also leads to a moderate contractile dysfunction characterized by an elevated left ventricular end-diastolic pressure, and also leads to enhanced peroxynitrite formation in rat hearts [47]. Thus, overwhelming evidence supports the negative impact of high-cholesterol diet on IPC in the myocardium [37-38]. Interestingly, these effects of cholesterol seem to be independent of its atherosclerotic action [38, 40, 45, and 48]. A few studies have attempted to explore the molecular mechanisms by which high cholesterol diet inhibits the endogenous adaptive capacity of the myocardium, but the exact factors responsible for this effect remain obscure. A variety of mechanisms, i.e. inhibition of the mevalonate pathway [48], decrease of NO bioavailability and increase of free

radical and peroxynitrite formation [40, 47] have been proposed to play a role in the cardiac effects of hyperlipidemia. Increased superoxide formation, when combined with NO at a diffusion limited reaction rate, leads to elevated peroxynitrite levels in the cells [49]. Peroxynitrite then may activate matrix metalloproteinases (MMPs), contributing to I/R-induced cardiac damage and to the attenuation of IPC by high-cholesterol diet [41, 48]. Furthermore, it has been shown that hyperlipidemia disrupts the NO signaling pathway downstream of NO generation [46]. However, the traditional biochemical and pharmacological approaches have been insufficient to explain the key cellular events in the heart of hyperlipidemic subjects.

4. 2. 2. Ischemic preconditioning as well as hyperlipidemia modifies gene expression pattern of rat hearts

Recent studies have examined gene expression changes in atherosclerotic plaques in human and animal blood vessel samples [50]. Using DNA microarray analysis verified by quantitative real-time polymerase chain reaction our group has shown for the first time that hyperlipidemia [51] altered gene expression pattern of the rat heart. Similarly in separate study our group has shown with DNA microarray that IPC [52] modifies I/R-induced gene expression pattern in the rat heart. On the other hand, there is no information available regarding the influence of a high-cholesterol diet on the regulation of genes involved in NO and free radical signaling in the myocardium in response to IPC. In addition, expression of genes that participate in the mevalonate pathway may also be altered as a result of cholesterol-enriched diet, I/R and IPC. Therefore, in our first study we have monitored transcript levels in the mouse heart to gain insight into the regulation of I/R injury and IPC in the myocardium in mice on cholesterol-enriched diet.

4. 2. 3. Hypercholesterolemia and hypertriglyceridemia: separate risk factors of coronary heart disease

The focus of research so far has been mainly on the coronary effects of cholesterol, i.e. coronary sclerosis, and the possible direct effect of hypercholesterolemia on the heart was neglected. Very few studies looked at the cellular effects of cholesterol-enriched diet on the myocardium, however, intracellular lipid accumulation in cardiomyocytes and several alterations in the structural and functional properties of the myocardium have been observed [53, 54]. Our group has shown that cholesterol-

enriched diet-induced hyperlipidemia leads to an increase in cardiac peroxynitrite formation and a decrease in the bioavailability of NO which contributes to the deterioration of cardiac performance [47]. Further studies are required to explore whether hypercholesterolemia or hypertriglyceridemia is responsible for the oxidative/nitrosative stress mediated detrimental effect of experimental hyperlipidemia [47]. The link between elevated cholesterol and coronary heart disease has been clearly established and the National Cholesterol Education Program clinical guidelines for the treatment of hypercholesterolemia identify low density lipoprotein (LDL) cholesterol as the primary treatment target [55]. Nevertheless, recent research activities identify novel and independent risk factors for coronary heart disease. Hypertriglyceridemia increase the risk of acute coronary events, and some clinical trials found high serum triglycerides to be an independent risk factor for cardiovascular disease [56-57].

4. 2. 4. Transgenic mouse models of hyperlipidemia

In rodents the serum lipid profile in rodents is very different from that of humans. These rodents carry most of the cholesterol on high density lipoprotein (HDL) and, since they have low LDL cholesterol levels, they are protected against hypercholesterolemia and do not develop atherosclerosis without dietary or genetic manipulations [58]. In 1992 the first line of gene targeted animal models, namely apolipoprotein E - knockout mice was developed. This model develops atherosclerotic lesions on laboratory chow diet. The LDL receptor - deficient model has elevated LDL levels, but no lesions, or only very small lesions form on the chow diet, however, robust lesions do form on western type diet [59]. Transgenic mice expressing human apolipoprotein B-100 (apoB-100) showed slightly elevated total cholesterol and markedly elevated triglycerides when compared to wild type (WT) mice fed normal diet [60]. The human apoB transgene leads to the production of the full-length apoB-100 protein [59]. The lipoprotein profile of apoB-100 transgenic mice, especially when fed a high-fat diet is similar to that seen in humans, i.e. higher proportions of VLDL and LDL fractions compared to HDL [60-62]. Serum triglycerides are elevated in these mice, however, serum triglycerides returns to normal level in response to high fat diet [60]. As we discussed previously studies are required to explore whether hypercholesterolemia or hypertriglyceridemia is

responsible for the detrimental effect of high cholesterol diet. Thus, these mice may provide a possibility to investigate the different effect of hypercholesterolemia and hypertriglyceridemia on cardiac oxidative/nitrosative stress and contractile function.

4. 3. Cholesterol lowering drugs and cardioprotective adaptations

Lipid-lowering using the HMG-CoA reductase inhibitor statin therapy forms the cornerstone of medical therapy in the primary and secondary prevention of cardiovascular disease. Statins inhibit the synthesis of mevalonate, the rate-limiting step in endogenous cholesterol biosynthesis (Fig. 2.).

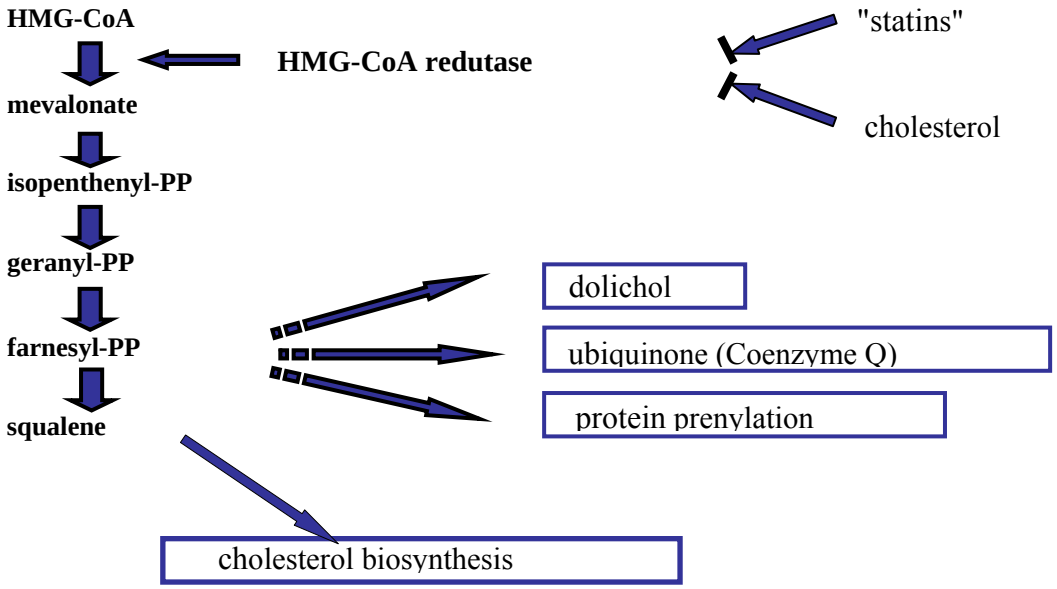

Figure 2.: The mevalonate pathway

In addition, to the improvements in lipid profile and reduction in cardiovascular morbidity and mortality [63-64], the beneficial effects elicited by this class of drugs may be attributed to their diverse variety of non-lipid lowering pleiotropic effects, including improved endothelial function, reduced oxidative stress, less platelet adhesion, and increased atherosclerotic plaque stability [65-66]. A less appreciated effect of statin therapy that has been reported in experimental studies is its cardioprotective effect with respect to its ability to directly protect the myocardium from the detrimental effects of acute ischemia-reperfusion injury [67]. On the other hand, it is well known that chronic treatment with statins may exhibit a number of extrahepatic adverse effects, such as myopathy and rhabdomyolysis [68]; however, the mechanisms of these side effects are not entirely clear. Therefore, the possible interactions of statins with adaptive cardioprotective mechanisms are of great importance. Some experimental studies have reported infarct-limitation with statin therapy administered at the onset of myocardial

reperfusion [67]. The mechanism through which elements of the reperfusion injury salvage kinase pathway are actually activated by statin therapy is, however, unclear. Another study from Hausenloy's group suggested that the direct cardioprotective effect elicited by statin therapy was due to the activation of the reperfusion injury salvage kinase pathway, the term given to a group of pro-survival kinases, the activation of which at the onset of myocardial reperfusion confers powerful cardioprotection in the animal heart [69]. In that study, atorvastatin administered at the onset of myocardial reperfusion reduced myocardial infarct size in isolated perfused murine hearts. The study demonstrated that the protective effect was mediated through the activation of the phosphatidylinositol-3-kinase-Akt-eNOS signal transduction pathway, given that the infarct-limiting effects were abrogated in the presence of a phosphatidylinositol-3-kinase inhibitor and were lost in mice lacking eNOS [67]. Subsequent studies have also implicated the recruitment of the reperfusion injury salvage kinase pathway in cardioprotection obtained with statin pretreatment of hearts [70, 71].

