Thesis for degree of Doctor of Philosophy

Pathological role and mechanisms of injuries induced by ischemia/reperfusion

Judit Varadi

Supervisor: Dr. Arpad Tosaki

Program director: Dr. Lajos Gergely

University of Debrecen

Health Science Center

Faculty of Pharmacy

Department of Pharmacology

2007

Introduction

Cardiac ischemia is one of the most frequent causes of death in Hungary. In case of impaired oxygen supply can cause serious injuries. Interest of the complications of myocardial ischemia has resulted in much emphasis being placed upon the characterization and treatment of supraventricular-, ventricular arrhythmias, and recovery of post-ischemic function. Common acquired reasons of ventricular arrhythmias and cardiac failure include cardiac ischemia and reperfusion resulting from the sudden disruption and restoration of blood flow to the heart, structural heart disease like cardiomyopathy, metabolic abnormalities, like abnormal serum potassium, sodium, calcium, or magnesium levels, and pharmacological medications.

Two gaseous molecules, nitric monoxide and carbon monoxide have been identified playing an important role in signal transduction in various cells and tissues and diseases. The main endogenous source of CO is heme oxygenase, which exists in constitutive (HO-2 and HO-3) and inducible (HO-1) isoforms. Heme serves as substrate for HO-1 and HO-2 in the formation of CO, free ferrous iron and biliverdin. HO-1 represents a pivotal inducible defensive system against stressful stimuli, including UVA radiation, carcinogenesis, ischemia-reperfusion damage, endotoxic shock, several other conditions characterized by production of oxygen-derived free radicals. As part of its physiological and cytoprotective actions, heme oxygenase-derived CO appears to play an important role as neurotransmitter, regulator of sinusoidal tone, inhibitor of platelet aggregation and suppressor of acute hypertensive responses.

The development of CO-releasing molecules (CORMs), compounds that carry and release CO into biological systems, is in line with the concept of delivering this gas for therapeutic purposes in a controlled and safe manner. Transition metal carbonyls such as tricarbonyldichlororuthenium (II) dimer (CORM-2) and tricarbonylchloro(glycinato)ruthenium(II) (CORM-3) as well as

sodium boranocarbonate (CORM-A1) have been used extensively in various models of disease to simulate the pharmacological activities that are typical of CO gas and/or HO-1 induction. Indeed, CORM-3 and other CO carriers have been reported to promote vasodilatation, regulate mean arterial pressure, prolong graft survival after transplantation by exerting anti-inflammatory actions and improve kidney function following cold storage and drug-induced toxicity. The mechanism(s) responsible for the protective and pharmacological actions of CORMs appear to involve guanylate cyclase and potassium channel activation but other pathways have recently emerged as important cellular targets that mediate the biological effects exerted by these CO carriers. The versatile properties of CORMs are currently under intense investigation and different classes of compounds are being developed with the aim of exploiting their therapeutic potential in the treatment of cardiovascular disorders and inflammatory states. The water-soluble CORM-3, which releases CO very rapidly in aqueous solutions, has been the most studied so far and its cardioprotective actions have been demonstrated in models of ischemia-reperfusion injury and myocardial infarction. These data are consistent with the fact that HO-1 can markedly protect cardiac tissue against reperfusion injury induced by oxidative stress and that HO-1-derived CO can significantly attenuate post-ischemic ventricular fibrillation.

Since its discovery, apoptosis deregulation has been shown to play an important role in the onset of numerous diseases including myocardial ischemia and reperfusion. Ischemic injury is thought to initiate apoptosis, but for substantial cell death to occur, reperfusion is necessary. Tissue transglutaminase selectively accumulates in cells undergoing apoptosis both *in vivo* and *in vitro.* Transglutaminases are a family of thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of a covalent bond between the γ–carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε–amino group of lysine in certain proteins. The reaction results in post-translational modification of proteins by establishing ε–(γ–glutamyl)lysine

cross-linkages and/or covalent incorporation of polyamines and histamine into proteins. Eight distinct, enzymatically-active transglutaminases have so far been described; one of them, TG2 is ubiquitously expressed in mammalian tissues. The enzyme has been implicated in a variety of cellular processes including cell death. In addition to its cross-linking activity, TG2 also acts as GTP-binding protein mediating intracellular signaling via the alpha-1b-adrenergic receptor in cardiac cells. These subcellular effects of tTG, i.e. cross-linking and pro-apoptotic activities, have also been hypothesized to play a role in some human pathological conditions. For instance, it has been associated with pathogenetic mechanisms of some human degenerative diseases i.g. cardiovascular disease.

