# FUNCTIONAL AND MOLECULAR EFFECTS OF HYPERCHOLESTEROLEMIA ON THE MYOCARDIUM: THE ROLE OF CONNEXIN-43 AND MICRORNA-25

**Summary of PhD. Thesis**


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**List of full papers directly related to the subject of the thesis**

I. **Varga ZV**, Kupai K, Szűcs G, Gáspár R, Pálóczi J, Faragó N, Zvara A, Puskás LG, Rázga Z, Tiszlavicz L, Bencsik P, Görbe A, Csonka C, Ferdinandy P, Csont T.: MicroRNA-25-dependent up-regulation of NADPH oxidase 4 (NOX4) mediates hypercholesterolemia-induced oxidative/nitrative stress and subsequent dysfunction in the heart. J Mol Cell Cardiol. (2013) 62:111-21. [IF: 5.148]

II. Görbe A, **Varga ZV**, Kupai K, Bencsik P, Kocsis GF, Csont T, Boengler K, Schulz R, Ferdinandy P.: Cholesterol diet leads to attenuation of ischemic preconditioning-induced cardiac protection: the role of connexin 43. Am J Physiol Heart Circ Physiol. (2011) 300:H1907-13. [IF: 3.708]

Cumulative impact factor of the papers directly related to the thesis: **8.856**

**Other full papers**

I. A. Gorbe, **Z.V. Varga**, N. Klincumhom, T. Eschenhagen, J. Paloczi, M. K. Pirity, A. Dinnyes, S. Rungarunlert, R. Madonna, T. Csont, P. Ferdinandy: Cytoprotection by the NO-donor SNAP against ischemia/reoxygenation injury in mouse embryonic stem cell-derived cardiomyocytes. Mol Biotechnol. (2013) (in press accepted manuscript). [IF:2.262]

II. Kocsis GF, Sárközy M, Bencsik P, Pipicz M, **Varga ZV**, Paloczi J, Csonka C, Ferdinandy P, Csont T.: Preconditioning protects the heart in a prolonged uremic condition. Am J Physiol Heart Circ Physiol. (2012) 303:H1229-36. [IF:3.629]

III. Faragó N, Zvara A, **Varga Z**, Ferdinandy P, Puskás LG.: Purification of high-quality micro RNA from the heart tissue. Acta Biol Hung. (2011) 62:413-25. [IF: 0.593]

IV. Csont T, Görbe A, Bereczki E, Szunyog A, Aypar E, Tóth ME, **Varga ZV**, Csonka C, Fülöp F, Sántha M, Ferdinandy P.: Biglycan protects cardiomyocytes against hypoxia/reoxygenation injury: role of nitric oxide. J Mol Cell Cardiol. (2010) 48:649-52. [IF: 5.499]

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# 1. Introduction

Elevated cholesterol level is still the leading risk factor for heart diseases. Although complications caused by hypercholesterolemia may affect several organs, cardiac effects are responsible for the majority of hypercholesterolemia-related morbidity and mortality.

## 1.1 Biological effects of hypercholesterolemia on the heart

### *1.1.1 Direct myocardial effects on the regulation of gene expression*

Cholesterol may exert several direct effects on the myocardium that seems to be independent of the development of atherosclerosis. Cholesterol may regulate gene expression at both transcriptional and posttranscriptional levels. Previously, our research group also has shown that hypercholesterolemia markedly affects cardiac gene expression patterns. Nevertheless, the factors (epigenetic, transcriptional, posttranscriptional, and posttranslational) that may regulate these cholesterol-induced gene expression changes are not entirely known.

Recently, microRNAs have emerged as powerful posttranscriptional regulators of gene expression. MicroRNAs are small RNA molecules, regulating eukaryotic gene expression at the post-transcriptional level by binding to the 3'UTRs. MicroRNAs are known to play important roles in many physiological and pathological processes in the heart, however, the role of cardiac microRNAs in metabolic disease states, especially in the myocardium in hypercholesterolemia is not known.

### *1.1.2 Myocardial oxidative stress in hypercholesterolemia*

Despite the high prevalence and significance of metabolic diseases, the mechanisms of impaired cardiovascular function in these diseases are not fully understood. Increasing evidence suggests that the dysfunction is highly associated with elevated reactive oxygen species (ROS) levels. ROS are highly reactive and unstable molecules causing irreversible and deleterious reactions with biological macromolecules. Therefore, excessive production of ROS may lead to pathologic cellular alterations. Cardiac conditions such as hypertension, diabetes mellitus, coronary artery disease, cardiomyopathies, heart failure, and hypercholesterolemia are associated with altered metabolism of ROS, resulting in chronically increased oxidative stress.

