

# THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY

## ISCHEMIA/REPERFUSION-INDUCED INJURY IN THE MYOCARDIUM: MECHANISM AND PREVENTION

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# 1. INTRODUCTION

It has been proposed that most cases of sudden cardiac death may result from ischemia and reperfusion-induced ventricular fibrillation (1-2). Interest in the development and pharmacological control of reperfusion-induced ventricular fibrillation has been stimulated by the realization that such arrhythmias may occur under a number of pathological and clinical circumstances including the spontaneous relief of coronary artery spasm (3). There is considerable controversy over the mechanisms responsible for the induction of these arrhythmias and a number of different factors have been suggested (4-5), but the two major mechanisms proposed and generally accepted to explain reperfusion-induced injury and ventricular fibrillation are: (i) calcium overload, and (ii) free radical formation (6). Many human diseases are associated with the overproduction of free radicals that inflict cell damage. Examples of oxidative stress-related diseases include reperfusion injury, such as that which occurs after tissue ischemia or stroke, and inflammatory processes, such as arthritis (7-8). Heme oxygenase (HO) catalyzes the rate-limiting step in the oxidative degradation of heme to biliverdin and carbon monoxide (CO) (9). Two isozymes of HO have been identified and cloned; HO-1, an inducible form, and HO-2, a constitutive form

(10-11). Studies demonstrate that HO-1 is induced in response to various interventions causing oxidative stress including ultraviolet irradiation, hypoxia, and ischemia (7, 12-15).

In a previous study, we observed a reduction in HO-1 mRNA expression and enzyme activity in ischemic/reperfused fibrillating myocardium but not in nonfibrillating hearts (16). Therefore, in the present study, we decided to approach the question from a different angle. The aforementioned finding has led us to speculate that a reduction in HO-1 mRNA expression and enzyme activity may be seen in nonischemic electrically fibrillated myocardium. If this were so, we would stress that the prevention of HO-1 mRNA downregulation could play a crucial role in the development of reperfusion-induced VF. Our study is concerned with the possibility that reperfusion-induced VF may be initiated by modification of the expression of heme oxygenase mRNA and enzyme activity.

Although many mechanisms have been proposed to explain the causes of arrhythmias, no work has been done, to our knowledge, in order to clarify the mechanism(s) of VF at a gene expression level in ischemic/reperfused myocardium. The long QT disease and idiopathic ventricular fibrillation, as known up to now, are the only cardiac disorders based

on genetic mutation and cause sudden cardiac death from ventricular arrhythmias (17-20, 33). Thus, the first part of our study may offer a further understanding of the arrhythmogenic mechanism(s) at a molecular level and identify the responsibility of heme oxygenase mRNA for arrhythmogenesis in ischemic and reperfused hearts.

In the second part of our study we investigated the role of oxidative stress in spontaneously hypertensive rat myocardium.

Spontaneously hypertensive rats (SHR) constitute an experimental model for hypertension with similarities for hypertension in humans. Hypertension results in left ventricular hypertrophy (21), a well recognized adaptation mechanism of the heart to reduce stress induced processes. Thus, myocardial hypertrophy in response to chronic pressure overload may reduce cardiac stress during the compensation state (22). While a number of studies provided evidence suggesting changes in cardiac function with aging in SHR, questions as to cardiac functional state, presence or absence of cardiac decompensation and genetic control have remained unsolved (23). During the transition from the compensated state to cardiac failure in SHR, numerous pathological changes, including left ventricular dilation (22, 24),

cardiac pump dysfunction (25), cardiac depression (26), fibrosis (27) and apoptosis (28) have been documented. Although it is now possible to study the molecular and cellular basis of pathological processes in the myocardium, the application of these approaches and hence the understanding of the transition to heart failure is still incomplete due, in part, to the limited availability of suitable animal models.

