Dissertation for the Doctor of Philosophy Degree

Role of the thiol redox control in myocardial ischemia-reperfusion injury

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Ad	Adenovirus
AF	Aortic Flow
Akt	Protein kinase B
AP-1	Activator protein 1, redox regulated transcription factor
ASK1	Apoptosis signal-regulating kinase 1
Bax , Bad	Pro-apoptotic proteins
Bcl-2	Anti-apoptotic protein
CAM	Cell adhesion molecules
cAMP	Cyclic adenosine 5'-monophosphate
CF	Coronary Flow
Dn	Dominant negative
eNOS	endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
EV	Empty vector
FGF2	Fibroblast growth factor 2
FGFR	Fibroblast growth factor receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glrx	Glutaredoxin
GSH	Glutathione
GSHPx	Glutathione peroxidase
GSSG	Oxidized glutathione, glutathione disulfide
πGST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HIF1α	Hypoxia inducible factor-1
HR	Heart rate
IAP-2	Inhibitors of apoptosis protein-2
IHD	Ischemic heart disease
I-R	Ischemia-Reperfusion
IκB	Inhibitory κB
JNK	c-Jun N-terminal kinase
LAD	Left anterior descending coronary artery

LPS	Lipopolysaccharides
LVDP	Left Ventricular Developed Pressure
LVdp/dt	maximum first derivative of developed pressure
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MI	Myocardial infarction
MnSOD	Manganese-Superoxide dismutase
α-MyHC	α-myosin heavy chain
NFκB	Nuclear transcription Factor κB
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
$O_2^{\bullet -}$	Superoxide anion radical
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
p.f.u.	Plaque forming unit
PI-3-kinase	Phosphatidyl-inositol -3 - kinase
PLA_2	Phospholipase A_2
PMN	Polymorphonuclear leukocyte
PR39	Peptide regulator 39 of angiogenesis
Prdx	Peroxiredoxin
PTP	Mitochondrial PTP
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SH-	Sulfhydryl groups
SOD	Superoxide dismutase
TBS	Tris-buffered saline
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TTC	Triphenyl-tetrazolium-chloride
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling

Introduction

Cardiovascular diseases represent the most prevalent serious disorders in the developed countries. The American Heart Association has reported that in 2002, 62 million Americans suffer from one or more cardiovascular diseases (including hypertension). In the United States, more than 12 million persons have ischemic heart disease (IHD), approximately 6 millon have angina pectoris, and 7 million have sustained a myocardial infarction (MI). In Hungary, approximately 51% of mortality is caused by cardiovascular diseases according to report of Hungarian Epidemiology Society and the second leading cause of death is cancer with 26%**.** Several risk factors may contribute to development of ischemic heart disease such as hyperlipidemia, hypertension, obesity, smoking, diabetes, inactivity, family history, stress, age and sex. IHD arises when there is an imbalance between the myocardial oxygen demand and blood supply. Faulty functioning of the coronary circulation, most commonly due to fatty atherosclerotic plaques or blood clots, causes a reduction in blood flow, and subsequently ischemia and/or MI (*Hearse, 1988; Katz, 1990*). MI related to infarction is a disease of multiple pathways with variable outcomes. Both ischemia and reperfusion contribute to cell and tissue damage after cardiac infarction. Myocardial ischemia/reperfusion initiates maldistribution of ions and various signaling mechanisms leading to oxidative injury and inflammatory responses which include liberation of cytokines (*Ockaili et al., 2005*) and free radicals (*McCord, 1985*), up- and downregulation of various genes and their proteins (*Abbott et al., 1999; Szendrei et al., 2002; Das et al., 2005*), and cell death by apoptosis (*Liu et al., 2005*) and/or necrosis (*Kingma et al., 1987*). The treatment of myocardial infarction is currently mostly directed at restoration of blood flow to the previously ischemic area, and reduction of oxygen demand of the heart. However, during reperfusion of cardiac tissue, depending on the duration of the previous ischemic event, the heart undergoes additional damage due to the activation of various pathways, functional and physiological impairments, leading to cell death.

Probably the two most important consequences of ischemia/reperfusion-induced cardiac injury are (i) heart failure and (ii) ventricular fibrillation leading to sudden cardiac death. Sudden cardiac death occurs in 1,2 million cases each year in the industrialized countries of North America and the European Union. (*Davies, 2001; Rosengren et al., 2004; Cesairo and Dec, 2006*). Thus, interventions for the salvage of the myocardium following myocardial ischemia are essential for minimizing the myocardial damage that leads to left ventricular dysfunction and the subsequent risk for heart failure and sudden cardiac death.

Reactive oxygen species (ROS) playing a dual role as both deleterious and beneficial species (*Valko et al., 2006*). At moderate concentrations, they play an important role as regulatory mediators in signaling processes and cellular processes, including gene expression, growth, and regulated forms of cell death (*e.g.*, apoptosis) (*Dröge, 2002*). However, at high concentrations they are deleterious for living organisms and damage all major cellular constituents (*Valko et al., 2001; Ridnour et al., 2005*). ROS have been implicated in tumorigenesis/carcinogenesis (*Cerutti, 1985*), in the aging process (*Cadenas and Davies, 2000*), and in the progression of cardiovascular diseases, neurodegenerative disorders, rheumatoid arthritis, and inflammatory diseases of the lung (*Dröge, 2002*).

In physiological conditions, free radicals and reactive nonradical species derived from radicals are generated in small but measurable concentrations (*Sies, 1993*) during the normal cellular metabolism or in response to environmental stress and are normally inactivated by endogenous scavenging systems (*Moensa et al., 2005*). However under pathophysiological conditions, the production of ROS in excess of an endogenous cellular capacity for their detoxification and/or utilization cause disruption of „redox homeostasis" and issue in a non-homeostatic state referred to as „oxidative stress" (*Pryor et al., 2006*).

Oxidative stress have been implicated in myocardial I-R injury via several mechanisms and multiple pathways such as e.g., (i) the modification or degradation of cellular biochemicals, including DNA, protein, lipids, and carbohydrates, (ii) the activation of pro-inflammatory nuclear transcription factors such as NFκB and AP-1 which may upregulate death proteins or produce inhibitors of survival proteins (*Ockaili et al., 2005*)**,** (iii) the release of cytochrome-*c* from mitochondria and activation of caspases, p53, and kinases, including apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (*Ueda et al., 2002*) (iv) the peroxidation of membrane lipids and formation of lipid radicals (*Singal et al., 1983; Radi et al., 1991; Leifert et al., 1999*) which may contribute to (v) calcium overload (*Tani et al., 1989; Piper et al., 1998*), (vi) contractile dysfunction and hypercontracture (*Suzuki et al., 1991; Bolli et al., 1999*), (vii) reperfusion arrhythmias (*Bernier et al., 1986; Hearse, 1988*) and ultimately all of which leads to (viii) the death of cardiomyocytes.

Cells or tissues are in a stable state if the rates of ROS production and scavenging capacity are essentially constant and in balance („Oxidant-antioxidant balance" or „redox balance"). This state is achieved by mechanisms called „redox regulation". The process of „redox regulation" protects living organisms from oxidative stress and maintains „redox homeostasis" by controlling the redox status in vivo (*Dröge, 2002; Valko et al., 2007*).

It now becomes increasingly clear that the major role in redox regulation is played by modification of sulfhydryl groups (SH-) in signal proteins, which, from one hand, involves ROS, and from the other such thiol-containing molecules as glutathione (GSH), glutaredoxins (Glrxs), thioredoxins (Trxs), and peroxiredoxins (Prdxs).

To date, no investigations have addressed the potential role of Glrx-2 and Prdx6 in cardiac disorders and myocardial ischemia-reperfusion. Therefore, our researches have been mainly focused to these thiol-containing antioxidants molecules.

- **Glutaredoxins**

Since Glrx-2 catalyzes the reversible glutathionylation of mitochondrial complex 1 (*Beer et al., 2004*), it is expected to play a significant role in reducing the oxidative environment of the cell and decrease the cellular injury. Glutaredoxins (Glrxs) belong to the members of the thioredoxin superfamily of thiol/disulfide exchange catalysts, and hence known as „thiol-transferases". Glrxs are similar to thioredoxins in structure and size; they also contain a redox active Cys-X1-X2-Cys (Cys-Pro-Tyr-Cys) sequence. Contrary to the Trx/thioredoxin reductase system, glutaredoxin lacks a specific reductase for reduction of its oxidized form; this role belongs to the GSH/glutathione reductase system. An important property of glutaredoxins is their ability to reduce mixed disulfides (*Holmgren, 1989*). The Glrx system consists of Glrx, GSH and NADPH-dependent GSH reductase. Glrxs are able to catalyse reactions not only via a dithiol mechanism (as Trxs do), but also via a monothiol mechanism (*Bushweller et al., 1992; Holmgren et al. 1995*), which is required for the reduction of protein GSH mixed disulphides (deglutathionylation). (*Holmgren, 1985*; *Lillig et al., 2004*)

Dithiol mechanism:

$R\text{-}S_2 + Grx\text{-}(SH)_2 \rightarrow R\text{-}(SH)_2 + Grx\text{-}S_2$

$Grx\text{-}S_2 + 2\ GSH \rightarrow Grx\text{-}(SH)_2 + GSSG$

Monothiol mechanism:

$R\text{-}S\text{-}SG + Grx\text{-}(SH)_2 \rightarrow R\text{-}SH + Grx\text{-}S\text{-}SG$

$Grx\text{-}S\text{-}SG + GSH \rightarrow Grx\text{-}(SH) + GSSG$

where R-S-SG is a mixed disulphide with GSH.

Glrxs are predominantly localized in the cytoplasm; however, they can also be detected in the nucleus and mitochondria (*Rodríguez-Manzaneque et al., 2002*). Two mammalian Glrxs have

been identified: *Glrx1* localizes in the cytosol and *Glrx2* localizes primarily in mitochondria, but can also localize in the nucleus (*Lundberg et al., 2001*). Cytosolic Glrx1 is involved in multiple cellular processes, e.g., protein disulphide reduction as in ribonucleotide reductase, dehydroascorbate reduction (*Wells et al., 1990*), regulation of transcription factors (*Bandyopadhyay et al., 1998; Hirota et al., 2000*) and apoptosis (*Chrestensen et al., 2000; Daily et al., 2001*). Similar to Trx-1 (*Saitoh et al., 1998*), Glrx1 can regulate apoptosis through ASK-1 (apoptosis signal-regulating kinase-1), which is a mitogen-activated protein kinase kinase kinase (MAPKKK) that binds to the reduced Glrx1 (Song *et al., 2002*). Upon oxidation, Glrx1 becomes detached from ASK-1 thereby potentiating a survival signal while activation of ASK-1 will generate a death signal. Recently, Glrx1 has been identified in mammalian hearts (*Mazzocco et al., 2002*), however, its role in ischemic heart disease remains unknown.

The second mammalian Glrx2 was identified more recently (*Gladyshev et al., 2001*). Glrx2 encodes two isoforms; a nuclear Glrx2 exists in addition to a mitochondrial Glrx2. Glrx2 is a very efficient catalyst of protein deglutathionylation. The oxidized active site of Glrx2 is also a substrate for thioredoxin reductase (TrxR) (*Johansson et al., 2004*). TrxR efficiently reduces both the active site disulphide and the Glrx2-S-SG intermediate formed in the reduction of glutathionylated proteins, thus supporting both monothiol and dithiol reactions. Glrx2 combines both the characteristics of a Trx and a Glrx. At sufficiently oxidizing conditions the active site in Glrx2 cannot be reduced by GSH. The direct reduction via TrxR enables Glrx2 to reduce glutathionylated proteins and a series of low-molecular-mass substrates even during conditions of oxidative stress (*Johansson et al., 2004*). Mitochondria are the major intracellular source of ROS, which are produced by complex I and complex III during mitochondrial respiration (*Indo et al., 2007*). ROS alter the ratio of GSH/GSSG, which can change the activity of many key proteins by formation of mixed disulphides of GSH with critical cysteine residues (glutathionylation) (*Wang et al., 1997; Lind et al., 1998; Barrett et al., 1999; Klatt et al., 1999; Zech et al., 1999; Pineda-Molina et al., 2001*). ROS formation by complex I is increased upon glutathionylation of two thiols in its NADH-binding pocket (*Taylor et al., 2003*). Glrx2 is an efficient catalyst of monothiol reactions with high affinity for glutathionylated proteins (*Johansson et al., 2004*), and Glrx2 catalyses the reversible glutathionylation of complex I and other proteins of the inner mitochondrial membrane over a wide range of GSH/GSSG ratios (*Beer et al., 2004*). In addition, Glrx2 protects the cells from apoptosis by preventing the loss of cardiolipin, and inhibit cytochrome-*c* release and caspase activation (*Lillig et al., 2004; Enoksson et al., 2005*).

Glrx2 has also been characterized as an iron–sulfur protein. This unusual Glrx can bind a (2Fe–2S) cluster that bridges two molecules of Glrx2. Dimeric holo Glrx2 is enzymatically inactive, but cluster degradation and monomerization of Glrx2 activates the oxidoreductase. The cluster is stabilized by GSH, but destroyed by GSSG and ROS. It has therefore been proposed that the cluster serves as a redox sensor for the activation of Glrx2 during conditions of oxidative stress (*Lillig et al., 2005*).

- **Peroxiredoxins**

Peroxiredoxins (Prdx) or thioredoxin peroxidases are a recently described superfamily of nonseleno-proteins (*Rhee et al., 1999*) belong to thioredoxin superfamily (*Fujii et al., 2001; Turoczi et al., 2003*) and found in a wide range of living organisms from bacteria to mammals (*Hofmann et al., 2002; Wood et al., 2003*). Peroxiredoxins are antioxidant enzymes, and perform the same function as catalases and glutathione-dependent peroxidases, however, their catalytic activity is significantly lower than that of the latter. Beside of thioredoxin reductase (TrxR) the peroxiredoxin is another thioredoxin related protein capable of directly reducing peroxides such as H_2O_2, organic hydroperoxides, and peroxynitrite. They use thiol groups as reducing equivalents to scavenge oxidants (*Rhee et al., 1999*). In the process they are oxidized as they homo or heterodimerize with other family members through disulfide bonds formed between conserved cysteine residues. Trx then reduces the oxidized Prdx back to the active monomeric form. In the process Trx becomes oxidized and is reduced by TrxR (*Chae et al., 1999; Moran et al., 2001; Nordberg et al., 2001; Powis and Montfort, 2001*).

To be able to perform peroxide reduction, Prdxs contain redox active cysteine residues in their catalytic sites. According to the protein contains one or two conserved cysteine residues, Prdxs can be divided into two subgroups, namely 1-Cys or 2-Cys peroxiredoxins (*Rhee et al., 2001; Flohé et al., 2003*). To date, six members of the peroxiredoxin superfamily have been known (Prdx1-Prdx6) in mammalian tissues: Five members are 2-Cys enzymes (Prdx1-Prdx5) that use thioredoxin as an electron donor (*Rhee et al., 2001; Hofmann et al., 2002*). Peroxiredoxin 6 (Prdx6) is the only mammalian member of the 1-Cys peroxiredoxin group. Prdx6, however - unlike the other mammalian members of the family - utilizes glutathione (GSH) as physiological reductant (*Kang et al., 1998; Fisher et al., 1999*). Prdx6 is expressed in all major organs, including the heart (*Fisher et al., 2005*), it is enriched in lung and especially in Clara and alveolar type II epithelial cells (*Kim et al., 1998*). Prdx6 is bifunctional because besides its peroxidase activity, protecting cells from oxidative damage, it also has Ca-

independent phospholipase A_2 (PLA_2) activity. According to above Prdx6 has two separate active centers for these two activities: (i) GPx catalytic center and (ii) PLA_2 catalytic center (*Fisher et al., 1999; Chen et al., 2000*). There are some differences between Prdx6 and other members of Prdx family: such as (i) 2-Cys enzymes form a disulfide either internally (Prdx5) or through homodimerization (Prdx1–4), whereas Prdx6 forms a disulfide with GSH mediated by πGST. (ii) Prdx6 can catalyze the reduction of phospholipid hydroperoxides, (*Fisher et al., 1999*), whereas this activity has not yet been demonstrated for the other peroxiredoxins. It is worth to mention that the ubiquitous cytosolic GSH peroxidase (GSHPx-1) also does not have this activity (*Fisher et al., 1999*). (iii) Prdx6 also has PLA2 activity, whereas the sequence associated with this activity is not present in other peroxiredoxins (*Chen et al., 2000*). A role of Prdx6 in neurodegenerative diseases has also been recognized (*Kim et al., 2003*). Changes in Prdx are associated with the development of Pick disease, dementia in Lewy body disease, in sporadic Creutfeldt-Jacob morbidity, and in atherogenesis (*Power et al., 2002; Krapfenbauer et al., 2003; Wang, Phelan et al., 2004*). Prdx6 is elevated in connection with Pick disease, a neurodegenerative illness related to nuclear palsy and temporal dementia in the central nervous system in relation with saitohin Q allele of human tau gene (*Verpillat et al., 2002*). Overexpression of Prdx6 was shown to protect the lung against hyperoxia-induced injury in mice (*Wang, Feinstein et al., 2004*). Conversely, Prdx6 knockout mice were shown to be hypersensitive to hyperoxia, providing evidence that Prdx6 is an important antioxidant enzyme under in vivo conditions.

- **New trend in the cardioprotection: PR39 Gene therapy**

Gene therapy may represent an alternate mode of pharmacological intervention to combat cardiovascular diseases. Gene therapy is a technique in which a normal allele of a gene is administered into a cell in order to modify the genetic repertoire of the target cell which either cannot express its own copy or produces a defective copy. A variety of cells have been used for this purpose which include fibroblasts, keratinocytes, hepatocytes, endothelial cells, and myocytes (*Brody and Crystal, 1994*).

There are five important components which must be considered in the development of gene therapy: (i) the isolation and cloning of a target gene; (ii) the development of a proper vector for gene transfer; (iii) the identification of a target cell; (iv) in vivo gene delivery, and (v) the identification of potential therapeutic targets. Both viral and nonviral vectors have been used for the purpose of gene therapy (*Schwartz and Moawad, 1997*), but viral vectors have

been proven to be the most effective (*Rowland et al., 1995*). Two types of vectors have been used for the purpose of gene delivery: (i) retrovirus and (ii) adenovirus. Retrovirus contains RNA genomes that are reverse transcribed after infection yielding a double-stranded cDNA copy of the genome flanked by identical elements (long terminal repeats) which contains the regulatory sequences necessary for the expression of intervening genes. Retroviral vectors are produced by deleting viral genes from the provirus and replacing with the target gene of therapeutic potential. Their use has been limited because of very unstable in vivo and their capacity for foreign DNA is limited to less than 10 kb. In contrast to retroviruses, adenoviruses contain relatively larger double stranded DNA of 36 kb and 150 kb with broad host range. Usually these viruses do not integrate into the host cell genome. They enter cells through specific surface receptors and travel to the nucleus. However, adenoviruses do not require host cell proliferation for gene transfer and subsequent expression.

PR-39 is a proline and arginine-rich (PR) macrophage-derived antibacterial peptide, exhibits a broad spectrum of biological activities that include inhibition of phagocyte NADPH oxidase (*Shi et al., 1996*), as well as proteasome activity (*Gaczynska et al., 2003*). Li et al., (2000) and Gao et al., (2000) have shown that the inhibition of proteasome activity results in decreased degradation of hypoxia inducible factor-1 (HIF1α), and IκBα proteins. The stabilization of HIF1α protein and the upregulation of HIF1α –dependent gene expression make PR39 an angiogenic master switch peptide (*Li et al., 2000*). Adding to the salutary effect of PR39 on tissues exposed to inflammation or ischemia, the increased IκBα levels seen in response to PR39 activity, inhibited NFκB activation, which leads to abrogation of NFκB-dependent gene expression in cell culture and mouse models of acute pancreatitis and myocardial infarction (*Gao et al., 2000*). In a next step, they tested that PR-39 inhibited myocardial ischemia–reperfusion (I–R) injury by blocking proteasome-mediated IκBα degradation (*Bao et al., 2001*). The biological properties of PR-39 make this peptide a reasonable candidate for cardioprotection. Indeed, in addition to our own work, several other reports exist in the literature pointing to the cardioprotective ability of PR-39. PR-39 has been shown to block high K^+-induced reactive oxygen species (ROS) production in cultured endothelial cells and isolated perfused rat lungs (*Al-Mehdi et al., 1998*), and it inhibits leukocyte recruitment into inflamed tissue and thus attenuates myocardial reperfusion injury in a murine model of cardiac I–R (*Hoffmeyer et al., 2000*). A more recent study showed that PR-39 also inhibits apoptosis in hypoxic endothelial cells (*Wu et al., 2004*).

