Ph.D. THESIS

THE PUTATIVE ROLES OF GENES IN PROTECTION AGAINST THE MYOCARDIAL INJURY INDUCED BY ISCHEMIA/REPERFUSION

written by

Tibor Turoczi

Supervisor: Prof. Dr. Arpad Tosaki

Ph.D. program leader: Prof. Dr. Lajos. Gergely

UNIVERSITY OF DEBRECEN, MEDICAL AND HEALTH SCIENCE CENTER

FACULTY OF PHARMACY

DEPARTMENT OF PHARMACODYNAMICS

2004

Abbreviations:

AP-1:	Activating protein-1
AF:	Aorta flow
ATPS6	ATPase subunit 6
CF:	Coronaria flow
CDDP:	Cis-diammine-dichloroplatinum
COXB III:	Cytocrhrome oxidase B subunit 3.
CO:	Carbon monoxide
Dp/Dt:	Cardiac contractility
GADPH:	Glyceraldehyde 3-phosphate dehydrogenase
GSHPx-1:	Glutathione peroxidase
HFE:	Hereditary haemokromatozis
HO-1:	Hemoxigenase-I.
KHB:	Krebs-Henselit buffer
HTLV-I:	Human-T cell leukemia virus
ISA/RE:	Ischemia/reperfusion
KO:	Knock out
LVDP:	Left ventricle „develop" pressure
LVEDP:	Left ventricle end-diastolic pressure
MAPK:	Mitogen activated protein kinase
MDA:	Malonaldehyde
NF-κβ:	Nuclear factor-κβ
NO:	Nitrogen monoxide
PC:	Preconditioning
PPI:	4-amino-5-(4-methylphenyl)-7-(*t*-butyl)-pyrazolo-3, 4-D-Pyrimidine
ROS:	Reactive oxygen species
SOD:	Szuperoxid dismutase
STAT5A/STAT6:	Signal transductions and activator factor
HF:	Heart frequency
TTC:	Triphenyl-Tetrazolyum-Clorid
Trx:	Thioredoxin
TUNEL:	Terminal-Deoxynucleotidil-Transferase-Deoxyuridine Nick end Labeling
VF:	Ventricular fibrillation
(+/+):	Homozygouse wild type
(+/-):	Heterozygouse
(-/-):	Knock out

THE PUTATIVE ROLES OF GENES IN PROTECTION AGAINST THE MYOCARDIAL INJURY INDUCED BY ISCHEMIA/REPERFUSION

INTRODUCTION

The ischemic cardiac diseases have relevant role nowadays, causing severe health problem for people. The cardiac diseases involving myocardial infarction, coronaria diseases have great impact on human life and performance. Therefore, the question regarding the protection of ischemic myocardium is so important, because it is still having a leading role in statistic of those diseases causing death. Knowing of the molecular mechanism of the ischemia and reperfusion is relevant tool for designing and developing of new pharmacy drugs, and possible treatment for those patiens who suffering in this type of illness. The harmful pathological process is induced by ischemic/reperfusion resulting in generation of the free radicals, can emphasize this field to be examined more detailed. The generation of free radicals is physiological process, which occurring under normal circumstances, which is significant part of molecular mechanisms of the cell physiology. The generation of free radicals is double edge sword, because it can initiate beneficial effects on cell involving expression of the protector gene product called anti-oxidant protein, but has other adverse action. This action is harmful, because previously described that free radicals can potentiate damage in cell membranes, and cardiac performance. This effect results in disrupting of the normal cell barrier function, followed by the changing in intracellular environments. Alteration in molecular structures of the myocardial contractile element can lead to impairment of the cardiac function, and deterioration of the sensibility of cardiac protein including troponin C. Free radicals also possessing such effect like altering the physiological ion distribution in the intracellular and extracellular spaces leading to development of cardiac arrhythmia including ventricular fibrillation and conducting disturbance.

I. The physiological role of iron and HFE mutation

The molecular iron is an important component of the human body because it is taking part in assembling of red blood cells and several enzymes including cytochrome oxidase. This metal is also a catalyst for chemical reactions associated with the products of oxygen species, which lead to oxidative stress and cell damage. The iron level within the cell must be precisely regulated to promote essential functions and provides an appropriate abundance to maintain

adequate sores and yet minimizes the risk of potential toxicity. The body iron homeostasis is regulated primarily by duodenal and upper small intestinal absorption and is responsive to body iron stores. The hereditary haemochromatosis, the prototype disorder of iron overload due to misregulated iron homeostasis in humans, is caused by an inappropriate increase in iron absorption in the duodenum and upper small intestine. This type of illness is associated with a homozygous mutation in HFE, the so-called haemochromatosis gene. Loss of the functional HFE protein product is characterized by iron deposition in parenchymal organs like liver, pancreas, heart, joints, and skin. The HFE protein is similar to major histocompatibility complex class 1-type proteins, a group of proteins that bind to β2-microglobulin. There are two missense mutation were identified in the HFE gene which is located on the short arm of chromosome 6. The single mutation of G to A at nucleotide 845 results in the substitution of tyrosine for cysteine at amino acid 282. The second mutation of C to G at nucleotide 187 results in a substitution of aspartate for histidine at amino acid 63. The disturbance of the iron homeostasis caused severe iron-overloading in the heart because of the massive expression of the extracellular matrix component. This process is resulting in heart fibrosis, which could change the structure of myocardial conductance system causing cardiac arrhythmia and ventricle fibrillation.

II. Redox-signaling and Thioredoxin (Trx)

It has become increasingly clear that redox signaling regulates diverse cellular functions in mammalian systems. The biochemical basis of the redox signaling is that oxidizing conditions are maintained by the stabilizing disulfides in the extracellular surface, while the intracellular environment is maintained in the reduced state with the help of free sulfhydryl groups. The principle disulfide reductase is responsible for maintaining intracellular milieu in the reduced state and it is a low molecular weight redox-active protein with two cysteine residues in its active sites, thioredoxin. Trx is ubiquitously present in mammalian cells and organs including hearts. Several reports exist in the literature indicating that Trx is induced by oxidative stress. Accumulating evidence suggests that myocardial ischemic/reperfusion injury may also be redox regulated. Reactive oxygen species (ROS) affect the cellular stress response to virtually all cellular compartments at regulatory levels. The long-held thought that ischemia/reperfusion mediated production of ROS is detrimental to the cardiac cells has undergone drastic changes in recent years from the discovery that the same ROS can function as signaling molecules and take part in the repair process. Many redox sensitive transcription factors and genes are likely to be involved in the redox-signaling process. Trx appears to play

a crucial role in the redox regulation of the ROS signaling during and/or following ischemia/reperfusion. First, Trx may be an important component of the cellular defense against cardiac injury. Oxidized Trx was found to be released into plasma of the patients undergoing cardiopulmonary bypass surgery. Endurance training by swimming accompanied by a reduction of ischemia/reperfusion-induced oxidative stress with a concomitant increase in Trx reductase resulted in a protection against myocardial ischemia/reperfusion injury. Human Trx attenuated hypoxia reoxygenation injury of murine endothelial cells in a thiol free condition suggesting Trx protection of myocardial injury through a novel redox-signaling pathway. To determine the real function of Trx in myocardial protection could be important, because the mechanims of it's effect is still unknow.