Little is known concerning the role and expression of superoxide dismutase (SOD) and catalase during oxidative stress caused by reactive oxygen species in the SHR myocardium. To improve our understanding of SOD and catalase expression in heart tissue subjected to oxidative stress we treated the myocardium obtained from SHR and WKY (Wistar-Kyoto rats) with H<sub>2</sub>O<sub>2</sub> and assessed the expression and activities of SOD, catalase and glutathione peroxidase (GPx). Although many mechanisms have been proposed to explain the causes of oxidative stress, its development in the SHR myocardium has not previously been addressed at a gene expression/regulation level. The aim in this part of the study was to instigate such a molecular approach by determining SOD- and catalase expression in the oxidatively stressed SHR myocardium to improve our

understanding of the stress response in dilative cardiomyopathy.

In the third part of our studies, the effect of a natural free radical scavenger, an extract of grape seed, was studied in ischemic/reperfused myocardium.

It is known that free radicals play an important role in reperfusion-induced cardiac arrhythmias (29-30). Garlick et al (31) and Zweier (32) showed that abrupt reperfusion of the ischemic myocardium led to a massive formation of oxygen free radicals. Thus, direct evidence suggests that formation of oxygen free radicals could be important in the development of reperfusion-induced ventricular arrhythmias, and agents known to scavenge or inhibit the formation of free radicals could prevent reperfusion-induced arrhythmias including ventricular tachycardia and ventricular fibrillation, and improve the recovery of ischemic and reperfused myocardium. Ventricular arrhythmias remain an important source of mortality in ischemic heart diseases, therefore every effort must be made to increase the recovery of cardiac function and minimize sudden cardiac death caused by VF.

An inverse relation between moderate alcohol consumption and the risk of cardiac disorders has been emphasized in several studies (33-35). There is considerable controversy over the

mechanisms responsible for the alcohol-induced cardiac protection, and a number of different factors have been suggested. These include the inhibition of platelet aggregation (33) and neointimal hyperplasia (36), antithrombogenesis (37), increased level of high density lipoprotein (34), and free radical production (38-39). Flavonoids, which increase the antioxidant capacity of cells and tissues (40-42), are probably responsible for the antioxidant property of red wine, and as a consequence, wine drinkers have a lower risk of cardiovascular disease or death from coronary artery disease (43). Thus, the beneficial effect of red wine has been attributed to the antioxidants present in the polyphenol fraction of red wine (44) including resveratrol, catechin, and proanthocyanidins. In vitro studies emphasized that proanthocyanidins are potent scavengers of peroxy and hydroxyl radicals which are generated in the ischemic and reperfused myocardium suggesting that the cardioprotective effects could be attributed, at least in part, to hydroxyl and peroxy radical scavenging properties of proanthocyanidins (38-39).

We hypothesized that grape seed proanthocyanidins could play an important role in the scavenging of free radicals, and as a consequence, the incidence of reperfusion-induced

arrhythmias could be reduced. Postischemic cardiac function was also compared between the proanthocyanidin-fed and the untreated control groups. The role of oxygen free radicals and their production were also studied and measured by ESR in hearts obtained from proanthocyanidins pretreated rats.

## 2. METHODS

*Animals:*

Male Sprague-Dawley rats (320-350 g body weight) were used for all studies. All rats 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).

*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, the aorta was cannulated, and the heart was perfused according to the Langendorff method for a 5-min washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). The perfusion medium consists of a modified Krebs-Henseleit bicarbonate buffer ([millimolar concentration] NaCl 118, KCl 4.7, CaCl<sub>2</sub> 1.7, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 0.36, MgSO<sub>4</sub> 1.2 and glucose 10). The Langendorff preparation was switched to the working mode as previously described by Tosaki and Braquet (45). Aortic flow was measured by calibrated rotameter. Coronary flow rate was measured by

timed collection of the coronary perfusate that dripped from the heart.