Although the role of IκBα stabilization in response to PR39 has been established as a cardioprotective effect of PR39 in I–R, the effect of PR39 on HIF1α protein levels in this

setting has not been studied. Hence, we studied HIF1α levels and cardioprotection in response to PR39 gene therapy, and because the cardioprotective effects of PR39 potentially are mediated by its established enhancement of fibroblast growth factor 2 (FGF2) signaling (*Li et al., 2002*), we also sought to determine the importance of FGF receptor (FGFR) signaling in ischemia/reperfusion.

- **Glucocorticoids**

Beneficial effects of glucocorticoids have been known for many decades, and increasing knowledge of the mechanisms of glucocorticoid action indicates that pretreatment with glucocorticoids could have organ-protective effects in various diseases (*Sellevold and Jynge, 1985; Engelman et al., 1989; Toft et al., 1997; Spanier and McDonough, 2000*). However, the action mechanism(s) of glucocorticoids, in ischemic/reperfused myocardium, is not clear up to date. Glucocorticoids given acutely are beneficial against inflammation in various experimental models (*Feola et al., 1976*), and they have even been employed to reduce the inflammatory effects of extracorporeal circulation during open-heart surgery (*Yamanoi et al., 1991; Ildan et al., 1995; Teoh et al., 1995; Sakurai et al., 1997*). Furthermore, they are able to reduce ischemia-reperfusion-induced myocardial apoptosis in immature hearts (*Pearl et al., 2002*). In addition, glucocorticoid administration reduces myocardial intercellular adhesion molecule-1 (*Toft et al., 1997*) and monocyte chemoattractant protein-1 mRNA expression compared with control piglets (*Pearl et al., 2002*). The reduction of neutrophil adhesion and activation proteins in neonatal myocardium was associated with less apoptotic cell death after glucocorticoid administration. Glucocorticoid-induced attenuation of myocardial apoptosis might have important implications for maintaining long-term ventricular function after ischemia and reperfusion (*Pearl et al., 2002*).

We investigated whether pretreatment with dexamethasone could protect working rat heart function when subjected to global ischemia and reperfusion. We hypothesize, that protection might be due to, beside the reduction of cytochrome-*c* release from mitochondria to cytoplasm, the induction of cardioprotective “de novo” proteins. Because actinomycin D is a protein synthesis inhibitor at the transcriptional level, in further experiments we investigated whether actinomycin D could interfere with the dexamethasone-induced cardioprotection.

AIMS OF THE STUDIES

In our experiments, we used isolated working mouse and rat hearts to study the role of thiol-containing compounds and bioactive agents in ischemia-reperfusion induced injury and potential role of redox-signaling mechanisms in cardioprotection. For this reason our researches have been focused for four different areas:

I. In the first parts of our work, we investigated Prdx6-/- mice. In view of the prevailing view that reperfusion of ischemic myocardium generates ROS (*Burwell et al., 2006; Das, 2001*) and GSH is a potential target of ROS attack, we hypothesized that Prdx6 could play a role in ischemia-reperfusion injury.

We investigated:

- Whether Prdx6 have any crucial role in cardioprotection?
- Targeted disruption of peroxiredoxin 6 gene how can influence the post-ischemic cardiac functions, the infarct size, and cardiomyocyte apoptosis?
- Does it have role in cellular injury? Does it have effects on malondialdehyde (MDA) formation?

II. In the second parts of our research, we worked with glutaredoxin-2 (Glrx2) transgenic mice and we intended to determine the potential role of Glrx2 in cardiac disorders and the specific role of Glrx2 in myocardial ischemia and reperfusion.

In our mind the following questions are come up:

- Whether myocardial overexpression of Glrx2 in the heart could rescue the cardiac cells from apoptosis and necrosis induced by ischemia and reperfusion?
- Besides we intended to examine the effects of Glrx2 overexpression on recovery of myocardium contractile performance, myocardial infarct size and cardiomycyte apoptosis.
- Does it have influence of Glrx2 overexpression on the caspase activity, cytochrome-*c* release and cardiolipin content of the heart?
- Does it have effects on antioxidant activity (GSH/GSSG ratio) and lipid peroxidation (malondialdehyde MDA formation)?

We also sought to determine the pattern of survival signal triggered by Glrx2 overexpression.

III. In the third parts of our experiments, we intended to study the beneficial effects of PR39 gene therapy in the setting of I-R injury. Because the cardioprotective effects of PR39 potentially are mediated by its established enhancement of fibroblast growth factor 2 (FGF2) signaling (*Ray et al., 2001*), we also sought to determine the importance of FGF receptor (FGFR) signaling in I–R.

We investigated:

- Whether the PR39 and FGFR1-dn gene therapy could improve the ventricular recovery and are able to decrease the infarct size as well as cardiomyocyte apoptosis?
- Is it able to reduce of ROS activity?
- Is there any effect of PR39 gene therapy on HIF1α expression? What are effects of PR39 gene therapy on HIF1α expression?

IV. In the last parts of our work, we investigated the pharmacological effects of pretreatment with dexamethasone in working rat heart model.

We asked:

- Whether pretreatment with dexamethasone could protect working rat heart function when subjected to global ischemia and reperfusion?
- Does it have influence of pretreatment dexamethasone on the translocation of cytochrome-*c* from mitochondria to cytoplasm?
- Whether actinomycin D (as a protein synthesis inhibitor) could interfere with the dexamethasone-induced cardioprotection?

MATERIALS AND METHODS

Animals and materials

1. Generation of Prdx6 -/- mice

The full-length BAC genomic clone (clone BACM-153C17 from a 129/SvJ mouse genomic library) containing functional 1-cys Prdx gene was used to construct the targeting vector as described by Krapfenbauer et al., (2003) elsewhere.

1.1 Expression analysis of the Prdx6 protein in the hearts of Prdx6 -/- and wild-type mice

Western blot analysis was performed to detect Prdx6 protein in the Prdx6 -/- and wild-type mouse hearts.

2. Generation of glutaredoxin-2 (Glrx2) transgenic mice

The human Glrx2 transgene was constructed by placing a full-length cDNA fragment, which codes for the human mitochondrial Glrx2 (IMAGE clone 512859, NCBI accession # AA062724), downstream to the 5′ flanking sequence and promoter of the mouse α-myosin heavy chain (α-MyHC) gene (*Chen et al., 2001*). The entire expression sequence, including the genomic sequence of the mouse α- MyHC gene, the human Glrx2 cDNA, and the SV40 splice and polyadenylation sites, was released from the plasmid by digestion with enzymes *Cla*I and *Not*I and purified after separation on an agarose gel. The DNA fragment was then microinjected into the pronuclei of fertilized eggs harvested from female B6C3 (C57BL/6×C3H) F1 mice mated with male B6C3 F1 mice according to the standard method (*Hogan et al., 1994*). Only one line of transgenic mice was generated. The mice used in the studies were generated by breeding the female (hemizygous) transgenic mouse with male B6C3 hybrid mice. Transgenic mice were identified by Southern blot analysis of mouse tail DNA. Both male and female mice were used in the studies and nontransgenic littermates were used as controls for transgenic mice.

2.1 Activity assay of total glutaredoxin in homogenates of heart mitochondria

Mitochondria were isolated from mouse hearts according to the method described previously by Xiong et al., (2006). The mitochondria from each mouse heart were then homogenized in 0.5 ml of lysis buffer (50 mM potassium phosphate buffer, pH 7.8, 0.5% Triton X-100, and 3% glycerol) containing protease inhibitor cocktail (P-8340, Sigma, St.

Louis, MO) and 1 mM phenylmethylsulfonyl fluoride with a Polytron homogenizer followed by sonication. The homogenates were then clarified by centrifugation at 20,000×g for 15 min and stored at −70 °C. The method described by Mieyal et al., (1991) was used to determine the Glrx in homogenates of isolated heart mitochondria. Briefly, 0.1 ml of homogenate was added to 0.8 ml of reaction mixture and then incubated at 30 °C for 5 min. The reaction was initiated by adding 0.1 ml of 20 mM 2-hydroxyethyl disulfide to the reaction mixture and absorbance at 340 nm was followed for 30 s. The activity of Glrxs was determined by the rate of NADPH oxidation per minute per mg of protein using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

3. Generation of adenoviral constructs

Adenovirus-serotype 5-based vectors were produced by recombination in 293 cells. Briefly, the transgene of interest was cloned into a shuttle plasmid that contained CMV promoter and SV40 polyadenylation sequences flanked by adenovirus-5 gene sequences from the E1 region. This shuttle plasmid was then co-transfected into 293 cells along with a plasmid containing the entire Ad5 genome, minus the E3 region, plus ampicillin resistance and stuffer sequences that made this construct too large to be packaged into an adenovirus capsid. After recombination, the transgene expression cassette was inserted into the Ad5 genome and the ampicillin resistance and stuffer sequences were deleted. The resultant double-stranded DNA was small enough to be packaged into the Ad5 capsid, and the missing E1 function was provided in trans by the 293 cells. After transfection, the 293 cells were overlayed with nutrient agarose and plaques were picked ~10–12 days later. Each plaque was amplified in 293 cells and the insertion of the transgene documented by PCR. Positive plaques then underwent large-scale amplification in 293 cells. After amplification, the adenoviral constructs were purified by double CsCl gradient ultracentrifugation, followed by dialysis against phosphate buffered saline. The final titers were generally in the 10^{12}–10^{13} particle/ml range, and the particle to plaque forming units (p.f.u.) ratios were generally ~10–100:1.

3.1 Time course of PR-39 expression after adenoviral gene transfer in healthy mouse heart

Mice were anesthetized, intubated, and ventilated with 2% isoflurane in oxygen and a left lateral thoracotomy was performed. Adenoviral constructs encoding PR-39 were suspended in PBS and a single injection of 109 p.f.u. in 20 μl was performed. The injection site was in the anterior wall of the left ventricle approximately 3 mm below the auricle of the left atrium

between the LAD (left anterior descending coronary artery) and the first diagonal branch. The injection needle was introduced 1 mm into the myocardium at a very shallow angle (10–20°) in cranial direction. Deposition of the suspension was verified by slight bulging and blanching of the epicardial surface. A total of twelve mice were injected, and three mice were euthanized at each of the following time points: immediately after injection, and 3, 7, and 14 days after injection. After isolation of RNA, expression of PR39 was assayed by reverse transcriptase polymerase chain reaction (RT-PCR).

Methods

1. Isolated working heart preparation

Mice (25–34 g) and rats (320-350 g) were anesthetized with pentobarbital sodium (60 mg/kg body wt ip.) and anticoagulated with heparin sodium (500 IU/kg body wt ip) injection. After ensuring sufficient depth of anesthesia, thoracotomy was performed, and aorta of heart was identified. After we excised the heart from the chest by the aorta, the lung and connective tissue were removed and the whole heart was transferred to ice-cold (4°C) modified Krebs–Henseleit bicarbonate solution, which contained (in mM) 118 NaCl, 4.7 KCl, 1.7 $CaCl_2$, 24 $NaHCO_3$, 1.2 KH_2PO_4, 12 $MgSO_4$, and 10 glucose, until contraction had ceased. Both the aorta and pulmonary vein were cannulated as quickly as possible and perfused in retrograde Langendorff mode against constant perfusion pressure of 100 cm H_2O (10 kPa) for a standardization period. Immediate start of retrograde perfusion helped to wash out the blood and its component from the vascular system. Perfusate temperature was maintained at 37°C and saturated with 95% O_2 and 5% CO_2 gas mixture during the entire experiment. The duration of the retrograde perfusion was 10 min; after this procedure, the heart was switched to an antegrade perfusion mode. In the antegrade perfusion mode, the buffer enters the cannulated left atrium at pressure equivalent to 10 cm H_2O (1 kPa) and passes to the left ventricle, from which it is spontaneously ejected through the aortic cannula. Baseline measurements of heart rate (HR), coronary flow (CF), aortic flow (AF), left ventricular developed pressure (LVDP), and its first derivative (LVdp/dt) were recorded and the coronary perfusate was collected for further MDA assay. After this periode the antegrade perfusion line was closed, and the heart was subjected to 30 min ischemia. Before the initiation of 2 h reperfusion, the heart was perfused in retrograde mode to avoid the development of a high incidence of ventricular fibrillation. The cardiac function was registered at 15, 30, 60, and 120 min of reperfusion.

2. *Experimental protocols*

2.1 *Experimental protocol for Prdx6 -/- mice study*

In the first study used isolated working Prdx6 -/- and wild-type mouse hearts. The isolated hearts were randomly divided into two groups: Prdx6 -/- or wild type. For baseline control, isolated hearts were perfused with Krebs-Henseleit bicarbonate buffer for 2 h 45 min.

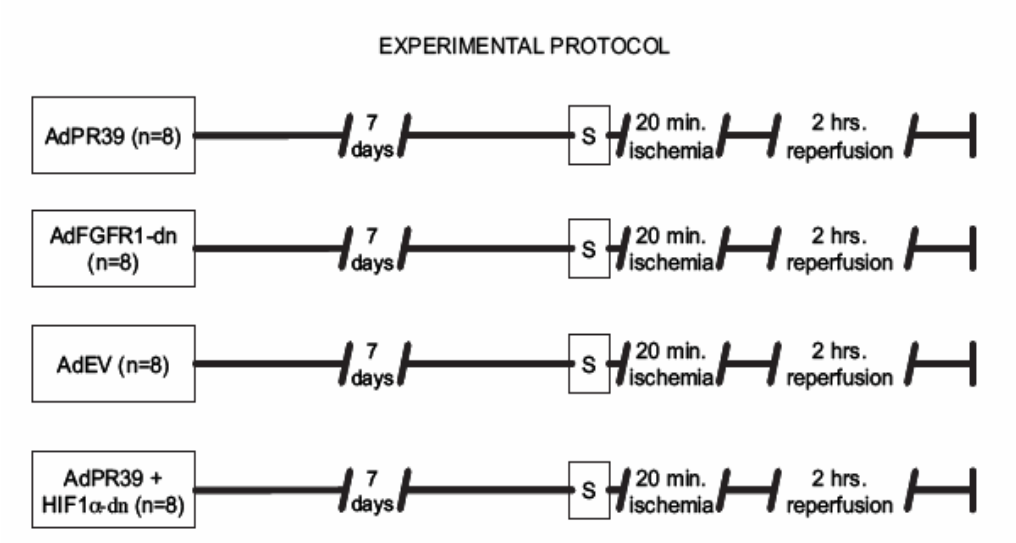
2.2 *Experimental protocol for studying cardioprotection of Glrx2 overexpression*

In the second study we used isolated working hearts from Glrx2 transgenic mice and non-transgenic littermates (wild type). The mice were randomly divided into six groups: (i) wild type control; (ii) wild type ischemia; (iii) wild type ischemia/reperfusion; (iv) Glrx2 transgenic control; (v) Glrx2 transgenic ischemia; and (vi) Glrx2 transgenic ischemia/reperfusion. All hearts were then perfused by working mode and haemodynamic measurements were performed.

2.3 *Experimental protocol for studying myocardial protection against ischemia–reperfusion injury with PR-39*

To study the cardioprotective effect of PR39 and the role of FGFR1 signaling in the setting of I-R injury, 36 mice were randomly allocated to one of four experimental groups, eight animals per group. One group was treated with adenoviral construct encoding PR39 (AdPR39) as described, a second group received adenoviral construct encoding dominant negative FGF receptor-1 (AdFGFR1-dn), and the control groups received either adenoviral constructs encoding empty vector (AdEV) or AdPR39 in combination with 4 μg of a plasmid encoding a dominant negative mutant of HIF1α (AdPR39 + HIF1α-dn) (Fig. 1). A left lateral thoracotomy was performed and the viral constructs were administered as a single injection of 109 plaque forming units (p.f.u.) in 20 μl phosphate buffered saline (PBS) and 8 μl PBS containing 4 μg of plasmid was injected. The injection site was in the anterior wall of the left ventricle approximately 3 mm below the auricle of the left atrium. Deposition of the suspension was verified by slight bulging and blanching of the epicardial surface. Seven days after gene transfer the mice were euthanized, the hearts were excised and positioned in an *ex vivo* working heart set up to be subjected to I–R injury. Hemodynamic measurements were carried out before a 20 min period of ischemia, followed by a 2 h of reperfusion.

Fig. 1. *Experimental protocol for PR39 gene therapy study.*



2.4 Experimental protocol for dexamethasone study

Rats were treated with 2 mg/kg of intraperitoneal injection of dexamethasone, and 24 hours later, hearts were isolated and subjected to 30 min of normothermic global ischemia followed by 2 hours of reperfusion. In additional studies, rats were treated with 0.5 mg/kg of an intravenous injection of actinomycin D, a protein synthesis inhibitor, one hour before the dexamethasone injection. The doses and administration of dexamethasone and actinomycin D were selected to our previously published study (*Tosaki et al., 1985*). The same concentration range of dexamethasone was used in the study of Spanier and McDonough (2000), and Engelman et al., (1989) in a model of cardiac ischemia/reperfusion. Myocardial function was measured before ischemia and after 60 and 120 min of reperfusion in each group.

3. Measurement of the cardiac function and arrhythmias

An epicardial electrocardiogram (ECG) was recorded by a computer system throughout the experimental period by two silver electrodes attached directly to the heart. The ECGs were analyzed to determine the incidence of ventricular fibrillation (VF). The heart was considered to be in VF if an irregular undulating baseline was seen on the ECG. The heart was considered to be in sinus rhythm if normal sinus complexes occurring in a regular rhythm were apparent on the ECG. Before ischemia and during reperfusion, heart rate, left ventricular develop pressure (LVDP) (the difference between the maximum systolic and diastolic pressure), and the first derivative of develop pressure were recorded by Gould p23XL transducer. The signal was amplified by using Gould 6600 series signal conditioner and

monitored on Cordat II real-time acquisition system (*Maulik et al., 1999; Ray et al., 2001; Turoczi et al., 2003*). The aortic flow was measured by a flow meter. The coronary flow was measured by time collection of the coronary effluent dripping from the heart.

4. Measurements of the infarct size

At the end of each experiment, the heart was infused with 10% solution of triphenyltetrazolium (TTC) in phosphate buffer through the aortic cannula for 20 min (*Imamura et al., 2002*). The left ventricle was removed and sliced into 1-mm thickness of cross-sectional pieces and weight. Each slice was scanned with computer-assisted scanner. The risk area of the whole myocardium was stained in red by TTC, while the infarct zone remained unstained by TTC. These were measured by using computerized software, and these areas were multiplied by the weight of the each section; these results were summed up to obtain the total of the risk zone and infarct zone. The infarct size was expressed as the ratio of the infarct zone to the risk zone.

5. TUNEL assay for the assessment of apoptotic cell death

Immunohistochemical detection of apoptotic cells was carried out using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), in which residues of digoxigenin-labeled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase II, an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3′-OH ends of double- or single-stranded DNA (*Maulik et al., 1999; Maulik et al., 2000*). The incorporated nucleotide was incubated with a sheep polyclonal anti-digoxigenin antibody followed by an FITC-conjugated rabbit anti-sheep IgG as a secondary antibody as described by the manufacturer. The sections were washed in PBS three times, blocked with normal rabbit serum, and incubated with mouse monoclonal antibody recognizing cardiac myosin heavy chain followed by staining with TRIRC-conjugated rabbit anti-mouse IgG. The fluorescence staining was viewed with a confocal laser microscope. Apoptotic cells were counted and expressed as a percentage of total myocyte population.