5. Aims

We have aimed to increase our knowledge on myocardial stress adaptation, focusing on further understanding the role of hyperlipidemia in this phenomenon.

Our aim was in study I to explore the effect of high-cholesterol diet on I/R- and IPC-induced cardiac gene expression pattern in mouse hearts, focusing on genes involved in NO and free radical signaling and the mevalonate pathway.

In study II we aimed to study the mechanism of hyperlipidemia-induced oxidative/nitrosative stress in a transgenic mouse model and to test if high serum cholesterol or high serum triglyceride leads to deterioration of myocardial function. Therefore we used human apoB-100 transgenic mice fed normal chow as a hypertriglyceridemia model and human apoB-100 transgenic mice fed cholesterol-enriched diet as a hypercholesterolemia model.

In study III we also aimed to investigate if acute or chronic applications of the cholesterol lowering statins interact with the adaptive mechanisms of IPC and IPost.

6. Materials and Methods

6. 1. Experimental design

6. 1. 1. Study 1

In order to explore the effect of high-cholesterol diet on I/R- and IPC-induced cardiac gene expression pattern, male CFLP mice were fed laboratory chow enriched with 2% cholesterol or standard chow for 8 weeks. At the end of the 8-week diet period, body weights of the animals were 35–45 g, and there was no significant difference between groups. Hearts from normal and cholesterol fed mice were then isolated and perfused in Langendorff mode with oxygenated, normothermic Krebs–Henseleit buffer. Three different perfusion protocols were applied (n = 6–8 in each group) (Fig 3.). Hearts were subjected to either an IPC or a non-IPC protocol followed by test I/R. After 5 min equilibration, IPC was induced by three intermittent cycles of 5 min no-flow ischemia, separated by 5 min aerobic perfusion.

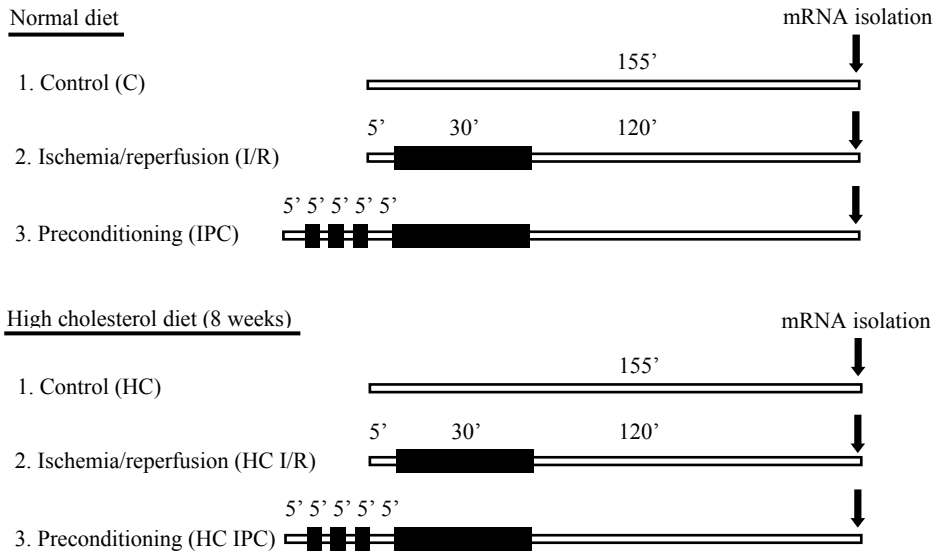

Figure 3. Experimental protocol. Aerobic perfusion (open line), global ischemia (black bars).

Non-preconditioned and preconditioned hearts were then subjected to 30 min global no-flow ischemia followed by 120 min reperfusion. A time-matched control group was aerobically perfused for 155 min. Heart rate and coronary flow were monitored throughout the perfusion protocol in all groups. At the end of the perfusion protocols,

the ventricles from each group were cut off and freeze-clamped, crushed and powdered at the temperature of liquid nitrogen and were stored at -80^{o}C until RNA preparation.

6. 1. 2. Study 2

To study the mechanism of hyperlipidemia-induced oxidative/nitrosative stress in apoB-100 transgenic mouse model and to test if high serum cholesterol or high serum triglyceride leads to the deterioration of myocardial function WT and apoB-100 transgenic mice were fed either a laboratory chow enriched with 2% cholesterol or a standard chow for 17–19 weeks. At the end of the diet period, hearts were isolated for measurement of cardiac function and biochemical markers of oxidative/nitrosative stress. Blood samples were collected from separate animals after 30 min fasting and stored at − 70 °C until assayed for serum lipids and malondialdehyde.

In order to check if peroxynitrite causes cellular damage and myocardial dysfunction due to hypercholesterolemia, separate groups of transgenic mice fed cholesterol-enriched or normal diet were injected twice intraperitoneally with 20 mg/kg FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]), a peroxynitrite decomposition catalyst, 24 h and 1 h prior to isolation of the hearts [47].

6. 1. 3. Study 3

In order to explore the effect of the cholesterol-lowering statins on the adaptive mechanisms of IPC and IPost and on the activation of the main pro-survival kinases and cardiac coenzyme Q9 level, male Wistar rats were randomly assigned to three groups (Fig.4.): i) vehicle-treated group was treated with 1% methylcellulose *per os* for 12 days followed by ex vivo drug-free perfusion protocols, ii) chronic lovastatin-treated group was given *per os* 15 mg/kg/day lovastatin dispersed in 1% methylcellulose for 12 days followed by ex vivo drug-free perfusion protocols and iii) acute lovastatin-treated group was treated with 1% methylcellulose *per os* for 12 days followed by ex vivo perfusion protocol in the presence of 50 μmol/L lovastatin. The doses of lovastatin have been selected according to our and other's previous literature data [72]. After the 12 day per os treatments, rats were anesthetized with diethyl ether, heparin (500 U/kg i.v.) was given, and the hearts were isolated and perfused at 37°C in Langendorff mode with oxygenated Krebs-Henseleit buffer. Hearts from the 3

groups were further subdivided to 3 subgroups and subjected to either a non-conditioning, IPC, or IPost perfusion protocol, respectively (Fig.4.).

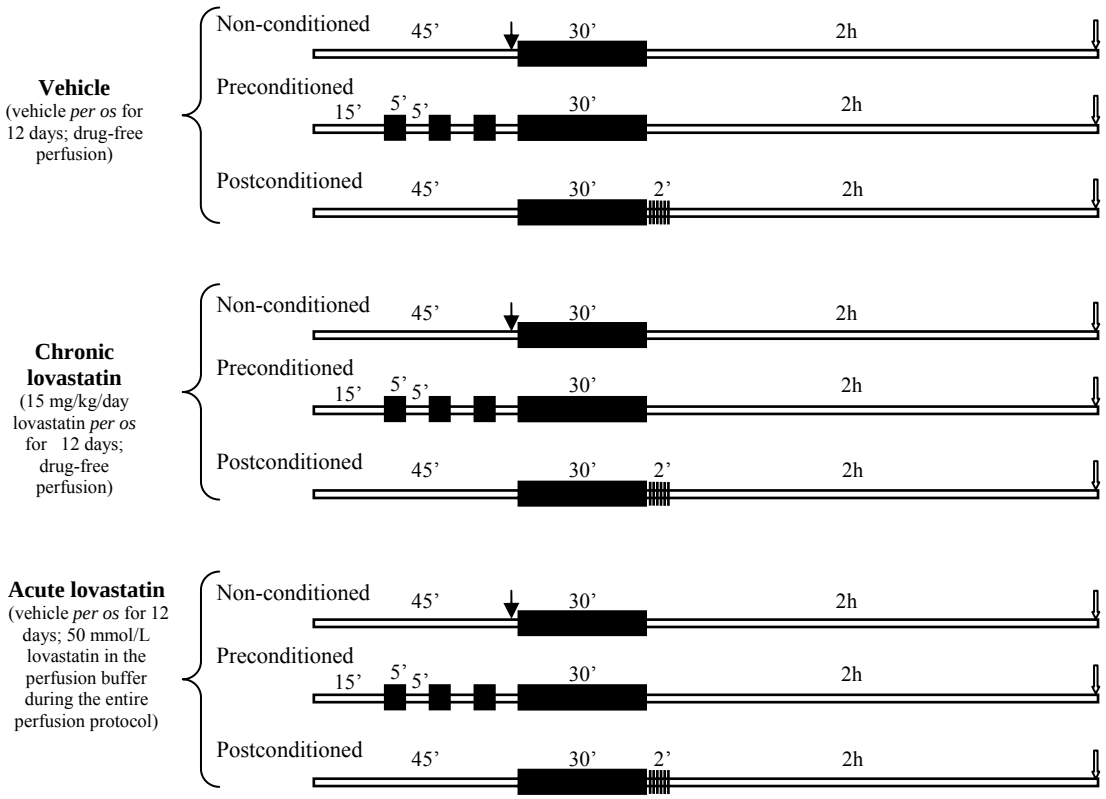

Figure 4. Experimental protocol. Aerobic perfusion (open line), coronary occlusion (black bars). Black arrow: time of sample collection for biochemical measurements. Open arrow: time of infarct size measurement.