*Measurement of heart function, and arrhythmias for studying the hemeoxygenase expression:*

An epicardial ECG was recorded by a polygraph throughout the experimental period by two silver electrodes attached directly to the surface of isolated hearts. The ECGs were analyzed to determine the incidence of VF. The heart was considered to be in VF if an irregular undulating baseline was seen on the ECG. The heart was considered to be in sinus rhythm if normal sinus complexes occurring in a regular rhythm were apparent on the ECG. VF was considered to be reversible if VF reverted to regular sinus rhythm within the first 5 min of the reperfusion period. If VF was persisting through the first 10 min of reperfusion period, the VF was considered to be irreversible. At the end of the first 10 min of Langendorff reperfusion (irreversible fibrillated myocardium), isolated hearts were electrically defibrillated using a single 20 V square-wave pulse of 1 ms duration, switched to the working mode, then nonfibrillated ischemic and reperfused hearts were reperfused for an additional 110 min. The defibrillation was repeated 2 to 4 times if it was necessary. Before ischemia and during reperfusion (n=6 in each group),

heart rate (HR), coronary flow (CF) and aortic flow (AF) rates were registered. Left ventricular developed pressure (LVDP) and the first derivative of LVDP (LVdp/dt<sub>max</sub>) were also recorded (Experimetria, Budapest, Hungary). In ischemic/reperfused myocardium, HO-1 mRNA expression, and enzyme activities were determined at the end of 120 min of reperfusion in the myocardium.

In additional studies, without the ischemic/reperfused protocol, in order to simulate the period of 10 min of irreversible VF, isolated hearts (n=6-12) were electrically fibrillated (20 Hz, 1200 beats/min) using 5 V square-wave pulses of 1 ms duration for 10 min in Langendorff mode. Then hearts were defibrillated, switched to working mode, and perfused for additional 110 min, and cardiac function was registered at the 60<sup>th</sup> min and 120<sup>th</sup> min of perfusion. HO-1 mRNA expression and enzyme activities were determined at the end of 120 min of perfusion.

#### *Total RNA isolation:*

Total RNA was isolated from rat heart tissue (100 mg) by homogenization in 1 ml of TRIzol reagent (Gibco BRL, Life Technologies, Eggenstein, Germany), a guanidium thiocyanate method (46), as described in the manual. 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 (Beckman DU 640, Beckman Instruments Inc., Fullerton, CA).

#### *RT-PCR:*

Total RNA (2 µg) of each samples were subjected to random primed first-strand cDNA synthesis (reverse transcription) in 40 µl reaction assays composed of (in mM) 50 Tris-HCl, 75 KCl, 3 MgCl<sub>2</sub>, 10 DTT, 1 dNTP's (each), 0.005 random hexamer, 0.5 IU/µl Mo-Mu-LV reverse transcriptase (Gibco BRL, Life Technologies, Eggenstein, Germany). Tubes with mixtures were heated for 60 min at 42 °C. The first-strand cDNA preparations were used as templates for PCR. The sequence of the primers was the following: HO-1 sense: 5'-AAG GAG GTG CAC ATC CGT GCA-3'; HO-1 antisense: 5'-ATG TTG AGC AGG AAG GCG GTC-3'; HO-2 sense: 5'-ATG GCA GAC CTT TCT GAG CTC-3'; HO-2 antisense (47): 5'-CTT CAT ACT CAG GTC CAA GGC-3'; GAPDH sense: 5'-TCC TGC ACC ACC AAC TGC TTA GCC-3'; GAPDH antisense (48): 5'-TAG CCC AGG ATG CCC TTT AGT GGG-3'. The reaction mixture contained 1x PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCB], 1.5 mM MgCl<sub>2</sub>, 100 µM dNTP's (each), 100 µM primers and 0.025U/µl

Taq polimerase (Gibco BRL, Life Technologies, Eggenstein, Germany). The number of PCR cycles was adjusted carefully to avoid saturation of the amplification system. All tubes were incubated at 95 °C for 1 min, at 55 °C for 1 min, and at 72 °C for 2 min for HO-1 and HO-2, at 95 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min for GAPDH and the numbers of cycles were 28, 35 and 22, respectively. The reaction mixtures were kept at 72 °C for 10 min. Amplification products were visualized on 2% agarose gels using ethidium bromide, identified by their sizes, the length of HO-1 fragment was 568, of HO-2 was 554, of GAPDH was 377 bp, respectively.