Aimes of the studies

1. In the first part of our study we approached the importance of CO signaling from guanylate cyclase angle. We investigated the effect of exogenous CO perfused *via* the oxygenated perfusion buffer on cardiac function (heart rate, coronary flow, aortic flow, and left ventricular pressure), infarct size, and incidence of reperfusion-induced ventricular fibrillation in association with cGMP, cAMP levels, and guanylate cyclase activities in ischemic/reperfused hearts. We make an attempt to describe a possible mechanism of action *via* the cGMP/guanilate cyclase activation as a regulator of cardiac function *iv vitro*.
2. The aim of the second part of the study was to investigate the effect of CORM-3 on reperfusion-induced ventricular fibrillation and myocardial dysfunction following ischemia in isolated perfused rat hearts and evaluate the recovery of the electrophysiological activity of the heart in response to CO. We examined the effect of CORM-3 on the imbalance of myocardial ion contents (Na^+, K^+, and Ca^{2+}) and its possible relationship with the

recovery of post-ischemic cardiac function by CO liberated from CORM-3.

3. Considering the central role played by mithocondria in apoptosis, we investigated the relationships existing TG2 and ischemia/reperfusion induced apoptosis in hearts. In the third part of our study we registered the cardiac functional parameters and measured the high energy phosphate-content by HPLC methods of the WT and $TG2^{-/-}$ mice hearts before and after ischemia/reperfusion.

Male, Sprague-Dawley (320-350 g body weight) rats were used for the first and second studies. $TG^{-/-}$ and wild type of mice were used for the investigation of apoptosis. 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

1. Investigation of the effect of exogenous CO

Isolated working heart preparation

Rats 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 the Langendorff method (buffer was oxygenated with the mixture of 95 % O_2 and 5 % CO_2 at 37 ^{o}C) 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 (sodium chloride 118, potassium chloride 4.7, calcium chloride 1.7, sodium bicarbonate 25, potassium biphosphate 0.36, magnesium sulfate 1.2 and glucose 10 millimolar concentrations). Following the washout period, the Langendorff preparation was switched to the working mode with a left atrial filling pressure of 1.7 kPa (17 cm H_2O) and aortic afterload pressure of 10.0 kPa (100 cm H_2O). Aortic flow was measured by an in-line calibrated rotameter. Coronary flow rate was estimated by timed collection of the coronary perfusate that dripped from the heart.

Determination of infarct size

For infarct size determination hearts were perfused at the end of each experiment with 25 ml of 1 % triphenyl tetrazolium solution in phosphate buffer (Na_2HPO_4 88 mM, NaH_2PO_4 1.8 mM) via the side arm of the aortic cannula, and then stored at -70 ^oC for later analysis. Frozen hearts were sliced transversely (Schultz et al., 1997) in a plane perpendicular to the apico-basal axis into 2-3 mm thick sections, weighted, blotted, placed in between microscope slides and scanned on a Hewlett-Packard Scanjet 5p single pass flat bed scanner (Hewlett-Packard, Palo Alto, CA, USA). Using the NIH Image 1.61 image processing software, each digitalized image was subjected to equivalent degrees of background subtraction, brightness and contrast enhancement for improved clarity. Infarct zones of each slice were traced and the respective areas were calculated in terms of pixels (Dickson et al., 2001). 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 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.

Measurement of VF, VT, and cardiac function

Throughout the experimental period, an epicardial ECG was recorded by attaching two silver electrodes directly to the myocardium and data collected using a computer acquisition system (ADInstruments, Powerlab, Castle Hill, Australia). ECGs were analyzed to determine the incidence and duration of ventricular fibrillation (VF) and ventricular tachycardia (VT). The first 10 min of reperfusion was done in Langendorf ("nonworking") mode in order to avoid the development of reperfusion-induced VT and VF during the "working" heart reperfusion. After the initial 2 min of VT or/and VF (sustained VF) in Langendorff reperfusion, hearts were defibrillated (if it was necessary), reperfused for additional 8 min in Langendorff mode, and switched to "working heart" reperfusion, and myocardial function was recorded. The heart was considered to be in VF if an irregular undulating baseline was apparent on the ECG. VT was defined as five or more consecutive premature ventricular complexes. The data (sec) of VT, VF and sinus rhythm show their durations within the first 120 sec of nonworking Langendorff reperfusion. Before ischemia and during reperfusion, heart rate (HR), coronary flow (CF) and aortic flow (AF) rates were registered. Left ventricular developed pressure (LVDP) was also recorded by a computer acquisition system (ADInstruments, Powerlab, Castle Hill, Australia) using a catheter inserted into the left ventricle via the left atria and mitral valve.