6. Measurement of malondialdehyde for assessment of oxidative stress

Malondialdehyde (MDA) was measured as MDA-2,4- dinitrophenylhydrazine (MDA-DNPH) derivative by HPLC (*Cordis et al., 1995*). In brief, coronary effluents were collected and derivatized with 2,4-dinitrophenylhydrazine (DNPH). The aqueous phase was extracted with pentane, blown down with nitrogen, and reconstituted in 200 µl of acetonitrile. Aliquots of 25

μl in acetonitrile were injected onto a Beckman Ultrasphere C18 (3 μm) column. The products were eluted isocratically with a mobile phase containing acetonitrile/water/acetic acid (40:60:0.1, vol/vol/vol) and measured at three different wavelengths (307, 325, and 356 nm) by using a Waters M-490 multichannel UV detector. The peak for MDA was identified by cochromatography with a DNPH derivative of the authentic standard, peak addition, UV pattern of absorption at the three wavelengths, and by gas chromatography-mass spectroscopy (GS-MS). The amount of MDA was determined by performing peak area analysis using the Maxima software program.

7. Western blot analysis

Left ventricles from the hearts were homogenized in a buffer containing 25 mM Tris–HCl, 25 mM NaCl, 1 mM orthovanadate, 10 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. High-molecular-weight markers (Bio-Rad, Hercules, CA, USA) and 100 μg total membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in running buffer (25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3). After electrophoresis, the separated proteins were transferred onto 0.45-μm polyvinylidene difluoride membrane in transfer buffer (25 mM Tris base, 192 mM glycine, 20 % (v/v) metanol, pH 8.3). The membrane was blocked in TBS-T buffer ((50 mM Tris, pH 7.5, 150 mM NaCl) and 0.1 % (v/v) Tween-20, and 5 % (w/v) non-fat dry milk)) and incubated overnight at 4°C with primary antibodies. Then the membrane was washed several times with TBS-T buffer according to the manufacturer's instructions and incubated with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. Blots were developed using ECL-PLUS detection system according to manufacturer instruction. The resulting blots were digitized, subjected to densitometric scanning using a standard NIH image program, and normalized against loading control. There is no antibody available that recognizes PR39 on Western blots, thus PR39 expression was confirmed by RT-PCR.

8. Determination of caspase 9 and caspase 3 activities

Caspase activity was evaluated by the use of caspase 9 and caspase 3 colorimetric assay kit obtained from R&D Systems (*Uchiyama et al., 2004*). In sortly, cells that are suspected to or have been induced to undergo apoptosis are first lysed to collect their intracellular contents. The cell lysate can then be tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroanaline (pNA). The cleavage

of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm.

9. NFκB analysis

To determine DNA binding of NFκB, nuclear proteins were isolated from the heart as described previously (*Maulik et al., 1999*). About 150 mg of left ventricle from the heart tissue was homogenized in ice-cold Tris-buffered saline (TBS) and centrifuged at 3000×g for 5 min at 4 °C. The pellet was resuspended by gentle pipetting in 1.5 ml of ice-cold hypotonic buffer containing 10 mM HEPES, (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 1 μM each of aprotinin, pepstatin, and leupeptin. The solution was allowed to swell on ice for 15 min after addition of 100 mM of 10% Nonidet P-40. This homogenate was centrifuged for 30 s at 4 °C in a microcentrifuge tube. The supernatant contained the cytoplasmic protein. The nuclear pellet was resuspended in a solution containing 20 mM HEPES, (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1 μM each of aprotinin, pepstatin, and leupeptin. Protein concentration was estimated using the Pierce Protein Assay kit (Pierce Chemical Co, Rockford, IL).

NFκB oligonucleotide (AGTTGAGG-GGACTTTCCCAGG) (2.5 μl of 20nm/μl) was labeled using T4 polynucleotide kinase as previously described. The binding reaction mixture contained in a total volume of 20.2 μl, 0.2 μl DTT (0.2 M), 1 μl BSA (20 mg/ml), 4 μl poly(dI-dC) (0.5 μg/μl), 0.2 μl buffer D+, 4 μl buffer F, 2 mM ^{32}P-oligo (0.5 ng/μl) and 7 μl extract containing 10 μg protein. The composition of buffer D+ was 20 mM HEPES, (pH 7.9), 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40 while buffer F contained 20% Ficoll 400, 100 mM HEPES, (pH 7.9), and 300 mM KCl. Ten milliliter of the solution was loaded onto a 4% acrylamide gel and separated at 80 V until the dye hit the bottom. Autoradiographic results were evaluated quantitatively by an image analyzer.

10. Assessment of GSH/GSSG ratio

The concentrations of GSH and GSSG were determined by the well-established methods by measuring the enzymatic recycling procedure using glutathione reductase and 5,5′-dithiobis (2-nitrobenzoic) acid using standard kits.

11. Determination of cardiolipid content of the heart

Mitochondria were prepared by subcellular fractionation of the heart by well-established method. Cardiolipin degradation was estimated by the measurement of cardiolipin content in

the mitochondria by staining with 10-N-nonyl acridine orange [NAO] (Molecular Probes) as described previously (*Nomura et al., 2000*). One hundred nanomolars of NAO was added to the mitochondrial preparation, incubated for 30 min, and NAO binding was determined with fluorescence activated cell sorter analysis.

12. Construction of HIF-1α dominant negative mutant

Mouse HIF1α cDNA, full length, in pSPORT expression vector (CMV, NotI-SalI) and the plasmid template were mixed and amplified by SP6 reverse primer (5'-GGCCTATTTAGGTGACACTA-3') and HIF1α internal primer encompassing amino acids 760 to 768 along with an inserted Sph1 site (5'-GTCTGCATGCTAAAATCCTTT-CACTCGTTTCCAG-3'), using VentR DNA polymerase (New England Biolab, Ipswich, MA). The amplified DNA fragment was digested by EcoRI and SphI, ligated back to the parental plasmid also digested with EcoRI and SphI. The resultant plasmid thus contained the HIF1α cDNA encoding amino acids 1–768, including the nuclear localization signal, the 5'-UTR, and the entire 3'UTR downstream of the SphI site. It was devoid of the amino acids 769 to 836 containing the C-terminal transactivation domain. The efficacy of the emutant was tested by using one C2C12 cell line stably transfected with a HIF1α (5 X) responsive promoter-reporter (luciferase) construct. Cells were grown in 60 mm dishes and transfected either with 2 μg empty vector DNA or the dominant negative construct, and cell lysates were assayed for luciferase activity.

13. Statistical analysis

Values for myocardial functional parameters, MDA, GSH/GSSG ratio, enzymes activities, apoptotic cardiomyocytes, and infarct sizes are expressed as the mean ± standard error of the mean (SEM). In the Prdx6-/- study, the statistical analysis was performed by one-way ANOVA for any differences between the mean values of all groups. Differences between data were analyzed for significance by performing Student's t-test.

In the Glrx2 and dexamethasone study, one-way analysis of variance was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of all groups were compared with those of the drug-free control group by multiple t-test followed by Bonferroni correction. Because the incidence of reperfusion-induced ventricular fibrillation followed a nonparametric distribution, therefore chi-square test was used for the statistical analysis of VF.

In the PR39 study, all parameters are also expressed as the mean ± standard error of the mean (SEM). Analysis of variance test was first carried out, followed by Bonforni's correction, to test for any differences between the mean values of all groups. If differences between groups were established, the values of the treated groups were compared with those of the control group by a modified *t*-test. The results were considered significant if $p < 0.05$.

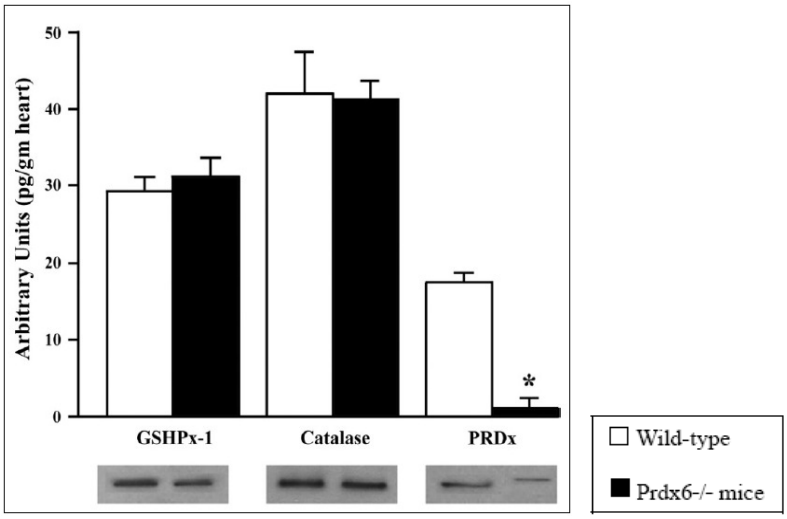
RESULTS

I. Part: Role of the Prdx6 in the ischemia-reperfusion injury

1.1 Characterization of Prdx6 $^{-/-}$ mice

The Western blot was performed for the detection of glutathione peroxidase-1 (GSHPx-1), catalase, and peroxiredoxin 6 (Prdx6) proteins. As shown in Fig. 2, Prdx6 $^{-/-}$ mouse hearts had no expression of Prdx6 while the wild-type mouse hearts possessed significant amount of Prdx6. Similar to GSHPx, Prdx6 scavenges both hydroperoxides and H_2O_2 (the latter can also be removed by catalase); it is thus presumed that Prdx6, GSHPx, and catalase together make an antioxidant module. We thus tested whether deletion of Prdx6 had any effects on catalase and GSHPx activities. Noticeably, in Prdx6 $^{-/-}$ mouse heart GSHPx-1 and catalase levels remained unchanged vis-a`-vis the wild-type hearts (Fig. 2.).

Fig. 2. *Western blot analysis for the detection of glutathione peroxidase-1 (GSHPx-1), catalase, and peroxiredoxin 6 (Prdx6) proteins.*

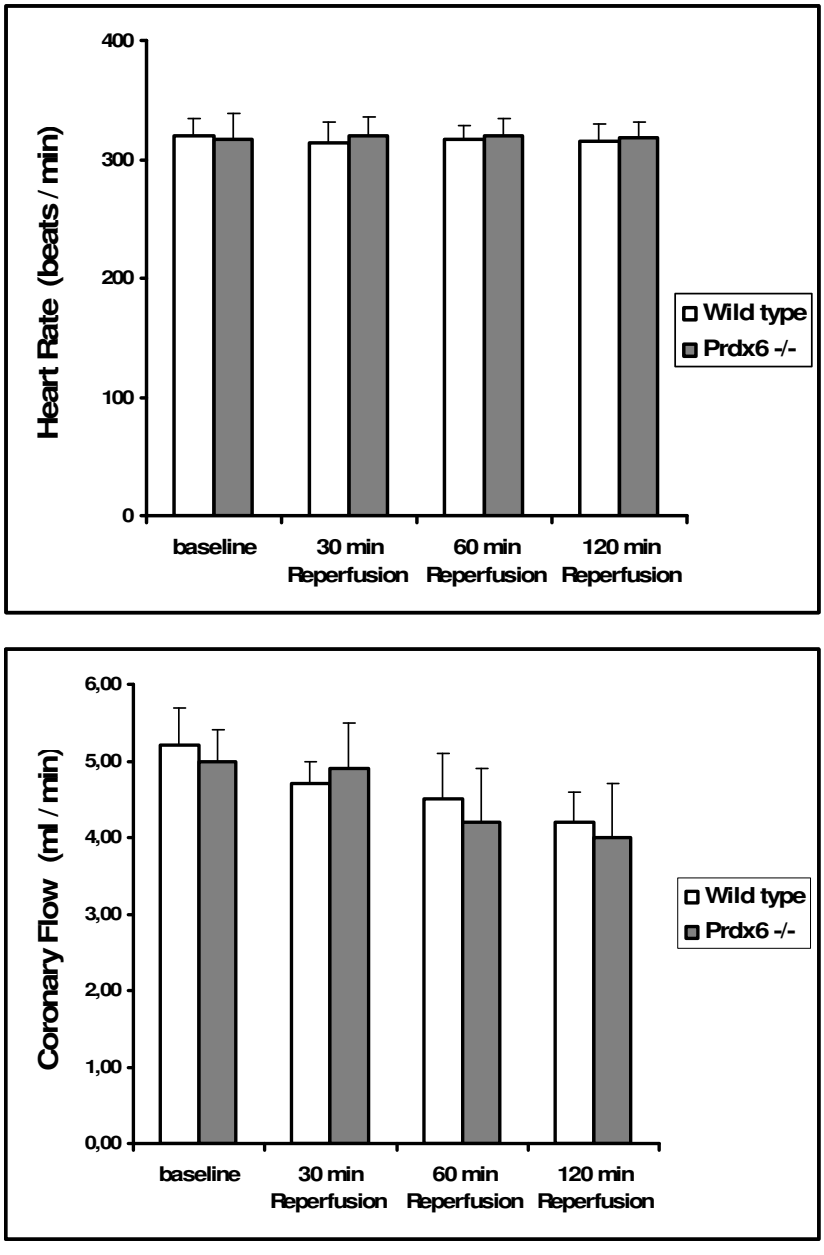


The results of densitometric scanning are shown on the top of the protein blots. Results are expressed as means ± SEM of 3 separate hearts per group. Prdx6 was not detected in the Prdx6$^{-/-}$ mouse hearts. *$p < 0.05$ vs. wild type.

1.2 Effects of ischemia-reperfusion on the left ventricular function of wild-type and Prdx6⁻/⁻ mouse hearts

Throughout the study, the heart rates and coronary flows were not different between the two groups (Fig. 3.), suggesting that Prdx6⁻/⁻ had no effects on these parameters.

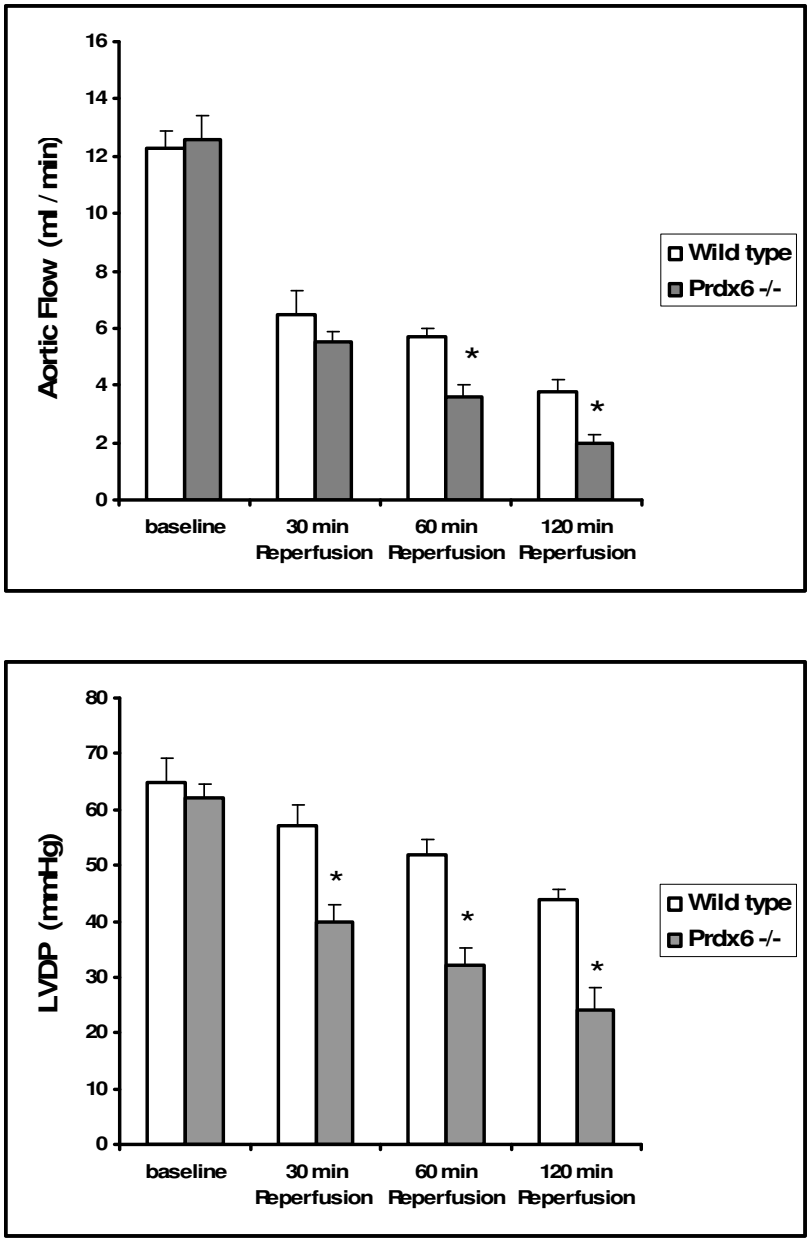
Fig 3. *Effects of Prdx6-/- on Heart Rate and Coronary Flow*

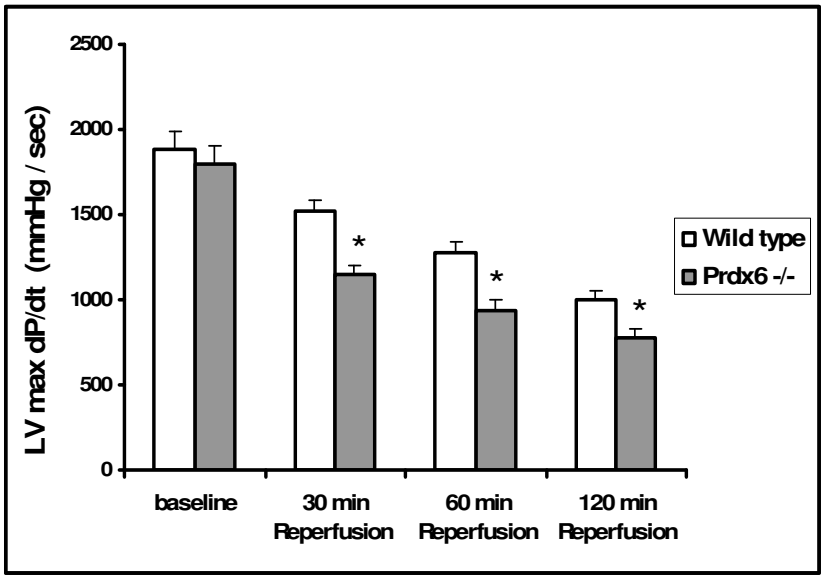


Results are expressed as means ± SEM of n = 6 animals per group.

The aortic flow (AF) and LVDP, as well as the maximum first derivative of LVDP, were significantly lower in the Prdx6$^{-/-}$ mouse hearts vs. wild-type hearts during the entire reperfusion period, except for AF, which was significantly lower only at 60 min (3.6 ± 0.4 ml/min vs. 5.7 ± 0.3 ml/min) and 120 min of reperfusion (2.0 ± 0.3 ml/min vs. 3.8 ± 0.4 ml/min) (Fig 4).

Fig 4. *Effects of Prdx6-/- on aortic flow (AF) and LVDP, as well as the maximum first derivative of LVDP*





Results are expressed as means ± SEM of n = 6 animals per group. LVDP, left ventricular developed pressure; $LV_{max}dP/dt$, maximum first derivative of LVDP; Prdx6$^{-/-}$ mice, peroxiredoxin 6 gene knockout mice. *$p < 0.05$ vs. wild type.