III. Signal transducer and activator of transcription factor

STAT3 has recently been found to play an important role in cardioprotection induced by ischemic preconditioning. Preconditioning can potentiate a Janus kinase (JAK)-STAT signaling by rapidly phosphorylating JAK and activating STAT3, resulting in a survival signal to the myocardium. Inhibition of JAK2 with tyrphostin AG 490 abolishes cardio-protective effects of preconditioning as evidenced by increased myocardial infarct size and cardiomyocyte apoptosis. In another related study, we found a rapid activation of two components of JAK-STAT signaling pathway, STAT5A and STAT6, in the rat heart subjected to ischemia and reperfusion. These activated STATs bound to a conserved nucleotide sequence (ST domain) in the promoter of the angiotensinogen gene and upregulated the level of its mRNA. Treatment of the hearts with the AT1 blocker, losartan, resulted in loss of the STAT-angiotensinogen promoter binding activity and upregulated level of angiotensinogen mRNA. Because angiotensin has been shown to play a role in preconditioning and because these STATs are required for angiotensin II-mediated signaling in the heart, we hypothesized that STAT5A and STAT6 might also play a role in preconditioning. There are two highly related STAT5, STAT5A and STAT5B, of which the former was found to be activated in the heart in response to ischemia and reperfusion. The main function of STAT5A is prolactin signaling, and it is required for mammary gland development. STAT5B is also located downstream of growth hormone signaling, which has recently found to play a crucial role in angiogenic response during hypoxic or ischemic preconditioning. STAT6 is an interleukin-4 (IL-4)-responsive transcriptional activator and is required for induction of IL-4-dependent gene expression.

IV. Roles of mithocondrial genes (COXB III., ATPS6) and HO-1 against ventricular fibrillation induced by oxidative stress.

It is widely believed that under circumstances, the act of reperfusion could induce lethal myocardial injury. The most serious consequence of reperfusion injury is the occurrence of arrhythmia and different ventricular conductance disturbance. The reperfusion induced arrhythmias were observed in all species, this phenomenon was frequently noted during cardiac surgery and thrombolytic treatment. Diabetis mellitus is often associated cardiovascular complications including diabetic cardiomyopathy. Diabetic myocardium exhibits a variety of abnormalities in sarcolemmal ion transport, including depression of Na^+-H^+ and Na^+-Ca^{2+} exchange process. Thus, the expression of some mitochondrial genes [e.g., cytochrome oxidase B subunit III (COXBIII) and ATPase subunit 6 (ATPS6)] may relate to the function of various ion channels and ion exchange processes, and could play a role in arrhythmogenesis in ischemic/reperfused myocardium because arrhythmias are known to be related to disturbances in ion metabolism. The microsomal heme oxygenase (HO) catalyses the oxidative degradation of heme to biliverdin, which is subsequently converted to bilirubin. Mammalian heme oxygenase catabolizes cellular heme to biliverdin, carbon monoxide (CO), and free iron, is represented by three isoforms, HO-1, HO-2, and HO-3 encoded by separate genes. HO-3, which in its primary structure resembles HO-2, is marginally active. Evidence has recently accumulated suggesting that CO generated by HO may be a physiological signaling molecule and, on the other hand, HO is thought to provide an antioxidant defense mechanism, on the basis of its marked up-regulation in stressed cells. In previous studies, we observed a reduction in HO-1 mRNA expression, its protein, and enzyme activity in ischemic/reperfused fibrillating hearts but not in nonfibrillating myocardium. The aforementioned findings have led us to speculate that the vulnerability of HO-1 knock out subjects' myocardium to VF may be related to HO-1 mRNA, its protein expression, enzyme activity, and HO-1-related endogenous CO production. If this were so, we would stress that the absence of HO-1 mRNA, its protein and HO-1-mediated endogenous CO production could play a crucial role in the development of reperfusion-induced VF.

THE AIM OF THIS STUDY

1. **We intend to study the correlation of the HFE mutation (autosomal, recessive) and the iron content on the diet relating infarct size, number of apoptotic cell and myocardial function. Using wild type and HFE KO animals, feeding with two type of diet enriched by different contents of iron (30-ppm, 300-ppm). In the second part of this study we examined the state of the component of the redox-system, looking for the answer at the question, that how the iron therapy can influence the antioxidant enzyme contents and all kind of process initiated by the oxidative stress.**

2. **We elucidated the protective effect of the Trx on ischemia/reperfusion induced cardiac changing. Using by molecular biology methods to determine the existence of the Trx mRNA by Northern blot analysis. Measuring the function of Trx wild and transgenic mice and Sprague-Dawley rat heart to figure out the scavenging effect of Trx on the myocardium. Using CDDP, which is an anticancer agent, to test the inhibitory action of Trx in rat preventing the heart from cell injury caused by ischemic/reperfusion. We studied also infarct size, cardiomyocytes apoptosis, and functional parameters.**

3. **We focused on STAT5A and STAT6 signal transduction protein putative role in ischemic preconditioning by using STAT5A and STAT6 knock out (KO) and corresponding wild type mice and Sprague-Dawley rats. We did functional study to understand these proteins impact on myocardial performance, subjected them to normal ischemia/reperfusion (30 min. ISA+2 hours RE.), preconditioning (4PC+30 min ISA+ 2 hours RE). To determine the phosphorylation of them, we applied 3 phosphorylase inhibitors called tyrphostin, PPI, and LY-294002.**

4. **We performed experiment to understand the roles of mithocondrial genes (ATPS6, COXBIII.) and HO-I in prevention of myocardium against ventricular fibrillation. Using HO wild type and KO mice, we did functional study to evaluate all beneficial effects of CO induced by oxidative stress. In case of mithocondrial enzymes we used diabetic hearts model to determine these genes anti-arrhythmatic effect in sick heart.**

MATERIALS AND METHODS

I. HFE STUDY

Animals and iron diet:

The knock out mice were developed by targeted disruption of the HFE gene. Northern blot analysis of HFE mRNA from the HFE KO mouse hearts and wild-type hearts showed complete absence of the HFE gene in the HFE KO mouse hearts. Four groups of mice were used for this study. HFE KO and C57 wild-type mice were kept either on low-iron diet (30 ppm) or on high-iron diet (300 ppm), and experiments were performed with these mice after 3 months.

Isolated working mouse heart:

All animals used in this study received humane care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals. Mice were first anesthetized with sodium pentobarbital (200 mg/kg body weight intraperitoneal injection, Abbott Laboratories) and anticoagulated with heparin (500 U/kg body weight, Elkins-Sinn Inc) in same way. The heart was excised and immediately immersed in ice cold perfusion buffer. The aorta was cannulated and retrograde perfusion in the Langerndorff mode was initiated at a perfusion pressure of 60 mmHg. The perfusion buffer used in this study consisted of a modified Krebs-Henseleit Bicarbonate buffer [KHB: composed of (in mmol/L) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 10 glucose, and 1.7 $CaCl_2$, gassed with 95% O_2 and 5% CO_2, filtered through a 5-mm filter to remove any particulate contaminants, pH 7.4] that was maintained at a constant temperature at 37°C and was gassed continuously for the entire duration of the experiment. After 10 minutes of retrograde perfusion, the heart was switched to ortograde perfusion mode where KHB buffer entered the cannulated left atrium at a pressure equivalent to 10 cm of water, and passed to the left ventricle from which it was spontaneously ejected through the aortic cannula against a pressure equivalent to 50 cm of water.

Cardiac function:

We monitored and registered the main cardiac function including left ventricle develop pressure (difference between the systolic and end-diastolic pressure), left ventricle end-

diastolic pressure, cardiac contractility (first derivative of LVDP), heart rate, coronary flow (CF), aortic flow (AF), cardiac output (CF+AF).

Estimation of iron content of heart:

The amount of non-heme iron in the hearts was analyzed after digestion in an acid mixture containing 3 mol/l HCl and 10% trichloroacetic acid for 24 hours at 65°C. About 500 ml of the acid extracts were mixed with 1.6 ml of bathophenanthroline chromogen reagent and the absorbance read at 535 nm with a Beckman DU-100000 spectrophotometer.

Evaluation of the MDA formation:

Malonaldehyde (MDA) was measured as MDA-DNPH derivative by HPLC. MDA was assayed as described previously, to monitor the development of oxidative stress.