Induction of ischemia, reperfusion and CO treatment

After 10 min of aerobic perfusion of the isolated rat heart, both the aortic outflow and pulmonary inflow lines were clamped at a point close to the origin of the aortic and pulmonary cannulas. Reperfusion could be initiated by unclamping and removing the line clamps. Langendorf perfusion was used, in order to avoid the high incidence of irreversible VF, during the first 10 min of perfusion in all experiments. Various concentrations of CO were applied, and

hearts were exposed to CO perfusion via the perfusion buffer 10 min before the induction of ischemia and during the first 10 min of reperfusion.

Myocardial cAMP and cGMP level

Concentrations of cAMP and cGMP were measured using radioimmunoassay kits obtained from Amersham in non-ischemic, ischemic and ischemic/reperfused myocardium. For cGMP assay, freeze-clamped myocardium was freezed-dried and 12-20 mg of dry tissue was extracted in 5% trichloroacetic acid. Extracted samples were ether-washed three times for 5-6 -min wash cycles. Then, samples were diluted 1:10 and acetylated for the ^{125}I cGMP assay. The IC_{50} for cGMP assay was 30 pmol in each tube. For cAMP assay 15- to 25-mg freeze-dried samples were extracted with perchloric acid, neutralized, and measured. The IC_{50} for cAMP assay was 1.79 nmol in each tube.

Guanylate cyclase activity

Myocardial tissue preparation was used to measure guanylate cyclase activity. Myocardium (about 50 mg in each sample) was disrupted in homogenization buffer at 4°C with a glass-glass homogenizer. The cytosolic fraction was prepared by recovering the supernatant after centrifuging the homogenate at 100,00 g for 60 min at 4°C. For preparation of the particulate fraction, the pellet was resuspended in homogenization buffer containing 1% triton X-100 and incubated for 15 min 4°C, and the supernatant was recovered after centrifugation at 100,000 g for 60 min 4°C. Guanylate cyclase activity was measures in aliquots containing 15 μg of protein in each tube as described previously and expressed in fmol.

2. The effect of different concentration CORM-3 treatment for the ischemic/reperfused rat heart

Experimental protocol

After a 10-min aerobic perfusion of the heart, the atrial inflow and aortic outflow lines were clamped at a point close to the origin of the aortic cannula. Reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines. To prevent the myocardium from drying out during normothermic global ischemia, the thermostated glassware (in which hearts were suspended) was covered and the humidity was kept at a constant level (95 %-100 %) and controlled by a hydrometer. A basic requirement for our studies was that untreated hearts exhibit a high vulnerability to reperfusion-induced arrhythmias. This gave a maximum scope for the demonstration of any antiarrhythmic activities of CORM-3 in treated subjects. This was achieved using a 30 min period of normothermic global ischemia followed by 120 min of reperfusion. In CORM-3 treated groups, after the washout of blood from the myocardium, the drug was administered for 10 min by infusion (Harvard apparatus 22, Southnatic, MA, USA) of a concentrate into a sidearm of the aortic cannula giving a final concentration of 10 μM, 25 μM, and 50 μM CORM-3, respectively, in the perfusion buffer. This procedure was employed in order to prevent any oxidation of CORM-3 prior to its infusion. Thus, CORM-3 was infused for 10 min before the initiation of the ischemic period, and the infusion was only maintained until the onset of ischemia.

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

In brief, hearts were rapidly cooled to 0-5 ^oC 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. The wash out technique is used, in different tissues, for the measurement of cellular ion contents by various laboratories. Following the washout, hearts were dried for 48 hours at 100 ^{o}C, and made ash at 550 ^{o}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 Ca^{2+} at 422.7 nm in air-acetylene flame using an atomic absorption spectrophotometer (Perkin-Elmer 1100-B).

