

THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY

**Ischemia and reperfusion in the myocardium: molecular
mechanisms of reperfusion-induced ventricular fibrillation**

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Introduction

Heart disease and cardiac surgery frequently result in periods of myocardial ischemia. Restoration of blood supply and reoxygenation of the myocardial tissue causes characteristic tissue damage, known as reperfusion injury, manifested by symptoms including ventricular arrhythmias and deterioration in cardiac functions. Reperfusion arrhythmias occur within seconds of the onset of reflow and have been observed in all species studied, including humans, in whom they regularly occur during cardiac surgery and thrombolytic therapy.

Diabetes mellitus is often associated with cardiovascular complications including coronary artery lesions and diabetic cardiomyopathy. Postmyocardial infarction prognosis also appears to be worse in diabetic patients, who exhibit a higher incidence of congestive heart failure and sudden cardiac death compared to nondiabetics. Diabetic myocardium exhibits a variety of abnormalities in sarcolemmal ion transport, including depression of $\text{Na}^+\text{-H}^+$ and $\text{Na}^+\text{-Ca}^{2+}$ exchange processes, and inhibition of Ca^{2+} and $\text{Na}^+\text{-K}^+$ ATPase. Sarcoplasmic reticular function also appears to be defective in diabetic myocardium with depressed ATP-dependent Ca^{2+} transport and Ca^{2+} -stimulated ATPase activity via a decreased ATP synthesis and electron transfer chain activity. 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.

Cells, tissues, and organs of humans and other mammals respond to low oxygen tension in part by fine tuning the expression of a group of physiologically relevant genes, proteins, and enzymes. The microsomal heme oxygenase (HO), originally identified by Tenhunen et al, catalyses the oxidative degradation of heme to biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. 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.

Aims of the studies

The overall aims of our studies were to investigate pharmacological approaches capable of reducing ischemia/reperfusion-induced damage.

- I. In the first part of our investigation we would like to determine whether diabetes affects cardiac susceptibility to reperfusion injury. We compared (i) the expression of mitochondrial genes (COXBIII and ATP6, selected out of 40 mitochondrial genes by a preliminary screening) using molecular biology techniques, (ii) their relationship to VF, and (iii) myocardial function in ischemic/reperfused, diabetic and nondiabetic hearts. In additional investigations, (iv) in isolated hearts obtained from nondiabetic and 8-week diabetic rats were subjected to electrically-induced VF in order to study the VF-induced COXBIII and ATP6 expression.

- II. In the second part of our experiments we studied the role of HO-1 mRNA expression related cellular defense mechanism and endogenous CO production, upon reperfusion, in nonfibrillated and fibrillated hearts obtained from wild type (+/+), HO-1 heterozygous (+/-), and homozygous (-/-) mice. We would like to investigate the role of HO-1 mRNA and its protein expression and HO-1 related CO production on reperfusion-induced arrhythmias, infarct size, cellular ion contents and cardiac function.

Male Sprague-Dawley rats (330-380 g) and male wild type (+/+), heterozygous (+/-), and homozygous mutant (-/-) HO-1 knockout mice (25-35 g), were used for all studies. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health (NIH Publication No. 86-23, revised 1985).

Methods

Isolated “working heart” preparation

Rats/mice were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg body weight) and then given intravenous heparin (500 IU/kg). After thoracotomy, the heart was excised and placed in ice cold perfusion buffer. Immediately after preparation, the aorta was cannulated, and the heart was perfused according to Langendorf method for a 5-min washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). The perfusion medium consisted of a modified Krebs-Henseleit bicarbonate buffer: 118 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl₂, 25 mM NaHCO₃, 0.36 mM KH₂PO₄, 1.2 mM MgSO₄, and 10 mM glucose. The left atrium was cannulated and the Langendorff system was switched to the working mode with a left atrial filling pressure of 17 cm of buffer (1.7 kPa) and aortic afterload pressure of 100 cm (10 kPa) of buffer as previously described. Aortic flow was measured by a calibrated rotameter, and coronary flow rate was measured by a timed collection of the coronary perfusate that dripped from the heart.

Induction of diabetes

Diabetes mellitus was induced by i.v. injection of streptozotocin (55 mg/kg). Nondiabetic age-matched controls were injected with an equivalent volume of vehicle. Diabetes was confirmed by the presence of hyperglycemia.