Previously, we have shown that in different animal models of hypercholesterolemia (2% cholesterol-fed rats and apoB100 transgenic mice) there is apparent cardiac oxidative/nitrative stress. We also implicated in our studies that increased expression of NADPH oxidases are the major source of ROS in hypercholesterolemia.

In metabolic stress situations (such as diabetes mellitus) cardiac dysfunction and oxidative stress are well correlated, suggesting the role of oxidative stress as a culprit in metabolic stress induced myocardial dysfunction. Our research group has previously published that hypercholesterolemia-induced myocardial oxidative/nitrative stress significantly contributes to the development of cardiac dysfunction, since contractile alterations can be diminished by pharmacologic attenuation of cardiac nitrative stress (e.g. with a peroxynitrite decomposition catalyst). However, the exact underlying molecular mechanisms in relation to hypercholesterolemia and oxidative stress are still not entirely clear.

### *1.1.3 Direct myocardial effects on endogenous ischemic adaptation*

The heart is known to have a remarkable adaptive ability to withstand myocardial ischemia/reperfusion injury. The phenomenon in which brief periods of ischemic episodes protect the heart against the subsequent lethal ischemia/reperfusion injury was termed ischemic preconditioning (IPre). Later it was described, that the ischemia/reperfusion-induced myocardial injury could be further reduced, if the myocardial reperfusion process was delayed or modified to a staged or gradual form of myocardial reperfusion. This phenomenon is known as ischemic postconditioning (IPost). Although, IPre and IPost has a marked cytoprotective effect in a variety of species, including humans; several studies described (including studies from our research group) that the effectiveness of cardioprotection may be attenuated or blunted in some co-morbid disease states such as high blood pressure, diabetes, heart failure, and hyperlipidemia.

In the last decades, an enormous amount of information has been generated on the molecular mechanisms involved in Ipre and Ipost A large number of stimuli (ROS, NO, adenosine, cardiac pacing) are capable to trigger a state of increased resistance to ischemia/reperfusion, and several interrelated signal transduction pathways (Erk and Akt signaling, cGMP-PKG pathway, etc.) have been identified. However, the end-effectors of cardioprotective interventions remained largely unknown so far, although they may represent promising therapeutic targets against ischemia/reperfusion injury. The end-effectors have also central role in necrotic cell death due to ischemia/reperfusion injury. Calpains and matrix metalloproteinases, the mitochondrial permeability transition pore, the sodium/calcium exchanger of the cardiomyocytes and gap junctions formed by connexin43 (Cx43) hemi-channels have been considered so far as potential end-effectors.

Gap junctions form channels between adjacent cells that are composed of connexin (Cx) protein subunits that may allow direct intercellular communication. Several studies implicated the role of Cx43 formed gap junction mediated cell coupling and/or uncoupling in the mechanisms of cardioprotective interventions, but these early studies were mainly inconclusive and controversial. Boengler et. al described the presence of Cx43 hemi-channels in the mitochondria of cardiomyocytes (mouse, rat, pig, and human) and showed that decreased mitochondrial import of Cx43 to below 50% of normal attenuates the infarct size reduction by ischemic or diazoxide-induced preconditioning in mice and rat hearts. However, the role of mitochondrial Cx43 in the attenuation of cardioprotection in cardiac co-morbidities is still unclear.

# 2. Aims

Here in this thesis our objective was to obtain a deeper insight into the functional and molecular effects of hypercholesterolemia on the myocardium.

Our first aim was to investigate the possible causes of hypercholesterolemia-induced oxidative/nitrative stress and subsequent myocardial dysfunction.

Therefore we have assessed:

- whether cardiac microRNAs are affected by experimental hypercholesterolemia.
- if microRNAs participate in the regulation of myocardial oxidative/nitrative stress in hearts of hypercholesterolemic rats
- potential protein targets of microRNAs affected by hypercholesterolemia

Our second aim was to analyze the direct molecular effects of hypercholesterolemia on the end-effectors of cardioprotective mechanisms. Although, mitochondrial Cx43 plays an essential role in cardioprotection, changes in cardiac mitochondrial Cx43 in hypercholesterolemia have not yet been analyzed. Therefore, we aimed to determine total and mitochondrial Cx43 levels in rats fed a cholesterol-rich diet. Furthermore, ischemic and preconditioned rat hearts were examined whether changes in mitochondrial Cx43 might be involved in attenuated cardioprotection in hypercholesterolemia.