*Northern blot:*

Thirty µg of total RNA were transferred to a nylon membrane (Qiabrane, Qiagen, Hilden, Germany) according to Sambrook et al (49). The purified GAPDH probe was given by Zador et al (48). Purified cDNAs were labeled with  $\alpha^{32}\text{P}$ -dCTP by random hexamer priming. Blots were prehybridized for 2 hours in 50% formamide, 5x Denhardt's solution, 100 µl herring sperm DNA, and 0.5% SDS, 0.9 M sodium chloride, 0.09 M sodium citrate at 42 °C. Blots were hybridized with cDNAs, labeled to a specific activity of  $3 \times 10^8$  cpm/µg in hybridization fluid at 42 °C

overnight. Hybridized blots were washed in 0.3, 0.15, 0.015 M sodium chloride, 0.03, 0.015, 0.0015 M sodium citrate, respectively, pH 7.0, and 0.1% SDS, for 20 min at 50 °C. Kodak Biomed MR-1 films were exposed for 3-4 days with an intensifying screen at -80 °C. Blots were washed and reprobred with different cDNAs using the hybridization and washing conditions described above.

*HO activity assay:*

One hundred mg of tissue were homogenized in 10 ml of 200 mM phosphate buffer and centrifuged at 19 000g at 4 °C for 10 min. The supernatant was removed and re-centrifuged at 100 000g at 4 °C for 60 min, and the precipitated fraction was suspended in 2 ml of 100 mM potassium phosphate buffer. Biliverdin reductase was crudely purified by the technique of Tenhunen et al (50). HO activity was assayed as described by Yoshida et al (51). Reaction mixtures consisted of (final volume 2 ml) 100 µM potassium phosphate (pH 7.4), 15 nM hemin, 300 µM bovine serum albumin, 1 mg biliverdin reductase, and 1 mg microsomal fraction of the myocardium. The reaction was allowed to proceed for 1 hour at 37 °C in dark in a shaking water bath and was stopped by placing the test tube on ice. Incubation mixtures were then scanned scanning

spectrophotometer, and the amount of bilirubin was calculated as the difference between absorbance at 464 and 530 nm (52). Protein content was determined according to Lowry et al (53) in the microsomal fractions.

*Studies with spontaneously hypertensive (SHR) rat hearts:*

Male SHR and age-matched non-hypertensive genetic control WKY were used at the age of 18 months.

After an initial 15 min equilibration period with regular buffer, hearts were subjected to 30 min perfusion of buffer containing 25  $\mu$ M of H<sub>2</sub>O<sub>2</sub>, followed by a 30 min washout period. In additional studies, after 15 min equilibration period, hearts were subjected to 30 min global ischemia (the atrial inflow line was closed) followed by 30 min reperfusion. Control hearts were subjected to 60 min perfusion without H<sub>2</sub>O<sub>2</sub>. At the end of each experiment, all hearts were immediately freeze-clamped and stored in liquid nitrogen.

*Measurement of cardiac function in H<sub>2</sub>O<sub>2</sub>-free and H<sub>2</sub>O<sub>2</sub>-treated hearts:*

Preload and afterload were kept constant throughout the experiments. Heart functional parameters were recorded after the 15 min equilibration period, immediately after the H<sub>2</sub>O<sub>2</sub> infusion and

after the 30 min washout period. CF was measured by collecting effluent from the right atrium in a measuring cylinder for a timed period and AF by a calibrated rotameter. HR and left ventricular end-diastolic pressure LVEDP were determined directly from the pressure curve. LVDP was calculated as the difference between the peak systolic pressure and LVEDP. The maximum and minimum of the first derivatives of left ventricular pressure (+LVdp/dt<sub>max</sub>, -LVdp/dt<sub>max</sub>) were also recorded.