Assessment of antioxidant enzymes in the heart:

The levels of the key antioxidant enzymes, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GHSPx) were measured using standard enzymatic assay methods. SOD was determined by its inhibitory action on the superoxide dependent reduction of ferricytochrome by xanthine/xanthine oxidase by following the rate of cytochrome C reduction at 417 nm. Catalase was assayed by following the decomposition of H_2O_2 directly by the decrease in extinction at 240 nm. The assay of GSHPx-1 was based on the coupling of the enzyme to NADPH via GSH reductase, and the rate of NADPH oxidation was measured.

Assessment of cell death due to necrosis and apoptosis:

Immunohistochemical detection of apoptotic cells was performed using TUNEL staining in conjunction with mouse monoclonal antibody recognizing cardiac myosin heavy chain followed by staining with TRIC-conjugated rabbit anti-mouse IgG (200:1 dilution) to specifically detect apoptotic cardiomyocytes. Myocardial infarct size was detected by standard TTC staining method using the NIH Image 1.6.1 image processing software system.

Statistical analysis:

The values for myocardial functional parameters, MDA, risk and infarct volumes, and infarct sizes were all expressed as the mean±SEM. For the statistical analysis, analysis of variance (ANOVA) followed by Scheffe's test was first performed to test for any differences between the groups. If differences were established, differences between data were analyzed for

significance by performing a Student's *t* test. The results were considered significant if $*p<0.05$

RESULTS I.

Iron absorption in the heart:

As expected, hearts of the HFE KO contained increased levels of iron compared with those of the wild-type heart and the wild-type mouse fed high-iron diet had relatively, but not significantly, higher amount of iron in the heart. Significantly higher amount of iron was found in the HFE KO mouse hearts fed both low and high-iron diet.

Reactive oxygen species in the heart:

At baseline, the MDA level of hearts of wild-type mice was minimal; the mice fed 300 ppm iron diet being slightly, but not significantly higher. At the end of 30 minutes of ischemia and 2 hours of reperfusion, MDA content of the wild-type mouse hearts increased significantly in both groups compared with baseline values. A dramatic increase in MDA content was found in the HFE knock out mouse hearts, both at the baseline levels and at the end of reperfusion.

Levels of antioxidant enzymes in the hearts of HFE KO and wild-type mice:

Because antioxidant enzymes comprise the primary defense line of hearts, we measured the key antioxidant enzymes, SOD, catalase, and GSHPx-1, in hearts at baseline and at the end of ischemia/reperfusion. The results indicate that Mn-SOD and GSHPx-1 levels, but not Cu/Zn-SOD and catalase levels, were lower in the HFE knock out mouse hearts fed high-iron diet at the baseline level. At the end of ischemia/reperfusion, levels of all antioxidant enzymes tested, except for Cu/Zn-SOD, which showed lowering in activities only for high-iron diet–fed hearts, were lower compared with wild-type controls.

Recovery of myocardial contractile performance:

There were no statistically significant differences between the wild-type and the knock out groups with regard to baseline cardiac parameters. The left ventricle develop pressure was dramatically decreased in HFE KO hearts to be given high-iron supplemented diet (300-ppm). This tendency was observed at R15' (63.40±2.77 versus 69.73±4.0 mmHg in the control). The left ventricular end-diastolic pressure of HFE KO animals fed with 300-ppm iron-containing food displayed elevation of pressure at R30' (14.13±0.72 versus 11.77±0.43 mmHg), R60' (17.66±0.67 versus 13.28±0.32 mmHg), and R120' (20.53±0.97 versus 14.27±0.40 mmHg).

The HFE KO hearts with 30-ppm caused also higher significant LVEDP values at 60, and 120 time points of recovery period compared to wild-type heart function. The dp/dt values representing the maximum first derivative of left ventricle develop pressure showed significant reduction in both of HFE KO groups at each time point of reperfusion period as compared with wild-type controls. Heart rate remained unchanged and preserved close to the baseline values for both HFE KO and wild-type animals for the entire duration of experimental period. The aortic flow displayed gradual depression throughout the reperfusion period as compared with baseline values. HFE KO 300-ppm group indicated significant impairment compared with wild-type group R30' (0.30±0.01 versus 0.47±0.04 mL/min), R60' (0.2±0.02 versus 0.30±0.02 mL/min), and R120' (0.13±0.01 versus 0.23±0.03 mL/min). The coronary flow showed the same pattern as the aortic flow at each point of reperfusion related to control value with exception of R30'of HFE KO 30-ppm heart function.

Myocardial infarction:

Infarct size was noticeably increased in the knock out mouse heart transverse sections compared with the wild-type control sections (44.65±1.37% of HFE KO 300-ppm, 38.90±1.93% of HFE KO 30-ppm versus 32.75±1.85% of wild type 300-ppm, 30.00±1.45% of wild type 30-ppm). Significant difference was observed between wild-type 300-ppm and HFE KO 30-ppm and HFE KO 300-ppm heart cross-sections compared with wild-type 30 ppm iron.

Apoptotic cell death:

As shown both wild-type and HFE KO hearts displayed significant number of apoptotic cardiomyocytes after 30 minutes of ischemia and 2 hours of reperfusion. The results indicate significantly higher numbers of apoptotic cardiomyocytes in the HFE KO mouse hearts compared with corresponding wild-type hearts. The number of apoptotic cells was higher in hearts fed diet containing high iron.

II. THIOREDOXIN STUDY

Animals:

The full-length human thioredoxin 1 (hTrx1) cDNA clone digested by EcoRI-NotI was inserted into the EcoRI plus SpeI digested-human β-actin expression vector by ligation of cohesive ends at 5'-end and of blunt ends at 3'-end of the cDNA. The entire expression sequence containing the human β-actin promoter, hTrx1 cDNA, and SV40 late

polyadenylation site was separated from the plasmid vector by digestion with restriction enzymes KpnI and SalI, and purified from agarose gel. The DNA fragment was then microinjected into fertilized mouse eggs harvested from mating of B6D2 (C57BL/6 × DBA/2) F1 hybrid mice. One transgenic founder was identified by Southern blot analysis of mouse tail DNA. The experimental animals including transgenic mice and nontransgenic littermates were derived from breeding of the homozygous transgenic founder with B6D2 F1 hybrid mice.

Confirmation of the hTrx expression in mice:

The total RNA was isolated and blot preparation, analysis performed according to the procedures described by Thomas, and was hybridized with the hTrx1 cDNA. To evaluate the elevated presence of the hTrx protein in myocardial tissue, we did Western blot analysis where heart samples were homogenized in 50 mM potassium phosphate buffer, pH 7.8, containing 0.1% Triton X-100 and 1 mM phenylmethyl-sulfonyl fluoride and protein contents were determined with the use of a bicinchoninic acid protein assay kit. Twenty-five micrograms of total heart proteins were separated on a 12.5% SDS polyacrylamide gel and then transferred to nitrocellulose membrane. The protein blot filter was first reacted with an immunoaffinity purified polyclonal goat anti-hTrx1 antibodies and then reacted with horseradish peroxidase-conjugated rabbit anti-goat IgG antibodies. Visualization of the secondary antibodies was performed with the SuperSignal chemiluminescent substrates from Pierce.