# 3. Materials and Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (National Institutes of Health publication 85-23, revised 1996), and it was approved by a local ethics committee of the University of Szeged.

**Experimental design**

In order to investigate the possible molecular mechanism of hypercholesterolemia-induced oxidative/nitrative stress and consequent myocardial dysfunction, male Wistar rats (250 g) were fed normal or 2% cholesterol- and 0.25% cholate-enriched rat chow for 12 weeks. At the end of the diet period, venous blood was taken for determination of serum lipids. Hearts of anesthetized (sodium pentobarbital; 60 mg/kg i.p.) and heparinized (sodium heparin; 500 U/kg i.v.) rats were then isolated and perfused according to Langendorff with an oxygenated Krebs-Henseleit buffer at 37°C for 10 minutes. Tissue samples of the ventricular myocardium (n=10-12 in each group) were rapidly frozen in liquid nitrogen for further biochemical analysis (microRNA assays, NADPH oxidase Western blots, nitro-tyrosine ELISA). Some of the hearts were fixed in 4% formaldehyde and were used for histological analysis. In a separate set of experiments, isolated hearts were perfused in a "working" mode according to Neely for 30 minutes following the 10-min Langendorff perfusion. Hemodynamic parameters including heart rate, coronary flow, aortic flow and ventricular pressure parameters were measured at the end of "working" perfusion. Another set of animals were used for heart catheterization to record ventricular pressure in vivo.

In order to investigate the possible role of mitochondrial Cx43 in impaired cardioprotection due to hypercholesterolemia, male Wistar rats (250-300 g) were fed normal (n=18) or 2% cholesterol-enriched rat chow (n=18) for 12 weeks. At the end of the diet, animals were anesthetized with diethylether and given 500 U/kg heparin. Hearts were than isolated and perfused according to Langendorff with Krebs-Henseleit buffer. Hearts from normal (n=6) and cholesterol-fed rats (n=6) were perfused aerobically for 10 min, these hearts were rapidly frozen in liquid nitrogen for further biochemical experiments (Western blots, immunohystochemistry). Then hearts in both diet-groups were subjected to a no-flow ischemia-induced preconditioning protocol (3 x 5-min ischemia and 5-min reperfusion) or a time-matched non-preconditioning protocol each followed by test ischemia/reperfusion (30-min global normothermic ischemia followed by 5-min or 120 min reperfusion, respectively n=6 in each group). Tissue samples from

these perfusion groups were used for mitochondria isolation and Western blots, and in separate experiments myocardial infarct size was determined.

**3.2 Measurement of lipid parameters**

Serum cholesterol and triacylglycerol levels were determined by colorimetric enzymatic assays (Diagnosticum). To analyze α (HDL), pre-β (VLDL), and β-lipoproteins (LDL) lipoproteins were separated on agarose gels.

**3.3 Immunohistochemistry and determination of infarct size**

Formaldehyde-fixed myocardial samples were embedded in paraffin, and, after conventional histological processing the samples were subjected to immunohistochemical analysis for NOX4 protein. Cx43 level of intercalated discs were detected also by immunohistochemistry. At the end of the perfusion protocols, hearts were frozen, sliced, and incubated at 37°C in 1% triphenyltetrazolium chloride to delineate infarcted tissue. Slices were then fixed and infarct size was quantified by planimetry.

**3.4 Isolation of mitochondria and analysis of mitochondrial Cx43 content**

In order to assess mitochondrial Cx43 content, mitochondria were isolated from hearts of hypercholesterolemic and control animals following 10 min aerobic perfusion, or from preconditioned and ischemic control hearts. Cx43 level in isolated mitochondria and in total heart homogenates was determined by Western blotting.

**3.5 Characterization of oxidative/nitrative stress**

***3.5.1 Cardiac superoxide production by lucigenin-enhanced chemiluminescence***

Cardiac superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence in normal and cholesterol-fed groups. Chemiluminescence was measured at room temperature in a liquid scintillation counter.

***3.5.2 Cardiac free 3-nitrotyrosine ELISA***

Cardiac free 3-nitrotyrosine level was measured by enzyme-linked immunosorbent assay (ELISA; Cayman Chemical) from normal and cholesterol-fed heart tissue samples.