In all groups test I/R was achieved by 30 min occlusion of the left coronary artery. Non-conditioned hearts were subjected to time-matched aerobic perfusion followed by test-I/R followed by a 2h reperfusion. Preconditioned hearts were subjected to 3 intermittent periods of I/R of 5 min duration each followed by test I/R. IPost was achieved by 6 cycles of 10 s I/R periods after the 30 min test coronary occlusion. At the end of the 2 hour reperfusion, infarct size was measured in all groups. Separate series of hearts from vehicle, acute and chronic lovastatin groups were perfused for 45 min, freeze clamped and used for biochemical measurements (Fig. 4.). Heart rate and coronary flow was monitored throughout the perfusion. Area at risk and infarct size was determined at the end of the protocol.

6. 2. Isolated heart perfusion

Cardiac performance was assessed in isolated hearts. Mice were heparinized with i.p. 100 U heparin in study 1 and study 2, rats were heparinized i.v. with 500 U/kg heparin in study 3 and in each study animals were anesthetized with diethyl ether. Hearts were then isolated, the aorta was cannulated and initially perfused in Langendorff mode (at a constant pressure of 60 mmHg, 37 °C) with Krebs–Henseleit buffer oxygenated with 95% O_2–5% CO_2 (pH = 7.4) containing in mmol/L 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 11.1 glucose. For perfusion of mouse hearts Krebs-Henseleit buffer contained 1.75 mmol/L $CaCl_2$ for rat hearts the buffer contained 1.2 mmol/L $CaCl_2$. The perfusion protocol for study 1 is in Fig. 3, and the perfusion protocol for study 3 is in Fig. 4.

In study 2, during the initial retrograde perfusion the left atrium was cannulated and the perfusion was then switched to a recirculating working mode (10 min, 37 °C, preload 15 mmHg, afterload 60 mmHg). Coronary flow, aortic flow, and left ventricular pressure were measured at the end of working perfusion. At the end of perfusion the hearts were snap frozen in liquid nitrogen and stored at − 70 °C until they were used for biochemical assays.

6. 3. RNA isolation

In study 1, two RNA pools were prepared from each group (n = 3, randomly selected from each group) and used in replica experiments. This means two biological replicas, but as each data point was gathered twice (technical replicates); altogether transcript mRNA levels were calculated from 4 measurements. Total RNA was purified from 25 mg tissue from each heart with Nucleo Spin RNA purification kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The entire heart was powdered as we were interested in global RNA expression changes in the heart reflecting complex physiological changes including the damaged tissue. Total RNA was used for quantitative real-time polymerase chain reaction (qRT-PCR). The quantity and quality of RNA from each sample was assessed by gel electrophoresis as well as spectrophotometry (NanoDrop spectrophotometer, NanoDrop, USA).

6. 4. The quantitative real-time polymerase chain reaction

QRT-PCR was performed on a Rotor Gene 2000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers and SYBR Green protocol to analyze the gene expression changes. In brief, 10 μg of total RNA from each pool was reverse transcribed in the presence of oligo dT (deoxythymidylic acid) primer in a total volume of 20 μl. After dilution of the mix with 80 μl of water, 2 μl of this mix was used as template in the qRT-PCR. Reactions were performed in a total volume of 20 μl (8 pmol/each forward and reverse primer, 1×Bio-Rad SYBR Green buffer, Bio-Rad Hungary, Budapest, Hungary) with the following protocol: 10 min denaturation at 95°C, and 45 cycles of 25 s denaturation at 95°C, 25 s annealing at 59°C, and 25 s extension at 72°C. Fluorescent signals were gathered after each extension step at 72°C. Curves were analyzed by the Rotor Gene software using dynamic tube and slope correction methods ignoring data from cycles close to baseline. Relative expression ratios were normalized to hypoxanthine-guanine phosphoribosyltransferase and ribosome protein S18 and calculated with the Pfaffl method [73]. The PCR primers used in this study are shown in Annex I. Primers were designed using the Array Express software (Applied Biosystems, Foster City, CA, USA). All the PCRs were performed four times in separate runs.

6. 5. Determination of serum lipids

In study 2, serum lipids including total cholesterol, triglycerides, LDL and HDL cholesterol were measured in triplicate, using commercially available colorimetric assay kits applying enzymatic determinations (Diagnosticum Ltd., Budapest, Hungary) adapted to 96-well plates. Accuracy of the assays was monitored by using Standard Lipid Controls (Sentinel, Milan, Italy). Results are expressed as mmol/l of serum. To analyze the ratios of α, pre-β, and β-lipoproteins in the serum of WT and transgenic mice fed cholesterol-enriched or normal diet, lipoproteins were separated on agarose gel, using Paragon Electrophoresis System Lipoprotein Electrophoresis Kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions.

6. 6. Measurement of superoxide production

To assess if hypercholesterolemia or hypertriglyceridemia leads to increased formation of cardiac superoxide, in separate experiments, superoxide production in

freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence in WT and apoB-100 transgenic mice fed either normal or cholesterol-enriched diet. Approximately 50 mg cardiac tissue was placed in Krebs–Henseleit buffer containing 5 μmol/L lucigenin. Chemiluminescence was measured at room temperature in a liquid scintillation counter (Tri-Carb 2100TR, Packard Instrument Company, Meriden, CT). Cardiac superoxide production was expressed as counts per min/mg wet tissue weight.

6. 7. Measurement of cardiac NO by electron spin resonance spectroscopy

To measure cardiac NO content in study 2, in vivo spin-trapping of NO was applied, followed by electron spin resonance spectroscopy analysis of ventricular tissue samples. The spin trap diethyl-dithio-carbamate (500 mg/kg), 50 mg/kg $FeSO_4$, and 200 mg/kg sodium citrate were administered i.p. under ether anesthesia. Thirty minutes after injections of diethyl-dithio-carbamate and a mixture of $FeSO_4$ and citrate, ventricular samples were placed into quartz electron spin resonance spectroscopy tubes, frozen in liquid nitrogen until assayed for electron spin resonance spectra of the NO–Fe^{2+}(diethyl-dithio-carbamate)$_2$ complex. Fe^{2+}(diethyl-dithio-carbamate)$_2$ has high affinity for NO while forming NO–Fe^{2+}(diethyl-dithio-carbamate)$_2$. Electron spin resonance spectra was recorded with a Bruker ECS106 (Rheinstetten, Germany) spectrometer at a temperature of 160 K.

6. 8. Measurement of serum malondialdehyde levels

In order to estimate the level of systemic lipid peroxidation serum malondialdehyde was measured in study 2. In this assay malondialdehyde reacts with thiobarbituric acid, and as the reaction is not entirely specific for malondialdehyde, the assay is called the thiobarbituric acid-reactive substance assay. Serum samples were mixed thoroughly with 1.2 volumes of a stock solution of 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and 0.25 N HCl and heated for 30 min at 95°C. After cooling and centrifugation at 1000xg for 10 min, the supernatant containing thiobarbituric acid-reactive substance were extracted in butanol and assayed spectrophotometrically at 535 nm. Freshly diluted tetramethoxypropane which yields malondialdehyde was used as the external standard. Results were expressed as nmol malondialdehyde/mL serum.

6. 9. Measurement of cardiac activities of NAD(P)H oxidase, xanthine oxidoreductase (XOR), superoxide dismutase (SOD), and NO synthase

The frozen ventricles collected in study 2 were ground at the temperature of liquid nitrogen. NAD(P)H-stimulated superoxide production was assessed by lucigenin-enhanced luminescence in freshly prepared ventricular homogenates. To estimate background levels of superoxide formation ventricular homogenate (30 μL) was added to 1 mL Krebs–Henseleit–HEPES buffer containing 5 μmol/L lucigenin. Chemiluminescence was measured with a liquid scintillation counter. To measure NAD(P)H-stimulated superoxide generation, either 100 μmol/L NADH or 100 μmol/L NADPH were added to the tubes and changes in luminescence after each addition were measured. 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 per minute/mg protein. Protein concentration was measured in the homogenates by the bicinchonic acid method using bovine serum albumin as a reference standard (Sigma St. Louis, MO). NADH or NADPH alone did not evoke lucigenin chemiluminescence in the absence of homogenate.

Activity of XOR (xanthine oxidase and xanthine dehydrogenase), one of the dominant sources of superoxide in rat hearts, was determined from ventricular homogenates by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR activity) and absence (XO activity) of the electron acceptor methylene blue.

Total activity of SOD in ventricular homogenate was measured by a spectrophotometric assay using a kit (Randox Laboratories, UK) according to the instructions of the manufacturer.

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. Powdered frozen ventricular tissue was placed in four volumes of ice-cold homogenization buffer and homogenized with an Ultra-Turrex disperser. The homogenate was centrifuged (1000 x g for 10 min at 4 °C) and the supernatant was kept on ice until enzyme activity assays. Samples were incubated for 25 min at 37 °C in the presence or absence of either EGTA (1 mM) or EGTA plus NG-monomethyl-L-arginine (1 mM) to determine the level of Ca^{2+}-

dependent and Ca^{2+}-independent NO synthase activities expressed in pmol/min/mg protein.

6. 10. Measurement of infarct size

In study 3, coronary artery was reoccluded at the end of the perfusion protocols and 4 ml of 0.1% Evans-blue dye was injected into the aorta to delineate the area at risk zone. Stained hearts were frozen, sliced, and incubated at 37^{o}C in 1% triphenyltetrazolium-chloride for 10 min to delineate infarcted tissue [74]. Slices were then fixed and quantified by planimetry. Infarct size was expressed as a percentage of the area at risk zone.