Indices measured

Heme oxygenase-1 studies:

An epicardial ECG was recorded throughout the experimental period by two silver electrodes attached directly to the heart. The ECGs were analyzed to determine the presence or absence of reperfusion-induced VF. Hearts were considered to be in VF if an irregular undulating baseline was apparent on the ECG. Aortic and coronary flow rates were measured by a timed collection of the aortic and coronary effluents that dripped from the heart. Before ischemia and during reperfusion, heart rate (HR), coronary flow (CF) and aortic flow (AF) rates were registered. Aortic pressure (AOP), and the first derivative of aortic pressure (AOPdp/dt) were measured by a computer acquisition system.

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. The incidence of reperfusion-induced VF, in our model, is very high (about 80%-90%) in both nondiabetic and diabetic subjects. Therefore, in order to get 6 nonfibrillated nondiabetic and 6 nonfibrillated diabetic hearts, 31 and 41 hearts were needed to be studied, respectively, and subjected to 30 min ischemia followed by 2 hours reperfusion. In hearts developed VF (25 out of 31 nondiabetics and 35 out of 41 diabetics) upon reperfusion were further divided into two subgroups (6 hearts in each subgroup): (i) the duration of reperfusion-induced VF was less than 3 min, and (ii) longer than 10 min. If the duration of VF was less than 3 min (spontaneous defibrillation occurred), the VF was defined as reversible VF. If the duration of VF was sustained until the end of the first 10 min of reperfusion, the VF was considered to be irreversible. In this latter group, after 10 min of VF, hearts were electrically defibrillated and reperfused for an additional 110 min. Heart rate (HR), coronary flow (CF), aortic flow (AF), and left ventricular developed pressure (LVDP) were also recorded.

Total RNA isolation:

Total RNA was isolated from heart tissue (100 mg) by homogenization in 1 ml of TRIzol reagent (Gibco BRL, Life Technologies, Eggenstein, Germany), a guanidium thiocyanate method, as described in the manual. The RNA pellets were dissolved in 100 μ l diethyl pyrocarbonate water, the concentrations were calculated from the absorbance at 260 nm measured by ultraviolet spectroscopy (Beckman DU 640, Beckman Instruments Inc., Fullerton, CA).

Northern blot

Hybridizations were performed as previously described. RNA was prepared from the left ventricle (about 100 mg), and 5 μ g 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 ³²P-labeled probes in a hybridization oven using an aqueous exclusion rate-enhancing solution (QuickHyb, Stratagene). 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 μ g) 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 μ g random 9-mer primers (Stratagene). Quantitation was done as described by Feldman et al [21]. Aliquots corresponding to 0.1 μ g 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 μ Ci [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.

Imaging of mitochondrial genes

Radioactivity within bands of interest was estimated by phosphorimage analysis (Molecular Dynamics Inc) corrected for background, and normalized to hybridization with GAPDH cDNA as described below. Storage phosphor screens were placed over the sample in a sample cassette and stored at room temperature for 48 hours. During incubation, radiation from the labeled RNA in the blot excites electrons in the phosphor to a metastable excited state. The screen was then placed in a Molecular Dynamics PhosphorImager, in which it was scanned with a laser beam inducing further excitation of the previously excited electrons to an unstable state, the decay of which is accompanied by the emission of visible light. The intensity of emitted light was measured and stored in a data file as a function of the position of the scanning laser, which corresponds to the location of exciting radiation on the blot. Each band in an image displayed on the screen was marked by a rectangle and the band or spot intensity within the marked area was then integrated.

cDNA library construction and analysis of mitochondrial genes

Ten μg poly(A)⁺ RNA from control and ischemic hearts (pooled samples) were used for the construction of a cDNA library in a pUC-derived plasmid vector. All necessary reagents, including the complete E. Coli cells, were from GibcoBRL (SuperscriptTM System for Plasmid Cloning). First strand synthesis of cDNA was carried out using a special oligo(dT)-blunt end Not-I primer-adapter instead of the oligo(dT) primer. After RNA-primed synthesis of second strand cDNA, Sal-I adapters were added followed by digestion with Not-I to create the compatible terminus at the poly(dT) end of the ds-cDNA, and five nanograms were used for oriented ligation into a Not-I/Sal-I-precut pSPORT 1 plasmid and 1/4 of the ligation reaction used to transform 100 μl competent MAX efficiency DH5 α E. Coli cells. After determination of transformation efficiency, cells were plated at $1\text{-}2 \times 10^3$ CFU/85 mm plate for 10-12 hours. After 4 h at 37 C° shaking with 4 ml/plate TB medium overlay followed by pooling of the medium, overnight liquid culture originated from 5.2×10^5 CFU was kept frozen for repeated library platings for screening.