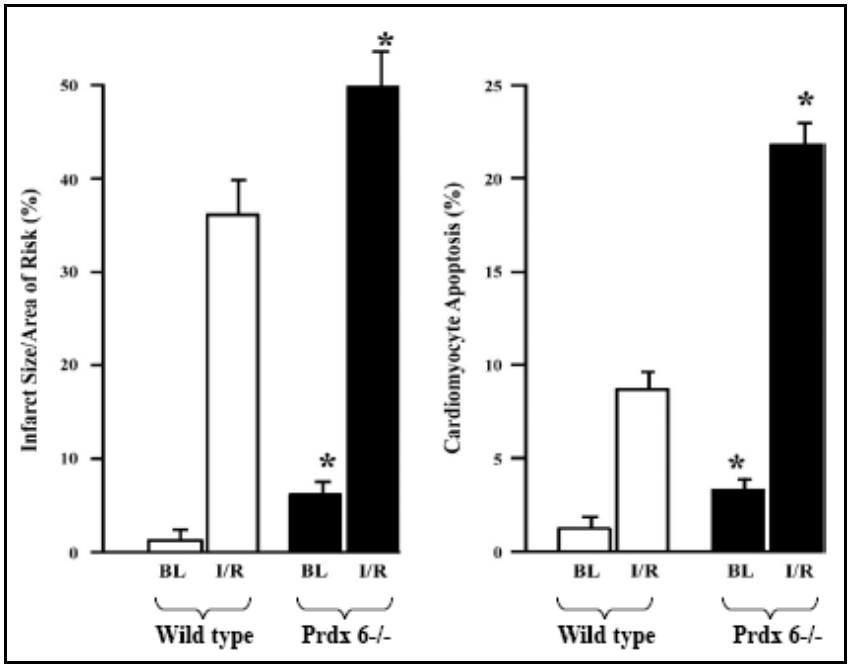
1.3 Myocardial infarction and cardiomyocyte apoptosis

Myocardial infarct size expressed as infarct size/area of risk was significantly higher for the Prdx6$^{-/-}$ mouse hearts compared with that in wild-type controls (Fig. 5, *left*). However, both groups had a similar level of area of risk. Myocardial infarcted tissue was scattered throughout each ventricle in each heart from both groups. The white area that was not stained by TTC indicated irreversible ischemic injury. Mean value of infarct size in the Prdx6$^{-/-}$ group was 49.9 ± 1.7% vs. 36.5 ± 1.4% for the wild-type group.

The apoptotic cardiomyocytes visualized by double-antibody staining (TUNEL in conjunction with a myosin heavy chain to detect myocytes) were present in significantly higher quantities (21.5 ± 0.9%) in the Prdx6$^{-/-}$ group compared with the wild-type (8.5 ± 0.8%) group (Fig. 5, *right*).

Infarction was not developed or apoptosis was not detected in the hearts perfused for the same time period without subjecting them to ischemia-reperfusion (results not shown).

Fig. 5. *Myocardial infarct size* (left) *and cardiomyocyte apoptosis* (right)



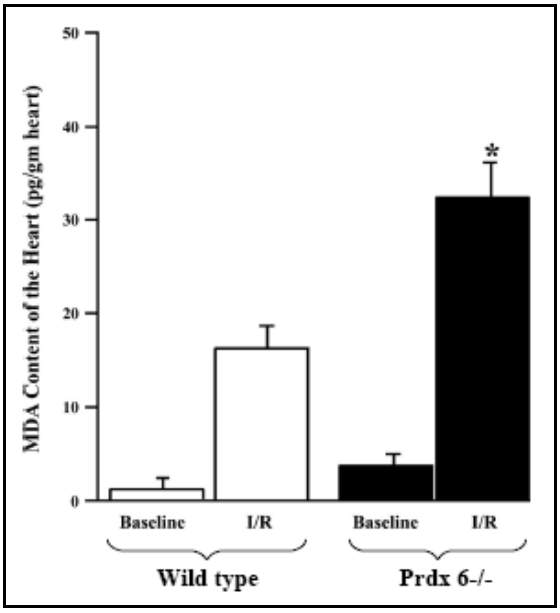
Results are shown as means ± SEM of 6 animals per group. Open bars, wild-type mice. Filled bars, Prdx6$^{-/-}$ mice. BL, baseline; I/R, ischemia-reperfusion. *$p < 0.05$ vs. wild-type.

1.4 ROS activity and oxidative stress

MDA is the presumptive marker for lipid peroxidation and oxidative stress developed from ROS generated during the reperfusion of ischemic myocardium.

MDA content of the heart determined at the end of each experiment showed significantly higher amount of MDA (39.6 ± 0.8 pg/g) compared with that in hearts from the wild-type controls (26.0 ± 0.9 pg/g), indicating development of higher amount of oxidative stress in the Prdx6$^{-/-}$ hearts (Fig. 6).

Fig. 6. *Malonaldehyde (MDA) formation* in the hearts of wild-type (open bars) and Prdx6$^{-/-}$ mice (filled bars) at baseline and at the end of ischemia/ reperfusion.



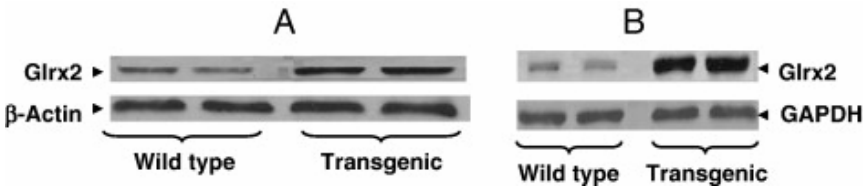
Results are shown as means ± SEM of 6 animals per group. *$p < 0.05$ vs. wild-type.

II. Part: Role of the Glrx-2 in the cardioprotection

2.1. Characterization of the transgenic mice overexpressing Glrx2 in the mitochondria of cardiomyocytes

A more detailed description on generation and characterization of the human Glrx2 transgenic mice will be documented elsewhere (Diotte et al., unpublished data). Briefly, the human Glrx2 transgene driven by the 5′ flanking sequence and promoter of the mouse α-MyHC gene is mainly expressed in the heart and to a much lesser extent in lungs of transgenic mice. Since the human Glrx2 cDNA contained in the transgene codes for a mitochondrial Glrx2 protein, we measured Glrx activity in homogenates of isolated heart mitochondria. The specific Glrx activity was increased by 192% in heart mitochondria of Glrx2 transgenic mice compared to that of non-transgenic mice (13.7± 0.9 vs. 4.7±0.9 nmol NADPH/min/mg protein, respectively. $p < 0.001$, $n > 5$).

Fig. 7. (Left) *Western blot analysis and* (right) *Northern blot analysis.*



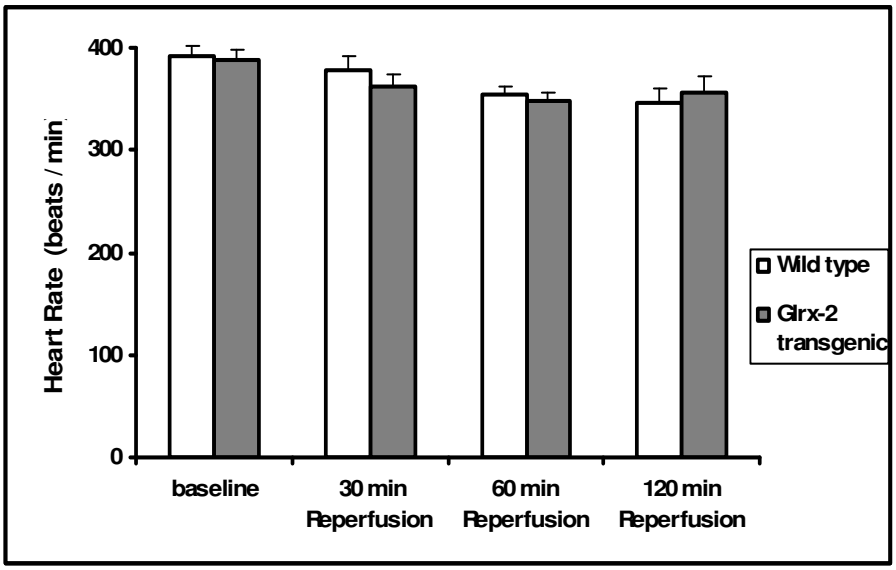
Homogenates of mouse ventricles were electrophoresed, transferred, and probed with antibodies against β-actin and glutaredoxin-2. One hundred micrograms of protein was loaded for each sample. In the transgenic mice a significant increase in the amount of glutaredoxin-2 occurred in the heart.

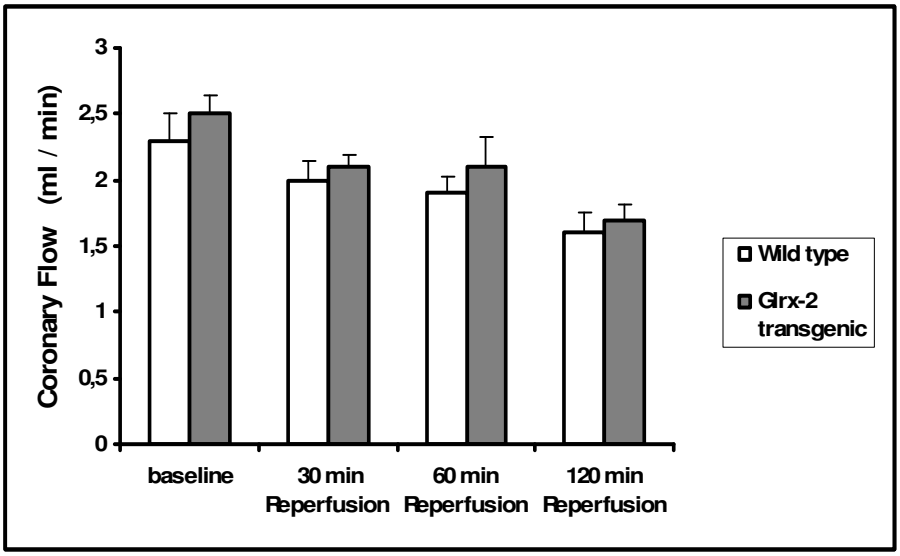
For the Northern blot analysis RNA samples were isolated from mouse heart ventricle and subsequently hybridized to 32P-labeled probes corresponding to glutaredoxin-2 and GAPDH used to demonstrate loading controls. In the transgenic mice a significant increase in the amount of glutaredoxin-2 occurred in the heart.

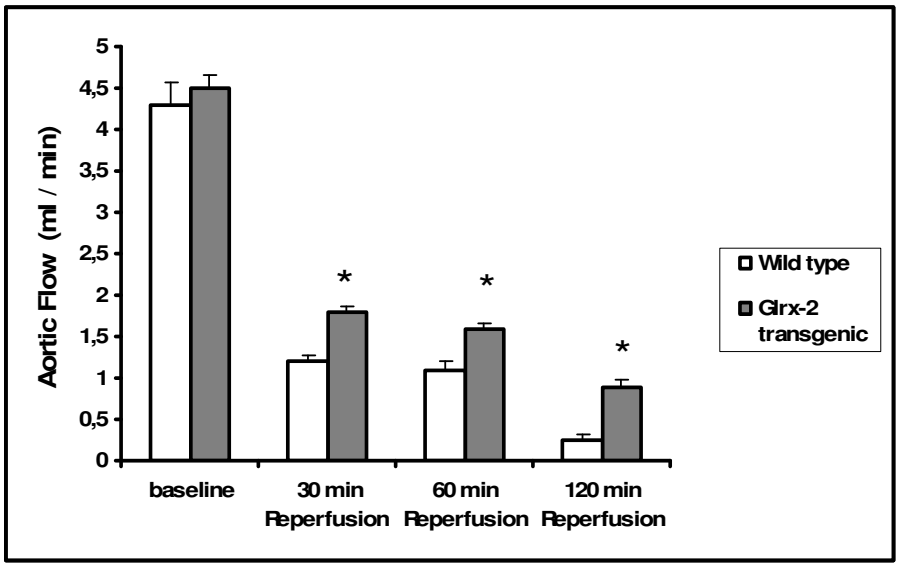
2.2. Recovery of myocardium contractile performance

In all groups the cardiac function including aortic flow (AF), LVDP, and LVdp/dt were significantly depressed during the progression of reperfusion. The cardiac function consistently displayed improved recovery (except for coronary flow) for the hearts from Glrx2 transgenic mice as compared to that of wild type mice. (Fig. 8) Aortic flow (AF), LVDP, and LVdp/dt are the function of cardiomyocytes while coronary flow is the function of endothelial cells. Thus, these results would tend to suggest that Glrx2 overexpression improved myocyte function without significantly affecting endothelial cell function.

Fig. 8. *Recovery of myocardium contractile performance in Glrx-2 transgenic mice as compared to wild type mice*




Coronary Flow (ml / min)
3
2,5
2
1,5
1
0,5
0
baseline
30 min Reperfusion
60 min Reperfusion
120 min Reperfusion
Wild type
Glrx-2 transgenic


Aortic Flow (ml / min)
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
*
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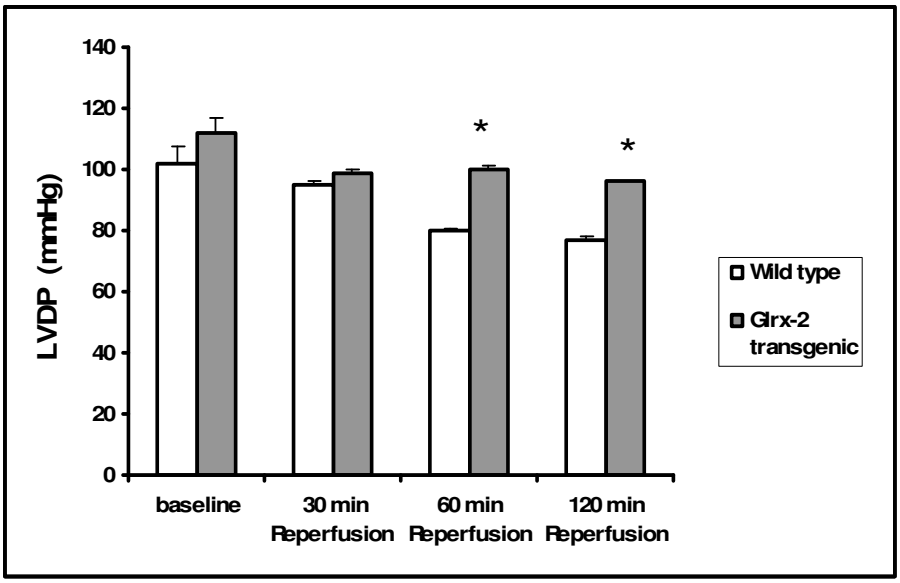
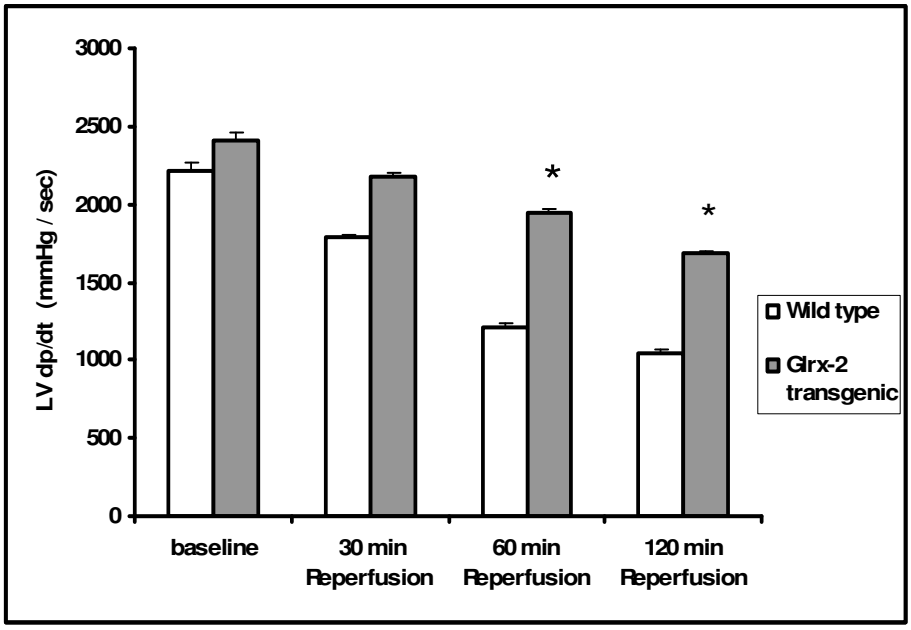
baseline
30 min Reperfusion
60 min Reperfusion
120 min Reperfusion
Wild type
Glrx-2 transgenic


LVDP (mmHg)
140
120
100
80
60
40
20
0
*
*
baseline
30 min Reperfusion
60 min Reperfusion
120 min Reperfusion
Wild type
Glrx-2 transgenic



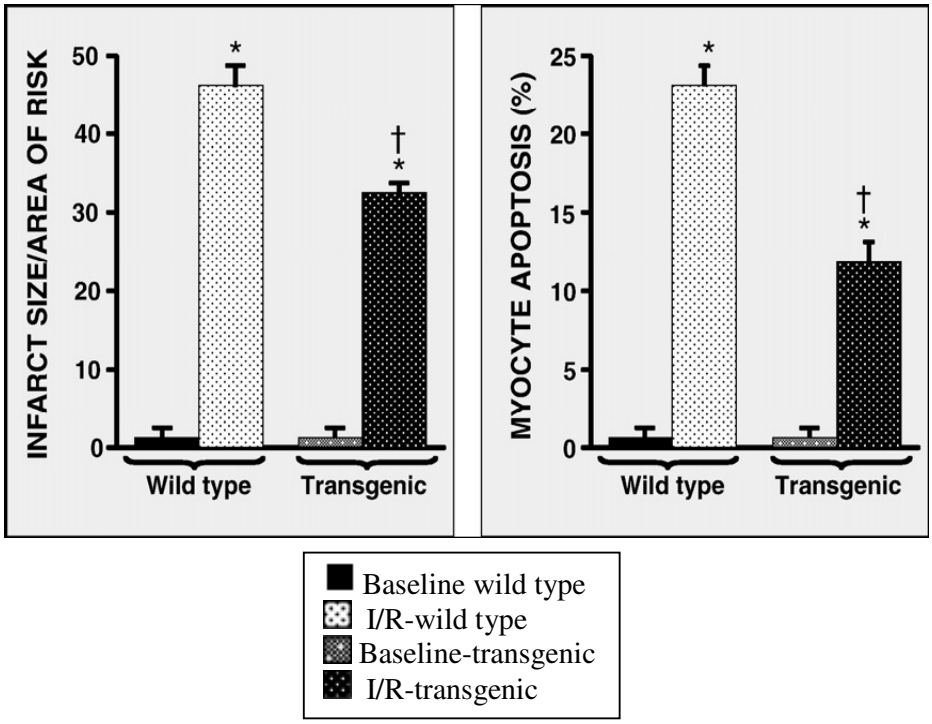
Results are expressed as means ± SEM of $n = 6$ animals per group. LVDP, left ventricular developed pressure; LVdP/d*t*, first derivative of LVDP; Glrx2 transgenic mice: Glutaredoxin-2 transgenic mice. *$p < 0.05$ vs. wild type (non-transgenic mice).

2.3. Myocardial infarct sizes and cardiomyocyte apoptosis in wild type and Glrx2 transgenic mice

Infarct size expressed as percent infarction to total area at risk was noticeably decreased in Glrx2 transgenic mouse hearts (32.5 ± 1.3%) compared to the wild type control (46.2 ± 2.6%) (Fig. 9, left). There was no infarction if these hearts were perfused for the same time period (time-matched) without subjecting them to ischemia/reperfusion.

Cardiomyocyte apoptosis (Fig. 9, right) followed an identical pattern. The apoptotic cardiomyocytes were present in significantly lower quantities in the Glrx2 transgenic mouse hearts (12.1 ± 1.2%) compared to the wild-type (23.0 ± 1.1%) group.

Fig. 9. *Myocardial infarct size* (left) *and cardiomyocyte apoptosis* (right) of the hearts from wild type and Glrx2 transgenic and knockout mice subjected to ischemia/ reperfusion.



Results are shown as means ± SEM of six hearts per group. *$p < 0.05$ vs. baseline; $^{\dagger}p < 0.05$ vs.wild type.

2.4. Effects of Glrx2 overexpression on cytochrome-c release and caspase activation

To specifically determine the role of mitochondria of cardiomyocyte apoptosis, immunoblots of cytochrome-*c* were examined. A significant increase in cytochrome-*c* was found after ischemia/reperfusion in the hearts of wild type animals, but this amount did not increase in the hearts of Glrx2 overexpressed hearts (Fig. 10).