*Polymerase chain reaction for MnSOD and ZnSOD:*

For RT-PCR (reverse transcriptase-polymerase chain reaction), total RNA (2  $\mu$ g) was subjected to random primed first-strand cDNA synthesis in 40  $\mu$ l reactions composed of (in mM) 50 Tris-HCl, 75 KCl, 3 MgCl<sub>2</sub>, 10 DTT, 1 dNTPs (each), 50 ng random hexamer, 0.5 IU/ $\mu$ l Mo-Mu-LV reverse transcriptase (Superscript Pre-Amplification System; Gibco BRL, Life Technologies, Eggenstein, Germany). The reactions were incubated for 60 min at 42 °C and terminated at 65 °C for 15 min. The first-strand cDNA's were subsequently amplified by PCR; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal control. The sequences of the primers were as follows: Zn- and

MnSOD sense, 5'-GAC AAA CCT GAG CCC TAA GGG-3'; MnSOD antisense, 5'-CTT CTT GCA AAC TAT G-3' (54); GAPDH sense, 5'-TCC TGC ACC ACC AAC TGC TTA GCC-3'; GAPDH antisense, 5'-TAG CCC AGG ATG CCC TTT AGT GGG-3' (48). The PCR reaction mixture contained 1x PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM MgCl<sub>2</sub>, 100 μM dNTP's (each), 100 μM primers and 0.025 U/μl Taq polymerase in a final volume of 50 μl. The number of PCR cycles was adjusted to avoid saturation of the amplification system [95 °C for 30 seconds, at 52 °C for 1 min, and at 72 °C for 30 s (27 cycles) for MnSOD and 95 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min (22 cycles) for GAPDH], with a final elongation at 72 °C for 10 min. Amplification products were visualized on 2% agarose gels containing ethidium bromide (1 μg/ml): MnSOD fragment, 361 bp; GAPDH fragment, 377 bp. A ΦX174 RF DNA Hae III digest was used as marker. The products were quantified by means of EASY (Enhanced Analysis System, Herolab GmbH, Wiesloch, Germany).

*Northern blot and dot blotting for catalase cDNA in normotensive and hypertensive hearts:*

For Northern blotting, 30 μg per lane of total RNA were subjected to agarose gel

electrophoresis in the presence of glyoxal, transferred to a nylon membrane (Qiabrane, Qiagen GmbH, Hilden, Germany) according to Sambrook et al (49) and U.V. cross linked (Stratalinker; Stratagene, Heidelberg, Germany). The rat catalase cDNA containing plasmid, pRCA38 (55) was from Dr. S. Furuta, Nagano/Japan and the purified GAPDH probe from Dr. E. Zador, Szeged/Hungary. cDNA probes were prepared by subjecting the gel purified fragments (GeneClean II Kit; Bio 101 Inc., Vista/Ca., USA) to random hexamer primed labelling with [ $\alpha^{32}$ P]-dCTP (Stratagene, Heidelberg, Germany). Blots were prehybridized in Quikhyb solution (Stratagene, Heidelberg, Germany) for 2 h at 42 °C. The cDNA probes ( $3 \times 10^8$  cpm/μg) were denatured (95 °C for 3 min) and added to the hybridization liquid. After overnight hybridization at 42 °C, the filters were sequentially washed in 2x-, 1x- and 0.1xSSC (containing 0.1% Na-dodecylsulfate [SDS]) for 20 min at 50 °C (SSC: 0.15 M NaCl, 0.015 M Na-citrate, pH 7.0). The filters were exposed (3-4 days at -80 °C with intensifying screen) on Kodak Biome MR-1 film. Blots were stripped (100 °C for 10 min in 0.1% SDS) for rehybridization with different cDNA probes. Northern blots showed differences between certain groups, but exact quantification was impossible. Therefore,

dot blotting was used for further quantification of catalase mRNA. Twenty  $\mu\text{g}$  of total RNA from each sample were dissolved in a final concentration of 23% formamide, 3% formaldehyde, 10x SSC vacuum blotted to the membrane, washed twice with 10xSSC and cross linked as above.