Subtractive screening of mitochondrial genes

Subtracted cDNA probes were prepared by modification of the technique described by Rhyner et al. Briefly, 5 μg of poly(A)⁺ RNA extracted from control, ischemic and reperfused diabetic, and nondiabetic myocardium was subjected to reverse transcription for 1 hour at 42 °C using 1,000 units of a RNase H M-MLV reverse transcriptase (SuperscriptTM II, GibcoBRL), 1 mg random 9-mer primers (Stratagene), dNTP mixture (0.5 mmol/liter final concentration with 1 μCi [α -³²P]dCTP tracer), 100 units placental RNase inhibitor (Promega) and appropriate buffer in a 125 μl iter reaction. The cDNA was subjected to two rounds of hybridization at 68 °C in 30 μl iter of 2 mol/L phosphate buffer, pH 6.8 with a tenfold excess (w/w) of poly(A)⁺ RNA from control tissue, and the non-hybridized cDNA separated after each round of hybridization by hydroxyapatite chromatography on thermo-jacketed (60 °C) columns using 0.05 mol/L phosphate buffer. The cDNA enriched in specifically expressed sequences was desalted, purified, and subjected to random-primed second strand synthesis for radiolabeling to high specific activity ($< 0.5 \times 10^9$ cpm/ μg). Replicas from cDNA library platings at $1\text{-}2 \times 10^3$ CFU/plate (85 mm plates) were made by colony lifts on nylon filters (CL/P, Bio-RAD) and screened by hybridization with this probe.

DNA sequencing and gene identification

The Sanger dideoxy-mediated chain-extension/chain-termination method was used with denatured plasmid DNA as substrate (pUC 19-derived pSPORT 1 vector; GibcoBRL). The M13/pUC 'universal' and T7/T3 α sequencing primers and the Sequenase ver 2.0 kit (USB) were routinely used in ³⁵S-labeled reactions and bands separated on 47 cm 6% polyacrylamide/urea gels with double loading (2.5 and 5 hours running at 2000 V). Pearson-Lipman algorithms were used for similarity searches to DNA sequences in the GenBank and EMBL databases.

Determination of infarct size:

Hearts for infarct size measurement were perfused, at the end of each experiment, with 15 ml of 1 % triphenyl tetrazolium (TTC) solution in phosphate buffer (Na₂HPO₄ 88 mM, NaH₂PO₄ 1.8 mM) via the side arm of the aortic cannula then stored at -70 °C for later analysis. Frozen hearts were sliced transversely (18) in a plane perpendicular to the apico-basal axis into 2-3 mm thick sections, weighted, blotted dry, placed in between microscope slides and scanned on a Hewlett-Packard Scanjet 5p single pass flat bed scanner. Using the NIH Image 1.61 image processing software, each digitized image was subjected to equivalent degrees of background subtraction, brightness and contrast enhancement for improved clarity and distinctness. Infarct zones of each slice were traced and the respective areas were calculated in terms of pixels. The areas were measured by computerized planimetry software and these areas were multiplied by the weight of each slice, then the results summed up to obtain the weight of the risk zone (total weight of the left ventricle, mg) and the infarct zone (mg). Infarct size was expressed as the ratio, in percent, of the infarct zone to the risk zone.

Determination of CO content

We measured tissue CO content using gas chromatography as described by Cook et al. Briefly, hearts were removed at various time points (before the induction of ischemia and after 5 min, 15 min, 30 min, and 120 min of reperfusion, respectively) from the perfusion set up and homogenized in 4 volumes of 0.1 M phosphate-buffer (pH: 7.4) using an X520 homogenizer (Ingenieurburo CAT, M. Zipperer GmbH, Staufen, Germany). The homogenates were centrifuged at 4 °C for 15 min at 12,800 g and the supernatant fractions were used for the determination of tissue CO. The reaction mixtures contain: 150 μ l of supernatant, 60 μ l of NADPH (4.5 mM) and 50 μ l of 3.5/0.35 mM methemalbumin, and for blank samples 60 μ l phosphate buffer was used instead of NADPH. The samples were preincubated at 37 °C for 5 min, then the headspace was purged and the incubation was continued for 1 hour in dark at 37

°C. One thousand µl of the headspace gas from each vial was injected into the gas chromatograph using a gastight syringe (Hamilton Co., USA) in hydrogen gas flow with a speed of 30 ml/min. Analysis took place during the next 150 seconds on a 200 cm stainless-steel column with a 0.3 cm inner diameter. The detector was a thermal conductivity detector with an AC current of 80 mA. The individual value was expressed in mV, than peak's area was integrated and expressed in arbitrary units. The column was packed with Molselect 5 Å and maintained at 30 °C.