3. The role of TG2 enzyme in ischemia/reperfusion injury

Experimental procedures

We worked with four experimental groups of mice. The first and second group consisted of wild type animals, the third and fourth consisted of transglutaminase knocked out mice. Mice were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg body weight) and then given intravenous heparin (500 IU/kg). Hearts were quickly excised and placed in ice-cold Krebs-Hanseleit solution. The aorta was cannulated on a 1.6 mm platina cannula and perfused in a nonrecirculating Langendorf apparatus with Krebs- Hanseleit solution. After equilibration the hearts of the first and the third groups were subjected to perfusion only for 10 minutes. The dissections of the second and the fourth groups were subjected to ischemia for 30 minutes, then reperfusion for 120 minutes. The perfusion medium consisted of Krebs-Hanseleit bicarbonate buffer: 118 mmol sodium chloride, 4,7 mmol potassium chloride, 1,7 mmol calcium chloride, 25 mmol sodium bicarbonate, 0,36 mmol potassium biphosphate, 1,2 mmol magnesium sulfate, and 10 mmol glucose. The solution was oxigenised by 95 %O_2 / 5%CO_2 at 37°C.

Sample preparation

The samples were frozen immediately in liquid nitrogen and then stored at -80°C. Later they were manually powdered in mortar under liquid nitrogen. To heart-muscle powder 50 µl of 60% methanol per mg of wet tissue was added. Extraction of metabolites occurred overnight at -80°C. The samples were mixed, centrifuged at 12,000g, at 4°C for 20 min and the supernatants were separated. Subsequently, the samples were frozen at –80°C until further analysis or immediately diluted with eluent 1:1, and analysed on the same day.

Chromatographic methods

For ATP, ADP and AMP analyses the mobile phase consisted of a 215 mmol/l KH_2PO_4, 2,3 mmol/l TBAHS, 5% methanol aqueous solution and was titrated to pH 6.5 with 8 M KOH. For CrP analysis the mobile phase consisted of a 14,7 mmol/l KH_2PO_4, 1,15 mmol/l TBAHS, and the aqueous solution was titrated to pH 5.3 with 8 M KOH. The mobile phase was pumped at 1ml/min. For ATP, ADP and AMP separations the UV absorption was measured at 254 nm and for PCr determination at 210 nm. The data were collected with Eurochrom 2000 software and analyzed on Microsoft Excel 2000 software.

Statistical analysis

Cardiac function (HR, CF, AF, LVDP), infarct size, myocardial ions, adenosine phosphates concentration, and the duration of arrhythmias and sinus rhythm were expressed as the means ± SEM. Two-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 the control group (not treated/wild type of mice) were compared to those of the drug-treated/$TG2^{-/-}$ groups by Dunnett's test at each time point. Because of the nonparametric distribution of the incidence of VF and VT, the chi-square test was used to compare individual groups. A change of $p<0.05$ was considered to be significant.

Results

1. Investigation of the effect of exogenous CO

Effect of CO on tissue cAMP, cGMP levels and guanyl cyclase activities

- Ischemia resulted in a small but significant increase in cAMP and cGMP levels after 10 min and 30 min of ischemia. However these tissue nucleotide levels returned to normal after 40 min of ischemia.
- CO administered to the perfusion buffer in a concentration of 0.001% significantly increased the tissue levels of cAMP and cGMP from their ischemic control values 0.30±0.03 nmol/g wet weight and 25.8±3.0 pmol/g wet weight to 0.52±0.05 nmol/g wet weight (#$p<0.05$) and 111.5±11.2 pmol/g wet weight (#$p<0.05$), respectively. The same increase in cAMP and cGMP levels were observed in hearts treated with the concentration of 0.01% of CO.
- The results show that there is no significant difference between heart exposed to CO at a concentration of 0.001% and 0.01%.
- Hearts exposed to 0.001% and 0.01% of CO, guanylate cyclase activity was increased from its ischemic/reperfused CO control value of 184±25 fmol/mg protein to 442±38 fmol/mg protein (#$p<0.05$).

Effect of CO on infarct size and the incidence of reperfusion-induced VF

- Infarct size was reduced in the presence of 0.001% and 0.01% CO from its ischemic/reperfused control value of 39% ± 5% to 21% ± 3% (*$p<0.05$) and 18% ± 4% (*$p<0.05$), respectively. A significant reduction in infarct size was not detected between hearts treated with 0.001% and 0.01% of CO. Further increase in CO concentration to 0.1% in the perfusion buffer, significant reduction was not observed in infarct size limitation, and returned to control value.