***3.5.3 In situ detection of superoxide***

*In situ* detection of superoxide was performed by fluorescent microscopy using the oxidative fluorescent dye dihydroethidium (DHE).

### *3.5.4 Measurement of protein carbonylation*

In order to confirm the effect of oxidative stress on cardiac proteins, total myocardial protein carbonylation was measured using the Oxyblot protein oxidation detection kit (Merck-Millipore) according to the manufacturer's protocol.

### *3.5.5 Quantitative analysis of NOX mRNAs by QRT-PCR*

To investigate the expression of NOX enzymes at the transcript level, QRT-PCR was performed on a RotorGene 3000 instrument with gene-specific primers and SybrGreen protocol. Relative expressions were calculated and normalized to rat HPRT and Cyclophyllin genes. The final relative gene expression ratios were calculated as delta-delta Ct values.

### *3.5.6 Measurement of NOX proteins by western immunoblotting*

In order to investigate whether hypercholesterolemia leads to an increased expression of NOX4 at the protein level in the heart, western blot was performed. Primary antibodies against either NOX4 (Novus Biologicals), NOX2 (Merck-Millipore), or NOX1 (Novus Biologicals) were used. Loading control was done by determining GAPDH content of each sample.

## 3.6 Primary neonatal rat cardiomyocyte cell culture transfection

To investigate the mechanisms by which microRNA-25 down-regulation affects NOX4 expression, neonatal rat cardiomyocyte cultures were prepared from newborn Wistar rats by triptic digestion. To knock-down endogenous expression of microRNA-25, a microRNA-25 inhibitor was used, while to up-regulate microRNA-25, a microRNA-25 mimic was used. As a negative control, a corresponding microRNA inhibitor or mimic control was used. The transfection was carried out according to the recommendations of the manufacturer. Expression level of microRNA-25 following transfections was assessed by QRT-PCR.

## 3.7 Measurement of oxidative stress in microRNA-25 inhibitor or mimic transfected cells by fluorescent microplate reader

Transfected neonatal cardiomyocytes were loaded either with 2′,7′-dichlorofluorescin-diacetate (DCF-DA) or dihydroethidium (DHE). After loading the dyes were removed, and the cells were covered with D-PBS and each well was scanned in a fluorescent microplate reader.

## 3.8 Measurement of oxidative stress in microRNA-25 inhibitor or mimic transfected cells by fluorescent microscopy

To further prove our hypothesis in vitro, neonatal cardiomyocytes were plated on glass coverslips, and transfected with a microRNA-25 mimic or inhibitor, or with their corresponding

controls. The transfected cells were loaded with dihydroethidium (DHE). Fluorescence in the transfected cells was detected by a fluorescent microscope.

### 3.9 Luciferase reporter assay

To investigate whether microRNA-25 directly regulates NOX4 expression, human NOX4 3′-UTR sequence was inserted downstream of a Renilla luciferase open reading frame. The luciferase construct was transfected into HEK293 reporter cells together with either a mimic of microRNA-25 or with an inhibitor of microRNA-25 or with a non-targeting sequence (mimic/inhibitor control), respectively.

### 3.10 MicroRNA isolation, microarray, stem-loop QRT-PCR, and microRNA target prediction

In order to investigate the role of microRNAs in the development of myocardial dysfunction due to hypercholesterolemia, heart samples (n=6) from both diet groups were powdered in liquid nitrogen. Steps of microRNA isolation were done according to the protocol of the microRNA isolation kit (Roche, Germany) with modifications. Pooled RNA samples were assayed on the microarrays. To confirm microarray results, quantitative real-time PCR (QRT-PCR) was used. The microRNA databases and target prediction tools TargetScan and microRNA.org were used to identify potential microRNA-25 targets.

### 3.11 Statistical analysis

Values are expressed as mean±SEM. One- or two-way analysis of variance (ANOVA) was used to evaluate differences in between treatment groups. Otherwise Student's t-test was used. Statistical analysis of microRNA microarrays were by using the Feature Extraction software of Agilent Technologies. Using two tailed two sample unequal variance Student t-test, the p-value was determined and used to find the significant gene expression changes. Gene expression ratio with p-value $< 0.05$ and $\log_2$ ratio $< -0.6$ or $\log_2$ ratio $> 0.6$ (~1.5 fold change) are considered as repression or overexpression, respectively. Changes in gene expression were plotted as $\log_2$ ratios of signal intensity values.