Measurement of cellular Na⁺, K⁺, and Ca²⁺

Cellular electrolytes were measured as described previously. In brief, hearts were rapidly cooled to 0-5 °C by submersion in, and perfused for 5 min with an ice-cold ion-free buffer solution containing 100 mmol/L of trishydroxy-methyl-amino-methane and 220 mmol/L of sucrose to washout ions from the extracellular space and to stop the activity of membrane enzymes responsible for membrane ion transports. Five min of cold washing of the heart washes out > 90 % of the ions from the extracellular space. Following the washout, hearts were dried for 48 hours at 100 °C, and made ash at 550 °C for 20 hours. The ash was dissolved in 5 ml of 3 M nitric acid and diluted 10-fold with deionized water. Myocardial Na⁺ was measured at a wavelength of 330.3 nm, K⁺ was measured at 404.4 nm, and Ca²⁺ at 422.7 nm in air-acetylene flame using an atomic absorption spectrophotometer. The method for the determination of myocardial ion contents has been previously described.

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. Formalin fixed tissues were paraffin embedded and 5 µm sections were placed on poly-l-lysine coated glass slides (Sigma, St.Luis, USA). Following deparaffinization and rehydration, samples were used for quenching endogenous peroxidases and blocking nonspecific binding sites by 3 % H₂O₂ in normal goat serum. Sections were incubated for another 2 hours with HO-1 antibody at a dilution of 1:750 as described in the manufacturer's manual. After a few minutes PBS washing, slides were incubated for additional 30 min in the presence of purified biotinylated anti/rabbit IgG (Vector, USA) at a dilution of 1:200. Slides were rewashed in PBS and incubated with peroxidase conjugated streptavidin (Zymed, USA) for 30 min followed by red color

development using 3-amino-9-ethylcarbazole in 0.1 M of acetate buffer (pH 5.2). Sections were counter-stained with Gill's hematoxylin for 15-20 sec, rinsed with water, and dipped in 1 % of lithium carbonate. After draining off water, slides were placed in oven for 30 min at 80 C°, then covered with permount coverslips.

Experimental time course

Isolated hearts (n=6 in each group) were aerobically perfused for 30-minute of preischemic period, and during the first 10-min period standard perfusion medium was used in Langendorff mode. During the second 20-min period, isolated Langendorff preparation was switched to 'working' mode and control drug-free measurements of electrocardiogram, HR, CF, AF, AOP, AOPdp/dt were recorded. Global ischemia was then induced for 20 min followed by 2 hours of reperfusion. Because the isolated working heart preparation is more sensitive to various interventions and load-work compared to the 'nonworking' Langendorff preparation, therefore the initial 10 min of reperfusion was done in Langendorff mode in order to try to avoid the development of high incidence of ventricular arrhythmias. If VF was registered during the initial phase of reperfusion, and we needed to register cardiac function, the isolated heart was immediately electrically defibrillated using a single 20 V square-wave pulse of 1 ms duration. Northern and Western blot analysis, infarct size and myocardial electrolytes were done after 120 min of reperfusion.

Hearts were obtained from two populations of rats: (i) age-matched nondiabetics which were the same age as diabetic rats and were injected with citrate buffer 8 weeks prior to the study; (ii) diabetic rats which were injected with streptozotocin (55 mg/kg, i.v.) eight weeks prior to the isolation of the hearts and induction of 30 min normothermic global ischemia followed by 2 hours reperfusion. Myocardial function (HR, CF, AF, and LVDP) was measured before ischemia and after 2 hours reperfusion. In additional studies, in order to simulate the period of 10 min of irreversible VF, without the ischemic/reperfused protocol, isolated hearts were electrically fibrillated (20 Hz, 1200 beats/min) using a 5 V square-wave pulses of 1 ms duration for 10 min in Langendorff mode. Then hearts were defibrillated (if it was necessary), switched to working mode, and perfused for additional 110 min. mRNA expression was determined at the end of each experiment.