Protocol:

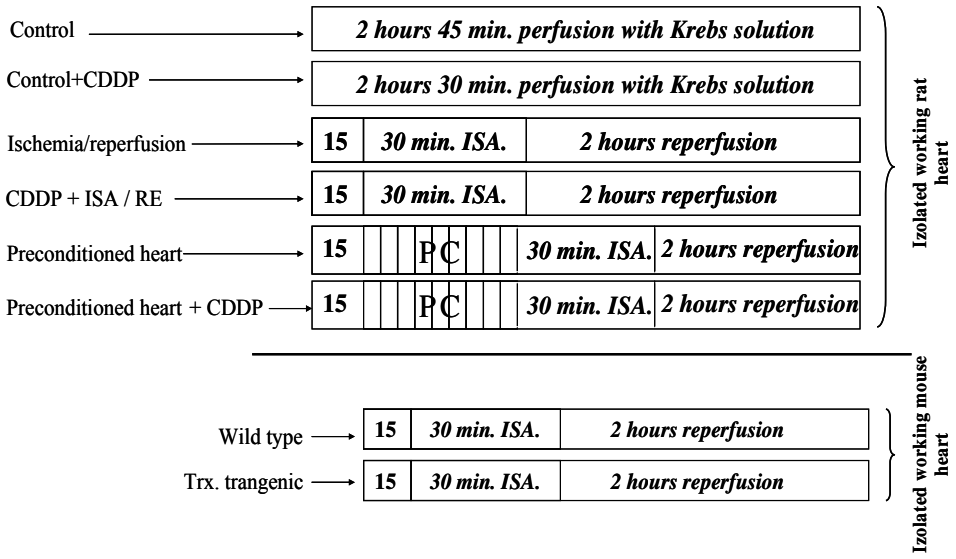

Figure 1. The experimental protocol

Isolated working rat and mouse hearts preparation:

This previously described the method of isolated working mice heart perfusion. The rat experiment's steps are similar to the study comparing to the mice heart except the anesthesia that was performed with 80 mg/kg b/w. intraperitoneal injection sodium pentobarbital. The afterload pressure in case of the rat perfusion was 100 mmHg (14 KPa), the preload pressure was equivalent to 17 mmHg (2,4 KPa).

Cardiac functions:

We measured them the same way according to the HFE study described above.

Determination of the infarct size:

We did it in the same described previously.

Northern blot analysis:

Isolation of RNA was performed as described by Hattori et al. For electrophoresis, Northern blot transfer and hybridization were used. As a loading standard, GAPDH mRNA was employed. The Northern blots were densitometrically scanned and the ratio of Trx1 over GAPDH mRNA was expressed. The images were then digitized and analyzed with the NIH image public domain image analysis software program.

Measurement of malonaldehyde for assessment of oxidative stress:

Malonaldehyde (MDA) was measured as MDA-DNPH derivative by HPLC. MDA was assayed as described previously, to monitor the development of oxidative stress.

TUNEL assay for assessment of apoptotic cell death:

The complete method was previously described.

Statistical analysis:

The values for myocardial functional parameters, MDA, risk and infarct volumes, and infarct sizes were all expressed as mean ± standard error of the mean (SEM). The statistical analysis was performed by one-way analysis of variance and Scheffe's multiple-comparison test for any differences between the mean values of all groups. Differences between data were analyzed for significance by performing a Student's *t*-test. The results were considered significant if $p < 0.05$.

RESULTS II.

Experiments with isolated rat hearts

Induction of Trx1 during myocardial adaptation to ischemic stress:

Northern blot analysis revealed that Trx was present in the intact aerobically perfused heart. As expected, CDDP blocked the expression of Trx1. The expression of Trx was also reduced in the ischemic/reperfused myocardium. Ischemic stress adaptation enhanced the expression of Trx, which was drastically reduced with CDDP.

Effects of Trx1 inhibition on the recovery of ventricular function:

We found that post-ischemic contractile function was not affected, when hearts were perfused for 2 h 45 min. with the KHB buffer. The cardiac function was also not affected, when the control hearts were preperfused for 15 min in presence of CDDP. As expected, recovery of post-ischemic cardiac function was reduced after 60 min. of reperfusion, and further reduced after 120 min. of reperfusion. Preperfusion with CDDP further reduced the recovery of cardiac function. Adapted hearts displayed significant recovery of post-ischemic ventricular function as compared to that after ischemia/reperfusion. The cardioprotective effects of ischemic adaptation were abrogated, when the adapted hearts were preperfused with CDDP.

Effects of Trx1 inhibition of myocardial infract size and cardiomyocyte apoptosis:

The ischemia/reperfusion increased myocardial infract size as well as apoptotic cell death. While CDDP itself had no effect on the heart, it increased infarct size and the number of apoptotic cardiomyocytes, slightly but not significantly. Myocardial infarct size and cardiomyocyte apoptosis were significantly reduced in the adapted myocardium. Cardioprotective effects of ischemic adaptation were partially abolished, when the adapted hearts were preperfused with CDDP.

Effects of Trx1 inhibition on ischemia/reperfusion-induced oxidative stress:

MDA content was increased progressively and steadily at the period of the reperfusion time in all groups of hearts. CDDP increased the MDA content even in the aerobically perfused heart and ischemic/reperfused hearts; but this increase was not significant. MDA content was significantly reduced in the adapted heart and this reduction of MDA was inhibited by CDDP.

Experiments with isolated mouse hearts

The hTrx1 is highly expressed in the heart of transgenic mice:

RNA blot analysis of RNAs from various tissues of transgenic mice revealed the hTrx1 mRNA is highly expressed in brain, heart, lung, skeletal muscle, spleen, and tongue. Two species of hTrx1 mRNA with sizes of 1.1 and 0.75 kb are expressed in the heart of transgenic mouse. Protein blot study shows that a single species of 12-kDa protein in transgenic heart reacts, strongly with the polyclonal anti-hTrx1 antibodies.

Recovery of myocardium contractile performance:

Due to high incidence ventricular fibrillation and conduction disturbance, two wild type and two transgenic hearts were excluded from this study. The LVDP displayed depressed recovery after ischemia compared to baseline values in both groups. However, the recovery of LVDP was significantly higher in the Trx1 group compared to wild-type controls. The recovery of d*p*/d*t* was significant at all levels. Significantly, difference in aortic flow was also noted during the reperfusion. There were no significant differences between the groups for coronary flow and heart rate.

Myocardial infarct sizes in wild-type and Trx1 transgenic mice:

Infarct size expressed as percent infarction to total area at risk was noticeably decreased in Trx1 transgenic mouse hearts (15.90 ± 0.29%) compared to the wild-type control (37.11 ± 0.18%).

II. STAT5A AND STAT6 STUDY

Knock out mice devoid of STAT5A and STAT6:

The knock out mice devoid of any copies of either STAT5A or STAT6 were obtained from the Jackson Laboratory (Bar Harbor, ME). The Western blot analysis demonstrates complete absence of STAT5A and STAT6 in the heart of these mice. Corresponding wild-type mice (B6129SF2/J101045 for STAT5A and BALB/cJ000651 for STAT6) were also obtained from the same supplier.

Isolated working mice and rat perfusion system:

It was performed by same manner as we previously described.

Protocol:

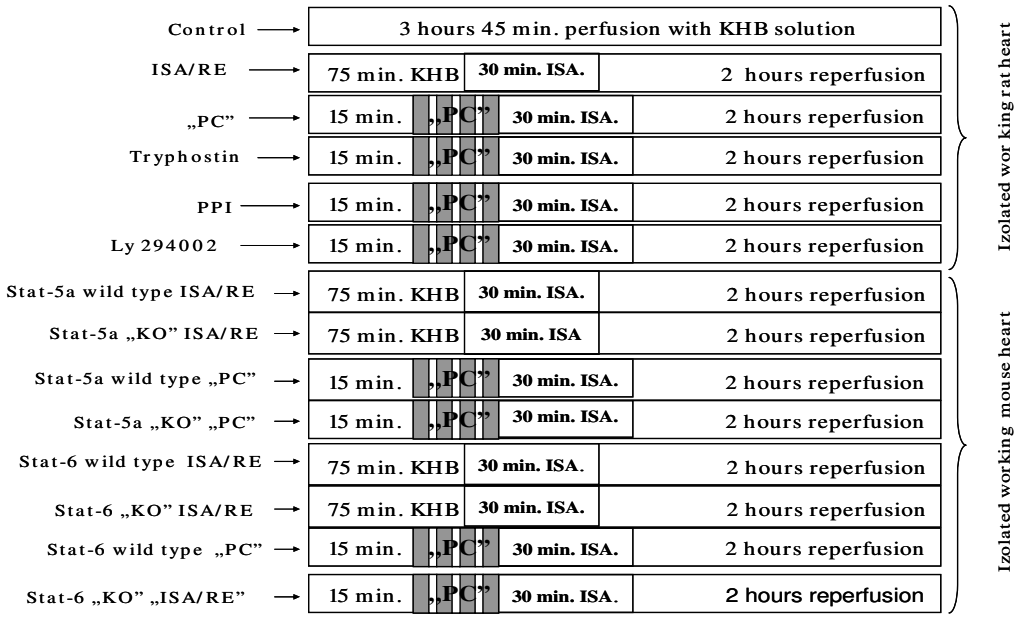

Figure 2. Experimental protocol

Measurement of the infarct size and apoptotic cardiomyocytes cell death by TUNEL method:

We did it according to HFE and Trx study.