- In the presence of 0.001% and 0.01% CO, a significant reduction in the incidence of reperfusion-induced VF was detected from its control value of 92% to 17% (*$p<0.05$) increasing the CO exposure to 0.1% in the perfusion buffer, 100% of hearts showed non-sustained or sustained reperfusion induced VF combined with VT or bradycardia and various rhythm disturbances. It is clear from these data that toxic effects of exogenous CO *via* the perfusion buffer appeared around 0.1% of CO concentration in the heart.

Effect of CO on cardiac function

- Cardiac function was not significantly changed before the induction of ischemia with the application of 0.001% and 0.01% of CO. However, the postischemic recovery of CF, AF and LVDP was significantly increased in the presence of both concentrations of CO, after 30 min ischemia followed by 60 min and 120 min of reperfusion, in comparison with the CO-free control group.
- HR did not show a significant change in hearts perfused with 0.001% or 0.01% of CO either before the induction of ischemia or during reperfusion. Its important to note, that hearts perfused with 0.1% of CO *via* the perfusion buffer, toxic effects of CO appeared on cardiac function before the induction of ischemia and during reperfusion.
- Application of 0.1% CO, high incidence of VF and VT, bradycardia, and ectopic beats were registered on the ECG making impossible to record cardiac function.

The effect of different concentration CORM-3 treatment for the ischemic/reperfused rat heart

Infarct size and arrhythmias

- Postischemic myocardium was associated with a 38 ± 1.3 % infarct size which was consistently reduced by increasing concentrations of CORM-3 to 33.5 ± 1.6 % (10 μM CORM-3), 23.3 ± 1.5 % ($p<0.05$, 25 μM CORM-3) and 20.7 ± 1.2 % ($p<0.05$, 50 μM CORM-3), respectively.
- The incidence of reperfusion-induced VF was significantly reduced by CORM-3 from 100 % to 33 % ($p<0.05$) and 8 % ($p<0.05$) with 25 μM and 50 μM CORM-3, respectively. The duration of reperfusion-induced VT and VF was reduced from their untreated drug-free control values of 14 ± 4 sec and 93 ± 12 sec to 5 ± 2 sec (*$p<0.05$) and 26 ± 8 sec (*$p<0.05$) in hearts treated with 50 μM of CORM-3, respectively. The duration of sinus rhythm was significantly increased with the application of 50 μM of CORM-3 from 13 ± 4 sec to 89 ± 15 sec ($p<0.05$).

Recovery in cardiac function

- After 30 min of ischemia followed by 120 min of reperfusion, AF was significantly improved from 9.8 ± 0.9 ml/min (untreated) to 18.7 ± 1.2 ml/min (*$p<0.05$) and 22.2 ± 1.9 ml/min (*$p<0.05$) with 25 μM and 50 μM CORM-3, respectively.
- A similar pattern of recovery was observed for CF and LVDP in the CORM-3-treated groups.
- Heart rate (HR) was not significantly changed during reperfusion in CORM-3-treated groups in comparison with the drug-free ischemic/reperfused control values. In addition, it is important to note that pre-ischemic values of HR, CF, AF, and LVDP in the untreated group were not statistically different to those of CORM-3-treated control values.

Effects of CORM-3 on myocardial ion contents

- It was found that CORM-3 did not significantly change myocardial Na^+, K^+, and Ca^{2+} contents in nonischemic myocardium. After 30 min of normothermic global ischemia followed by 120 min of reperfusion, the cellular contents of Na^+ and Ca^{2+} were significantly increased from their pre-ischemic values of 30 ± 3 μM/g dry weight and 1.5 ± 0.2 μM/g dry weight to 85 ± 5 μM/g dry weight and 4.1 ± 0.3 μM/g dry weight, respectively.
- The ischemia/reperfusion-induced K^+ loss was observed in the untreated group from 300 ± 9 μM/g to 238 ± 8 μM/g dry weight.
- In hearts treated with 25 μM and 50 μM CORM-3, a significant attenuation of post-ischemic Na^+ and Ca^{2+} increase was detected. Specifically, in hearts treated with CORM-3, the increase of cellular Na^+ and Ca^{2+} was significantly reduced from their post-ischemic untreated control values (85 ± 5 μM/g dry weight and 4.1 ± 0.3 μM/g dry weight to 57 ± 6 μM/g (*$p<0.05$) dry weight and 2.8 ± 0.5 μM/g (*$p<0.05$) dry weight, and 49 ± 5 μM/g (*$p<0.05$) dry weight and 2.3 ± 0.2 μM/g (*$p<0.05$) dry weight, respectively).