Statistics

The data for HR, CF, AF, AOP, AOPdp/dt, infarct size, myocardial electrolyte contents and tissue CO were expressed as the mean \pm 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

I. Subtractive screening:

We generated a specific cDNA library in plasmid pSPORT 1, using as starting material left ventricular tissue from rat hearts subjected to 30 min ischemia. To under-represent genes having allogenic variability, pooled poly(A)⁺ RNA is used for cloning. The size of the obtained library was estimated to be 340,000 primary recombinants, and the median insert size is between 1.5 and 2.0 kb. Single stranded tester cDNA, reverse transcribed from aliquots of the same pooled ischemic heart mRNA, was repeatedly subtracted with driver mRNA from nonischemic hearts to produce a subtracted probe. Out of 40 putative positive clones that we selected, rapid screening by hybridization of control and ischemic RNA with probes made from the corresponding cloned inserts revealed 28 clones of apparently overexpressed genes. When we processed DNA sequencing and database searching, these clones of ischemia-expressed genes were found to contain copies of 8 different genes. Partial sequencing from the 3' and 5' ends (200-300 bp) followed by similarity searches against sequences stored in the GenBank and identified 6 of these genes. They are genes that coded for cytochrome oxidase subunits I, II, and III, for ATP synthase subunit 6 and 8, and for cytochrome B.

II. 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). In the irreversible fibrillated and reversible fibrillated nondiabetic myocardium, upon reperfusion, a significant reduction in the expression of COXBIII mRNA was observed (^{*} $p < 0.05$), respectively. In diabetic ischemic and reperfused hearts, the upregulation of COXBIII was significantly increased (⁺ $p < 0.05$), respectively, in comparison with the diabetic nonischemic myocardium.

The upregulation of ATP5B (this was not so pronounced as in COXIII) was observed, although the data were not statistically significant, after 30 min ischemia and after 2 hours reperfusion in the nondiabetic/nonfibrillated myocardium. In irreversibly fibrillated and reversibly fibrillated hearts, upon reperfusion, a marked downregulation in ATP5B was observed. In diabetic ischemic and reperfused myocardium, the regulation of ATP5B was almost the same (nonsignificant change) as it was observed in nondiabetic/nonfibrillated hearts. In diabetic fibrillated hearts, the signals of ATP5B were completely undetectable.

After 30 min ischemia, the expression of COXIII mRNA was observed ([#] p<0.05) compared to the nonischemic control group. This expression was further increased ([#] p<0.05) after 30 min ischemia followed by 2 hours reperfusion in the nonfibrillated myocardium. However, in the ischemic/reperfused fibrillated group a significant reduction in the expression of COXIII mRNA was detected. The same significant reduction in COXIII expression was also observed in the electrically fibrillated myocardium. The trend in the changes of COXIII mRNA expression was also the same in diabetic subjects.

Ischemia/reperfusion-induced and electrically-induced VF resulted in a significant reduction in signal intensity in both nondiabetic and diabetic hearts.

The expression of ATP5B and COXIII was observed after ischemia in both nondiabetic and diabetic hearts. Upon reperfusion, the same expression was observed in nonfibrillated nondiabetic, and diabetic myocardium. In fibrillated hearts, upon reperfusion, the down-regulation of ATP5B (8±3 mRNA relative amount in nondiabetics) and COXIII (6±1 mRNA relative amount in nondiabetics) was observed in nondiabetic myocardium, but these mitochondrial genes were not detected by RT-PCR in diabetic fibrillated subjects.

III. 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, where ischemia/reperfusion-induced damage superimposed by reperfusion-induced VF, a further significant reduction in postischemic cardiac function was observed. These results are consistent with the known ability of VF to enhance reperfusion-induced injury, and in the presence of a superimposed acute ischemic event by VF, VF further increased the postischemic cardiac functional damage.

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. The postfibrillated recovery in diabetics resulted in a significantly improved cardiac function compared to those hearts subjected to ischemia/reperfusion in the presence of VF.

IV. 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. Thus, we have got the necessary numbers of HO-1 (-/-) mice, for the present study, in matings between HO-1 (+/-) and HO-1 (-/-) animals. Mice lacking HO-1 gene were slightly lighter (26 ± 3 g) than HO-1 (+/+) (30 ± 4 g) or HO-1 (+/-) (31 ± 3 g) age-matched littermates from birth to early adulthood (12-13 weeks), but were otherwise indistinguishable. However, we observed that, as early as 22 weeks of age, most HO-1 (-/-) mice became thin, losing weight, poorly groomed, and appeared less active than HO-1 (+/+) or HO-1 (+/-) animals. Premature mortalities among HO-1 (-/-) mice after 22 weeks of age were common. Therefore, in our studies, 16 to 18 weeks of age mice were used in all groups.