6. 11. Western blot analysis of survival kinases

Standard Western blot analysis was performed in study 3 from cardiac tissue homogenates to assess activation of survival kinases. In brief, 20 μg protein were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes (Amersham Biosciences) that were incubated with rabbit polyclonal IgG antibodies (Cell Signaling) against rat Akt (1:1000), phopho-Akt, (1:500, [Ser 473]), p42/p44 MAPK/ERK (1:1500), and phospho-p42/p44 MAPK/ERK (1:1500, [Thr 202/Tyr204]). After washing, the membrane was incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Chemicon) (1:5000). For detection, ECL Advance Western Blotting Detection kit (Amersham) was used. Intensities of the appropriate bands were analyzed by densitometry.

6. 12. Measurement of myocardial coenzyme Q9

The level of cardiac coenzyme Q9 was measured by an HPLC method following lipid extraction with n-hexane as described previously [75]. Coenzyme Q9 was detected at 275 nm using an UV/VIS detector following separation with a Gilson HPLC system on a C18 column (50734 LiChrospher endcapped, Merck). Calibration curves were made by using coenzyme Q9 standard (Sigma St. Louis, MO).

6. 13. Statistical analysis

In study 1 comparative CT (cycle time) method was used to quantify gene expression. The CT for hypoxanthine-guanine phosphoribosyltransferase and ribosome protein S18 was subtracted from the corresponding value of the gene of

interest for that sample to give a ΔCT value. ΔΔCT values were determined by subtracting the appropriate ΔCT value from the corresponding group's ΔCT values, for example control from cholesterol-enriched diet value (the corresponding group pairs are indicated in the figures). Relative mRNA levels were calculated from two biological and 2 technical measurements. In study 2 and study 3 data were expressed as means ± SEM and analyzed with one-way ANOVA followed by Tukey's post-hoc test. $*p < 0.05$ was used as the criteria for indicating a statistically significant difference.

7. Results

7. 1. Study 1

7. 1. 1. The effect of cholesterol-enriched diet on I/R- and IPC-induced gene expression pattern

In study 1 the effects of cholesterol-enriched diet on I/R- and IPC-induced cardiac gene expression pattern were examined in the mouse heart focusing on genes involved in NO, free radical signaling and in the mevalonate pathway. Figures 5-9. show pairwise comparisons of cardiac transcript levels assessed by qRT-PCR. Comparisons were analyzed between the different diet and perfusion protocols see in Fig. 3. Changes in expression were considered as significant alteration, when the fold change was higher than 2. On the following 5 figures dashed lines indicates the border of significance.

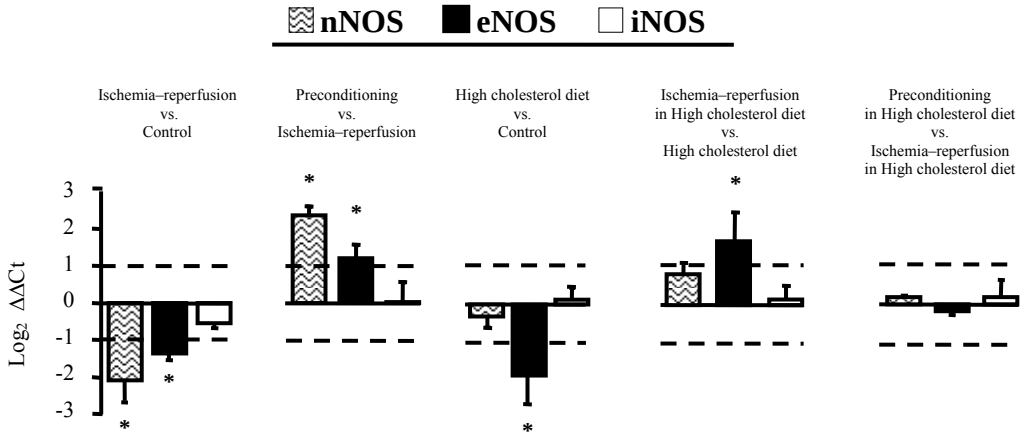

Figure 5. Pairwise comparison of cardiac mRNA levels of nNOS, eNOS and iNOS assessed by qRT-PCR.

The various isoforms of NOS enzymes were differentially regulated in response to IPC and IPC in cholesterol feeding. I/R decreased nNOS and eNOS mRNA levels in the normal fed animals, while IPC counteracted this effect by increasing gene expression of these genes (Fig. 5.). Interestingly, in case of eNOS the adverse result was observed in the hearts of cholesterol-fed animals, i.e.: I/R upregulated eNOS gene transcription but IPC had no effect. Moreover, cholesterol-enriched diet by itself decreased eNOS mRNA content (Fig. 5.). On the contrary, high-cholesterol diet and

IPC had no influence on iNOS expression (Fig. 5.). I/R also resulted in the diminished cardiac mRNA level of superoxide producing enzymes, such as NADPH oxidase (Phox1) and XO due to normal feeding when compared to laboratory chow fed time-matched controls, while IPC reversed this effect (Fig. 6.)

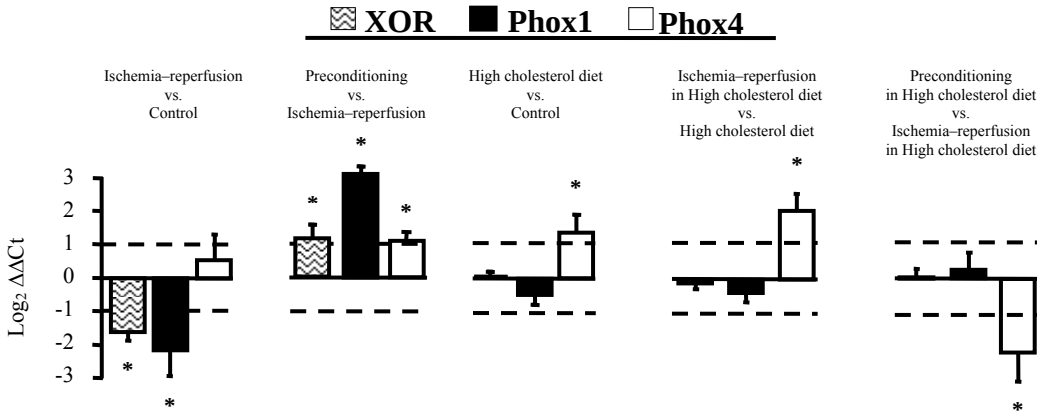

Figure 6. Pairwise comparison of cardiac mRNA levels of XOR, NADPHox1 (Phox1), and NADPHox4 (Phox4) assessed by qRT-PCR.

High-cholesterol diet conflict the effect of IPC on Phox1 and XO. Cholesterol-enriched diet by itself changed Phox4 mRNA content. Moreover, Phox4 mRNA content is the only from the examined superoxide producing isoenzymes that react on I/R and IPC in cholesterol-enriched diet.

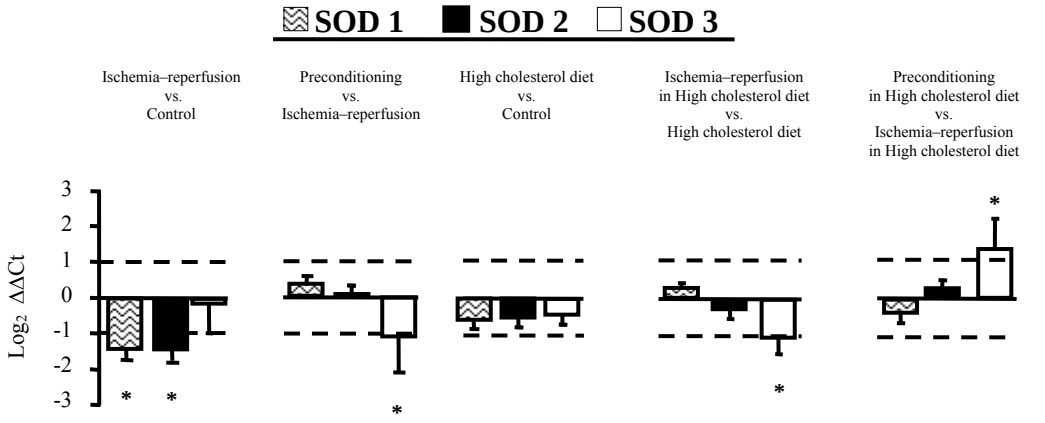

Figure 7. Pairwise comparison of cardiac mRNA levels of SOD1, SOD2, and SOD3 assessed by qRT-PCR.

I/R decreased mRNA levels of SOD1 and SOD2 only in the animals on normal diet, however, IPC did not affect the transcription of these antioxidant enzymes. Interestingly, I/R and IPC altered mRNA expression only for the SOD3 gene in the hearts of cholesterol-fed animals, however, these changes did not match the alterations seen in the hearts of mice fed a normal diet (Fig. 7.). As downstream targets of peroxynitrite, some MMPs and their tissue inhibitors were also studied. Basal cardiac expression of MMP9 was increased due to cholesterol-enriched diet. On the contrary, I/R decreased cardiac mRNA levels of MMP2 and tissue inhibitor of metalloproteinases (TIMP2) compared to the time-matched control group. IPC altered MMP9 gene expression only in the normal fed mice (Fig. 8.). The effect of I/R and IPC on MMP2, 9 and on TIMP2 in cholesterol-enriched diet is not seen.