Western blot analysis:

Left ventricles from hearts were homogenized in a buffer containing (in mM) 25 Tris HCl, 25 NaCl, 1 orthovanadate,10 NaF, 10 pyrophosphate, 10 okadaic acid, 0.5 EGTA, and 1 PMSF. Proteins (100 μg) of each heart homogenate were incubated with 1 μg of antibody against STAT5A and STAT6 (Santa Cruz Biotechnology; Santa Cruz, CA) for 1 h at 4°C. The immune complexes were precipitated with protein A-Sepharose, and immunoprecipitates were separated by SDS-PAGE and immobilized on polyvinylidene difluoride membrane. The membrane was immunoblotted with phosphotyrosine (PY20) clone antibodies to evaluate the phosphorylation of STAT5A and STAT6. The membrane was stripped and reblotted with specific antibodies against STAT5A and STAT6. The resulting blots were digitized and subjected to densitometric scanning using a standard NIH image program.

Statistical Analysis:

The values for myocardial functional parameters, number of apoptotic cardiomyocytes, and infarct sizes were all expressed as the means ± SE. The statistical analysis was performed by one-way analysis of variance for any differences between the mean value of all groups.

Differences between data were analyzed for significance by performing a Student's *t*-test. The results were considered significant if $P < 0.05$.

RESULT III.

Experiments with isolated rat hearts

Phosphorylation of STAT5A and STAT6:

Western blot analysis revealed that both STAT5A and STAT6 are extensively phosphorylated during preconditioning. The tyrphostin or PPI had no effect on the phosphorylation of either STATs (control), but they partially blocked the phosphorylation of STAT5A during preconditioning. Interestingly, PPI was quite effective in blocking the phosphorylation of STAT5A, but it had no effect on STAT6 phosphorylation, suggesting a role of src kinase in STAT5A phosphorylation only. Tyrphostin reduced the phosphorylation of both the STATs, suggesting the involvement of JAK2 in STAT phosphorylation.

Effects of inhibition of phosphorylation of STAT5A and STAT6 on myocardial infarct size and cardiomyocytes apoptosis:

Myocardial infarct size and cardiomyocytes apoptosis were significantly reduced in the preconditioned heart. Whereas none of these inhibitors including tyrphostin, PPI, and LY-294002 had any effect on the heart function, they partially abolished the cardioprotection afforded by preconditioning by increasing the infarct size and the number of apoptotic cardiomyocytes, suggesting a role of multiple signal transduction pathways in preconditioning. Inhibition of PI-3 kinase with LY-294002 completely abolished the cardioprotective abilities of preconditioning.

Experiments with isolated mouse hearts

Recovery of myocardium contractile performance and infarct size after ischemia/reperfusion:

The results indicate that the hearts from STAT5A knock out mice could not be preconditioned because there were no differences in the infarct size, the number of apoptotic cardiomyocytes, and cardiac functions between the ischemia-reperfusion group and the preconditioned group. In contrast, preconditioning significantly decreased the myocardial infarct size and reduced the number of apoptotic cardiomyocytes in these hearts indicating that STAT6 knock out hearts could be preconditioned.

IV. COXB III. , ATPS6, and HO-1

Isolated „working heart" preparation:

It was performed by same manner as we previously described.

Induction of diabetes:

Diabetes mellitus was induced by i.v. injection of streptozotocin (55 mg/kg).

Heme oxygenase-1 studies:

An epicardial ECG was recorded throughout the experimental period by two silver electrodes attached directly to the heart.

Mitochondrial gene expression studies:

Serum glucose was measured by a spectrophotometer using standard assay kits. ECG was registered by a recorder (Haemosys, Experimetria) throughout the experimental period by two silver electrodes attached directly to the heart.

Total RNA isolation:

Total RNA was isolated from heart tissue (100 mg) by homogenization in 1 ml of TRIzol reagent (Gibco BRL, Germany), a guanidium thiocianate method. The RNA pellets were dissolved in 100 µl diethyl pyrocarbonate water, the concentrations were calculated from the absorbance at 260 nm measured by ultraviolet spectroscopy.

Northern blot:

Hybridizations were performed as previously described. RNA was prepared from the left ventricle (about 100 mg), and 5 µg total RNA was subjected to electrophoresis in formaldehyde-containing 1% agarose gels and transferred to nylon membranes by standard capillary transfer. Hybridization was carried out with ^{32}P-labeled probes in a hybridization oven. After autoradiography and automated radiometric scanning, stripping and re-probing for housekeeping gene mRNA levels (GAPDH), the membranes' RNA was routinely employed for standardization of quantitative measurements.

Western blot:

Myocardial samples were homogenized in Tris-HCl (13.2 mM/L), glycerol (5.5%), SDS (0.44%), and β-mercaptoethanol. The same amount of soluble protein (50 µg) was fractionated by Tris-glycine-SDS-polyacrylamide gel (12%) electrophoresis, and Western blot

was carried out as described by Pellacani et al with the use of an antibody to recombinant rat HO-1 protein.

RT-PCR:

Aliquots containing 50 ng total RNA or 20 ng mRNA were digested with amplification grade DNAse I (GibcoBRL) to remove any DNA contamination and reverse transcribed into cDNA using 0.1 μg random 9-mer primers (Stratagene). Quantitation was done as described by Feldman et al [21]. Aliquots corresponding to 0.1 μg initial total RNA was amplified for 12-26 cycles using specific primers (5'-CGAACCTGAGCCCTAATA and 5'-GTAGCTCCTCCGATTAGA, 312 bp for ATPase 6; 5'-CTTAGCATCAGGAGTCTC and 5'-TATCATGCTGCGGCTTCA, 338 bp for cytochrome oxidase III) in an optimized reaction using the AmpliTaq PCR kit. Fragments were radiolabeled by the use of 10 μCi [^{32}P]dCTP per reaction and five 1/10 volume aliquots were taken every two cycles. Unincorporated nucleotides were separated on 3% agarose gel electrophoresis and radioactivity incorporated in amplified fragments measured by liquid scintillation.

Determination of infarct size:

We did same way, described previously.

Determination of CO content:

We measured tissue CO content using gas chromatography as described by Cook et al.

Measurement of cellular Na^+, K^+, and Ca^{2+}:

Cellular electrolytes were measured as described previously.

Immunohistochemistry:

Paraffin sections (5 μm) of left ventricular tissue were incubated in the presence of polyclonal antibody and purified liver HO-1 obtained from rats (Stress Gen Biotech., Canada). Reactions were visualized by immunoperoxidase color reaction in wild-type (+/+), heterozygous (+/-), and homozygous (-/-) HO-1 mutant mice.