V. 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. After 20 min of aerobic perfusion the same cytoplasmic HO-1 staining was observed in the myocardium obtained from (+/-) mouse heart. In the homozygous (-/-) mutant mouse myocardium, cytoplasmic HO-1 staining was not detected under 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. However, no HO-1 mRNA band was detected in the isolated homozygous mutant (-/-) aerobically perfused mouse heart.

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.

VI. 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). However, in reperfused/fibrillated (VF) wild type (+/+) myocardium, HO-1 enzyme detection by immunohistochemistry was reduced. The same pattern, in HO-1 enzyme detection, was obtained in the heterozygous (+/-) nonfibrillated (No-VF) and fibrillated (VF) hearts, indicating that there is no substantial difference between the localization of HO-1 enzyme in wild type (+/+) and heterozygous (+/-) mouse myocardium. Upon reperfusion, because of the absence of HO-1 enzyme in homozygous (-/-) mice, the localization of HO-1 enzyme was not possible in either nonfibrillated or fibrillated homozygous (-/-) myocardium. In homozygous (-/-) mouse studies, all hearts developed reperfusion-induced VF, therefore electric defibrillation was necessary to be done in order to measure myocardial function during reperfusion.

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. It is important to note that in reperfused/fibrillated wild type (+/+) mouse heart a reduction in HO-1 protein expression was observed in comparison with the nonfibrillated myocardium. HO-1 protein detection was not recorded in heterozygous (+/-) fibrillated myocardium.

VII. 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. However, in the presence of VF, endogenous CO production was not detected.

The time-course of endogenous CO production in (+/+) and (-/-) myocardium subjected to 20 min ischemia followed by 5, 15, 30, and 120 min of reperfusion:

The results clearly show that endogenous CO production was increased in reperfused/nonfibrillated myocardium (hearts were immediately defibrillated if it was necessary). However, a small amount of endogenous CO production was detected in homozygous (-/-) reperfused/nonfibrillated myocardium indicating that other pathways may also play some role in endogenous CO production, but these pathways could be relatively unimportant in comparison with the HO-1-dependent pathway.

VIII. 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 AOPdp/dt was observed in HO-1 knockout homozygous (-/-) mouse hearts in comparison with the corresponding values obtained in wild type (+/+) and heterozygous (+/-) mouse myocardium. These significant changes, the same trend, in cardiac function were also detected between groups after 20 min of ischemia followed by 30 min and 120 min of reperfusion. It is important to note that all hearts developed reperfusion-induced VF in the HO-1 knockout homozygous (-/-) group, therefore all hearts were electrically defibrillated during reperfusion. 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 $\mu\text{mol/g}$ dry weight) in the -/- group compared to the nonischemic +/+ value (278±7 $\mu\text{mol/g}$ dry weight). Reperfusion of the ischemic myocardium resulted in a same maldistribution of cellular ion contents in all groups which were further aggravated in the -/- myocardium.

Conclusions

I. Ischemic episodes elicit different changes in the expression of several genes, arrhythmogenesis, and cardiac function in the myocardium. The electrophysiology of arrhythmogenesis including the mechanism(s) of VF, e.g., re-entry, is very well defined, and only a few data, to our knowledge, are available to explain the mechanism of VF at genetic level. Efforts were made to study the mechanism of idiopathic VF indicating that mutations in cardiac ion-channel genes may contribute to the risk of fibrillation and sudden cardiac death. This idea led us to speculate that mitochondrial genes, because mitochondria are the locus of oxidative phosphorylation, may primarily play an important role in the regulation of arrhythmogenesis in ischemic/reperfused 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 ATP6, without the ischemic/reperfused protocol, in electrically fibrillated myocardium. Our data show an increase, studied and preselected by subtractive screening from 40 mitochondrial genes, in the expression of the two mitochondrial-encoded genes, COXBIII and ATP6, after 30 min ischemia, and a downregulation of these two genes after 2 hours reperfusion in fibrillated hearts. In nonfibrillated myocardium, upon reperfusion, the downregulation of the mitochondrial genes studied was not observed. It is relatively surprising that we found a significant up- and downregulation so quickly and while the heart is still under ischemic arrest, but this may be natural for mitochondrial-encoded genes since the mitochondrial genetic apparatus is similar to that of procaryotes. As in most other mammals, the rat mitochondrial genome contains only 13 protein coding areas (genes for Cox I, II, III, ATPase 6, 8, cytochrome B, and NADH 1, 2, 3, 4, 4L, 5, 6). In our studies, the development of reperfusion-induced VF was critically dependent upon the downregulation of COXBIII and ATP6. This finding was directly supported by data obtained in electrically fibrillated myocardium indicating that VF elicits the downregulation of COXBIII and ATP6. The observation in electrically fibrillated myocardium shows that VF is probably responsible for the reduction of signal intensity of these genes, but a fibrillation-induced moderate ischemia cannot be completely ruled out. Although electrical fibrillation-induced coronary vasoconstriction is unlikely to account for the initiation of mitochondrial gene regulation, there seems little doubt that, after 10 min electrical fibrillation, this factor can modify the postfibrillated recovery of hearts or support their maintenance compared to the recovery of