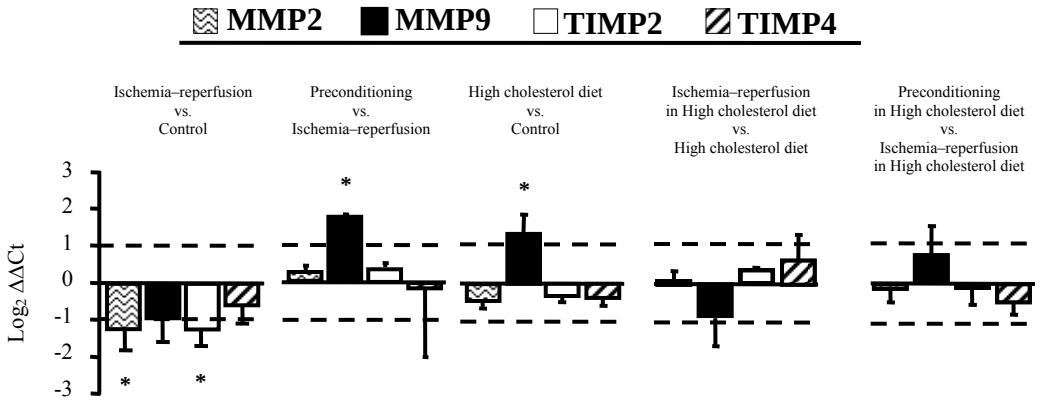

Figure 8. Pairwise comparison of cardiac mRNA levels of MMP2, MMP9, TIMP2, and TIMP4 assessed by qRT-PCR.

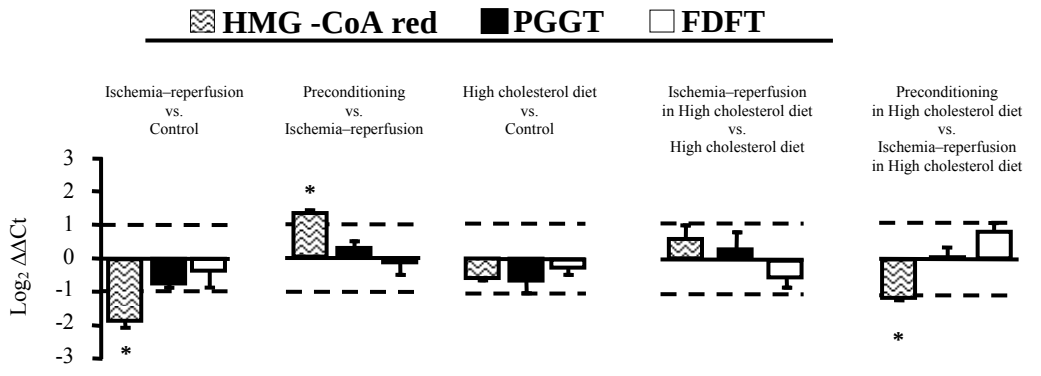

Figure 9. Pairwise comparison of cardiac mRNA levels of HMG-CoA reductase, Geranylgeranyl transferase (PGGT), and Farnesyl diphosphate farnesyl transferase (FDFT) assessed by qRT-PCR.

Among the genes coding for enzymes of the mevalonate pathway, interestingly, HMG-CoA reductase gene showed differential expression in the myocardium in response to IPC; I/R decreased mRNA levels, while IPC upregulated this gene when compared to the non-preconditioned mice on normal diet. On the other hand, this effect of IPC was not evident in the hearts of cholesterol-fed animals (Fig. 9.).

7. 1. 2. Hemodynamic data

Hemodynamic parameters including coronary flow and heart rate were not altered significantly by the different feeding regimens (for detailed data see Annex I), indicating that high-cholesterol diet did not result in the restriction of coronary circulation and in the development of myocardial I/R by itself.

7. 2. Study 2

7. 2. 1. Serum lipids

We characterized the lipid profile of apoB-100 transgenic and WT mice fed a cholesterol-enriched or normal diet. Neither cholesterol-enriched diet, nor apoB-100 transgene affected serum total cholesterol when compared to normal diet-fed WT mice (Fig. 10A). However, serum cholesterol was increased significantly due to cholesterol-enriched diet in the apoB-100 transgenic animals (Fig. 10A, $n = 7–8$ in each group).

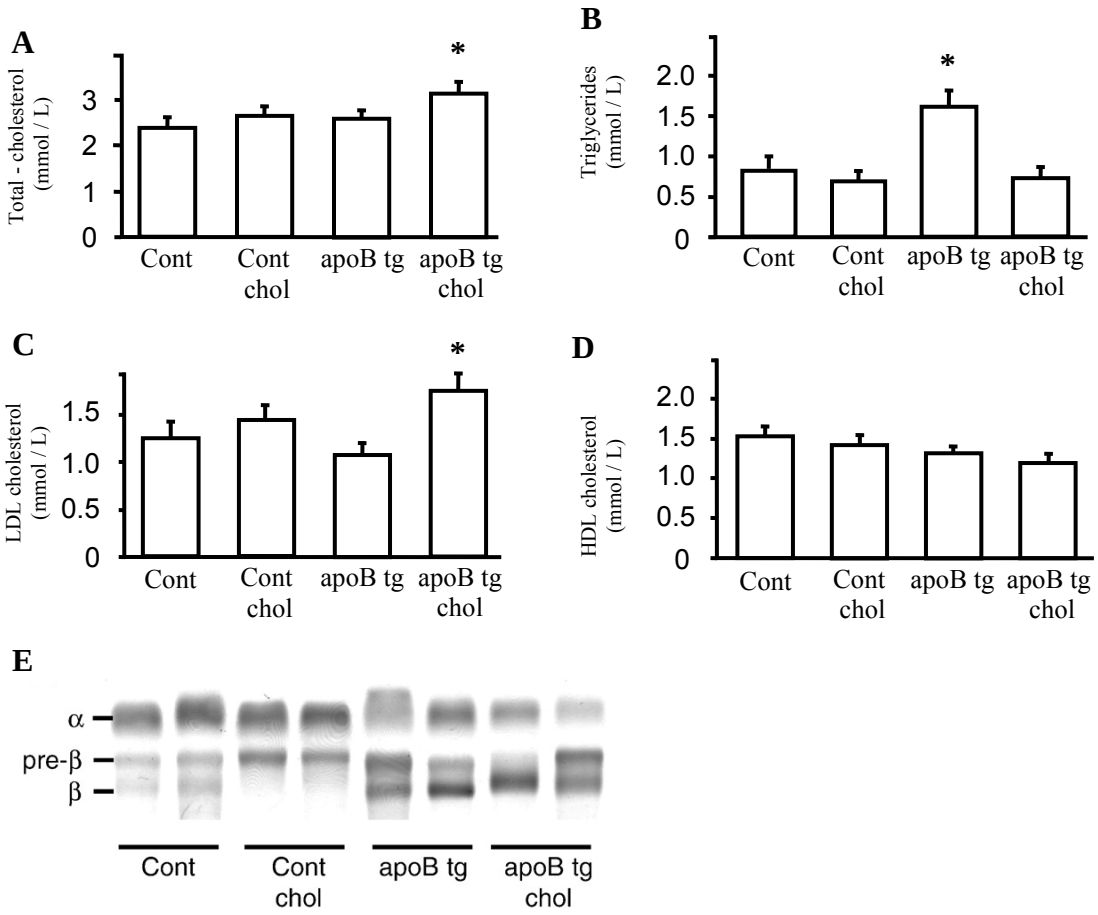

Figure 10. Serum total cholesterol (A), serum triglycerides (B), LDL-cholesterol (C), HDL-cholesterol (D) levels, lipoprotein electrophoresis (E) in apoB100 and WT (Cont) mice fed either cholesterol-enriched (chol) or normal diet. Results are means ± SEM ($n = 5–8$ in each group). $^{*}P < 0.05$ vs. controls.

Similarly, the level of LDL cholesterol was increased significantly only in the apoB-100 transgenic mice fed cholesterol enriched diet (Fig. 10C, $n = 5–8$ in each group). On the other hand, neither of the treatments affected HDL cholesterol levels significantly (Fig. 10D, $n = 5–8$ in each group). ApoB-100 transgene markedly increased the level of triglycerides in the serum compared to normal diet-fed WTs, however, serum triglycerides were reduced significantly by cholesterol in transgenic mice (Fig. 10B, $n = 7–8$ in each group). To analyze the ratios of α, pre-β, and β-lipoproteins in the serum of WT and transgenic mice fed cholesterol-enriched or normal diet, lipoproteins were separated with agarose gel electrophoresis (Fig. 10E). In the plasma of the apoB-100 transgenic mice, the amount of β-migrating lipoproteins increased dramatically with a parallel decrease in the α fraction. Cholesterol feeding slightly increased preβ-migrating lipoproteins in WTs, while caused only a slight shift of the β-fraction towards the preβ-fraction in the transgenic animals (Fig. 10E) without affecting the level of β-fraction.

7. 2. 2. Cardiac NO content, NOS activity

To study if cholesterol-enriched diet or human apoB-100 transgene affects NO content in mouse hearts, electron spin resonance spectroscopy following in vivo spin trapping was applied ($n = 5–6$ in each groups). Cardiac NO content did not change due to apoB-100 transgene or cholesterol-enriched diet in either group (Fig. 11A). Moreover, cardiac activity of NOS, the enzyme responsible for the endogenous synthesis of NO, was not altered in either groups (Fig. 11B, $n = 5–7$ in each groups).