Statistics:

The data for HR, CF, AF, AOP, AOP dp/dt, infarct size, myocardial electrolyte contents and tissue CO were expressed as the mean ± SEM. 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 wild type groups were compared to those of HO-1 heterozygous and homozygous groups, and the values of the diabetic groups were compared with those of the nondiabetic age-matched controls by multiple t-test followed by Bonferroni correction. A change of $p<0.05$ was considered to be statistically significant.

RESULTS IV.

The mRNA expression of the identified genes:

The upregulation of COXBIII was observed as early as 30 min following the onset of ischemia ($^{\#}p<0.05$ vs. controls) and after 120 min of reperfusion in nondiabetic and nonfibrillated hearts ($^{\#}p<0.05$ vs. controls).

Cardiac functions and serum glucose:

Cardiac function (AF and LVDP) was reduced in diabetic myocardium (serum glucose: 508±31 mg/dl) in comparison with the age-matched nondiabetic (serum glucose: 128±31 mg/dl) hearts. Reperfusion resulted in a relatively weak postischemic cardiac function in nondiabetic nonfibrillated myocardium. In ischemic/reperfused nondiabetic hearts further significant reduction in postischemic cardiac function was observed. In electrically fibrillated nondiabetic myocardium, the postfibrillated cardiac function was significantly improved in comparison with the ischemic/reperfused and fibrillated group. In diabetic ischemic/reperfused, fibrillated and nonfibrillated hearts, the trend in the recovery of postischemic cardiac function was the same as it was observed in the nondiabetic group, however cardiac function and contractility were significantly reduced in diabetic groups compared to the age-matched nondiabetic values.

HO-1 knockout mice:

The low HO-1 (-/-) mice survival percentage (about 18 %) was maintained in matings between HO-1 (+/-) and HO-1 (-/-) mice, which yielded 105 HO-1 (+/-) and 13 HO-1 (-/-) mice, however matings between HO-1 (-/-) males and HO-1 (-/-) females did not yield viable litters.

HO-1 in nonischemic mouse heart:

Immunohistochemistry:

Left ventricular cardiac biopsies were obtained from a wild type (+/+), a heterozygous (+/-),

and a homozygous mutant (-/-) mouse heart, respectively, perfused under aerobic conditions, then subjected to immunochemical reaction. Homogenous cytoplasmic staining of HO-1 in wild type (+/+) nonischemic myocardium was observed after 20 min of aerobic perfusion.

Northern blot:

Blots were hybridized with a rat HO-1 cDNA probe recognizing a major mRNA band of approximately 1.5 kb in both the (+/+) and (+/-) mouse myocardium, respectively.

Western blot:

HO-1 protein expression was detected with the use of recombinant rat HO-1 antibody as described in the Methods. Data clearly show that neither HO-1 mRNA nor its protein was detected in the homozygous (-/-) myocardium perfused under aerobic conditions.

HO-1 in ischemic/reperfused hearts:

Immunohistochemistry:

In hearts subjected to 20 min of ischemia followed by 120 min of reperfusion. Upon reperfusion, in wild type (+/+) control mice, HO-1 enzyme localization was detected in nonfibrillated/reperfused myocardium (No-VF).

Western blot:

HO-1 protein expression was detected in ischemic/reperfused nonfibrillated (No-VF) wild type (+/+) and nonfibrillated heterozygous (+/-) hearts. However, in HO-1 homozygous hearts (-/-), HO-1 protein was not detected either in ischemic/reperfused nonfibrillated (electrically defibrillated) or fibrillated myocardium.

Endogenous CO detection in mouse myocardium:

In wild type (+/+) and heterozygous (+/-) hearts subjected to 20 min of ischemia followed by 30 min and 120 min of reperfusion, a substantial increase in CO production was observed in comparison with the nonischemic wild type (+/+) myocardium. However, in the homozygous (-/-) mouse myocardium, endogenous CO production because of the lack of the function of HO-1 system was detected at a low level.

Cardiac function, infarct size and myocardial Na^+, K^+, and Ca^{2+} contents:

Before the induction of global ischemia a significant reduction in HR, CF, AF, AOP, and AOP dp/dt was observed in HO-1 knockout homozygous (-/-) mouse hearts in comparison

with the corresponding values obtained in wild type (+/+) and heterozygous (+/-) mouse myocardium. Infarct size was significantly increased from 37.6±4 % (wild type) and 38.5±4 % (HO-1 heterozygous) to 49.5±5 % ($p<0.05$) in the HO-1 homozygous group. Tissue Na^+ and Ca^{2+} contents are significantly elevated before the induction of ischemia in the -/- myocardium in comparison with the +/+ and +/- values. In addition, the cellular K^+ was significantly at a lower level (253±8 μmol/g dry weight) in the -/- group compared to the nonischemic +/+ value (278±7 μmol/g dry weight).

DISCUSSION

Discussion I.

In the first part of the study, we found that iron content of the diet interacted with the HFE polymorphism to increase ischemia/reperfusion injury in the heart. Iron overload is the most common metal-related toxicity that can result in cardiomyopathy and arrhythmias leading to heart failure. The exact mechanism(s) by which iron overload induces myocardial injury is not completely understood, but the results demonstrate the presence of increased granules of both Fe^{2+} and Fe^{3+} in the hearts of the chronically iron-loaded mice. These results support the notion that iron-catalyzed OH· radical formation (Fenton Reaction) may be primarily responsible for the formation of ROS, which then lead to cellular injury. The results of our study document that the amount of MDA, presumptive marker for lipid peroxidation and oxidative stress, was significantly higher in hearts of HFE KO mice compared with those present in the wild-type mouse heart. Consistent with these results, the amount of Mn-SOD, Cu/Zn-SOD, catalase, and GSHPx-1 activities were lowered in HFE KO mouse hearts as compared with those in the wild-type hearts. The results of the present study show that HFE KO mouse hearts are subjected to excessive oxidative stress and greater degree of ischemic reperfusion injury as evidenced by increased myocardial infarct size and cardiomyocyte apoptosis and reduced postischemic ventricular recovery. The role of ROS is further supported from the results that HFE KO hearts of the mice fed high-iron diet were subjected to greater degree of oxidative stress and as a result greater degree of myocardial injury. Mammalian hearts are protected from the cellular injury by their own defense system, which includes various intracellular antioxidants, such as glutathione, α-tocopherol, ascorbic acid, β-carotene; and antioxidant enzymes that include SOD, catalase, and glutathione peroxidase. These cellular compounds reduce/eliminate the oxidative stress by directly quenching the reactive oxygen species before they damage vital cellular components, and therefore, they can be considered as part of the first line of defense against the external stress. The levels of these antioxidant enzymes reduced dramatically in the heart of the HFE KO mice fed high-iron diet, suggesting that increased iron levels resulting in a higher amount of oxidative stress could be instrumental for the reduced level of antioxidant enzymes in these hearts. HFE mutation is common in certain ethnic portions of the population, that about 10% or these populations are heterozygotes, and that heterozygotes exhibit higher body iron levels than normal. Epidemiological investigation also supports the role of increased iron in long-term risk of

coronary events, including myocardial infarction. Further work should include examination of possible increased susceptibility of heart to other insults, and examination of other genetic polymorphisms that affect or modify the HFE gene in iron absorption. Future experiments should also investigate the impact of antioxidant administration on the ability of the high-iron diet to induce cardiac damage, as well as consideration of other dietary constituents that may modify the absorption and/or availability of iron such as phytate and vitamin C.

Discussion II.