*Catalase, MnSOD, and ZnSOD enzyme activities:*

For enzyme assays, 60 mg of tissue were homogenized in a Potter homogenizer in 10 volumes of ice-cold water followed by 2 x 5 s (with a break of 10 s) sonication on ice. Catalase activity (E.C. 1.11.1.6) was determined and expressed as  $k$  in  $\text{mg}^{-1}\text{min}^{-1}$  protein (first-order rate constant of the reaction) (48). GPx (E.C. 1.11.1.9) and SOD (E.C. 1.15.1.1) activities were determined using test kits (Randox, RS 506 and SD 125 respectively). MnSOD was determined after addition of 1 mM KCN to specifically inhibit CuZnSOD. Protein was determined by a protein assay kit (P 5656; Sigma, Deisenhofen, Germany).

*Statistical analysis:*

Data of myocardial function (HR, CF, AF,  $+\text{LVdp}/\text{dt}_{\text{max}}$ ,  $-\text{LVdp}/\text{dt}_{\text{max}}$ , LVEDP, LVDP), enzyme activities and mRNA levels were expressed as the mean  $\pm$  SEM. One-way analysis of variance was first

carried out to verify any difference between the mean values of all groups. Where differences were evident, the values of the SHR groups were compared with those of the WKY groups or with the respective treated or non-treated (SHR, WKY) groups by a modified t-test. A difference of  $p < 0.05$  was considered significant.

*Experimental time course to study the effect of grape seed proanthocyanidins:*

Before the isolation of hearts, rats were treated orally with 50 or 100 mg/kg/day of grape seed proanthocyanidins for 3 weeks because this treatment resulted in an effective cardiac protection in rats (39). A commercially available IH636 grape seed proanthocyanidin-extract (ActiVin, InterHealth Nutraceuticals, Benecia, CA, USA) has been used in our study. Gas chromatography-mass spectrometric analyses in conjunction with high performance liquid chromatography have demonstrated that this novel IH636 grape seed proanthocyanidin-extract contains 54% dimeric proanthocyanidins, 13% trimeric proanthocyanidins and 7% tetrameric proanthocyanidins, as well as a small amount of monomeric (less than 5%) and high molecular weight oligomeric (less than 5%) proanthocyanidins and flavonoids. Age-matched control rats received a daily dose of methylcellulose-saline solution for 3

weeks. Grape seed proanthocyanidins were homogenized in 2 ml of 1% methylcellulose solution then diluted with 0.9% NaCl to 10 ml, and 10 ml/kg of final volume was used for oral treatment of rats as a gavage in each day. At the end of the treated period, rats were anesthetized with an i.p. injection of pentobarbital sodium (60 mg/kg) then i.v. heparin was given. Hearts were excised and perfused with a drug-free buffer according to the Langendorff method for a 10-min washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). During the washout period, pulmonary vein was cannulated and the Langendorff preparation was switched to the working mode for an additional 5 min perfusion as previously described (45). Our study had two single objectives: the first was whether proanthocyanidins pretreatment could reduce the incidence of reperfusion-induced VF and VT and improve postischemic cardiac function. To achieve this, hearts (n=12 in each group) were subjected to 30 min global ischemia followed by 2 hours reperfusion.