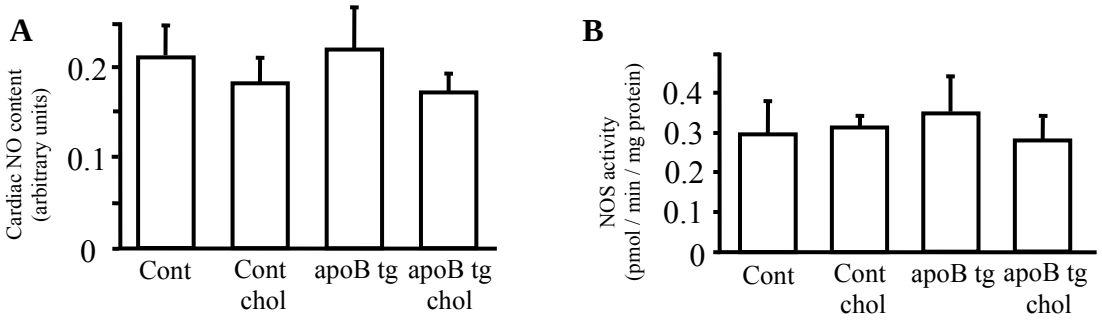

Figure 11. Myocardial NO content (A), and NOS activity (B) in apoB100 and WT (Cont) mice fed either cholesterol-enriched (chol) or normal diet. Results are means ± SEM ($n = 5–7$).

7. 2. 3. Systemic oxidative stress

As a marker of systemic lipid peroxidation due to oxidative stress, serum malondialdehyde levels were determined in apoB-100 transgenic and WT mice fed either cholesterol-enriched or normal diet (n = 5–6 in each group). Malondialdehyde levels were significantly increased in cholesterol-fed transgenic animals when compared to WT ones fed normal diet (Fig. 12A).

7. 2. 4. Cardiac superoxide, NAD(P)H oxidase and superoxide dismutase activity

Cardiac superoxide was markedly increased by cholesterol-enriched diet in the apoB-100 transgenic mice, but not in WTs (Fig. 12B).

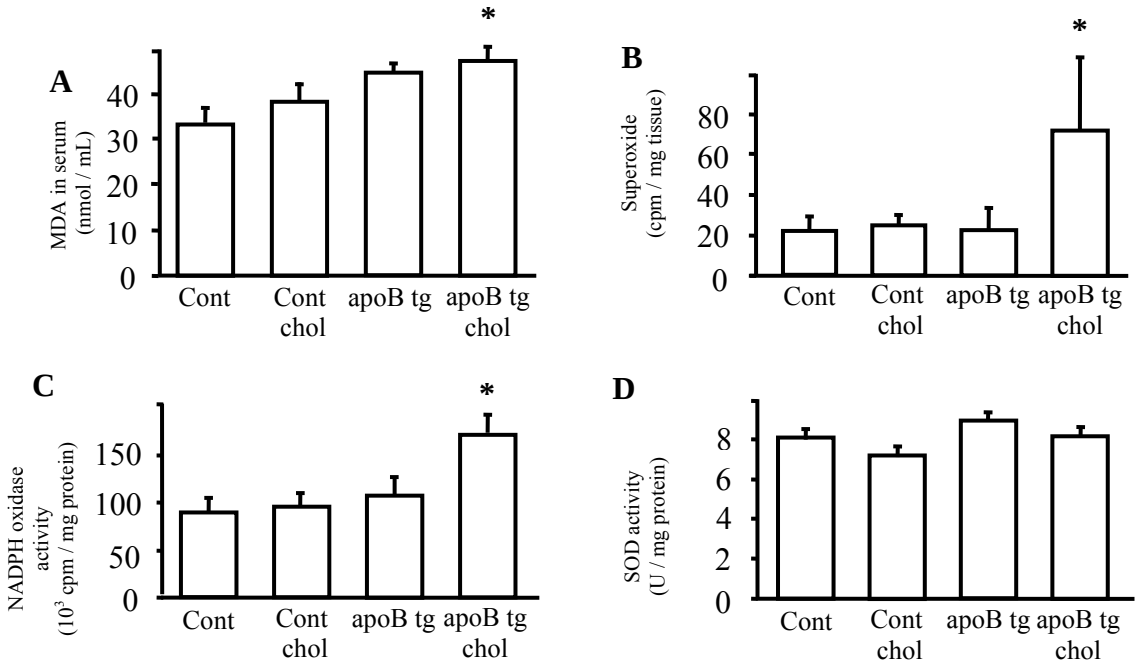

Figure 12. Serum malondialdehyde levels (MDA) as indicators of systemic lipid peroxidation (A), cardiac superoxide production (B), myocardial NADPH oxidase activity (C), myocardial superoxide dismutase (SOD) activity (D) in apoB100 and WT (Cont) mice fed either cholesterol-enriched (chol) or normal diet. Results are means ± SEM (n = 5–8). $P < 0.05$ vs. controls.

ApoB-100 transgene alone did not change cardiac superoxide in mice fed normal diet (Fig. 12B). To determine possible sources of cardiac superoxide, the activity of NAD(P)H oxidase and XOR was measured. Cholesterol-enriched diet significantly increased the activity of NADPH oxidase in human apoB-100 transgenic mice

without having an effect in WT ones (Fig. 12C, n = 6-8 in each group). Activities of neither NADH oxidase nor XOR have changed significantly in any of the treatment groups (n = 5-8 in each group, data not shown). To estimate the activity of the most important enzymatic antioxidant mechanism against superoxide, total cardiac superoxide dismutase activity was determined. Neither apoB-100 transgene, nor cholesterol-enriched diet affected total superoxide dismutase activity in the mouse heart (Fig. 12E, n = 6-8 in each group).

7. 2. 5. Myocardial dysfunction and peroxynitrite

Aortic flow was determined to estimate basal pumping capacity of the heart. ApoB-100 transgene did not alter aortic flow in mice fed normal diet (Fig. 13.). However, cholesterol-enriched diet significantly deteriorated aortic flow in the human apoB-100 transgenic mice without having an effect in WTs (Fig. 13, $n = 7–9$ in each group). To test if peroxynitrite is involved in cardiac dysfunction induced by

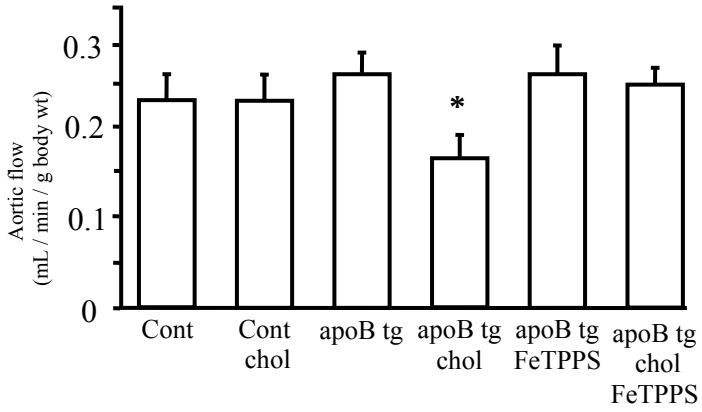

Figure 13. Aortic flow in isolated working hearts of apoB-100 transgenic and WT (Cont) mice fed either cholesterol-enriched (chol) or normal diet when pretreated or not with the peroxynitrite decomposition catalyst FeTPPS. Results are means ± SEM ($n = 5–7$). $^{*}P < 0.05$ vs. controls.

cholesterol-enriched diet in apoB-100 transgenic mice, a peroxynitrite decomposition catalyst, FeTPPS, applied 24 and one hour prior to isolation of the hearts, significantly improved aortic flow deteriorated by cholesterol-enriched diet in apoB-100 transgenic mice (Fig. 13, $n = 5–7$ in both groups). Neither the presence or absence of the apoB-100 gene, nor the cholesterol content of the diet had any significant effect on heart rate, coronary flow, left ventricular peak systolic pressure, or ±dp/dtmax (see in Annex II).

7. 3. Study 3

7. 3. 1. Infarct size

To assess if cholesterol lowering therapy interacts with myocardial adaptation the effect of acute and chronic administration of lovastatin on I/R and on the infarct size limiting effect of IPC and IPost was investigated on rat hearts after three types of perfusion protocols, (see Fig. 3.). Neither chronic nor acute lovastatin treatment affected baseline coronary flow, heart rate and area at risk (data not shown). Both IPC and IPost significantly decreased infarct size in the vehicle-treated groups (Fig. 14.). While chronic lovastatin treatment reduced infarct size in the non-conditioned group, it did not affect the infarct size limiting effect of IPC, but it inhibited the infarct size limiting effect of IPost. While acute lovastatin treatment failed to decrease infarct size significantly in the non-conditioned group, it abolished the infarct size limiting effect of IPC, however, it did not affect IPost.