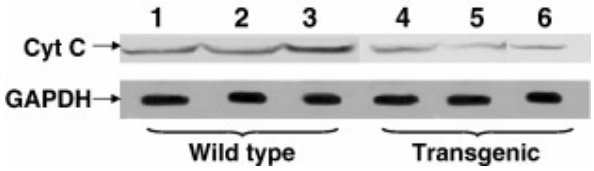
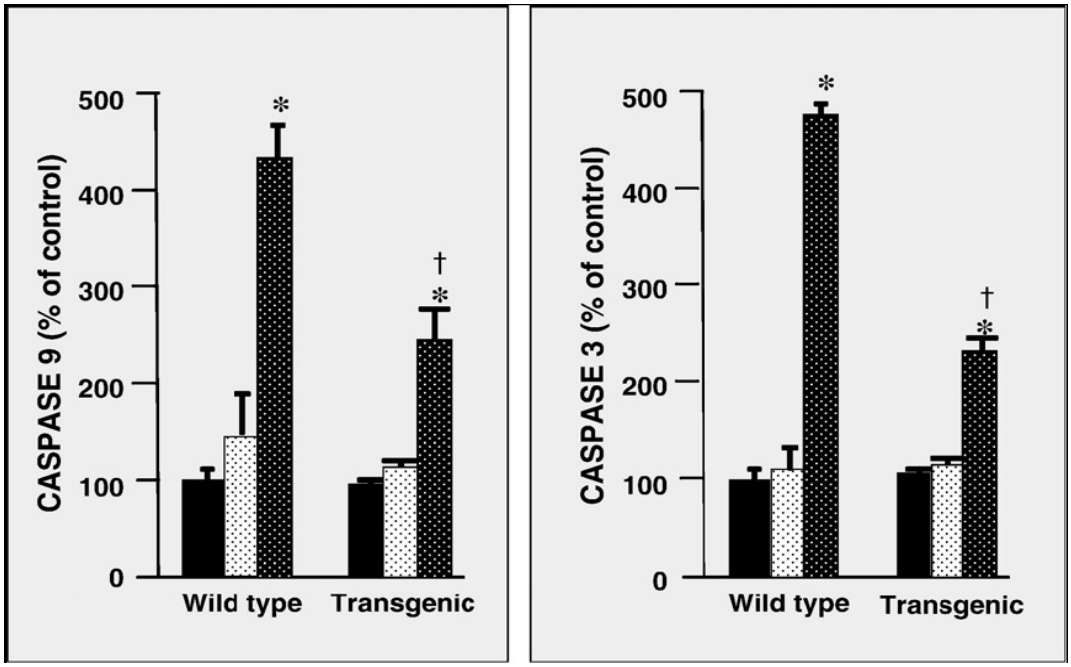


Fig. 10. *Western blot analysis cytochrome-c protein.*

Cytosolic fraction from mouse ventricles was electrophoresed, transferred, and probed with antibodies against cytochrome-*c* and GAPDH. In the transgenic mice, significantly reduced amount of cytochrome-*c* was found. 1: baseline-wild type; 2, 3: I/R-wild type; 4: baseline transgenic; 5, 6: I/R-transgenic.

Caspases measured with synthetic caspase substrates DEVD-pNA and LEHD-pNA revealed an increase in both caspase 3 (right) and caspase 9 (left) after the reperfusion of the ischemic hearts (Fig. 11). There was no increase in caspase activities after 30 min of ischemia in any of the hearts. The amount of increase in caspase activities was less for the Glrx2 overexpressed hearts compared to wild type hearts.

Fig. 11. *Activities of caspase 3 and caspase 9.*

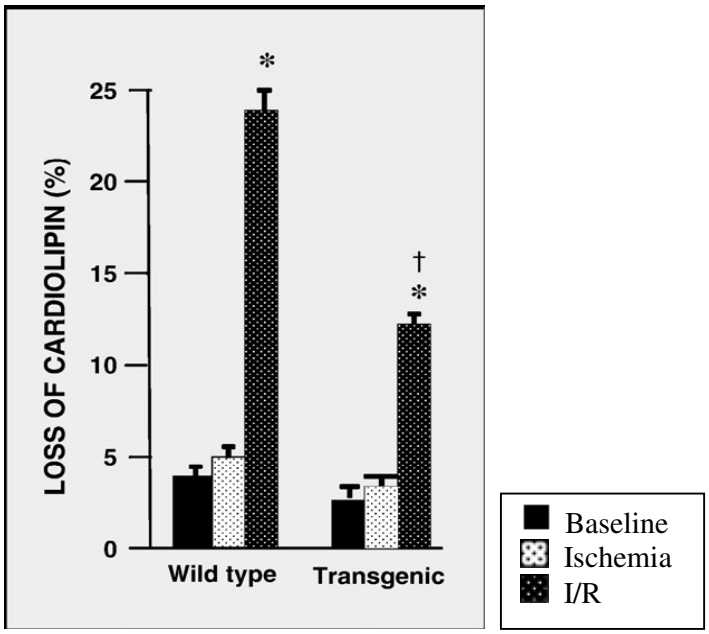


Results are shown as means ± SEM of six hearts per group. *p<0.05 vs. baseline; †p<0.05 vs. wild type. Baseline ■, ischemia ▩, I/R ▩.

2.5. *Effects of Glrx2 overexpression on cardiolipin content of the heart*

Since binding of cytochrome-*c* to the inner mitochondrial membrane is known to involve mitochondrial phospholipid, cardiolipin, we determined the effects of Glrx2 overexpression on the mitochondrial cardiolipin content as shown in Fig. 12.

Fig. 12. *Loss of cardiolipin from the inner membrane of the mitochondria.*



Results are shown as means ± SEM of six hearts per group. *p<0.05 vs. baseline; †p<0.05 vs. wild type.

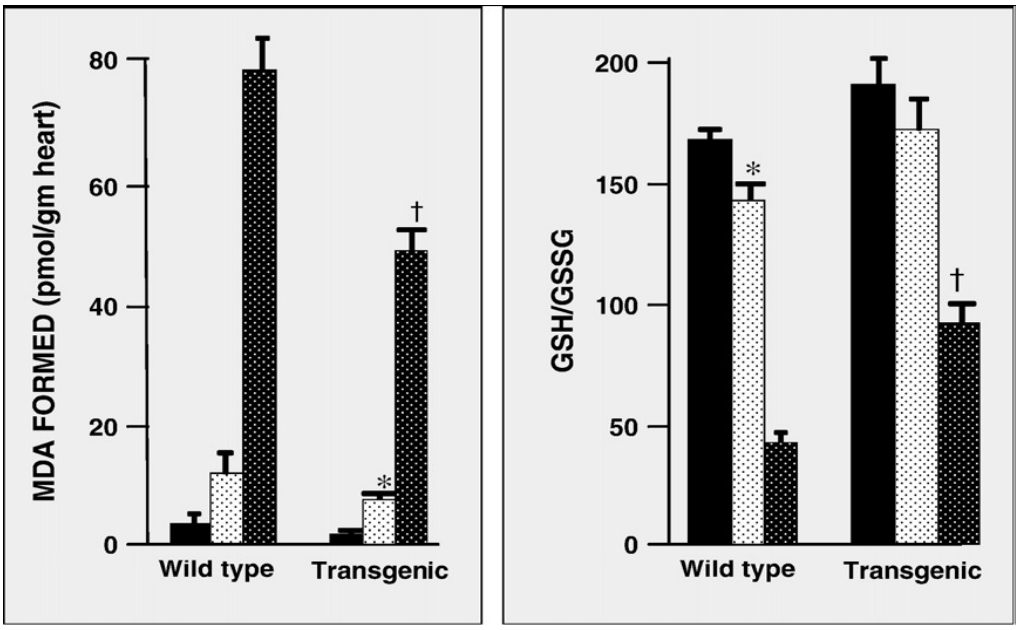
Ischemia/reperfusion induced significant loss of cardiolipin from the mitochondria; however, such loss of cardiolipin was significantly less in the Glrx2 overexpressed hearts compared to wild type controls.

2.6. Effects of Glrx2 overexpression ischemia /reperfusion-induced oxidative stress

We determined the amount of oxidative stress in the heart by measuring MDA content of the mouse hearts. MDA content was increased progressively and steadily as a function of the reperfusion time in all groups of hearts (Fig. 13, left). MDA content was significantly reduced at 60 min and 120 min of reperfusion in the Glrx2-overexpressing hearts compared to wild type controls.

The ratio of GSH/GSSG (Fig. 13, right) followed a similar pattern. Glrx2 overexpression increased GSH/GSSG ratio in ischemia reperfused hearts compared to that of wild type hearts.

Fig. 13. *Malonaldehyde content* (left) *and GSH/GSSG ratio* (right) of the hearts from wild type and Glrx2 transgenic mice subjected to ischemia/reperfusion.



The hearts were collected at the indicated times and MDA content, GSH and GSSG were determined as described earlier. Results are shown as means ± SEM of at least 4 hearts per group per time point. *$p<0.05$ vs. baseline; $^{\dagger}p<0.05$ vs. wild type. Baseline ■, ischemia ▩, I/R ▩.

2.7. Glrx2-mediated survival signals in the heart

Having confirmed cardioprotective role of Glrx2 in the heart, we attempted to determine the pattern of survival signal generated by Glrx2. We first examined if Glrx2 could induce the activation of Akt, the well-known component of survival signaling. As shown in Fig. 14, the Western blots revealed no changes in the amount of Akt after ischemia or reperfusion. The phosphorylation of Akt also remained unaltered after ischemia or reperfusion; however, significantly higher extent of Akt phosphorylation was noticed in Glrx2 overexpressed hearts.

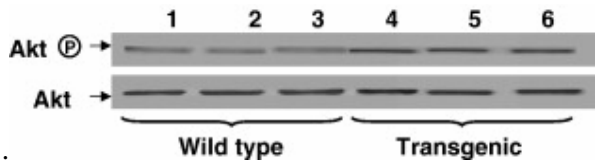


Fig. 14. *Effects of ischemia/reperfusion on the phosphorylation of Akt.*

The hearts were collected at the end of each experiment and Western blots were performed with specific antibodies against Akt or phospho-Akt. Akt served as control. Representative blots (of three experiment per group) are shown. *1, 2, 3*: wild type; *4, 5, 6*: transgenic. *1, 4*: baseline; *2, 5*: ischemia; *3, 6*: I/R.

The amount of proapoptotic Bax increased significantly after ischemia and reperfusion in the wild type hearts as compared to Glrx2 transgenic hearts (Fig. 15). The anti-apoptotic protein Bcl-2 was reduced significantly in both wild type and Grx2+/+ heart, but the amount of Bcl-2 remained much higher in the Glrx2 overexpressed hearts. The ratio of Bcl2/Bax remained significantly higher after ischemia/reperfusion in the Glrx2 overexpressed hearts.

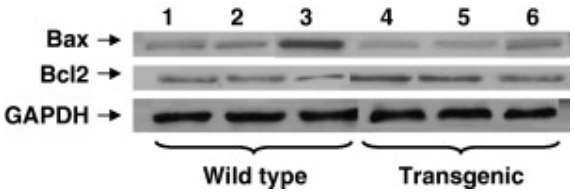


Fig. 15. *Effects of ischemia/reperfusion on the expression of Bax and Bcl-2.*

The hearts were collected at the end of each experiment and Western blots were performed with specific antibodies against Bax or Bcl-2. GAPDH served as control. Representative blots (of three experiments per group) are shown. *1, 2, 3*: wild type; *4, 5, 6*: transgenic. *1, 4*: baseline; *2, 5*: ischemia; *3, 6*: I/R.

We also measured the nuclear binding of the redox-sensitive transcription factor NFκB, which is known to be regulated by Glrxs. As shown in Fig. 16, NFκB binding activity increased for both groups after ischemia and further increased after reperfusion. The greater NFκB binding activities was noticed in the Glrx2 overexpressed hearts compared to wild type hearts.

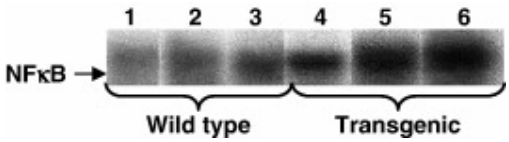


Fig. 16. *Effects of ischemia/reperfusion on the NFκB binding activity in the glutaredoxin transgenic heart.*

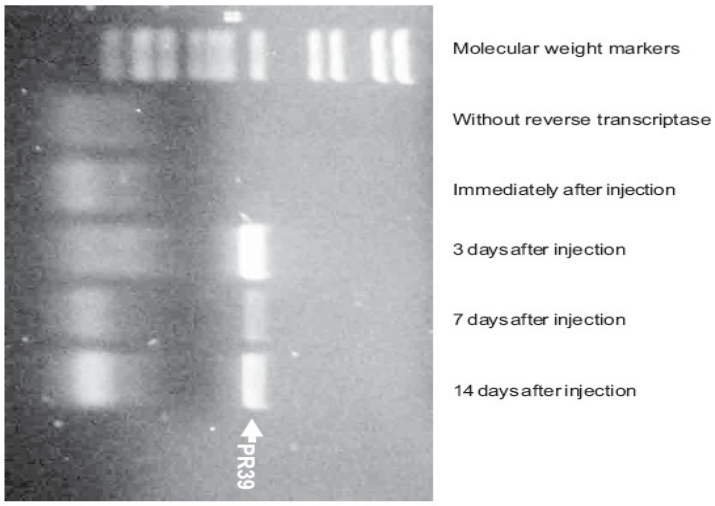
Nuclear extracts were isolated from wild type and transgenic hearts. These extracts were used for electrophoretic mobility shift assay as described in the Materials and methods section. *Lanes 1, 4*: baseline; *2, 5*: ischemia; *3, 6*: I/R. Results are representative of three experiments per group.

III. Part: Role of the PR39 gene therapy in the cardioprotection

3.1. Time course of PR-39 expression

As shown in Fig. 17. there was no PR39 expression immediately after injection, but robust expression of the transcript was found at all later time points. The complete absence of PR-39 immediately after injection is in concordance with the fact that PR-39 has not been identified in mice to date, and is in line with the absence of the PR-39 sequence in the mouse genome.

Fig. 17. *PR39 expression after intramyocardial injection of AdPR39 in normal mouse hearts.*



Lane 1: molecular weight markers. *Lane 2*: PCR reaction without reverse transcriptase. *Lane 3*: no PR39 expression immediately after injection (n = 3); robust PR39 expression at 3 days (n = 3, *lane 4*), 7 days (n = 3, *lane 5*), and 14 days (n = 3, *lane 6*) after injection.

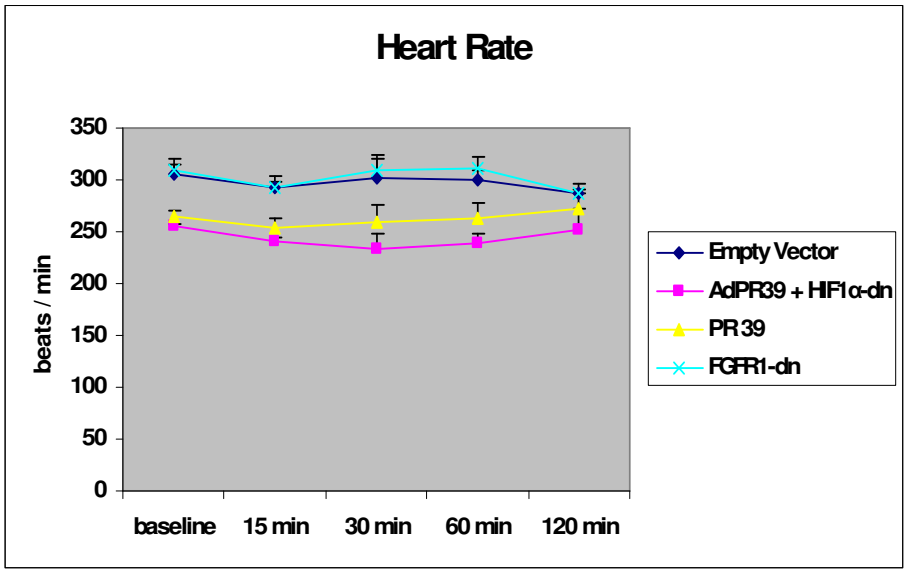
A left lateral thoracotomy was performed and adenoviral constructs encoding PR39 were administered as a single injection of 109 p.f.u. in 20 µl phosphate buffered saline. A

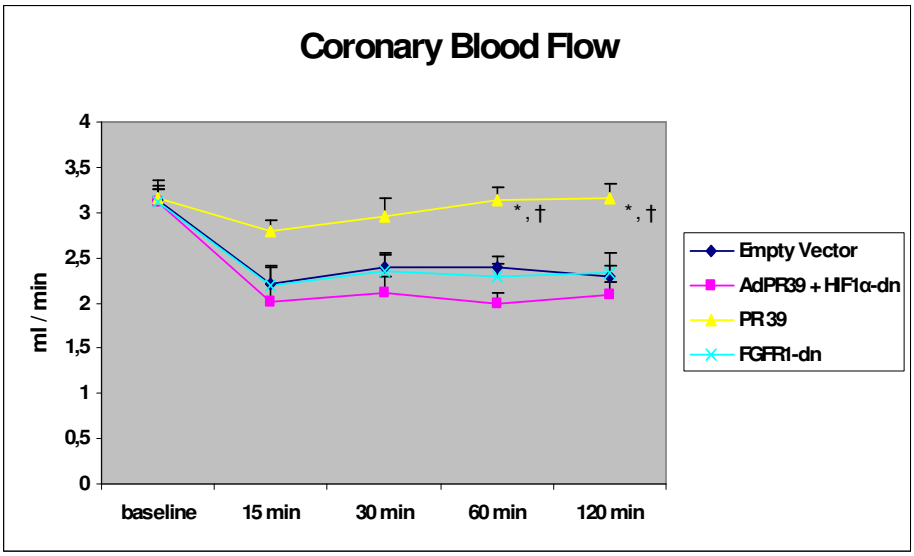
total of 12 mice were injected, and 3 mice were euthanized at each of the following time points: immediately after injection, and 3, 7, and 14 days after injection. The hearts were excised and placed in RNAlater. After isolation of RNA, expression of PR39 was assayed by RT-PCR.

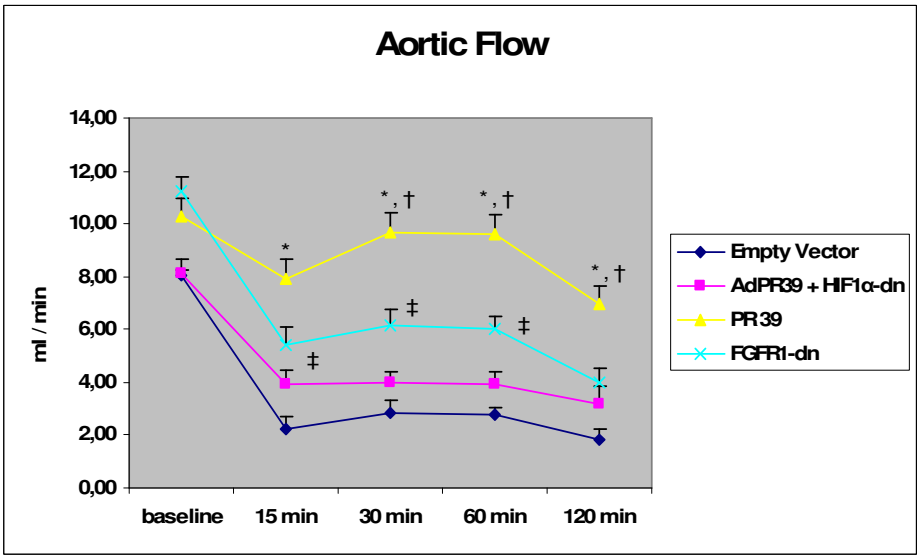
3.2 *Effects of PR-39 and dnFGFR1 gene therapy on ventricular recovery, infarct size, and cardiomyocyte apoptosis*

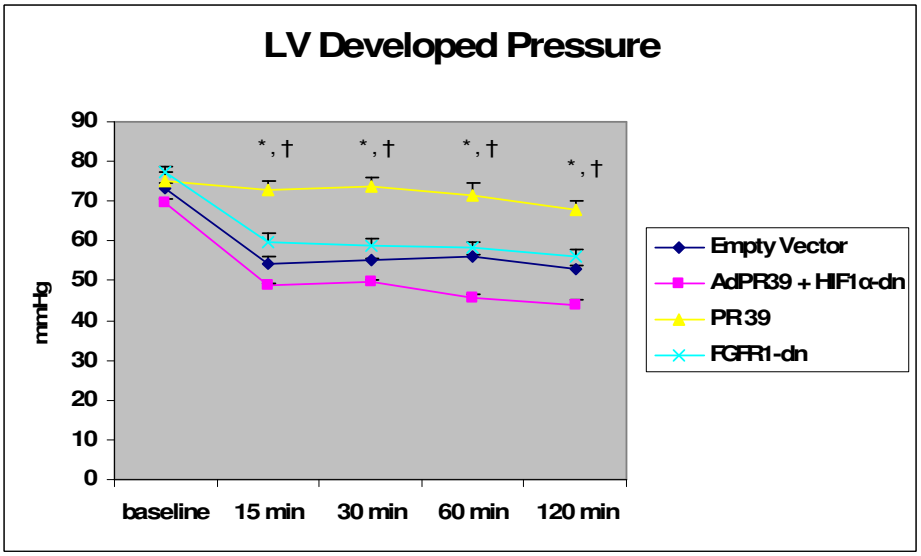
At baseline the haemodynamics parameters are comparable in all groups, there were no differences between groups, and heart rate remained the same in all groups throughout the experiment. Following I–R, in AdPR-39 hearts, aortic flow, LVDP, and LVdp/dt were maintained at baseline levels, whereas all values dropped significantly after I–R in the other groups (Fig. 18), except for aortic flow in the AdFGFR1-dn group, which remains significantly higher in the AdFGFR1-dn group than in the AdEV and the AdPR39 + HIF1α-dn control groups. The difference in aortic flow between AdPR39 and both control groups (AdEV and AdPR39 + HIF1α-dn) was statistically significant, whereas LVDP and LVdp/dt were significantly lower compared to AdPR39 hearts in all other groups (Fig. 18).