The second objective of our work was to study whether proanthocyanidins could attenuate the formation of oxygen free radicals measured by ESR in ischemic/reperfused myocardium (n=6 in each group). Thus, the effect of proanthocyanidins at different doses (50 mg/kg or 100 mg/kg) on free radical

production was tested in hearts subjected to 30 min ischemia followed by reperfusion. The samples for ESR studies were taken at the 3<sup>rd</sup> min of reperfusion to measure oxygen free radicals from the effluents of hearts because this time point of sampling gave a maximum signal intensity of free radical production in our model system (56). Pre-selected exclusion criteria for the present studies demanded that working hearts were excluded if: (i) ventricular arrhythmias occurred during the period prior to the induction of global ischemia, (ii) coronary flow and aortic flow were less than 19 ml/min and 35 ml/min, respectively, prior to the initiation of ischemia.

#### *Electron spin resonance (ESR) studies:*

Spin trap studies were performed by infusing the spin trap 5,5-dimethylpyrroline-N-oxide (DMPO), through the side arm located just proximal to the end of the heart perfusion cannula. In order to prevent light-induced degradation of DMPO solution, the infusion syringe was covered with aluminum foil. During the first 3 min of Langendorff reperfusion period, DMPO was directly infused into the untreated or proanthocyanidins treated heart at a rate of 1 ml/min of 100 mmol/liter stock solution. This resulted in a final perfusate DMPO concentration, depending on the coronary flow of each

heart, about 10-12 mmol/liter. To prevent spin-adduct decay, the effluent was immediately frozen in liquid nitrogen as it flowed from the myocardium with an effluent sampling time of 30 sec. ESR spectra were recorded in a flat quartz cell with a Bruker ECS106 spectrometer operating at X band (9.3 MHz) with a 100 kHz modulation frequency. The microwave power maintained at 10 mW to avoid saturation. Scans were traced with 0.2 mT (milli Tesla) of modulation amplitude with 2 min of scan time and with 300 msec of response time. Hyperfine coupling constants were measured directly from the field scan using  $Mn^{2+}$  as a marker for calibration.

*Indices measured:*

The ECGs were analyzed to determine the incidence of VF and VT (57). Hearts were 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, and this classification included repetitive monomorphic VT which is difficult to dissociate from rapid VT. The heart was considered to be in sinus rhythm if normal sinus complexes occurring in a regular rhythm were apparent on the ECG. Before ischemia and during reperfusion, HR, CF and AF rates were registered. LVDP was also recorded by the insertion of

a catheter into the left ventricle via the left atrium and mitral valve. The hemodynamic parameters were registered by Hemosys (Exprimetria, Budapest, Hungary) computer acquisition system.

*Statistics:*

The data for HR, CF, AF, LVDP, mRNA expressions, enzymes activities, and signal intensity of DMPO-OH adduct were expressed as the mean  $\pm$  SEM. One-way analysis of variance test was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of all groups were compared with those of the drug-free control group by multiple t-test followed by Bonferroni correction (58). For the distribution of discrete variables such as the incidence of VF and VT which follows a nonparametric distribution, an overall chi-square test for a 2xn table was constructed followed by a sequence of 2x2 chi-square tests to compare individual groups. A change of  $p < 0.05$  between the drug-free control and treated groups was considered to be significant.