## 4. Results

### 4.1 Cholesterol diet results in elevation of serum cholesterol level

Cholesterol-enriched diet for 12 weeks caused a significant increase in serum cholesterol level which was accompanied by an altered lipoprotein pattern. The 12-week long diet induced a decrease in HDL-fraction, while it had no influence on serum triacylglycerol level.

**4.2 Hypercholesterolemia leads to mild diastolic dysfunction**

Parameters of myocardial contractile function were measured both in vivo and in isolated perfused hearts. Left ventricular end-diastolic pressure (LVEDP) showed a significant increase in the cholesterol-fed group as assessed both in vivo and ex vivo, indicating impaired relaxation and diastolic dysfunction. In addition, there was a non-significant ($p=0.096$) decrease in $+dP/dt_{max}$ in the *ex vivo* perfused hearts of cholesterol-fed rats. Other examined parameters (heart weight, heart weight/body weight ratio, coronary flow, cardiac output, left ventricular developed pressure, -dP/dtmax) did not change significantly due to the diet.

**4.3 Hypercholesterolemia leads to increased myocardial oxidative and nitrative stress**

Myocardial oxidative stress was estimated by staining frozen myocardial sections with dihydroethidium. Increased nuclear red fluorescence was detectable in the hearts of cholesterol-fed rats as compared to control rats, indicating enhanced superoxide formation.

The amount of oxidatively modified myocardial proteins was elevated in the cholesterol-fed group as assessed by dinitrophenylhydrazine assay (Oxyblot assay). The intensity of the band with a molecular weight corresponding to actin (42 kDa) was significantly increased, suggesting that oxidation of contractile proteins are likely involved in hypercholesterolemia-induced myocardial dysfunction. The nitrative stress marker nitro-tyrosine was also elevated in the heart due to cholesterol-enriched diet. Moreover we have found elevated superoxide level in the hearts of cholesterol-fed rats as assessed by lucigenin enhanced chemiluminescence.

As we have previously proposed in our studies that NADPH oxidases might be involved in hypercholesterolemia-induced oxidative stress, in the present study we have examined, whether hypercholesterolemia leads to modulation of expression of the cardiac NOX enzymes. QRT-PCR analysis of myocardial samples obtained from cholesterol-fed and control animals showed no alterations in transcript levels of any of the NOX isoforms (NOX1, 2, and 4).

To determine whether hypercholesterolemia induces protein expression alteration in abundant cardiac NOX isoforms, we also examined the protein expression of NOX1, NOX2, and NOX4. Although, there were no alterations in the protein level of cardiac NOX1 or NOX2 due to cholesterol-enriched diet, our analysis revealed the up-regulation of NOX4 after 12 weeks of cholesterol-enriched diet, suggesting its role in hypercholesterolemia-induced oxidative stress.

Immunostaining for NOX4 showed a diffuse positive staining in cardiomyocytes in both diet groups on FFPE sections.

**4.4 Hypercholesterolemia leads to global microRNA expression changes**

In order to analyze the role of microRNAs in the direct myocardial effects of hypercholesterolemia, myocardial microRNAs were isolated from the left ventricles of cholesterol-fed and control rats and were analyzed on a microRNA microarray. Among the assessed 350 microRNAs, 120 showed detectable expression in both groups. One of the most pronounced and significant alteration with reasonable inter-sample variation was seen in the case of microRNA-25 (-1.13 log2 down-regulation). The cholesterol-enriched diet-induced down-regulation of microRNA-25 was further confirmed by QRT-PCR.

**4.5 Potential targets of microRNA-25**

To identify putative microRNA-25 targets, we have performed a bioinformatic analysis by searching for potential 3′ UTR binding sites in two different databases. Several proteins were listed, which were previously shown to be involved in myocardial physiology and/or pathophysiology. Out of these hypothetical targets, NADPH oxidase 4 (NOX4) seemed to be a promising one, as it has 3 conserved and 1 poorly conserved 3' UTR sites. To exclude the role of other cardiac NOX isoforms, we also screened microRNA-25 binding sites for NOX1, NOX2, and p22 phox, however, neither of these were predicted to be a potential target of microRNA-25.