Fig. 18. *Hemodynamic effects of PR39 and FGFR1-dn gene therapy,* before 20 min cardiac ischemia and during 2 h reperfusion in *ex vivo* isolated working mouse hearts.




Coronary Blood Flow
ml / min
4
3,5
3
2,5
2
1,5
1
0,5
0
baseline
15 min
30 min
60 min
120 min
*, †
*, †
Empty Vector
AdPR39 + HIF1α-dn
PR39
FGFR1-dn


Aortic Flow
ml / min
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
baseline
15 min
30 min
60 min
120 min
*
*, †
*, †
*, †
‡
‡
‡
Empty Vector
AdPR39 + HIF1α-dn
PR39
FGFR1-dn


LV Developed Pressure
mmHg
90
80
70
60
50
40
30
20
10
0
baseline
15 min
30 min
60 min
120 min
*, †
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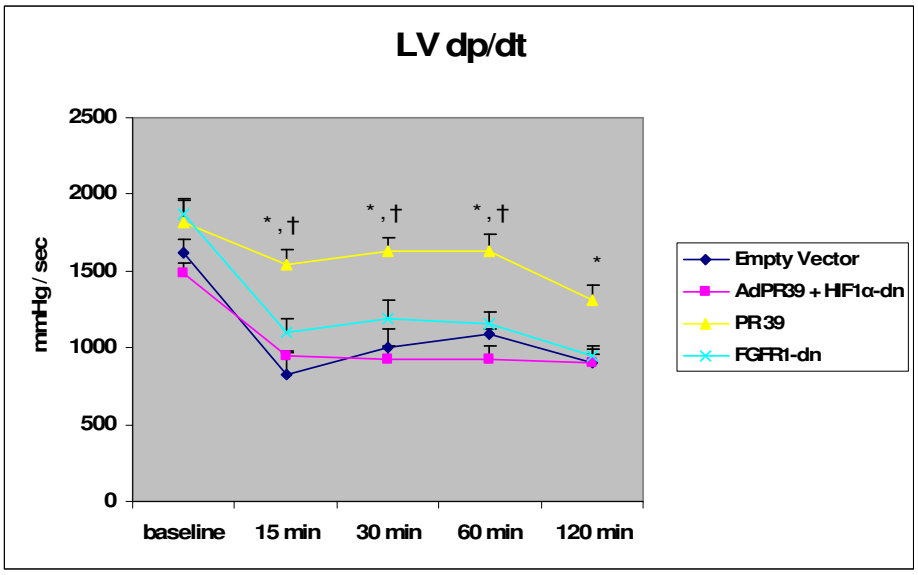
Empty Vector
AdPR39 + HIF1α-dn
PR39
FGFR1-dn



Results are expressed as means ± SEM of six animals per group. LV: left ventricle. *AdPR39 *vs*. AdEV and AdPR39 + HIF1α-dn: $p < 0.05$; †AdPR39 *vs*. AdFGFR1-dn: $p < 0.05$; ‡ AdFGFR1-dn *vs*. AdEV and AdPR39 + HIF1α-dn: $p < 0.05$.

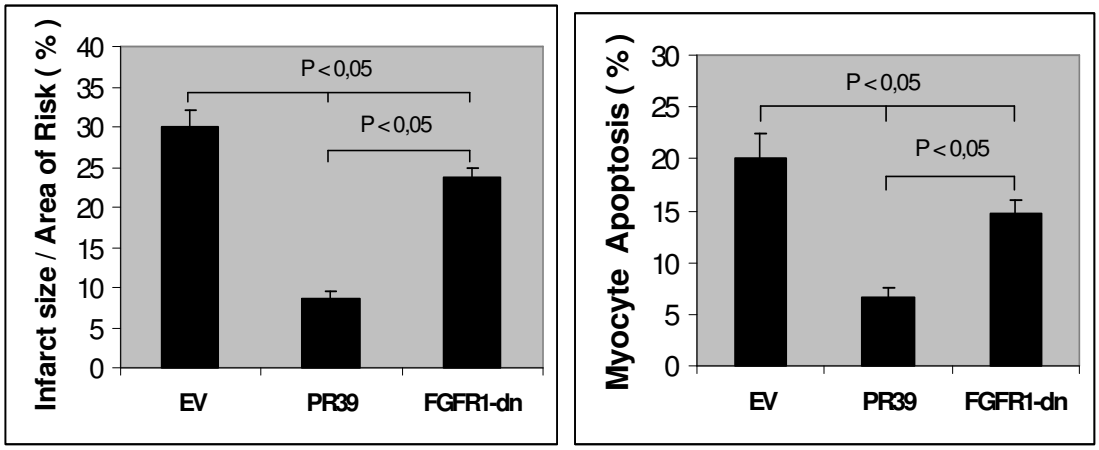
3.2.1 Effects of PR39 and FGFR1-dn gene therapy on MI size and cardiomyocyte apoptosis

MI was significantly smaller in the AdPR39 (8.7 ± 0.9 %), and AdFGFR1-dn (23.8 ± 1.1%) groups, compared to controls (AdEV 29.9 ± 2.2%, AdPR39 + HIF1α-dn 30.8 ± 2.7%). The MI size in the AdPR39 group was also significantly smaller compared to the other groups.

Cardiomyocyte apoptosis was also significantly reduced after AdPR39 and AdFGFR1-dn gene transfer (Fig. 19). In the AdPR39 group apoptosis was also significantly smaller compared to the AdFGFR1-dn group.

Interestingly, although the effect of FGFR1-dn gene transfer was clearly less pronounced than AdPR39 gene therapy, abrogation of FGFR1 signaling also conveyed cardioprotection after I–R. The hemodynamic profile of AdFGFR1-dn treated animals was more favorable than in the control group, infarcts were smaller, and cardiomyocyte apoptosis was reduced.

Fig. 19. *Effects of PR39 and FGFR1-dn gene therapy on myocardial infarct size and cardiomyocyte apoptosis.*



The results are expressed as means ± SEM of six animals per group. *EV*, group treated with adenoviral construct encoding empty vector; *FGFR1-dn*, group treated with adenoviral construct encoding dominant negative FGF receptor 1; *PR39*, group treated with adenoviral construct encoding PR39; *AdPR39 + HIF1α-dn*, group treated with adenoviral construct encoding PR39 and plasmid encoding dominant negative HIF1α.

3.3. Effects of PR-39 and dnFGFR1 gene therapy on the reduction of ROS activity

MDA formation is a presumptive marker for ROS activity in the biological system. PR-39 and FGFR1-dn gene therapy resulted in a significant reduction of ROS formation, as shown by significantly reduced the amounts of MDA compared to control group. In addition, the generation of ROS in the FGFR1-dn group was significantly greater than in the PR39 group (Fig. 20).

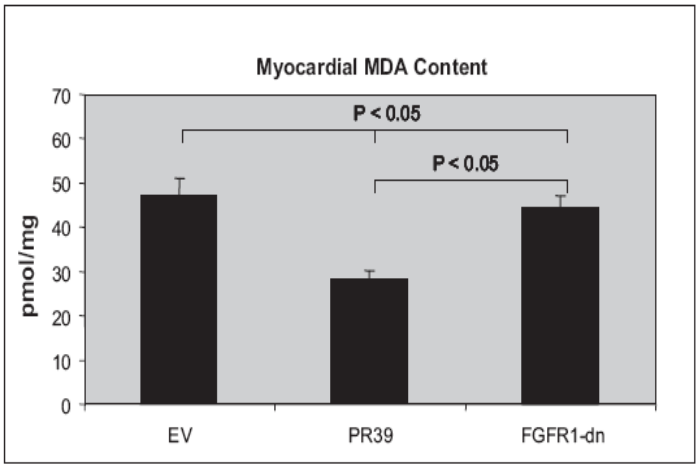


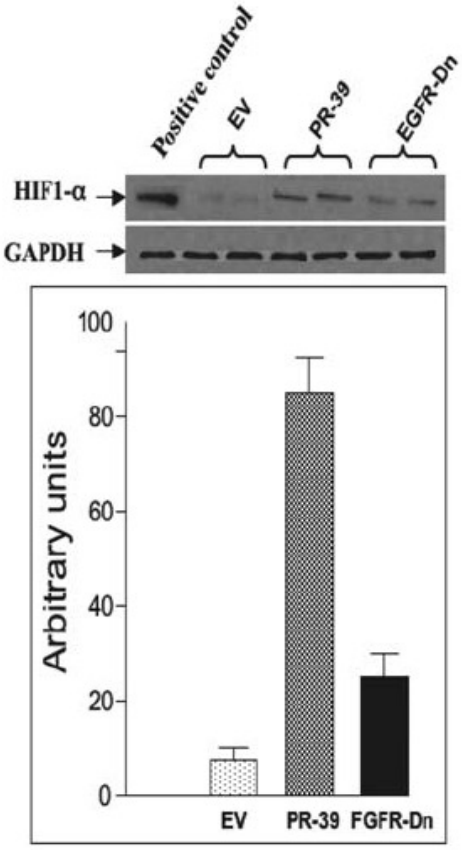
Fig. 20. *Effects of PR-39 and FGFR1-dn gene therapy on malonaldehyde (MDA) content of the heart.*

The results are expressed as means ± SEM of six animals per group. *EV*, group treated with adenoviral construct encoding empty vector; *FGFR1-dn*, group treated with adenoviral construct encoding dominant negative FGF receptor 1; *MDA*, malonaldehyde; *PR39*, group treated with adenoviral construct encoding PR39.

3.4. Effects of PR-39 and dnFGFR1 gene therapy on HIF1α expression

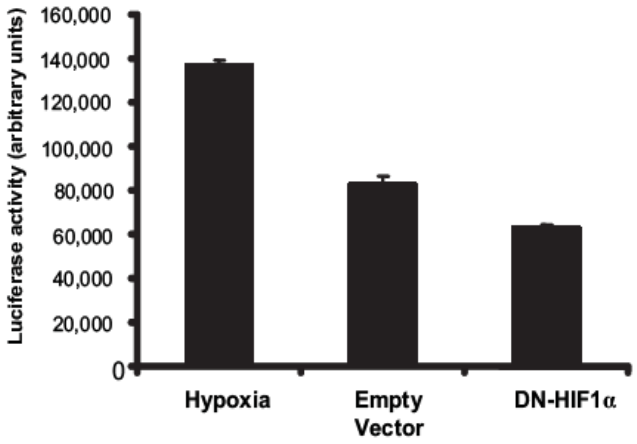
Western blot analysis of left ventricular myocardium showed increased levels of HIF1α protein in AdPR39 treated animals compared to animals treated with AdFGFR1-dn and AdEV (Fig. 21). Furthermore, the reduction in infarct size was abrogated when dnCMV-HIF1α was injected along with AdPR39 (AdPR39 + HIF1α-dn group), further confirming that the beneficial effect of PR39 was mediated by HIF1α.

Fig. 21. *Elevated myocardial HIF1α protein levels after PR39 gene therapy* but not after FGFR1-dn and EV transfer.



The results are expressed as means ± SEM of six animals per group. *EV*, group treated with adenoviral construct encoding empty vector; *FGFR1-dn*, group treated with adenoviral construct encoding dominant negative FGF receptor 1; *PR39*, group treated with adenoviral construct encoding PR39.

Fig. 22. *Confirmation of the dominant negative function of the HIF1α mutant.*



In non-transfected cells hypoxia caused about 20-fold induction of basal (normoxic) promoter activity. The results are expressed as means ± SEM of three experiments.

IV. Part: Role of the glucocorticoids against I-R induced injury

4.1 Effects of dexamethasone and actinomycin D preteatment on recovery of myocardium contractile performance

Table 1 shows no changes registered in heart function (heart rate, coronary flow, aortic flow, and left ventricular developed pressure) before the induction of ischemia in the dexamethasone (2 mg/kg), and actinomycin D (0.5 mg/kg) coadministered with dexamethasone (2 mg/kg) treated hearts in comparison with the drug-free control ischemic/reperfused values. A significant increase in aortic flow and left ventricular developed pressure were observed during reperfusion in the dexamethasone treated subjects. Thus, after 60 min of reperfusion, postischemic values of aortic flow and left ventricular developed pressure were significantly increased from their ischemic/reperfused control values of 10.7 ± 0.3 ml/min and 10.5 ± 0.3 kPa to 22.2 ± 0.3 ml/min ($p<0.05$) and 14.3 ± 0.5 kPa ($p<0.05$), respectively, in the dexamethasone treated group. The same improvement in the

recovery of aortic flow and left ventricular developed pressure was observed after 2 h of reperfusion in the dexamethasone treated myocardium. In rats treated with 0.5 mg/kg of actinomycin D, a protein synthesis inhibitor, injected i.v. 1 hour before the dexamethasone injection (2 mg/kg), suppressed the dexamethasone-induced cardiac protection in heart function in the isolated ischemic/reperfused myocardium. Actinomycin D alone did not change significantly cardiac function (heart rate, coronary flow, aortic flow, and left ventricular developed pressure) before the induction of ischemia and during reperfusion in comparison with the drug-free ischemic/reperfused control values.

Table 1. *Effects of dexamethasone pretreatment on ventricular recovery*

Parameter	Group	Baseline	Reperfusion	
			60 min	120 min
Heart Rate (beats/min)	I/R	309 ± 7	304 ± 2	300 ± 2
	DX + I/R	315 ± 8	303 ± 3	301 ± 2
	Act D + DX + I/R	312 ± 9	303 ± 1	300 ± 2
Coronary Flow,(ml/min)	I/R	27.0 ± 1.1	20.0 ± 0.5	17.8 ± 0.3
	DX + I/R	26.6 ± 1.0	20.5 ± 0.4	18.3 ± 0.3
	Act D + DX + I/R	26.3 ± 0.8	19.3 ± 0.5	17.5 ± 0.3
Aortic Flow (ml/min)	I/R	52.0 ± 1.5	10.7 ± 0.3	7.0 ± 1.2
	DX + I/R	51.4 ± 1.3	22.2 ± 0.3 *	19.3 ± 0.3 *
	Act D + DX + I/R	50.8 ± 1.1	10.3 ± 2.4	7.0 ± 0.4
LVDP (mmHg)	I/R	17.4 ± 0.4	10.5 ± 0.3	7.0 ± 0.4
	DX + I/R	17.8 ± 0.3	14.3 ± 0.5 *	12.3 ± 0.5 *
	Act D + DX + I/R	17.0 ± 0.5	10.6 ± 0.2	6.7 ± 0.4

n = 6 in each group, mean ± SEM, *p < 0.05 compared to the I/R group, HR: heart rate (beasts/min), CF: coronary flow (ml/min), AF: aortic flow (ml/min), LVDP: left ventricular developed pressure (kPa), I/R: ischemia/reperfusion.

4.2 Effects of dexamethasone and actinomycin D preteatment on reperfusion induced arrythmias

The ECGs were analyzed to determine the incidence of reperfusion-induced VF. Our data demonstrate that 24 hours dexamethasone pretreatment significantly reduced the incidence of reperfusion-induced VF (Fig. 23). Thus, in rats treated with 2 mg/kg of dexamethasone and hearts were isolated and subjected to 30 min ischemia followed by 2 h of

reperfusion, the incidence of reperfusion-induced VF was reduced from its control drug-free value of 100% to 33% ($p<0.05$). Actinomycin D completely interfered with the antiarrhythmic effect of dexamethasone, and in rats when actinomycin D was coadministered with dexamethasone, the incidence of reperfusion-induced VF was the same (100%) as we observed it in the drug-free ischemic/control group (Fig. 23).

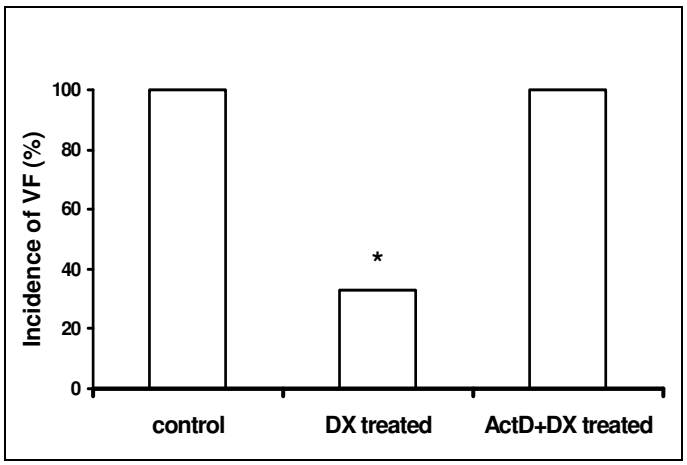


Fig. 23. *The effect of dexamethasone (DX) and actinomycin D coadministered with dexamethasone (ActD + DX) on the incidence (%) of reperfusion-induced ventricular fibrillation (VF).*

*p < 0.05, comparisons were made to the drug-free control group.

In the other study, we investigated the effects of various doses of sour cherry seed extract and we found that pretreatment - dose-dependent manner - was able to reduce the incidence of reperfusion induced VF in isolated rat hearts. (Fig. 24) (*Bak et al., 2006*)

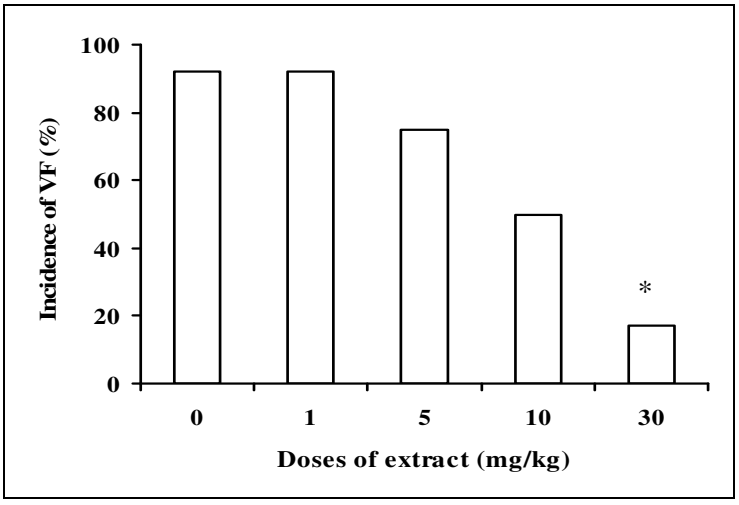


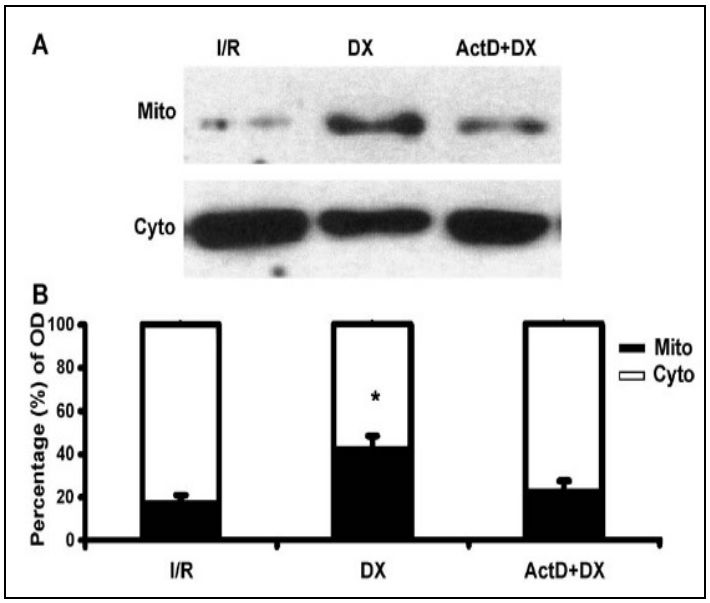
Fig. 24. *Effects of various doses of sour cherry seed extract on the incidence (%) of VF in isolated rat hearts subjected to 30 min of ischemia followed by 120 min of reperfusion.*

Isolated hearts (n=12 in each group) were obtained from rats treated orally with 0 mg/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, and 30 mg/kg of sour cherry seed extract, respectively, for 14 days. *$p<0.05$ compared to the untreated age-matched drug-free control values.