2. The role of TG2 enzyme in ischemia/reperfusion injury

Infarct size and cardiac function in TG2 knock out mice after the ischemia/reperfusion

- The infarct size was significantly increased ($p<0.05$) in the $TG2^{-/-}$ ischemic/reperfused hearts (47.2 ± 3.3%) as compared to the drug-free ischemic/reperfused wild-type group (37.2 ± 2.1%).
- The incidence of reperfusion-induced ventricular fibrillation was also significantly increased ($p<0.05$) in the $TG2^{-/-}$ ischemic/reperfused hearts

(83%) as compared to the wild-type ischemic/reperfused group (33%) resulting in a significant worsening in the recovery of the post-ischemic cardiac function in $TG2^{-/-}$ mice. For instance, after 40 min of ischemia followed by 120 min of reperfusion, AF was only 0.8 ± 0.1 ml/min in $TG2^{-/-}$ hearts, while it was 2.6 ± 0.4 ml/min in wild-type hearts ($p<0.05$).

- It is important to note, however, that already the pre-ischemic values of HR, CF, AF, and AOP of the $TG2^{-/-}$ control group were significantly ($p<0.05$) reduced when compared to those of the wild type control values.

Alteration of high energy phosphate-content in $TG2^{-/-}$ ischemic/reperfused hearts

- There was a significant reduction in the high-energy phosphate content of the $TG2^{-/-}$ mice already before ischemia which was further reduced following ischemia/reperfusion. The ATP levels decreased from 6,18 ± 2,52 to 3,81 ± 2,41 μmol/g wet weight in wild type mice, in case of $TG2^{-/-}$ mice ATP content decreased from 3,37 ± 1,32* μmol/g wet weight to 2,00 ± 1,20* μmol/g wet weight. The ADP levels did not show significant alteration. There is a decrease in the AMP concentration (from 0,073 ± 0,027 μmol/g wet weight to 0,024 ± 0,011 μmol/g wet weight in wild type and from 0,039 ± 0,011* to 0,029 ± 0,011* in TG2 knock out mice). In the CrP concentration we measured similar decrease (from 6,48 ± 0,51 to 5,20 ± 1,43 and from 4,55 ± 1,07* to 3,36 ± 1,11*μmol/g wet weight in the $TG2^{-/-}$ mice). These data imply that in $TG2^{-/-}$ hearts the maintenance of the high energy-phosphate content is impaired.
- No significant difference was found in the infarct size, the incidence of ventricular fibrillation, on the postischemic cardiac functional parameters between $AR^{-/-}$ and $AR^{+/+}$ strains, indicating that the increased sensitivity

of $TG2^{-/-}$ hearts to ischemia/reperfusion injury is not related to an impaired AR signaling.

Conclusions

I. The ischemia and recovery of post-ischemic myocardium during reperfusion result in the activation of multiple signaling pathways. In the present study we investigated the CO-related cGMP and cAMP signaling in isolated ischemic/reperfused hearts. Our results demonstrate that the presence of very low concentrations of CO in the perfusion buffer affords a significant protection against the ischemia/reperfusion-induced damage in insolated buffer perfused hearts. Presumably, the isolated buffer perfused heart could be an ideal experimental environment to study the direct effect of exogenous CO in cardiac function and metabolism because the blood and its elements are excluded from the model, thus the oxygen transport to cells and tissues is not directly damaged *via* the CO/blood/haemoglobin system. Our results show that low concentration of exogenous CO protect the isolated ischemic/reperfused heart and act by increasing cAMP and cGMP levels in the myocardium. A moderate increase in cAMP content could lead to arrhythmias by elevating cytosolic calcium levels in ischemic and post-ischemic myocardium. However the multiple increases in cGMP levels could mask and interfere with the arrhythmogenic effect of cAMP, leading to the suppression of reperfusion-induced VF. The significant increase in cGMP levels related to guanylate cyclase activities in CO-treated hearts, and suggests that CO-induced elevation *via* the guanylate cyclase-cGMP system is essential for cardioprotection in ischemic/reperfused myocardium.