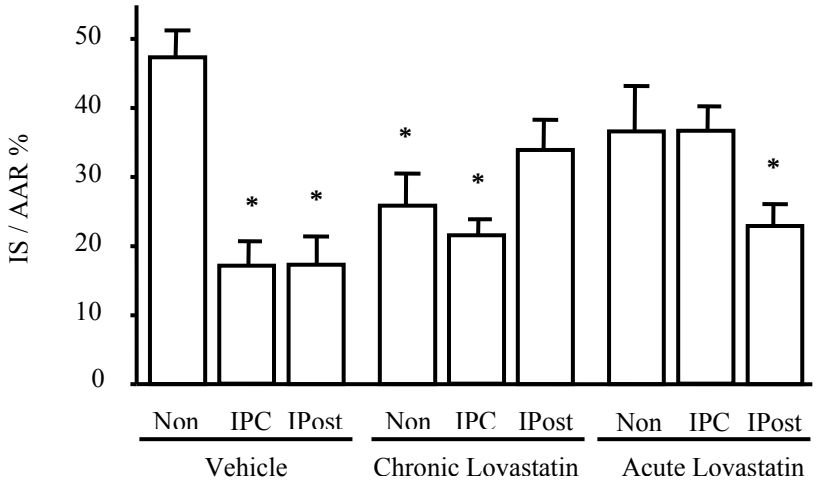

Figure 14. Infarct size (IS) expressed as a percentage of the area at risk (AAR) in isolated hearts of vehicle-, chronic lovastatin-, and acute lovastatin-treated rats subjected to non-conditioning (Non), preconditioning (IPC), or postconditioning (IPost) perfusion protocols. Values are mean ± SEM (n=8-12). *P<0.05 vs. vehicle-treated non-conditioned group.

7. 3. 2. Biochemical measurements

After 45 min perfusion cardiac level of coenzyme Q9, total and phosphorylated protein level of Akt and p42 MAPK/ERK were measured from rat hearts in order to get insight to the molecular mechanisms of different administration ways of lovastatin. Both chronic and acute lovastatin treatment significantly decreased myocardial coenzyme Q9 level (Fig. 15/A), and attenuated phosphorylation of Akt (Fig. 15/B, C) without affecting total-Akt protein level (Fig. 15/B, D). However, phosphorylation of p42 MAPK/ERK was increased only by acute lovastatin treatment and it was unaffected by chronic lovastatin treatment. Total p42 MAPK/ERK was not changed by either acute or chronic lovastatin (Fig. 15/B, E, F).

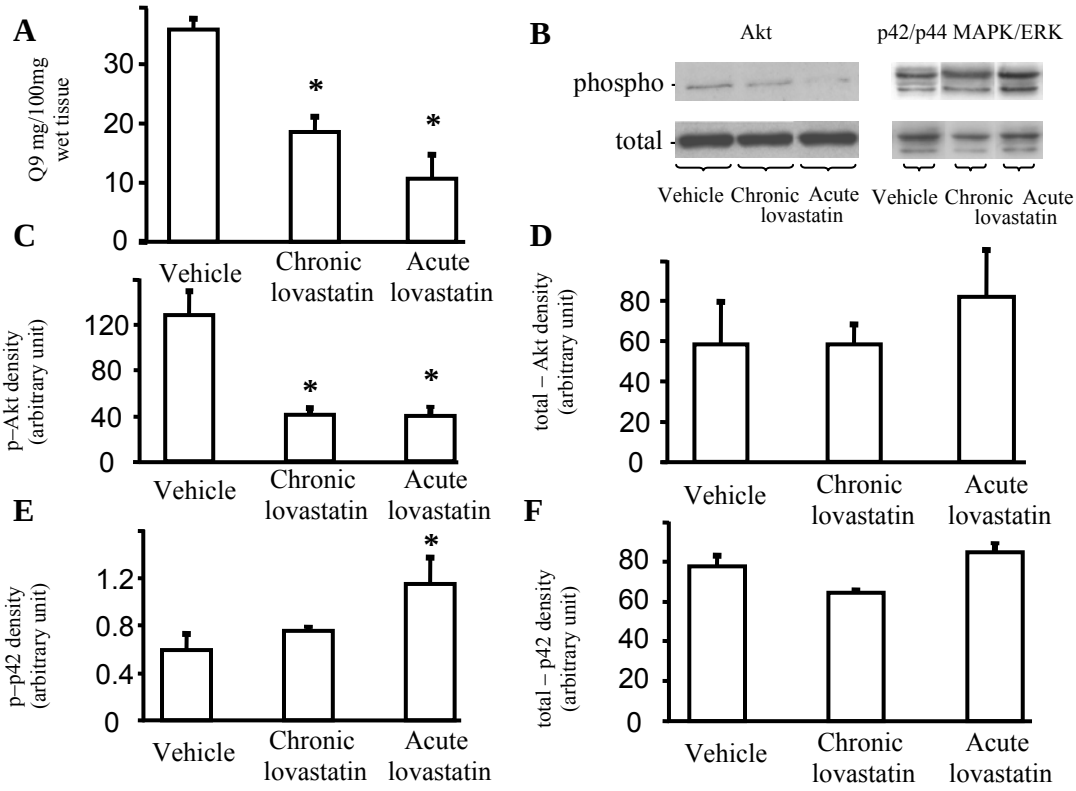

Figure 15. **A**, Myocardial coenzyme Q9, **C-F**, p-Akt, total-Akt, p-p42, total-p42 levels in vehicle-, chronic lovastatin-, and acute lovastatin-treated rat hearts. Values are mean ± SEM (n=6-8). *P<0.05 vs. vehicle-treated group. **B,** Representative Western blots demonstrating phoshorylated and total protein levels of Akt and p42/p44 MAPK/ERK.

8. Discussion

8. 1. New findings

1. Here we show the first time that gene expression pattern is altered in the myocardium of cholesterol-fed mouse in response to IPC. This effect is apparent in the regulation of NO signaling, oxidant producing enzymes, and the mevalonate pathway.

2. This is the first demonstration in human apoB-100 transgenic mice that hypercholesterolemia but not hypertriglyceridemia is responsible for the hyperlipidemia-induced, peroxynitrite-mediated cardiac dysfunction and that increased cardiac NADPH oxidase activity is a major source of increased oxidative stress in hypercholesterolemia.

3. Our novel finding is that acute lovastatin treatment abolishes the infarct size-limiting effect of IPC and that chronic lovastatin treatment attenuates the protective effect of IPost. We have also proposed that acute and chronic lovastatin treatment may modulate the cardioprotective mechanisms through different cellular pathways.

8. 2. The effect of cholesterol-enriched diet on I/R- and IPC-induced gene expression pattern

Our research group and others have shown that hypercholesterolemia blocks the cardioprotective effect of IPC [37, 48, 76-78]. In our first study we have conducted a comprehensive gene expression analysis focusing on the regulation of transcripts involved in free radical/NO signaling and in the mevalonate pathway to gain insight into the molecular mechanisms of this phenomenon. Here we show the first time that gene expression pattern is altered in the myocardium of cholesterol-fed mouse in response to IPC. This effect is apparent in the regulation of NO signaling, oxidant producing enzymes, and the mevalonate pathway.

We have demonstrated previously in rat myocardium that high-cholesterol diet alters the expression of several gene products [52]. In the current study, cholesterol-enriched diet decreased the expression of eNOS, while increased the mRNA level of MMP9 compared to normal fed mouse, indicating that 8 weeks of cholesterol feeding influences gene regulation of key enzymes that may participate in cardioprotection. In

fact, eNOS has been identified as one of the triggers of the second window of protection [39]. Thus, although a recent article using genetic and pharmacological approaches reported that eNOS is not required for the early phase of IPC [79], it is possible that lower eNOS expression attenuates the delayed adaptive response to an IPC stimulus in the myocardium of mouse on high-cholesterol diet. On the other hand, Giricz et. al. [80] have reported that cholesterol-enriched diet decreased cardiac NO content without any change in NOS activity or protein level. Moreover, I/R resulted in the opposite effect between the two feeding regimens; eNOS was decreased in normal hearts, while this transcript was elevated in the myocardium of the cholesterol-fed mouse. The most important outcome, however, that IPC failed to affect the transcript levels of NOS isoenzymes in the animals on high-cholesterol diet. This finding supports the notion that the adaptive mechanisms are impaired in the hyperlipidemic myocardium at the molecular level. However, IPC did not alter the expression of iNOS gene in the current study. As our IPC was acute, our finding is not on the contrary with the literature, that iNOS is an important player in late IPC [39]. It is feasible, however, that other molecules that regulate NO signaling may be altered. For example, Tang and colleagues [77] have demonstrated that tetrahydrobiopterin (BH4), an essential iNOS cofactor is responsible for the loss of the cardioprotective effect of late IPC in hyperlipidemic rabbits. Moreover, another study by the same group indicated that NO signaling is disrupted downstream of NO generation in the hearts of these animals [46]. Nevertheless, we show here that the regulation of the eNOS gene is disrupted in the cholesterol-fed animals, which may alter the trigger for IPC and therefore the adaptive response in these hearts.