4.3 Effects of dexamethasone and actinomycin D treatment on the ischemia/reperfusion-induced cytochrome-c release

In additional experiments, we investigated the effects of dexamethasone and actinomycin D treatment on the ischemia/reperfusion-induced apoptotic signal intensity measuring cytochrome-*c* release from mitochondria. As it was previously described, the mitochondrial cytochrome-*c* is released to the cytosol during apoptosis (*Borutaite et al., 2003; Vanden Hoek et al., 2003*). Using Western blot analysis and measuring the relative amounts of mitochondrial and cytosolic cytochrome-*c*, we found that dexamethasone pretreatment (2 mg/kg i.p.) prevented ischemia/reperfusion-induced apoptosis (Fig. 25.) measuring cytosolic cytochrome-*c* release, as a marker for apoptosis. Thus, 83% of total cytochrome-*c* signal was found in cytosol in the control drug-free ischemic/reperfused samples, and this value was decreased to 58 % in the dexamethasone treated subjects indicating a reduced release of cytochrome-*c* from the mitochondria to cytoplasm. In addition, the coadministration of dexamethasone and actinomycin D resulted in the same extent of cytochrome-*c* release to that seen in the case of ischemic/reperfused drug-free controls (78 % of total cytochrome-*c* was detected in cytosol). It seems, therefore, that the application of actinomycin D suspended the action of dexamethasone, at least in part, to prevent ischemia/reperfusion-induced cytochrome-*c* release-related apoptotic cell death.

Fig. 25. *The effect of dexamethasone (DX) and actinomycin D + dexamethasone (ActD + DX) pretreatment on ischemia/reperfusion-induced cytochrome-c release.*



A) Mitochondrial (Mito) and cytosolic (Cyto) fractions were prepared from hearts, which received either ischemia/reperfusion only (I/R), 2 mg/kg dexamethasone pretreatment before the initiation of ischemia/reperfusion, or 0.5 mg/kg actinomycin D plus 2mg/kg dexamethasone treatment before the initiation of ischemia/reperfusion (ActD + DX). Immunoblotting was carried out using a mouse anti-cytochrome *c* antibody.

B) The amounts of the cytochrome-*c* in the different fractions were quantitated by densitometry (optical density; OD), and expressed as the percentage of the total OD representing the sum of the OD values in the two fractions. The figure is a representative of four independent experiments providing similar data. *$p < 0.05$ compared to the ischemic/reperfused (I/R) value.

DISCUSSION

I. Part: Role of the Prdx6 in the ischemia-reperfusion injury

The most noticeable and salient feature of our studies is that despite the presence of significant amount of catalase and GSHPx in the hearts of Prdx6$^{-/-}$ mice, these hearts were susceptible to ischemia-reperfusion injury, suggesting a nonredundant role of Prdx6 in cardioprotection. The hearts of Prdx6$^{-/-}$ mice had reduced postischemic ventricular recovery and increased myocardial infarct size and exhibited a greater number of apoptotic cardiomyocytes compared with those values in wild-type hearts. These hearts also had a significantly higher amount of MDA compared with that present in wild-type mouse hearts.

Peroxiredoxins or thioredoxin peroxidases, belong to a relatively new family of antioxidant enzymes. Six peroxiredoxins (Prdx1–Prdx6) have yet been identified, of which Prdx6 is found in the cytosolic fraction together with Prdx1, Prdx2, and Prdx4. Prdx4 is also found in mitochondria and peroxisome, while Prdx3 exists only in mitochondria (*Rhee et al., 2005*). Prdx6 is abundantly present in most of the tissues, including the heart. The antioxidant activity of Prdx6 is attributed to its ability to reduce H_2O_2 and hydroperoxides. Unlike other members of the Prdx family, which have two catalytically active cysteines, Prdx6 contains only one NH_2-terminal conserved cysteine (Cys47) and is, therefore, termed as 1-Cys Prdx (*Rhee, Kang et al., 2005*). While oxidized Prdx1–Prdx5 are reduced through the electron transfer from thiol-containing donor thioredoxin, Prdx6 receives electron transfer from glutathione.

It has long been known that reperfusion of the ischemic heart produces ROS, thereby subjecting the hearts to an increased amount of oxidative stress. In normal hearts, because of the presence of an adequate amount of antioxidants, ROS are readily removed. In contrast, under pathophysiological conditions, $O_2^{\bullet-}$ undergoes a sequence of reactions producing H_2O_2 and hydroxyl radicals ($OH^{\bullet}$). SOD scavenges $O_2^{\bullet-}$ by catalyzing a dismutation reaction, where simultaneous oxidation ($O_2^{\bullet-}$ to O_2) and reduction ($O_2^{\bullet-}$ to H_2O_2) reactions take place (*Das and Maulik, 1994*). The heme-containing enzyme catalase transforms H_2O_2 into H_2O and molecular O_2.

Mammalian heart contains high amount of glutathione (GSH), which ensures the conversion of toxic lipid peroxides into nontoxic products utilizing the necessary reducing equivalents from the reduced GSH (*Rigobello et al., 2005*). GSH also detoxifies H_2O_2 and hydroperoxides that are produced in the ischemic myocardium. Oxidized glutathione is then

reduced to GSH through GSHPx, thereby maintaining the supply of reduced glutathione. The GSHPx reaction removes H_2O_2 at the expense of GSH. The maintenance of GSH levels thus appears to be crucial, and thiol (SH) groups are essential for the tissues to protect themselves against the ROS attack. Furthermore, the generated $OH^{\bullet}$ can attack the unsaturated lipids in the cell, causing lipid peroxidation and producing lipid hydroperoxides, which further exacerbates ischemia- reperfusion injury. GSHPx can also scavenge the hydroperoxides by converting them into hydroxy fatty acids. GSHPx reverses the thiol oxidation reaction, because GSHPx is a GSH-consuming enzyme. It has been observed that while transgenic mice overexpressing GSHPx were resistant to myocardial ischemia-reperfusion injury (*Yoshida et al., 1996*), whereas mice devoid of GSHPx were susceptible to the same (*Yoshida et al., 1997*).

Similar to GSHPx, Prdx6 can also remove both H_2O_2 and hydroperoxides. However, Prdx6 can reduce phospholipid hydroperoxides while GSHPx (the type I or cytosolic enzyme) does not have that ability (*Fisher et al., 1999*). We hypothesize that reduction of peroxidized membrane phospholipids by Prdx6 accounts for its unique antioxidant effect. In addition to GSH peroxidase activity, Prdx6 also possesses phospholipase A_2 (PLA_2) activity. A recent study showed a direct interaction between surfactant protein A and Prdx6, which provided a mechanism of regulation of the PLA_2 activity of Prdx6 by surfactant protein A (*Wu et al., 2006*). The same authors demonstrated that Prdx6 null mice had reduced degradation of internalized dipalmitoylphosphatidylcholine (DPPC) in the lung epithelium and a decreased rate of DPPC synthesis by the remodeling pathway (*Fisher et al., 2005*).

The results of this study showed the presence of significant amounts of catalase and GSHPx in the hearts of $Prdx6^{-/-}$ mice. It is interesting to note that despite the presence of catalase and GSHPx in these hearts, these hearts were subjected to an increased amount of oxidative stress and were vulnerable to cellular injury, suggesting a crucial role of Prdx6 in the ischemic reperfusion injury. Evidence is rapidly accumulating, suggesting a key role of Prdx6 in cellular injury. For example, transgenic mice overexpressing Prdx6 exhibited increased resistance to lung injury in hyperoxia (*Wang, Feinstein et al., 2004*). In this study, at 96 h of hyperoxia, transgenic mice had less epithelial cell necrosis, perivascular edema, and inflammatory cell recruitment, as well as lower thiobarbituric acid-reactive substances and protein carbonyls in lung homogenate, indicating increased cellular defense and providing evidence that Prdx6 functions as a lung antioxidant enzyme. In another study, the same authors showed an induction of Prdx6 in lung epithelial cells by oxidative stress (*Kim et al., 2003*). Increased lung expression of Prdx6 through adenoviral-mediated transfer of the Prdx6 gene protected against hyperoxic injury (*Wang, Manevich et al., 2004*). Examination of the lungs

indicated that Prdx6-overexpressing animals compared with wild type had less lipid peroxidation, less protein oxidation, less lung edema, and less lung inflammation when evaluated at 72 h of hyperoxia (*Wang et al., 2006*). Another recent study found that Prdx6$^{-/-}$ mice on a B6;129 background were significantly more susceptible to atherosclerosis compared with controls (*Wang, Phelan et al., 2004*). However, Prdx6$^{-/-}$ mice on either 129 or B6 backgrounds were neither more susceptible nor more resistant to atherosclerosis than were their normal counterparts. In another related study, mice with targeted mutation of Prdx6 were found to develop normally but were susceptible to oxidative stress (*Wang et al., 2003*). This study showed that Prdx6$^{-/-}$ macrophages had higher H_2O_2 levels and lower survival rates, more severe tissue damage, and higher protein oxidation rates despite the fact that there were no differences in the mRNA expression levels of GSHPx and catalase. It was also reported that Prdxs reduce hydrogen peroxide coupled with Trx (*Netto et al., 1996*), and protect against oxidative stress-induced cytochrome-*c* release and apoptosis differently from Bcl-2 (*Zhang et al., 1997*). The results of our study are therefore consistent with these reports that despite undiminished levels of catalase and GSHPx, Prdx6$^{-/-}$ hearts were more susceptible to ischemic injury.

In summary, the results of this study demonstrated a crucial role for Prdx6 in myocardial ischemia-reperfusion injury. Prdx6$^{-/-}$ mice devoid of Prdx6 exhibited reduced postischemic ventricular recovery and larger infarct size and a higher number of apoptotic cardiomyocytes compared with those in wild-type controls. It appears that these Prdx6$^{-/-}$ mouse hearts were exposed to a greater amount of oxidative stress as evidenced from the presence of higher amount of MDA in the hearts.

II. Part: Role of the Glrx-2 in the cardioprotection

Several salient features are apparent from the results of our second study. First, overexpression of Glrx2 resulted in cardioprotection as evidenced by improved post-ischemic ventricular recovery and reduction of myocardial infarct size and cardiomyocyte apoptosis. Reduction of apoptotic cell death appears to be due to the reduction of caspase activation and cytochrome-*c* in the mitochondria. Second, Glrx2 overexpression resulted in a reduced loss of cardiolipin, a target for cytochrome-*c* binding to the inner mitochondrial membrane. Third, Glrx2 overexpression reduced ischemia/reperfusion-mediated increased oxidative stress and

increased the amount of GSH/GSSG ratio in the heart. Finally, the survival signal triggered by Glrx2 overexpression appears to involve activation of NFκB, Akt, and Bcl-2.

Evidence is rapidly accumulating indicating a crucial role of mitochondria in predicting the life and death of cardiomyocytes. For example, mitochondria are critically involved in apoptotic cell death triggered by ischemia and reperfusion (*Kluck et al., 1997*). On the one hand, protooncogene product Bcl-2 located in mitochondria functions as a suppressor of apoptosis while the proapoptotic protein Bax readily translocated into the mitochondrial membrane after a death signal (*Pagano et al., 2007*). Bcl-2 prevents the release of many apoptogenic proteins from mitochondria to the cytosol thereby inhibiting apoptosis (*Miyamoto et al., 2007*). In heart cells, the ratio of Bcl-2 and Bax predicts whether these cells would survive or destined to die. When Bcl-2/Bax ratio is reduced, such as during ischemia/reperfusion, cytochrome-*c* is released in the cytosol, which triggers the formation of apoptosome complex leading to the activation of caspases, the end effectors of apoptosis (*Halestrap et al., 2000*). On the other hand, for the maintenance of mitochondrial integrity, membrane potential is likely to have influence on myocardial energy production and ultimate survival of the cells. Cellular injury is directly related to changes of mitochondrial architecture including an irreversible loss of the matrix contents and integral membrane protein constituents such as cytochrome-*c* (*Hengartner, 2000*). Once released, cytochrome-*c* triggers the formation of apoptotic complex, which readily activates caspase cascade initiated by caspase 9 leading to the activation of procaspase 3, the main executioner of apoptosis (*Cardone et al., 1998*).

The results of the present study showed reduction of cytochrome-*c* and caspase activation in the Glrx2 overexpressed heart suggesting critical involvement of mitochondria in the process of cardiomyocyte survival and death. These results are consistent with previous findings that Glrx2 plays an important role in attenuating apoptosis by preventing cytochrome-*c* release in Glrx2 overexpressed HeLa cells (*Enoksson et al., 2005*). Binding of cytochrome-*c* to the inner mitochondrial membrane involves mitochondrial phospholipid cardiolipin (*Hoch, 1992*). Reduced amount of cardiolipin was detected in the mitochondria after ischemia and reperfusion. Oxidative stress developed during ischemia/reperfusion is likely to reduce mitochondrial content of cardiolipin (*Fariss et al., 2005*). Moreover, cardiolipin is the mitochondrial target for the Bcl2 family protein Bid (*Liu et al., 2004*). Overexpression of Glrx2 significantly reduced the loss of cardiolipin from the mitochondrial membrane.

Glrx2 facilitates the maintenance of mitochondrial redox homoeostasis upon treatment with apoptotic agents, thereby preventing cardiolipin oxidation and cytochrome-*c* release, i.e. the induction of apoptosis.

Glutaredoxin-2 is a 16 kDa protein, which catalyzes reduction of protein disulfides via a dithiol reaction involving two redox active cysteine residues, or reduction of protein–GSH mixed disulfides through a monothiol mechanism utilizing only the N-terminal active site Cys residue (*Holmgren, 1989*). While mammalian Glrx1 lacks known translocation signals for transport to a subcellular compartment (*Padilla et al., 1995*), Glrx2 protein can be present in both the nucleus and mitochondria. Glrx2 possesses high affinity toward glutathionylated substrates, especially protein–GSH mixed disulfides, and accepts electrons from both GSH and thioredoxin reductase (*Johansson et al., 2004; Beer et al., 2004*). It should be noted that, under oxidative stress conditions when GSH/GSSG ratio is reduced, thereby limiting the availability of GSH, the active site thiols in Glrx2 can still be reduced by thioredoxin reductase and NADPH (*Aslund et al., 1997*), and hence it is likely that Glrx2 would protect the cells from oxidative stress. In our study Glrx2 rescued the hearts from ischemia/ reperfusion-mediated oxidative stress by reducing MDA formation and maintaining GSH/GSSH ratio. Our results support previous reports that silencing of Glrx2 by siRNA dramatically increased the sensitivity of cells towards oxidative stress induced by (ROS-inducing agents) doxorubicin and phenylarsine oxide (*Lillig et al., 2004; Lillig et al., 2005*), and overexpression decreases the susceptibility of cells to apoptosis induced by doxorubicin or the antimetabolite 2-deoxy-D-glucose (*Enoksson et al., 2005*). Another recent study has indicated that Glrx2 possesses GSH- and thioredoxin reductase-dependent peroxidase activity (*Fernando et al., 2006*). The authors noted that dual electron accepting capability of Glrx2 might be important to the cells, especially those under high oxidative stress conditions where cellular GSH level becomes low as in the present case. A related paper showed that the exposure of mitochondria to oxidized GSH/GSSG led to reversible oxidation of reactive protein thiols by thiol-disulfide exchange and both protein disulfide formation and glutathionylation were catalyzed by Glrx2 (*Beer et al., 2004*). The authors were able to demonstrate that Glrx2 played a central role in mitochondrial response to redox signal and oxidative stress by coordinated regulation of mitochondrial glutathione pool and thiols.

Overexpression of Glrx2 appears to rescue the cardiomyocytes in the ischemic reperfused heart through the PI-3-kinase-Akt survival pathway. Akt appears to be a critical regulator of PI-3-kinase-mediated cell survival and constitutive activation of Akt is sufficient to block cell death by a variety of apoptotic stimuli (*Datta et al., 1999*). The present study

showed that overexpression of Glrx2 potentiated an increased phosphorylation of Akt supporting a previous finding, which demonstrated that dual activation of Ras/PI-3-kinase and AP-1 cascades was an essential component of the Glrx2 mechanism of action (*Daily et al., 2001*). Once activated, Akt can phosphorylate and inactivate proapoptotic proteins such as Bad and procaspase 9 and activate antiapoptotic redox-sensitive transcription factor NFκB (*Brunet et al., 1999*), a finding consistent with our results that indicated an increase in Bcl2/Bax ratio and activation of NFκB in the Glrx2 overexpressed cells. A previous study showed nuclear translocation of NFκB by Glrx2 involving IκBα phosphorylation and degradation (*Brunet et al., 1999*).

In summary, our results demonstrate that overexpression of Glrx2 can rescue the heart cells from ischemia/reperfusion induced apoptosis through the activation of NFκB and Akt. There was a reduction of cytochrome-*c* and caspases as well as preservation of mitochondrial cardiolipin indicating a crucial role of mitochondria in the cardioprotection afforded by Glrx2.

III. Part: Role of the PR39 gene therapy in the cardioprotection

Ischemic reperfused heart represents a potential target for gene therapy because gene transfer can represent an alternate pharmacological approach to protect the heart from cellular injury. It was reported that direct injection of reporter genes into hearts subjected to coronary artery occlusion followed by reperfusion could result in gene expression comparable to the levels observed in non-occluded normal hearts (*Leor et al., 1996*).

Our study demonstrates the cardioprotective effect of AdPR39 gene transfer in I–R, because previous work in this setting was done in genetically modified mice (*Cordis et al., 1995*) and with PR39 peptide therapy (*Hoffmeyer et al., 2000*). We show that mouse hearts infected with adenovirus encoding PR39 were resistant to I–R as compared to empty vector and for the first time we show that HIF1α protein levels are elevated in I–R after AdPR39 gene transfer. Thus, PR39-mediated cardioprotection after I–R is conveyed not only through decreased degradation of IκBα (*Bao et al., 2001*), but also through prevention of HIF1α breakdown. This mechanism is confirmed in the mice that were co-transfected with AdPR39 and the HIF1α-dn plasmid. Interestingly and rather unexpectedly, the dominant negative mutant of FGF receptor-1 conveyed an intermediate degree of cardioprotection against I–R, as shown by a more favorable hemodynamic profile in AdFGFR1-dn treated animals versus controls, as well as significantly smaller MI, significantly less apoptosis, and significantly lower MDA levels

compared to the EV group. This is the first report demonstrating that PR39 gene therapy can reduce myocardial I–R through a mechanism involving conservation of HIF1α protein and a reduction of oxidative stress and it is in agreement with previous observations that PR39 reduces the formation of ROS in isolated perfused rat lungs (*Al-Mehdi et al., 1998*). Others have shown that PR39 mediated protection against I–R *in vivo* is accompanied by a reduction of neutrophil infiltration in the area at risk (*Korthuis et al., 1999; Hoffmeyer et al., 2000; Bao et al., 2001*). In the present study, the ability of PR-39 to reduce ROS activity cannot be attributed to its ability to reduce polymorphonuclear leukocytes (PMN) accumulation in the infarct zone, because this study was performed in buffer-perfused (devoid of PMN) isolated hearts. Although it was not shown, it is likely that PR-39 reduced mitochondrial generated ROS activity because concomitant with a reduction of MDA, PR-39 also decreased cardiomyocyte apoptosis.