ischemic/reperfused fibrillated myocardium. Thus, in comparison to hearts subjected to ischemia/reperfusion protocol, coronary flow failed to recover to its preischemic control value, while in electrically fibrillated hearts, coronary flow recovered to 80-95% of the prefibrillated value.

As speculated above, the mechanism underlying the role of mitochondrial mRNA expression in arrhythmogenesis may involve the encoded proteins which may be responsible for the regulation of ion control mechanisms. Our present study might indicate that the heart appears to be able to recover in terms of cardiac function, and COXBIII and ATP5A3 downregulation could be originated from VF. While these results may appear to be in conflict, it may well be that the injury induced by electrical fibrillation is heterogeneous and that irreversible injury is induced in an initially small population of myocardial cells. Thus, on termination of electrical fibrillation, the undamaged or reversible damaged cardiac cells become functionally close to the normal ones but continue to deteriorate in terms of mitochondrial mRNA expression. Such a proposition gains some support from our observations of mitochondrial mRNA expression in fibrillated, nonfibrillated, and electrically fibrillated hearts indicating that ischemia/reperfusion-induced injury in cardiac function is superimposed by VF-induced functional damage in the myocardium.

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 ATP5A3 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. Thus, our findings suggest that pharmacological stimulation of the expression of COXBIII and ATP5A3 during reperfusion could prevent the development of VF, and these genes could regulate the mechanism(s) of arrhythmogenesis in both ischemic/reperfused nondiabetic and diabetic myocardium

II. In the second part of our 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 (-/-) 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. Indeed, after ischemia/reperfusion, there was extensive HO-1 mRNA and protein downregulation, and enzyme activity reduction in fibrillated HO-1 heterozygous and homozygous mouse myocardium. The lack of HO-1 mRNA and its protein was observed in aerobically perfused, without an episode of ischemia/reperfusion, homozygous mouse myocardium. Cardiac function in HO-1 knockout homozygous hearts was significantly reduced under aerobic conditions and reperfusion in comparison with the wild type myocardium. Furthermore, upon reperfusion, all HO-1 knockout homozygous hearts developed reperfusion-induced VF indicating the importance of the presence of HO-1 mRNA, its protein, and enzyme activity as antiarrhythmic substances in the cardiac tissue. These changes in HO-1 mRNA, protein, and enzyme activity correlated to the HO-1 related endogenous CO production. Thus, endogenous CO production was not detected, or at a very low level, in fibrillated HO-1 knockout heterozygous and homozygous myocardium, while a substantial presence of endogenous CO was measured in ischemic/nonfibrillated myocardium.

The precise molecular mechanism(s) by which HO-1 and HO-1 related endogenous CO production confers myocardial protection is currently under extensive investigation. For instance, bilirubin protects myocardial cells against oxidative damage and improves the recovery of postischemic function in isolated rat hearts. Furthermore, elevated serum bilirubin concentrations are associated with reduced risks for the development of early familial coronary artery diseases. Thus, it is reasonable to believe that antioxidant effects of bilirubin contribute to cardiac protection, which has a relatively weak endogenous defense in comparison with those of other organs such as the liver and intestines. It has been recently suggested that CO could prevent the inflammatory response in hyperoxic injury reducing oxidative damage.

There is a substantial body of evidence supporting the existence of an association in cells between the HO system activity and function that are related to cGMP synthesis. The activation of soluble guanylyl cyclase (sGC) by CO leads to the accumulation of cGMP in cells indicating a direct relationship between endogenous CO production and cGMP concentration. Studies have in fact established that, under physiological conditions, maintenance of normal vascular tone and blood flow can be attributed in part to enzymatically

derived CO. Therefore, changes in vascular tone, endothelial permeability, and/or coagulation function observed under hypoxic or ischemic conditions may in part be a consequence of HO-1 induction and localized production of CO.