The results of the present study demonstrate that Trx plays an important role in myocardial ischemia/reperfusion injury. There are several salient features of the study including:

1. Trx1, which is ubiquitously present in the heart, is downregulated after ischemia/reperfusion but upregulated in the adapted myocardium;
2. Inhibition of Trx1 with CDDP abrogated cardioprotective effects of ischemic adaptation as evidenced from impaired post-ischemic ventricular recovery, increased myocardial infarct size, and cardiomyocytes apoptosis;
3. Inhibition of Trx1 enhances oxidative stress in the adapted myocardium;
4. Transgenic mouse hearts with extra copies of Trx1 are resistant to myocardial ischemic/reperfusion injury;

Myocardial ischemia/reperfusion is associated with increased ROS formation, which is significantly reduced by ischemic stress adaptation. Consistent with these results, an increase cardiomyocyte apoptosis in the ischemic/reperfused heart is prevented by preconditioning. A reduction of Trx expression as a result of Trx oxidation leads to the development of oxidative stress and induces apoptotic cell death. We have used cisplatin and CDDP to inhibit Trx1. CDDP, an antitumor agent, widely used in chemotherapy of human cancers, functions by causing cell cycle arrest and potentiating apoptosis. CDDP is a highly effective inhibitor for Trx1 system, including Trx and Trx reductase. We used 1 mM of CDDP, when Trx1 was almost completely blocked. At lower concentrations (0.5 and 0.75 mM) only partial amount of Trx1 was inhibited. Many redox-sensitive transcription factors including NFκB and AP-1 have been shown to be redox regulated by thioredoxin. Trx, thus, plays a crucial role in thiol-redox control of cell function through transcription regulation of target genes including NF-κB, which control numerous gene expressions. Several recent studies have demonstrated an

increase in DNA binding of NF-κB and AP-1 in the ischemic/reperfused myocardium. Trx has been shown to function as intracellular antioxidant by scavenging ROS, and can protect the cells against oxidative stress. The results of our study clearly demonstrated increased amount of MDA formation in the hearts, when Trx1 was inhibited with CDDP. Oxidative stress was significantly reduced in the ischemic/reperfused myocardium, when the heart was adapted to ischemic stress. CDDP increased the amount of MDA in the heart, suggesting that Trx1 is indeed instrumental for lowering the oxidative stress in the heart. Interestingly, myocardial adaptation to ischemic stress was accompanied by an overexpression of Trx1. The results of this study clearly document a crucial role of Trx1 in redox signaling in the ischemic myocardium. To further confirm cardioprotective function of Trx1, a line of hTrx1 transgenic mice was generated for elucidating the function of Trx1 in myocardial ischemia/reperfusion. Transgenic mouse hearts overexpressing Trx1 were resistant to ischemic/reperfusion injury as evidenced by improved post-ischemic ventricular recovery and reduced myocardial infarct size as compared to corresponding wild-type mouse hearts. These results are in consistent with previous report that the elevation of serum Trx1 level negatively correlated with the left ventricular ejection fractions in patients with heart diseases. The results of the present study, support the cardioprotective role of Trx and further document that Trx1 can be induced in the heart after adaptation to ischemic stress.

Discussion III.

The results of the present study demonstrate that STAT5A, not STAT6, plays an important role in preconditioning, although both of these STATs are activated in the heart after preconditioning. There are several salient features of the study including *1*) both STAT5A and STAT6 are activated in the hearts after ischemia and reperfusion but only STAT5A plays a role in preconditioning; *2*) STAT5A preconditioning is achieved with two different upstream signaling components, Src kinase and JAK, where Src kinase appears to play a predominant role; *3*) Src kinase-STAT5A signaling is linked to PI-3 kinase Akt-mediated surviving signals leading to the reduction of cardiomyocytes apoptosis; and *4*) STAT5A knock out hearts were resistant to preconditioning, whereas STAT6 knock out hearts are not affected by preconditioning stimulus. PC reduced myocardial infarct size as well as cardiomyocytes apoptosis, whereas these parameters were proportionately increased with the inhibitors of JAK2, Src kinase, or PI-3 kinase suggesting that both necrosis and apoptosis are independent contributors of myocardial infarction. The results of this study suggest a role of STAT5A, in

addition to previously reported STAT3, in ischemic preconditioning of myocardium. STAT activation occurred through the upstream signaling component JAK2, and JAK2-STAT3 signaling was instrumental for changing the ischemia/reperfusion-mediated death signal into preconditioning-mediated survival signal. The present study demonstrates that in addition to JAK2, Src tyrosine kinase is also involved in STAT5A signaling because the specific Src kinase inhibitor PPI mostly abolished the cardioprotective abilities of STAT5A signaling. A role of tyrosine kinases in preconditioning is widely recognized, and as mentioned above, Src kinase appears to play an essential role in transmitting preconditioning-mediated survival signal. In the present study, we demonstrate that JAK2 and Src kinase equally contribute in transmitting survival signal in the preconditioned myocardium. Inhibition of either JAK2 with tyrphostin or Src kinase with PPI blocks the activation of STAT5A and consequently blocks cardioprotective abilities of STAT5A preconditioning. Interestingly, Src kinase-STAT5A signaling appears to be linked with PI-3 kinase-Akt-mediated survival signals. Inhibition of Src kinase by PPI, but not the inhibition of JAK2 by tyrphostin blocked Akt phosphorylation, suggesting that STAT5A activation via JAK2 does not contribute to PI-3 kinase-Akt-mediated survival signal. Consistent with these reports, the present results demonstrate that inhibition of PI-3 kinase with LY-294002 blocked the phosphorylation of Akt and abolished the antiapoptotic abilities of preconditioning. Akt, a serine-threonine kinase, is a key effector of PI-3 kinase in the survival pathway against apoptosis. Akt can phosphorylate the proapoptotic protein Bad thereby inhibiting its proapoptotic function, which may account for the antiapoptotic effect of Akt.

Discussion IV.

I. Ischemic episodes elicit different changes in the expression of several genes, arrhythmogenesis, and cardiac function in the myocardium. We have examined the dependence of reperfusion-induced VF on mitochondrial mRNA regulation in perfused nondiabetic and diabetic rat hearts. In addition, we have analyzed the relationship between VF and mitochondrial gene expression focusing on COXBIII and ATPS6, without the ischemic/reperfused protocol, in electrically fibrillated myocardium. In our studies, the development of reperfusion-induced VF was critically dependent upon the downregulation of COXBIII and ATPS6. This finding was directly supported by data obtained in electrically fibrillated myocardium indicating that VF elicits the downregulation of COXBIII and ATPS6. In summary, it has been published that diabetic hearts respond on different ways to the

susceptibility of the myocardium in comparison with the nondiabetic myocardium. However, in our studies, significant changes, regarding the arrhythmogenic mechanism in connection with COXBIII and ATPS6 mRNA expression, were not observed between ischemic/reperfused diabetic and nondiabetic myocardium, indicating that these two mitochondrial genes may play a crucial role in arrhythmogenesis under diabetic as well as nondiabetic conditions.

II. In the second part of our last studies, we have endeavored to obtain more circumstantial evidence for the involvement of HO-1-related endogenous CO production and its direct measurement in the genesis of reperfusion-induced ventricular fibrillation. We now provide direct evidence by measuring cellular CO production suggesting that this is the case and the protective effect against the development of reperfusion-induced VF is attributable to the generation of endogenous CO through HO-1 mRNA induction. We found, in the present study, that infarct size was significantly increased in homozygous KO mouse hearts, and this increase in infarct size related to a significant elevation in myocardial Na^+ and Ca^{2+} accumulation and K^+ loss. We have shown that HO-1 and HO-1-mediated endogenous CO production play an important role in myocardial homeostasis by protecting the heart from ischemia/reperfusion-induced injury, including reperfusion-induced VF.

SUMMARY

The phenomenon of the ischemia/reperfusion has relevant role in the cardiac surgery and thrombolytic treatment, because the elevation of immediate O_2 tension causes severe free radicals generation-induced cell injury. To know the mechanisms of this type of process could be beneficial and useful tool in developing new therapeutic agents.