A recent study has indicated that PR39 inhibits apoptosis by inhibiting caspase-3 (*Ramanathan et al., 2004*), based on the observations of early LPS (lipopolysaccharides) induced apoptosis in macrophages. Macrophages that were treated with PR39 had significantly less caspase-3 activity compared to untreated controls. In the PR39-treated cells, IκBα degradation was inhibited and thus nuclear translocation of NFκB and NFκB-dependent gene expression were attenuated. The ability of PR39 to reduce ROS activity may also contribute to its antiapoptotic effects as shown here. In collaboration with others, we have shown that PR39 inhibits apoptosis in hypoxic endothelial cells by decreasing caspase-3 and increasing inhibitors of apoptosis protein-2 (IAP-2) expression (*Engelman et al., 1995*). Reduction of caspase-3 activity by PR39 was attenuated in IAP-2siRNA transfected cells, suggesting that PR39 mediated inhibition of apoptosis may also occur via IAP-2.

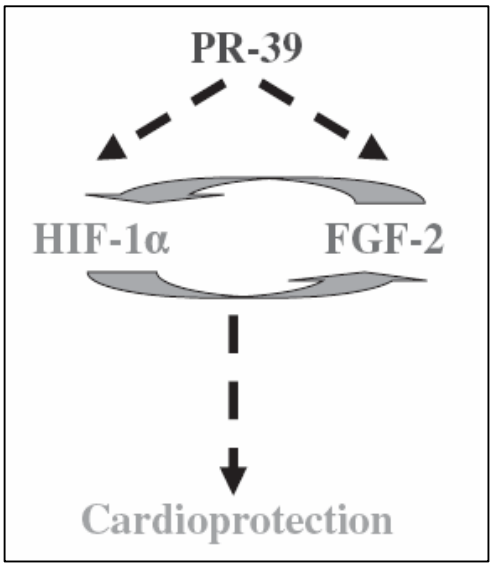
As mentioned earlier, PR39 is an angiogenic master switch peptide, thus increased neovascularization could have played a role in mitigating I–R in the PR39-treated animals, the more so because increased HIF1α levels in response to PR-39, augment myocardial vessel density in α-myosin heavy chain-PR39 transgenic mice (*Bao et al., 2001*). Here we confirm persistence of HIF1α in PR39-treated hearts, but there is no evidence of increased vessel density in these hearts, because the coronary blood flow levels were the same in all groups at baseline (Figs. 18 and 22). Rather, it is more likely that the metabolic and pro-survival responses induced by HIF1α are the major determinants of cardioprotection in the current experiments, together with the previously documented reduction of IκBα degradation. The fact that the abrogation of FGFR1 signaling conveys cardioprotection in the current study is intriguing and merits further investigation because FGF signaling has been shown to reduce

apoptosis and enhance cell survival (*Alavi et al., 2003*). It was also reported that intracoronary administration of an adenoviral vector encoding fibroblast growth factor could ameliorate ischemic reperfusion injury (*Giordano et al., 1996*). On the other hand, inflammation and cell death in I–R are mediated in part by increased FGF levels (*Meij et al., 2002; Detillieux et al., 2003*). Thus, in balance the beneficial anti-inflammatory effect of abrogated FGF signaling may outweigh the negative effect of a diminution of cell survival signals.

There are two contradictory hypotheses regarding the cellular redox status and HIF1α expression during hypoxia. First, cellular levels of ROS production are enhanced during hypoxia in an attempt to stabilize HIF1α (*Chandel et al., 2000*). The alternative hypothesis proposes that HIF1α expression is necessary to inhibit ROS generation during hypoxia (*Neumcke et al., 1999; Semenza, 2001*). Our study supports the second hypothesis as it shows a reduction of ROS in concert with HIF1α induction. Nevertheless, the molecular link between PR39 expression, induction of HIF1α, and cardioprotection needs further investigation. Although HIF1α has been attributed towards protection against ischemia reperfusion injury of the myocardium, the genetic network that mediates this effect is yet to be deciphered (*Wilhide and Jones, 2006*). Furthermore, recently the cardioprotective effects of adenovirus-mediated delivery of a constitutively stable hybrid form of HIF1α (HIF1α-VP16) cDNA have been reported (*Date et al., 2005*). However, follow-up analysis revealed that while hypoxic myocardium does not induce B-type natriuretic peptide (BNP), those receiving recombinant HIF1α induces BNP expression via an HIF1α responsive element in the BNP promoter, thereby raising a cautionary note of such approach (*Wilhide and Jones, 2006*). Taken together, our approach of induction of HIF1α by delivering AdPR39 provides an alternative approach of therapeutic activation of HIF1α in a natural context.

In summary, we demonstrate for the first time the cardioprotective ability of PR-39 gene therapy. PR-39 expression persisted up to 14 days following gene transfer and was associated with a reduction of oxidative stress and apoptotic cell death in concert with an increase in HIF1α protein levels. HIF1α-dependent protective metabolic and pro-survival responses rather than proangiogenic mechanisms are likely responsible for the cardioprotective effects in this study (Fig. 26). Figure 26. shows the proposed mechanism of action of cardioprotection by PR-39 gene therapy. Expression of PR-39 leads to the induction of HIF1α and FGF-2, which then synergize each others effect. Cardioprotection is provided by the integrative effects of both FGF-2 and HIF1α.

Fig. 26. *The proposed mechanism of action of cardioprotection by PR-39.*



Furthermore, in a novel observation we show that abrogation of FGFR1 signaling also conveys cardioprotection, albeit to a lesser degree than PR39 gene transfer. The consequences of this finding relative to the mechanism of PR39-mediated enhancement of FGF signaling and the role of FGF itself in I–R injury merit further investigation.

IV. Part: Role of the glucocorticoids against I-R induced injury

The effects of glucocorticoids are numerous and widespread in cells and tissues. Their various effects include alterations in carbohydrate, lipid, and protein metabolism, maintenance of electrolyte and fluid balance, preservation of normal function of kidney, skeletal muscle, endocrine, nervous, immune, and cardiovascular system. In addition, by mechanisms those are still not completely understood. It has been also proven that glucocorticoids have anti-inflammatory and immunosuppressive actions, one of the major pharmacological uses of this class of drugs, also provide a protective mechanism under physiological or pathological conditions, since many of the endogen mediators associated with pathological conditions could lead to cardiovascular collapse if unopposed by adrenal glucocorticoids. Several steroids that are classified predominantly as mineralocorticoids or glucocorticoids also possess significant activity on fluid and electrolyte handling influencing the function of cells and organs. Thus, the diverse actions of glucocorticoids are related in complex ways to those of various drugs affected the function of cardiovascular system.

Glucocorticoids have been the basis of pharmacotherapy aimed at reducing inflammation response after cardiopulmonary bypass surgery (*Sellewold and Jynge, 1985; Engelman et al., 1989; Pearl et al., 2002*). However, the mechanism(s) by which glucocorticoids improve postischemic cardiovascular function has not been yet elucidated in ischemic myocardium. In the present study, we approached the action mechanism(s) of glucocorticoids, dexamethasone, from a different angle including the suppression of cytochrome-*c* release that results, at least in part, in cardiomyocytes loss in ischemia/reperfusion. Although the specific roles of apoptosis and necrosis, the two causes of cell death, remain controversial. Studies have demonstrated that apoptosis, beside necrosis, significantly contributes to the death of cardiomyocytes with reperfusion (*Stephanou et al., 2002; Borutaite et al., 2003; Hochhauser et al., 2003*).

It is not clear and is not the goal of the present study to what extent of apoptosis and necrosis individually contribute to the development of postischemic injury, and probably both of them, a "necro-apoptotic" mechanism contributes to the development of reperfusion-induced damage. Cytochrome-*c* is an essential component of the mitochondrial respiratory chain. It is a soluble protein that is localized in the intermembrane space and is loosely attached to the surface of the inner mitochondrial membrane (*Gonzales and Neupert, 1990*). Cytochrome-*c* is a necessary component of cellular apoptotic program suggests that mitochondria may be involved in apoptosis by releasing cytochrome-*c*. One of the main mechanisms for triggering this apoptosis appears to be mitochondrial permeability transition followed by cytochrome-*c* release. Cytochrome-*c* release can result in caspase activation and thus apoptosis, but also results in mitochondrial dysfunction, which might contribute to contractile dysfunction or necrosis at reperfusion (*Borutaite et al., 2003*). In the present study, we demonstrate that under our experimental circumstances dexamethasone significantly improves cardiac function providing evidence that glucocorticoids could mediate reperfusion-induced injury via the mechanism of apoptosis signals, including the release of cytochrome-*c* from mitochondria to cytoplasm. Furthermore, the results of our study measuring of cytochrome-*c* release from mitochondria to cytoplasm suggest that pro-apoptotic signaling may play an important role in the development of reperfusion-induced damage. However, the application of dexamethasone may not afford alone a complete protection against postischemic damage via apoptotic or other mechanisms in our model. We demonstrate, under our experimental conditions, that dexamethasone significantly improves postischemic cardiac function, providing evidence that reduced release of cytochrome-*c*, a marker of apoptosis (*Zhao et al., 2003*), to cytoplasm attenuates reperfusion-induced injury. Furthermore, the results of this

study suggest that cytochrome-*c* release from mitochondria an important role in the development of reperfusion-induced damage, and the application of dexamethasone could afford a significant protection against postischemic injury, which can be blocked by a protein synthesis inhibitor, actinomycin D. Actinomycin D, as an antineoplastic drug, displays its cytotoxicity and induction of apoptosis against tumor cells (*Shang et al., 2001*). On the other hand, actinomycin D is an inhibitor of RNA synthesis, which can alleviate or block the apoptotic process and decrease the cytotoxicity induced by several stimuli such as the dihydrofolate reductase inhibitor aminopterin (*Chung et al., 2001*) and the prostaglandin derivative 15-deoxy-delta 12,14-prostaglandin J_2 (*Clay et al., 2001*). However, a surprising manifestation has also been observed that actinomycin D promotes induction of apoptosis by some specific stimuli, for example, tumor necrosis factor-related apoptosis-inducing ligand (*Griffith et al., 2002*) and the death receptor CD95 (*Glazyrin et al., 2002*). In addition to inhibiting RNA and "de novo" protein synthesis, actinomycin D intercalates DNA and produces double-strand DNA breaks as a topoisomerase II poison. DNA breaks can also occur through the generation of free radicals (*Ross and Bradley, 1981*). Actinomycin D by itself is enough to induce tumor cell apoptosis (*Muscarella et al., 1998*) although it can suppress RNA synthesis and encoded proteins during the process, and its inhibitory effect is believed to be the main mechanism of its anticancer activity. In our study, actinomycin D inhibits the cardioprotective effect of dexamethasone, probably via repressing RNA synthesis (*Quing et al, 2003*) and inhibiting glucocorticoid-induced "de novo protein" synthesis. Thus, dexamethasone-induced cardiac protection could be originated from "de novo" protein synthesis, which may include heat shock proteins (HSP), such as HSP 32 (*Bak et al., 2003*) and 72 (*Valen et al., 2000; Tekin et al., 2001*).

There are currently abundant data to indicate that different signal mechanisms contribute to apoptosis leading to postischemic cardiac failure, but it is reasonably to believe that different and multiple mechanisms rather than a single factor could significantly contribute to the development of cardiac apoptosis. This is supported and well explained for instance by an elegant study of Ma et al., (1999) showing that the administration of a p38 MAPK inhibitor completely blocked p38 MAPK activation, but this concentration failed to completely prevent the development of ischemia/reperfusion-induced apoptosis. Of course, other apoptotic signal mechanisms, not specifically studied and discussed in the present study, e.g., caspases (*Li et al., 2001; Scarabelli et al., 2001; Stephanou et al., 2001; Rodriguez et al., 2002*), TNF-α (*Birks et al., 2000; Kurrelmeyer et al., 2000*), p53 (*Leri et al., 1999*), transglutaminase (*Szegezdi et al., 2000; Nemes et al., 2001*), heat shock proteins (*Valen et al., 2000*), glucose and cellular ATP

contents also may play an important role in the development of apoptosis (*Jonassen et al., 2000; Schaffer et al., 2000; Elsasser et al., 2000*). Our present study suggests a mechanism of glucocorticoid-induced cardiac protection, however, more pharmacological studies must be done to verify the exact action mechanism of dexamethasone related to apoptosis and "de novo protein" synthesis in ischemic/reperfused myocardium.

SUMMARY

In our experiments, we have been used isolated working mouse hearts to study the role of redox-signaling mechanisms in cardioprotection.

In the first part of our research, we studied the potential role of the peroxiredoxin 6 (Prdx6) in I-R induced injury. Prdx6 is a novel peroxidase enzyme belonging to the Prdx family, which in mammals contains five more peroxiredoxins (Prdx1–Prdx5). Like glutathione peroxidase (GSHPx) and catalase, Prdx6 possesses H_2O_2- scavenging activities, and, like the former, it also removes hydroperoxides. Since significant amounts of catalase and GSHPx are present in the heart contributing toward the attenuation of H_2O_2 and hydroperoxides formed during I-R injury and thereby providing cardioprotection, we investigated whether Prdx6 also has any role in this process. In this study we used Prdx6$^{-/-}$ mice to assess the role of Prdx6 in ischemic injury. Western blot analysis revealed the absence of any Prdx activity in the Prdx6$^{-/-}$ mouse heart, while the GSHPx-1 and catalase levels remained unchanged. Randomly selected hearts from Prdx6$^{-/-}$ mice and wild-type mice were subjected to 30 min of global ischemia followed by 120 min of reperfusion at normothermia. The hearts from the Prdx6$^{-/-}$ mice were more susceptible to ischemic reperfusion injury as evidenced by reduced recovery of left ventricular function, increased myocardial infarct size, and higher amount of apoptotic cardiomyocytes compared with wild-type mouse hearts. These Prdx6$^{-/-}$ hearts were also subjected to a higher amount of oxidative stress as evidenced by the presence of higher amount of malondialdehyde. Our finding thus indicates a non-redundant role of Prdx6 in myocardial ischemic reperfusion injury as catalase, and GSHPx could not make up for the deficiency of Prdx6 activities.

In the second part of our experiments, we intend to determined the potential role of the glutaredoxin2 (Glrx2) in cardiac disorders. Mitochondrial Glrx2 has been recognized as an important redox regulator in mammalian organs including heart. This study examined if myocardial overexpression of Glrx2 in the heart could rescue the cardiac cells from apoptosis and necrosis induced by ischemia and reperfusion. The human Glrx2 transgene was created by placing a full-length cDNA fragment encoding human mitochondrial Glrx2 downstream to the 5′ flanking sequence and promoter of the mouse α-myosin heavy chain gene. The isolated hearts from Glrx2 transgenic mice and non-transgenic (wild type) littermates were subjected to 30 min of global ischemia followed by 2 h of reperfusion in working mode. The hearts from Glrx2 transgenic mice displayed significantly improved contractile performance and reduced myocardial infarct size and cardiomyocyte apoptosis. There was a reduction in

cytochrome-*c* release and activation of caspase 3 and caspase 9. Glrx2 overexpression also reduced the ischemia/reperfusion-mediated loss of mitochondrial cardiolipin, decreased the activities of reactive oxygen species (ROS) and preserved GSH/GSSG ratio. Glrx2 mediated survival signal appeared to be stemmed from PI-3-kinase-Akt survival signaling pathway and involved the activation of redox sensitive transcription factor NFκB and anti-apoptotic protein Bcl-2. Our results indicate a crucial role of mitochondrial Glrx2 in cardioprotection.

In the third parts of our work, we have been examined the cardioprotective abilities of PR39 gene therapy. PR-39, a proline-arginine-rich angiogenic response peptide, has been implicated in myocardial ischemic-reperfusion injury. In this study, male C57Bl/J6 mice were randomized to intramyocardial injection of 109 plaque forming units (p.f.u.) adenovirus encoding PR39 (PR39), FGFR1 dominant negative signaling construct (FGFR1-dn), empty vector (EV), or PR39 adenovirus plus 4 μg of plasmid endcoding a HIF-1α dominant negative construct (PR39 + HIF-1α-dn). Seven days later, hearts were subjected to 20 min of ischemia and 2 h. reperfusion *ex vivo* and aortic and coronary flow, left ventricular developed pressure (LVDP), and LVdp/dt were measured. Myocardial infarct (MI) size and cardiomyocyte apoptosis were measured by TTC staining and TUNEL, respectively. PR39 expression was robust up to 14 days after gene transfer and was absent after EV and FGFR1-dn. Hemodynamics showed no differences at baseline, and heart rate remained unchanged in all groups throughout the experiment. After I–R, hemodynamics remained unchanged in PR39 hearts, but deteriorated significantly in the other groups, except for aortic flow, which remained significantly higher in FGFR1-dn than in EV and PR39 + HIF-1α-dn ($p < 0.05$), although it was lower than in PR39 ($p < 0.05$). MI was 8.7 ± 0.9 % in PR39, 23.8 ± 1.1% in FGFR1-dn, 29.9 ± 2.2% in EV, and 30.8 ± 2.7 % in PR39 + HIF-1α-dn (PR39 *vs.* other groups: $p < 0.05$; FGFR1-dn *vs.* EV and PR39 + HIF-1α-dn: $p < 0.05$). In PR39, HIF-1α protein was higher than in FGFR1-dn and EV. Importantly, co-transfection of HIF1α-dn with PR39 completely abolished cardioprotection by PR39. Cardioprotection by PR39 is likely conveyed by protective metabolic and survival responses through HIF-1α stabilization and not by angiogenesis, because baseline coronary flow was the same in all groups. Abrogation of FGFR1 signaling conveyed an intermediate degree of cardioprotection.

In the last parts of our research, we investigated the contribution of dexamethasone treatment on the recovery of postischemic cardiac function and the development of reperfusion-induced arrhythmias in ischemic/reperfused isolated rat hearts. Electrocardiograms were monitored to determine the incidence of reperfusion-induced ventricular fibrillation. Dexamethasone pretreatment significantly reduces the occurrence of

ventricular fibrillation. Cytochrome-*c* release was also observed in the cytoplasm and it was interfered with dexamethasone pretreatment. The results suggest that the inhibition of cytochrome-*c* release is involved in the dexamethasone-induced cardiac protection and actinomycin D prevented the dexamethasone-induced cardiac protection.

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ACKNOWLEDGEMENTS

I would like to give expression to my sincere gratitude to my supervisor, Prof. Dr. Árpád Tósaki (University of Debrecen, Dept. of Pharmacology, Debrecen, Hungary) for his excellent leadership and supervision. I also would like to thanks for his support, patience and encouragement during my entire PhD studies.

I am also grateful to Prof. Dr Dipak K. Das (University of Connecticut School of Medicine, Farmington, CT, USA) and Prof. Dr. Joël de Leiris (University of Joseph Fourier, Grenoble, France) for their excellent guidance and great advices.

I am beholden to Dr. Bob Kaba Loemba (French Institute, Budapest) who has given me a wonderful opportunity to learn in France.

I would like to give my special thanks to all of my present and past colleagues and friends from Hungary (Dr. Edit Varga, Dr. Rudolf Gesztelyi, Dr. Béla Juhász, Zsuzsa Koszorús, Éva Tamás) from USA (Prof. Dr. Nilanjana Maulik, Dr. Samarjit Das, Dr. Malik Gautam) and from France (Andry Rakotovao, Dr. Michel de Lorgeril, Dr.Patricia Salen) for their help.

I am also beholden to Dr. Marie-Claire Toufektsian (University of Joseph Fourier, Grenoble, France) for her excellent guidance and great advices during my entire time in France.

Finally, I take this opportunity to acknowledge my family for their support and encouragement.

Dissertation based on following publications

1. **Nagy N**, Malik G, Fisher AB, Das DK. (2006) Targeted disruption of peroxiredoxin 6 gene renders the heart vulnerable to ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol.* 291 (6): H2636-40. **IF:** 3,724

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