How can heme oxygenase expression provide protection against ischemia/reperfusion-induced injury? Although the main subject of the present study is the delineation of HO-CO system in the ischemic/reperfused myocardium, some discussion of the putative mechanism of action of this pathway must also include some mention of nitric oxide synthase (NOS) system. These two pathway systems show many similarities: both HO and NOS have distinct constitutive and inducible isoforms, and both CO and NO stimulate soluble guanylyl cyclase to produce cGMP as the second messenger effector. The relative contribution of CO and NO to the activation of guanylyl cyclase in the cardiovascular system remain unknown, even under physiological conditions. In many pathological conditions such as hypoxia, thermal injury, and ischemia/reperfusion, CO-mediated effects may be predominant. For instance, it was found that an HO-inhibitor blocked cGMP production, whereas a NOS inhibitor had no effect. Pannen et al showed that in a rat model of hemorrhagic shock CO induced sinusoidal dilatation and protection, whereas NO did not appear to play a significant role. However, hyperthermic stress also elevates cGMP via CO but not NO. Recent studies have demonstrated that NO is a much more potent stimulator of soluble guanylyl cyclase producing about 300 to 400-fold increase in basal guanylyl cyclase activities in comparison with 5 to 15-fold increase generated by CO. It appears likely that, although in general NO is the more dominant activator of guanylyl cyclase, under certain conditions of altered redox state and thermal injury in specific tissues, CO may be physiologically more important. Because both NO and CO mediate their effects through the stimulation of guanylyl cyclase, in future investigation, it is necessary to demonstrate that an increased cardiac cGMP level is at least partly due to CO rather than NO in our model system.

In summary, we have shown using wild type, HO-1 knock out heterozygous, and homozygous mice that HO-1 and HO-1-related endogenous CO production play an important role in the adaptation of cardiovascular system to ischemia followed by reperfusion. The myocardium from HO-1 knockout mice exposed to ischemia/reperfusion was severely injured; the poor recovery of postischemic cardiac function was recorded, and all hearts developed reperfusion-induced VF. On the basis of these findings, we postulate that HO-1 may play a central role in cardiac physiology by protecting the heart from ischemia/reperfusion-induced damage.

Publication (the thesis is based on)

Original articles

1. **Szendrei L**, Turoczi T, Kovacs P, Vecsernyes M, Das DK, Tosaki A. (2002) Mitochondrial gene expression and ventricular fibrillation in ischemic/reperfused nondiabetic and diabetic myocardium. *Biochem Pharmacol* **63**: 543-552. **IF: 3,542**
2. 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. (2003) Heme oxygenase-1-related carbon monoxide production and ventricular fibrillation in isolated ischemic/reperfused mouse myocardium. *FASEB J* **17**: 2133-2135 **IF: 7,252**

Abstracts published in journals having IF

1. **Szendrei L**, Turoczi T, Das DK, Tosaki A. (2001). The role of mitochondrial gene expression in ventricular fibrillation. *J. Mol. Cell. Cardiol.* **33**: (6) p. A117. **IF: 4.091**
2. **Szendrei L.**, Turoczi T., Das D. K., Tosaki A. (2002) Reperfusion-induced ventricular fibrillation and mitochondrial gene expression in ischemic/reperfused hearts. *J. Mol. Cell. Cardiol.* **34** (6): A61 **IF: 4.091**
3. **Szendrei L.**, Bak I., Juhasz B., Turoczi T., Das D. K., Tosaki A. (2003) The role of heme oxygenase-1 and carbon monoxide in reperfusion-induced ventricular fibrillation. *J. Mol. Cell. Cardiol.* **35** (6): A45 **IF: 4.091**

Other publications (do not use for the thesis)

1. Kovacs P., Bak I., **Szendrei L.**, Vecsernyes M., Varga E., Blasig I. E., Tosaki A. (2001) Non-specific caspase inhibition reduces infarct size and improves post-ischaemic recovery in isolated ischaemic/reperfused rat hearts. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **364**: 501-507; **IF: 2.566**
2. Bak I., Papp G., Turoczi T., Varga E., **Szendrei L.**, Vecsernyes M., Joo F., Tosaki A. (2002) The role of heme oxygenase related carbon monoxide and ventricular fibrillation in ischemic/reperfused hearts. *Free. Rad. Biol. Med.* **33**: 639-648; **IF: 5.533**