**4.6 MicroRNA-25 down-regulation results in increased NOX4 expression, thereby causing oxidative/nitrative stress in the hypercholesterolemic heart**

Luciferase reporter assay was carried out, in order to prove the direct binding of microRNA-25 to NOX4 3'UTR. Co-transfection of a mimic of microRNA-25 and the luciferase construct (consisting of the 3'UTR region of the NOX4 mRNA) resulted in a decrease in the luciferase signal, indicating direct binding of microRNA-25 to NOX4 3'UTR. In addition, when the inhibitor of microRNA-25 was co-transfected with the luciferase construct, there was a non-significant increase in the luciferase signal ($p=0.062$).

To further investigate, whether binding of microRNA-25 to NOX4 3'UTR affects oxidative stress, cardiomyocytes were transfected with a mimic of microRNA-25 resulting in an up-regulation of microRNA-25 ($4.14\pm1.22$ $\log_2$ expression alteration). Dihydroethidium staining showed a decrease in superoxide level, suggesting that the direct binding of microRNA-25 to NOX4 3' UTR likely results in decreased NOX4 activity.

To prove that the down-regulation of microRNA-25 is responsible for increased oxidative stress by modulation of NOX4, we have transfected neonatal rat cardiomyocytes with a synthetic microRNA-25 inhibitor which induces knock-down of endogenous microRNA-25 level (-

6.96±3.07 log2 expression alteration). As a negative control, a non-targeting microRNA inhibitor control was used. MicroRNA-25 knock-down significantly increased the fluorescence intensity of the hydrogen peroxide-sensitive dye dichlorofluorescin-diacetate (DCF-DA) in cardiomyocytes. Similarly, the fluorescence signal of the superoxide-specific dihydroethidium (DHE) was also significantly increased in cells transfected with microRNA-25 inhibitor. The non-selective NADPH oxidase inhibitor, diphenileniodonium (DPI), dose-dependently attenuated the microRNA-25 inhibitor-induced oxidative stress.

Transfection of cardiomyocytes with microRNA-25 inhibitor showed an increased fluorescent signal as assessed by DHE histochemistry, whereas, a reduced signal was detected in the microRNA-25 mimic transfected cells.

To confirm that the knock-down of microRNA-25 results in increased oxidative stress as a result of NOX4 up-regulation, NOX4 protein level was determined in transfected cells. MicroRNA-25 inhibitor significantly increased NOX4 protein level in primary cardiomyocytes.

**4.7 Hypercholesterolemia attenuates the anti-ischemic effect of ischemic preconditioning**

To investigate the role of Cx43 in attenuated cardioprotection in hypercholesterolemia, we determined infarct size in normal and cholesterol-fed rats subjected to ischemia/reperfusion and preconditioning protocols. Infarct size was 29±2% in normal ischemic/reperfused heart, whereas it was significantly reduced to 9±2% following ischemic preconditioning. The protective effect of ischemic preconditioning was abolished in cholesterol-fed rats.

**4.8 Hypercholesterolemia leads to redistribution of sarcolemmal and mitochondrial Cx43**

To determine the role of Cx43 in the hypercholesterolemia-induced attenuation of cardioprotection, protein expression of Cx43 was determined in whole tissue homogenates from control and cholesterol-fed groups subjected to preconditioning or ischemia/reperfusion perfusion protocols. Total myocardial Cx43 content (sum of Po, P1, P2 signal intensities)
remained unchanged following 12-weeks of cholesterol-rich diet, and neither ischemia/reperfusion, nor preconditioning altered total Cx43 level. However, there was an increase in Po with ischemia which was partially reversed by preconditioning in both cholesterol and normal-fed rats. However, Cx43 localized at the intercalated discs was significantly decreased by hypercholesterolemia, as revealed by our immunohystochemical analysis.

To further investigate intracellular redistribution of Cx43 due to preconditioning and hypercholesterolemia, Cx43 protein levels were determined in mitochondrial fractions.

Mitochondrial total Cx43 content was decreased approximately by 50% in cholesterol-fed as compared to normal-fed rats. At 5 min reperfusion following 30 min global ischemia the total and dephosphorylated mitochondrial Cx43 content was increased which was significantly decreased by ischemic preconditioning in case of both normal and high-cholesterol diet.

# 5. Discussion

## 5.1 New findings

1. ***Cardiac microRNA expression is altered in the heart***

Here we report for the first time in the literature that diet-induced hypercholesterolemia affects myocardial microRNA expression pattern, suggesting the involvement of microRNAs in the posttranscriptional regulation of cardiac gene expression in hypercholesterolemic rats.

2. ***MicroRNA-25 regulates NOX4 expression in the heart***

We have identified and validated with several experimental approaches that NOX4 is a major microRNA-25 target that is responsible for oxidative/nitrative stress in hearts of hypercholesterolemic rats.