The application of CO under *in vitro* conditions when the blood and its elements are excluded from experimental models, such as in isolated rat hearts, gives the chance to study the direct effects of CO on cardiac function and metabolism. These, and some other conflicting results, may possibly be

explained on the basis of different experimental conditions, different concentrations and duration of CO was given before the induction of ischemia or at the time of reperfusion.

In conclusion, the mechanisms underlying CO-induced cardioprotection or toxicity remain to be elucidated. The interpretation of our results focusing on the protective effect of exogenous CO must be limited by our observation in the rat heart and by fact that we used an isolated preparation. The immediate extrapolation of our results, obtained in isolated rat hearts, to an actual clinical situation should be viewed with some caution because of the absence of blood and its elements in our model system.

II. Our results also give an insight regarding the effect of CORM-3 on the imbalance of myocardial ion contents (Na^+, K^+, and Ca^{2+}) and its possible relationship with the recovery of post-ischemic cardiac function by CO liberated from CORM-3. Cellular K^+ loss and impairment of ion-operated channels during myocardial ischemia are critical factors causing lethal ischemia/reperfusion damage and arrhythmias. In the ischemic/reperfused myocardium, an increase in intracellular Na^+ content and a depolarized membrane potential leads to Ca^{2+} entry into the cardiomyocytes in exchange for intracellular Na^+ *via* the Na^+-Ca^{2+} exchange pump.

The primary factor responsible for net K^+ loss is altered Na^+ fluxes, which cause intracellular Na^+ to accumulate and displace intracellular K^+. In order to determine how the treatment of hearts with CORM-3 affects myocardial susceptibility to reperfusion-induced damage, we have compared arrhythmias, heart function, infarct size, and ion shifts with CORM-3-free and CORM-3-treated hearts. We found that ischemia/reperfusion-induced cellular Na^+ gain leads to a net K^+ loss because of the need to maintain electroneutrality of charge movement and ion balance following the ischemic event. Apart form Na^+, K^+ is the most likely to maintain the charge-balancing role, because K^+ is a ubiquitous

intracellular cation possessing high membrane permeability activity. Our results clearly show that 25 μM and 50 μM CORM-3 significantly reduced ischemia/reperfusion-induced cellular K^+ loss and prevented Na^+ and Ca^{2+} gains. Indeed, hearts treated with CORM-3 were less susceptible to potentially life-threatening arrhythmias, as evidenced by the reduced incidence of VF and VT during reperfusion following 30 min of normothermic ischemia. Thus, the present study emphasizes the importance of ionic balance across the cell membrane, because different clinical conditions are frequently complicated by arrhythmias that originate from an ionic imbalance of the heart. The observed protection against the incidence of arrhythmias by CORM-3 was reflected by a significant reduction in infarct size and improvement in post-ischemic cardiac function.

Until recently, the hypothesis that ion channels could be a target of CO was perceived with great scepticism because it was assumed that channel proteins lack of any molecular domain that would validate a direct chemical interaction with CO. In fact, heme or metal centers in proteins are the preferential targets for CO making them the most probable "molecular switch" required by cells to transduce the signal elicited by this diatomic gas . Notably, calcium-sensitive potassium channels have been recently reported to covalently bind with iron protoporphyrin IX (heme); moreover, compelling evidence showed that heme is a potent, but subtle, allosteric regulator of human maxi-K^+ channels where the binding of gaseous molecules, including CO, is likely to play an important modulatory function. Although the direct mechanism(s) underlying the effect of CO on potassium levels observed in this study remains to be fully identified, the results presented here demonstrate that CO liberated from CORM-3 markedly influences the balance of important ions and, as a consequence of its effect, provides cardiac protection against tissue damage and myocardial dysfunction inflicted by ischemia-reperfusion.

The advent of CORMs, chemically engineered CO carriers that can be used in biological systems, has corroborated the important notion that small amounts of CO can be utilized to investigate more specifically the cellular effects of this diatomic gas and more studies will be directed to optimizing the therapeutic benefits role of CO in cardiac dysfunction and other diseases.