In this present work we focused on the free radical induced myocardial damages. In the first section we tried to establish correlation between the dietary iron content and HFE polymorphism using isolated working mice hearts. Our study revealed that HFE knock out mice feeding with 30 and 300 ppm iron showed susceptibility to ischemic/reperfusion induced cardiac injury. This phenomenon was evidenced by impaired left ventricular function, increased infarct size, and elevated cardiac apoptotic cell number. The antioxidant enzyme including SOD, catalase, and peroxidase indicated lower activity in HFE KO mice feeding with 30 and 300 ppm iron enriched diet.

In the second part of the thesis work, we made effort to examine the human Trx effect against the reperfusion-induced cell injury. Using Trx transgenic mice, that are containing extra copy of the Trx gene, were submitted 30 minutes ISA and 2 hours reperfusion. These transgenic mice displayed improved cardiac function, reduced infarct size and apoptotic cell numbers. The MDA contents, which is a marker of the oxidative stress was significantly reduced, indicating that Trx has important role in the cellular defense mechanisms. Preperfusion of the hearts with CCDP, an anticancer agent was used to determine its effect on the Trx in the cardioprotection. We found that all beneficial action of Trx was diminished with the administration of CDDP. The CDDP also was able to abolish the protective effects of precondition in connection with the Trx elevated expression in ischemic/reperfused hearts.

In the third part of our study we focused on the role STAT5A and STAT6 in ischemic preconditioning. All animals and their corresponding wild type mice were subjected to PC and 30 minutes ischemia followed by 2 hours reperfusion. Using different type of phosphorylase inhibitors to elucidate what kind of signal-trasduction pathways could be activated after PC. Our results demonstrated that the Src and Jak2 kinase activated STAT5A has a significant role in the ischemic preconditioning evidenced by decreased phosphorylation by PPI, and tyrphostin. In mice studies the STAT5A KO showed increased infarction and an elevation in the cardiac apoptotic cell numbers.

In the last part of our work we tried to find correlation between the mithocondrial genes (COXB III., ATPS6), HO-1 and arrhythmogenesis. Our data suggest that COXBIII, ATPS6,

and HO-1 may play a critical role in arrhythmogenesis, and the stimulation of COXBIII, ATPS6, and HO-1 mRNA expression may prevent the development of VF in both nondiabetic and diabetic ischemic/reperfused myocardium.

Our result shows that certain genes could be involved in ischemic/reflow induced myocardial cellular injury and process of ischemic preconditioning. The practical advantages of these genes are still unknown, but further experiments are needed to be performed. Further research work will be necessary to confirm the application of these genes in gene therapy process which might be useful tool in the treatment of cardiac diseases.

The dissertation is based on the following publications.

1. Bak I, Szendrei L**, Turoczi T,** Papp G, Joo F, Das DK, de Leiris J, Der P, Juhasz B, Varga E, Bacskay I, Balla J, Kovacs P, Tosaki A. Heme oxygenase-1-related carbon monoxide production and ventricular fibrillation in isolated ischemic/reperfused mouse myocardium. *FASEB J*. 2003 Nov; 17 (14): 2133-5. **Impact Factor: 7,252**

2. Szendrei L**, Turoczi T,** Kovacs P, Vecsernyes M, Das DK, Tosaki A**.** Mitochondrial gene expression and ventricular fibrillation in ischemic/reperfused nondiabetic and diabetic myocardium. *Biochem Pharmacol*. 2002 Feb 1; 63(3): 543-52. **Impact Factor: 3,542**

3. **Turoczi T,** Chang VW, Engelman RM, Maulik N, Ho YS, Das DK. Thioredoxin redox signaling in the ischemic heart: an insight with transgenic mice overexpressing Trx1. *J Mol Cell Cardiol*. 2003 Jun; 35(6): 695-704. **Impact Factor: 4,950**

4. **Turoczi T,** Jun L, Cordis G, Morris JE, Maulik N, Stevens RG, Das DK. HFE Mutation and Dietary Iron Content Interact to Increase Ischemia/Reperfusion Injury of the Heart in Mice. *Circ Res*. 2003 Jun 13; 92(11): 1240-6. **Impact Factor: 9.694**

5. Yamaura G, **Turoczi T,** Yamamoto F, Siddqui MA, Maulik N, Das DK. STAT signaling in ischemic heart: a role of STAT5A in ischemic preconditioning. *Am J Physiol Heart Circ Physiol*. *2003* Aug; 285 (2): H476-82. **Impact Factor: 3,369**

Abstract:

1. Cordis G**, Turoczi T**, Jun L, Morris JE, Maulik N, Stevens RG, Das DK. Detection of Oxidative Stress in Mouse Heart affected by HFE Mutation and Dietary Iron Content by estimating the Dinitrophenylhydrazine Derivative of Malonaldehyde. *J. Mol. Cell. Cardiol.,* 2003, **35** (6):A46 **IF: 4,091**

2. Cordis G**, Turoczi T**, Jun L, Morris JE, Maulik N, Stevens RG, Das DK. Detection of Oxidative Stress in Mouse Heart affected by HFE Mutation and Dietary Iron Content by estimating the Dinitrophenylhydrazine Derivative of Malonaldehyde. *J. Mol. Cell. Cardiol.,* 2003, **35** (6):A46 **IF: 4,091**

3. Szendrei L**, Turoczi T**, Das DK, Tosaki A. The role of the mitochondrial gene expression in ventricular fibrillation. *J. Mol. Cell. Cardiol.* 2001, **33:** (6) p.A117, **IF: 4,091**

4. Szendrei L**, Turóczi T**, Das DK, Tosaki A. Reperfusion-induced venrticular fibrillation and mitochondrial gene expression in ischemic/reperfused hearts. *J. Mol. Cell. Cardiol.* 2002, **34** (6): A61, **IF: 4,091**

5. Szendrei L, Bak I, Juhasz B, **Turoczi T**, Das DK, Tosaki A. The role of heme-oxygenase-1 and carbon monoxide in reperfusion–induced ventricular fibrillation. *J. Mol. Cell. Cardiol.* 2003, **35** (6): A45, **IF: 4,091**

6. **Turoczi T,** Jun L, Cordis G, Morris JE, Maulik N, Stevens RG, Das DK**.** HFE Mutation and Dietary Iron Content Interact to Increase Ischemia/Reperfusion Injury of the Heart in Mice. *J. Mol. Cell. Cardiol,* 2003, **35** (6):A39, **IF: 4,091**

7. **Turoczi T,** Jun L, Cordis G, Morris JE, Maulik N, Stevens RG, Das DK**.** HFE Mutation and Dietary Iron Content Interact to Increase Ischemia/Reperfusion Injury of the Heart in Mice. *FASEB J.,* 2003, **17** (5): A18 **IF: 7,252**

8. **Turoczi T,** Jun L, Cordis G, Morris JE, Maulik N, Stevens RG, Das DK**.** HFE Mutation and Dietary Iron Content Interact to Increase Ischemia/Reperfusion Injury of the Heart in Mice. *Circ.* 2003 Jun 13; **92** (11): 1240-6. **IF: 11.164**

The list of publications was not used in this study but correlated to the dissertation

1. Bak I, Papp G**, Turoczi T,** Varga E, Szendrei L, Vecsernyes M, Joo F, Tosaki A. The role of heme oxygenase-related carbon monoxide and ventricular fibrillation in ischemic/reperfused hearts. *Free Radic Biol Med*. 2002 Sep 1; 33(5): 639-48. **Impact Factor: 5.533**

2. Juhasz B**,** Der P**, Turoczi T,** Bacskay I, Varga E, Tosaki A. Preconditioning in intact and previously diseased myocardium: laboratory or clinical dilemma? *Antioxid Redox Signal*. 2004 Apr; 6 (2):325-33. **Impact Factor: 3,027**