3. ***Cardiac NOX4 is up-regulated due to hypercholesterolemia***

We have shown here for the first time that NOX4 is up-regulated in the heart due to hypercholesterolemia. Our present results show, that the microRNA-25 dependent up-regulation of NOX4 is likely a major contributor of increased oxidative/nitrative stress due to hypercholesterolemia.

4. ***Cx43 plays a role in the impaired cardioprotective effect of preconditioning in hypercholesterolemia***

We demonstrated for the first time the intracellular redistribution of Cx43 in hypercholesterolemia. Here we showed that cholesterol feeding did not affect the total expression of Cx43, while the mitochondrial total and dephosphorylated Cx43 content as well as gap junctional Cx43 were decreased concomitantly with a significant increase in cardiac superoxide levels. This was further associated with the loss of the protective effect of preconditioning in hypercholesterolemia.

## 5.2 The effect of hypercholesterolemia on myocardial microRNA expression

Here we reported, for the first time in the literature, that myocardial microRNA expression pattern in affected by hypercholesterolemia. There are only a few reports showing that cholesterol affects microRNA expression, however, these studies were carried out on the liver in cholesterol fed pigs and baboons. Interestingly, a recent report showed an epigenetic regulation of

microRNA-29b expression by oxidized-LDL, raising a new possible level of cholesterol-dependent gene regulation.

**5.3 MicroRNA-25 regulates NOX4 expression in the heart**

In our present study, cardiac microRNA-25 showed a pronounced down-regulation due to cholesterol-enriched diet. To date, mainly cancer related reports were published showing the involvement of microRNA-25 in apoptotic signaling and in cell invasion and migration. Here we used bioinformatic analyses to characterize microRNA-25 mRNA targets (targetome) and a luciferase reporter assay to validate the direct binding of microRNA-25 to the 3'UTR region of the most putative target, NOX4. We have identified and validated NOX4 as a major microRNA-25 target that is responsible for oxidative/nitrative stress in hearts of hypercholesterolemic rats.

**5.4 Cardiac NOX4 is up-regulated due to hypercholesterolemia**

One of the most interesting finding in this study was to show that hypercholesterolemia-induced myocardial dysfunction is mediated by a microRNA-dependent regulation of NADPH oxidase activity. The NADPH oxidase 4 isoenzyme (NOX4) is highly expressed in the heart including cardiomyocytes and localized in different intracellular organelles (e.g. mitochondria, ER, nucleus). There is increasing evidence now, showing that NOX4 is involved in many pathological cardiac and vascular processes, including diabetic cardiomyopathy, TNF alpha-induced endothelial cell apoptosis, and in stroke. Our present study also suggests that the reactive oxygen species produced by NOX4 may contribute to myocardial dysfunction induced by cholesterol-feeding. Our results further suggest that microRNA-25 may act as an endogenous regulator of NOX4 by fine-tuning its expression in physiologic and pathologic conditions as well, which was now confirmed in our present study in the hearts of hypercholesterolemic rats as well as in primary cardiomyocyte cultures.

Our present results show, that the microRNA-25 dependent up-regulation of NOX4 is likely a major contributor of increased oxidative/nitrative stress. Oxidative as well as nitrative stress has been shown to play a central role in several cardiovascular diseases, including hyperlipidemia-induced cardiovascular pathologies. Previous studies from our research group showed that diet-induced hyperlipidemia increases cardiac oxidative and nitrative stress and contributes to myocardial dysfunction. The present study confirms our previous reports, and reveals a previously unknown molecular regulatory pathway (i.e. hypercholesterolemia - microRNA-25 - NOX4 – oxidative stress – oxidized contractile protein – cardiac dysfunction),

providing more insight into the mechanism of hypercholesterolemia-induced myocardial dysfunction.

## 5.5 Cx43 plays a role in the impaired cardioprotective effect of preconditioning in hypercholesterolemia

### *5.5.1 Connexin43 re-distribution*

In the present study by investigating the role of Cx43 in the impairment of preconditioning in hypercholesterolemia, we have found that, hypercholesterolemia did not alter total myocardial Cx43 protein expression. In contrast, marked hyperlipidemia, which can be induced by 12-weeks cholesterol-enriched diet in rabbits caused down-regulation of total myocardial Cx43 protein and also its redistribution. In the present study, intracellular Cx43 distribution was altered as the gap junctional Cx43 content was decreased in cholesterol-fed rats. Altered Cx43 protein levels, Cx43 phosphorylation and/or Cx43 lateralization were already detected under a variety of pathophysiological conditions of the heart. However, the mechanism by which hypercholesterolemia may influence Cx43 localization and content is not known in detail. Based on literature data, increased cholesterol level and also oxidative stress could have an indirect effect on Cx43 expression and Cx43-formed channel activity, offering explanation why Cx43 content was reduced in myocardial intercalated discs and mitochondria of cholesterol-fed rats.