III. To assess the role of TG2 in programmed cell death, TG2 knock-out mice have been generated. Considering that the functional parameters of $TG2^{-/-}$ hearts were steady state much worse than that of the wild-types, and high-energy phosphate content was reported to affect the cardiac functional parameters before and after ischemia/reperfusion[2], we decided to test the high energy phosphate content of the wild-type and $TG2^{-/-}$ hearts before and after ischemia/reperfusion. The significant reduction in ATP, AMP and CrP content imply that in $TG2^{-/-}$ hearts the maintenance of the high energy-phosphate content is impaired. This defect could reflect a failure in mitochondrial ATP production.

These data provide evidence for a novel function of TG2 participating in the maintenance of the intact mitochondrial respiratory function, the absence of which leads to a serious failure in ATP production. Defects observed in $TG2^{-/-}$ mice can be due both to a lack of direct action of TG2 on the mitochondrial substrate proteins as well as to the absence of TG2-catalyzed post-translational modifications of some important mitochondrial regulatory proteins.

In conclusion, we present compelling evidence that under physiological circumstances TG2 acts at mitochondrial level. Future work should determine the TG2 mitochondrial substrates and which of the many TG2 enzymatic activities (i.e. transamidating, kinase and protein disulphide activity) is responsible for the physiological function described in this study.

Publications (the tesis is based on)

1. Bak I., **Varadi J.**, Nagy N., Vecsernyes M., Tosaki A., (2005) The role of exogenous carbon monoxide in the recovery of post-ischemic cardiac function in buffer perfused isolated rat hearts. *Cell Mol Biol* **51:**453-459. **IF: 1.018**

2. **Varadi J.**, Lekli I., Juhasz B., Bacskay I., Szabo G., Gesztelyi R., Szendrei L., Varga E., Bak I., Foresti R., Motterlini R., Tosaki A., (2007) Beneficial effects of carbon monoxide-releasing molecules on post-ischemic myocardial recovery. *Life Sciences* **80:**1619-1626. **IF: 2.512**

3. Szondy Zs., Mastroberardino P.G., **Varadi J.**, Farrace M.G., Nagy N., Viti I., Wieckowski M.R., Melino G., Rizzuto R., Tosaki A., Fesüs L. and Piacentini M., Tissue transglutaminase (TG2) protects cardiomyocytes against ischemia/reperfusion injury by relating ATP synthesis *Cell Death Differ* **13:**1827-1829. **IF: 7,785**

Other publications (do not used for the thesis)

1. Bak I., Lekli I., Juhasz B., Nagy N., Varga E., **Varadi J.**, Gesztelyi R., Szabo G., Szendrei L., Bacskay I., Vecsernyes M., Antal M., Fesus L., Boucher F., Joel de Leiris, Tosaki A., (2006) Cardioprotective mechanisms of Prunus cerasus (sour cherry) seed extract against ischemia/reperfusion-induced damage in isolated rat hearts. *Am J Physiol Heart and Circ Physiol* **291:**H1329-36 **IF: 3.56**

2. Szilagyi A., Fenyvesi F., Majercsik O., Pelyvas F.I., Bacskay I., Feher P., **Varadi J**., Vecsernyes M., Herczegh P., (2006) Synthesis and cytotoxicity of leinamycin antibiotic analogs *J Med Chem* **49:**5626-5630 **IF: 5.104**

Abstracts

Varadi J., Lekli I., Juhasz B., Foresti R., Motterlini R., Tosaki A., (2006) Cardioprotective mechanisms of sour cherry seed extract against ischemia/reperfusion-induced damage in isolated rat hearts *J Mol Cell Cardiol* **40**P117, p963 **IF: 3.872**

Bácskay I., Kocsán R., Fenyvesi F., Fehér P., Sipos T., Váradi J., Vecsernyés M., (2005) Cytotoxicity of different types of surfactant on HeLa cells. *E J Pharmaceutic Sci* **25:**S46-S47 **IF: 2.347**

Poster

Fenyvesi F., Kiss T., Bácskay I., Fodor Z., Fehér P., **Váradi J.**, Fenyvesi É., Szente L., Vecsernyés M., (2006) A Caco-2 transzport model és alkalmazásának lehetőségei ciklodextrinek felszívódást befolyásoló hatásának tanulmányozásában *Congressus Pharmaceuticus Hungaricus XIII.*