Although one would expect an increase in superoxide production as a result of I/R, it is possible that this phenomenon is not reflected at the transcript level, especially at the time point of our sampling, at 2 hours of reperfusion. This is also evident in the mRNA expression of antioxidant enzymes, which was reduced by I/R in the normal hearts, although there was no change in the myocardium of cholesterol-fed mouse. In fact, every gene whose expression was altered by I/R in the animals on normal diet exhibited decreased transcript levels, including MMP2. Therefore, these results suggest the failure of global transcription or a diminished mRNA stability as a result of I/R. Supporting this notion, I/R also resulted in a marked decrease of total mRNA level in isolated rat hearts [81]. Our IPC protocol was also without any effect

regarding the transcriptional regulation of superoxide producing enzymes in the animals on the cholesterol-enriched diet, while the same protocol was successful in the normal myocardium. A small increase of mRNA levels of superoxide producing enzymes in the preconditioned hearts may be necessary for cardiac adaptation. In fact, it has been reported that a low level of radical species is necessary for cellular signaling and may be required for a normal physiological response by an organism [82]. Interestingly, the effect of IPC was only seen in the cholesterol-fed animals for the SOD3 gene. It has also been reported that both IPC and pharmacological preconditioning upregulated MnSOD gene expression [83], thus the lack of change in this transcript as a result of IPC in the normal hearts was unexpected in our case. On the other hand, the overexpression of CuZnSOD did not protect against necrosis in the mouse heart [84], indicating that this isoenzyme does not play a primary role in the protection against I/R-induced tissue damage. It is possible, however, that gene expression and protein content/enzyme activity of these antioxidant enzymes are independently regulated. In fact, Nilakantan et. al. [85] reported that while SOD protein content and enzyme activity decreased no change in mRNA levels occurred in acutely rejecting cardiac allografts.

Cholesterol-enriched diet did not influence the gene expression of HMG-CoA reductase. This finding is in agreement with the existing literature; that is the feedback regulation of the rate-limiting enzyme of the cholesterol biosynthesis pathway does not occur at the gene expression level [86]. More importantly, I/R and IPC altered mRNA levels only in the normal hearts, thus cholesterol feeding diminished the gene regulatory response.

We only focused on transcript levels and have not analyzed protein expression and enzyme activity changes in this study, which may be differentially regulated during I/R in hyperlipidemia, therefore this limitation has to be taken into account. However, based on our results, it is feasible to speculate that high cholesterol-diet alters transcriptional signaling and/or transcription factor activity that may be necessary for IPC-induced cardioprotection. Therefore, future studies should investigate transcriptional signaling molecules and transcription factor activity in the hearts of hyperlipidemic animals in order to gain insight into possible interventions to restore IPC adaptation.

8. 3. Hypercholesterolemia increases myocardial oxidative and nitrosative stress thereby leading to cardiac dysfunction in apoB-100 transgenic mice

In study 2, we have systematically analyzed the plasma lipid, lipoprotein, and MDA levels, as well as the cellular mechanisms of cardiac oxidative and nitrosative stress, along with the estimation of myocardial contractile function in human apoB-100 transgenic and WT mice fed normal or high-cholesterol diet. We have shown that in apoB-100 transgenic mice hypercholesterolemia induced by cholesterol-enriched diet increases cardiac oxidative/nitrosative stress thereby leading to cardiac dysfunction, which can be prevented by pharmacologic attenuation of cardiac nitrosative stress. Furthermore, this is the first demonstration that hypercholesterolemia but not hypertriglyceridemia is responsible for the hyperlipidemia-induced, peroxynitrite-mediated cardiac dysfunction.

In this study, cholesterol-enriched diet resulted in an increase in serum total cholesterol and LDL cholesterol levels in the human apoB-100 transgenic mice without having an effect in WTs. HDL cholesterol did not change significantly between the groups. Similarly to previous findings [60] and [62], serum triglycerides were markedly elevated in apoB-100 transgenics fed normal diet compared to WT values. Cholesterol-enriched diet did not have an effect on serum triglycerides in WT mice, however, it did reduce elevated triglycerides back to normal values in apoB-100 transgenic animals. This unique lipid profile makes our transgenic model a suitable tool for the investigation of the distinct effects of hypercholesterolemia (in cholesterol-fed apoB-100 transgenic mice) and hypertriglyceridemia (in apoB-100 transgenic mice fed normal diet) on the heart. Another useful feature of this model is the more “human-like” distribution of lipoproteins in the cholesterol-fed transgenic mice than seen in WTs. In accordance with previous studies [60] and [87] we have found an increased LDL cholesterol/HDL cholesterol ratio in the cholesterol-fed apoB-100 mice that is more characteristic for the human rather than the murine lipid profile.

It is known that hyperlipidemia increases vascular oxidative stress [88-89]. Here we have found that oxidative/nitrosative stress in the heart was increased in cholesterol-fed apoB-100 mice compared to WTs. In order to assess cardiac oxidative/nitrosative stress, we have systematically analyzed myocardial formation of superoxide (measured by lucigenin chemiluminescence), NO, and peroxynitrite (indirectly by

FeTPPS treatment), as well as the activities of major enzymatic sources of these species (XOR, NAD(P)H oxidase, and NOS), and the most relevant antioxidant enzyme activities (SOD). In agreement with our previous study in a rat model of hyperlipidemia [47], we have shown here an increase in cardiac superoxide production. We demonstrate here that the activity of NADPH oxidase is responsible, at least in part, for the increased superoxide production seen in cholesterol-fed apoB-100 transgenic mice. We have further shown that increased oxidative/nitrosative stress was associated with a significant myocardial dysfunction in the transgenic mice fed cholesterol. The mechanism by which superoxide has contributed to the cardiac dysfunction likely involves the formation of the cytotoxic species, peroxynitrite. Pathological conditions resulting in increased production of NO, superoxide, or both often lead to increased formation of peroxynitrite, which causes cellular damage [49, 90-91]. We have previously reported that hyperlipidemia (marked hypertriglyceridemia and moderate hypercholesterolemia) induced by cholesterol-enriched diet resulted in enhanced formation of peroxynitrite in rat hearts [47]. Our present study shows that cholesterol-enriched diet in apoB-100 transgenic mice leads to increased myocardial oxidative/nitrosative stress and cardiac dysfunction. We have further shown here that the peroxynitrite decomposition catalyst FeTPPS improved cardiac contractile function deteriorated by cholesterol-enriched diet in apoB-100 transgenic mice, indicating that formation of peroxynitrite in these mice exerts a deleterious effect on cardiac function. Our data also indicates that the increased superoxide formation is responsible for the increased peroxynitrite in these hearts, since neither the activity of NOS, nor cardiac NO content showed any change among the treatment groups.

In summary, cardiac oxidative/nitrosative stress induced by hypercholesterolemia but not hypertriglyceridemia leads to myocardial dysfunction. Consequently, lowering of serum cholesterol or attenuation of oxidative/nitrosative stress by pharmacological tools may be effective potential targets to protect the heart in hypercholesterolemia.

It is plausible to speculate that lowering of serum cholesterol by pharmacological tools may protect the heart against oxidative and nitrosative stress in hypercholesterolemia, however, it needs further investigations. Some statins have been shown to decrease vascular oxidative and nitrosative stress, and we have previously found that e.g. inhibition of the mevalonate pathway did not influence

cardiac NO synthesis [80]. Nevertheless, a number of clinical trials and animal experiments have shown the beneficial effects of cholesterol-lowering drugs in the prevention of ischemic heart diseases [92].

8. 4. Lovastatin interferes with the infarct size-limiting effect of IPC and IPost

In cholesterol lowering therapy statins are the most widely used drugs. We have shown in study 3 that chronic application of lovastatin for 12 days markedly reduces infarct size in the ischemic/reperfused rat heart, but acute lovastatin treatment had no effect. This shows that acute and chronic lovastatin treatment may trigger different cellular mechanisms. As to the lack of acute effect of lovastatin, our results are in contrast to that of Di Napoli et al [72] showing that acute application of 25 μM simvastatin protects the ischemic/reperfused heart against contractile dysfunction, release of creatine kinase, and post-ischemic hyperpermeability. Interestingly, in that study protection by simvastatin became less evident at 50 μM and reverted to increased damage at 100 μM. Nevertheless, as to the protective effect of chronic lovastatin treatment, our results are in line with the outcome of clinical trials showing the reduction of cardiovascular events due to statins [93].

In this study, we have shown that lovastatin abrogates the cardioprotective effect of IPC when applied acutely but not when given chronically. The cardioprotective effect of IPost was attenuated when chronic lovastatin treatment was applied and acute lovastatin treatment had no effect. As chronic lovastatin showed cardioprotection by itself, the fact that IPost in the chronic lovastatin-treated group did not reduce infarct size shows, there may be an antagonism between the cellular mechanisms of IPost and that of chronic statin treatment. These results show that acute and chronic lovastatin treatment by interfering with different cellular mechanisms may modulate cardioprotective mechanisms.

Indeed, in study 3, both acute and chronic lovastatin administration inhibited basal cardiac level of the antioxidant coenzyme Q9, which has been previously shown to be a cardioprotective molecule and an important determinant of normal cardiac function [38, 75]. Furthermore, both acute and chronic lovastatin treatment significantly decreased phosphorylation of Akt on Ser473 in the heart in the present study. As phosphorylation of Akt is a cell survival signal [94-95], decreased Akt phosphorylation may contribute to lovastatin-induced deterioration of cardiac

adaptation. Acute and chronic lovastatin treatment showed differential effect on p42/p44 MAPK/ERK in the present study. Neither total nor phosphorylated p42/p44 MAPK/ERK protein levels were affected significantly by chronic lovastatin treatment. In contrast, acute lovastatin treatment significantly increased p42 MAPK/ERK phosphorylation, however, it did not affect total p42 MAPK/ERK level. These effects of lovastatin might play a role in its differential action on cardioprotective mechanisms. Our results indicate that both mevalonate pathway dependent and independent mechanisms can be involved in the interaction of statins with cardiac adaptation to I/R injury. Nevertheless, the upstream triggers for activation of survival pathways and the sequences of their activation are far from clear; and the interpretation of different experiments using different species and experimental protocols in cardiac ischemic adaptation is complicated (see Ferdinandy et al. [37]).

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