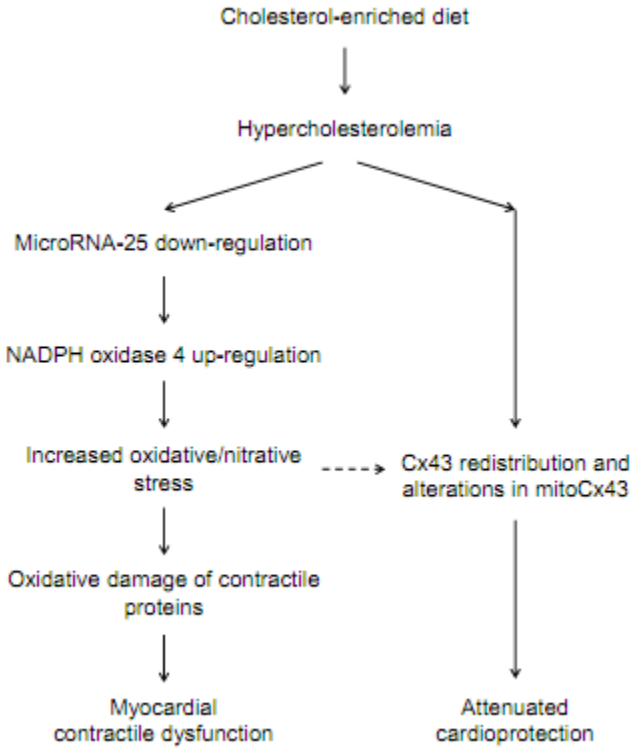
### *5.5.2 Role of Cx43 in the blunted cardioprotective effect of preconditioning due to hypercholesterolemia*

Our hypothesis was based on the observations of our collaborators, i.e. Cx43 is present at the inner membrane of subsarcolemmal mitochondria, increasing potassium influx into the mitochondrial matrix. In the present study, mitochondrial Cx43 content in hypercholesterolemic rat hearts was reduced to approximately 50% of the content measured in mitochondria obtained from rats fed a normal diet, and this decrease in mitochondrial Cx43 content was associated with the loss of ischemic preconditioning's cardioprotection. A similar association between a reduced mitochondrial Cx43 content and a loss of infarct size reduction by ischemic preconditioning was seen in aged hearts. Ischemic preconditioning increased mitochondrial Cx43 content before and at the end of the sustained ischemic period; however, in contrast to the previous studies, ischemic preconditioning reduced mitochondrial Cx43 content at 5 min reperfusion following the prolonged ischemic period in normal and hypercholesterolemic rats. Similarly, ischemic postconditioning limited the migration of phospho-Cx43 to mitochondria following the prolonged

ischemic period in isolated rat hearts. Such decrease in mitochondrial Cx43 following ischemia/reperfusion in pre- and postconditioned hearts could contribute to protection, since mitochondrial Cx43 was proposed as regulator of apoptosis in neonatal myocytes after simulated ischemia and reoxygenation.

# 6. Conclusions

Increased oxidative stress plays a central role in the development of cardiac functional disturbances caused by hypercholesterolemia. Our results clearly show that NADPH oxidases (especially NOX4) are major sources of ROS in the hypercholesterolemic myocardium, and that the expression of NOX4 is regulated and fine-tuned by a microRNA-dependent posttranscriptional mechanism. Furthermore we have shown here that intracellular Cx43 redistributions and mitochondrial Cx43 alterations might play a role in the attenuation of the cardioprotective effect of ischemic preconditioning in hypercholesterolemia.




These results provided information on the source of oxidative stress (microRNA25-NOX4) and on the mechanisms by which oxidative stress may interfere with cardioprotective interventions (Cx43) in the hearts of hypercholesterolemic rats. We hope that these results enhanced our knowledge on the myocardial effects of hypercholesterolemia and may open new perspectives in the treatment of hypercholesterolemic patients.

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