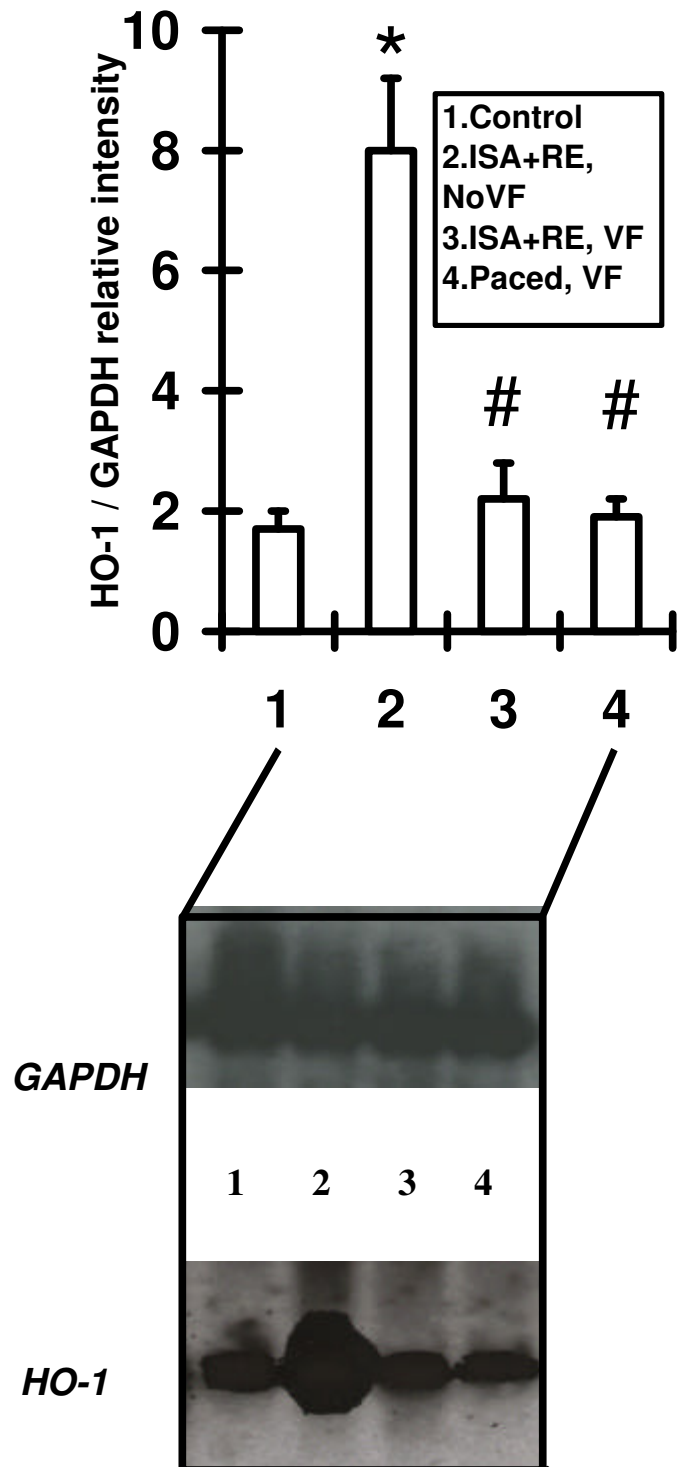
### 3. RESULTS

## RESULTS I.

In the following experiments we studied the expression of HO-1 in nonischemic control, ischemic/reperfused nonfibrillated, ischemic and reperfused fibrillated, and electrically fibrillated myocardium. Figure 1 shows the results of Northern hybridization performed by the probe for HO-1.

The expression of HO-1 mRNA (about four-fold) was observed in ischemic and reperfused nonfibrillated myocardium (Fig. 1, lane 2) in comparison with the nonischemic control heart (Fig. 1, lane 1). In hearts subjected to 30 min ischemia followed by 2 hours of reperfusion and VF was developed (Fig. 1, lane 3), the expression of HO-1 mRNA was not observed in comparison with the nonischemic control myocardium. In other words, HO-1 mRNA expression was significantly reduced in ischemic and reperfused fibrillated myocardium (Fig. 1, lane 3) in comparison with the ischemic/reperfused nonfibrillated tissue (Fig. 1, lane 2). In additional studies, hearts were electrically fibrillated in order to avoid the ischemia/reperfusion protocol, and HO-1 mRNA expression was studied. Thus, in electrically fibrillated myocardium (Fig. 1, lane 4), the expression of HO-1 mRNA was not observed in comparison with the

nonischemic control hearts. The electrically fibrillated myocardium (Fig. 1, lane 4)



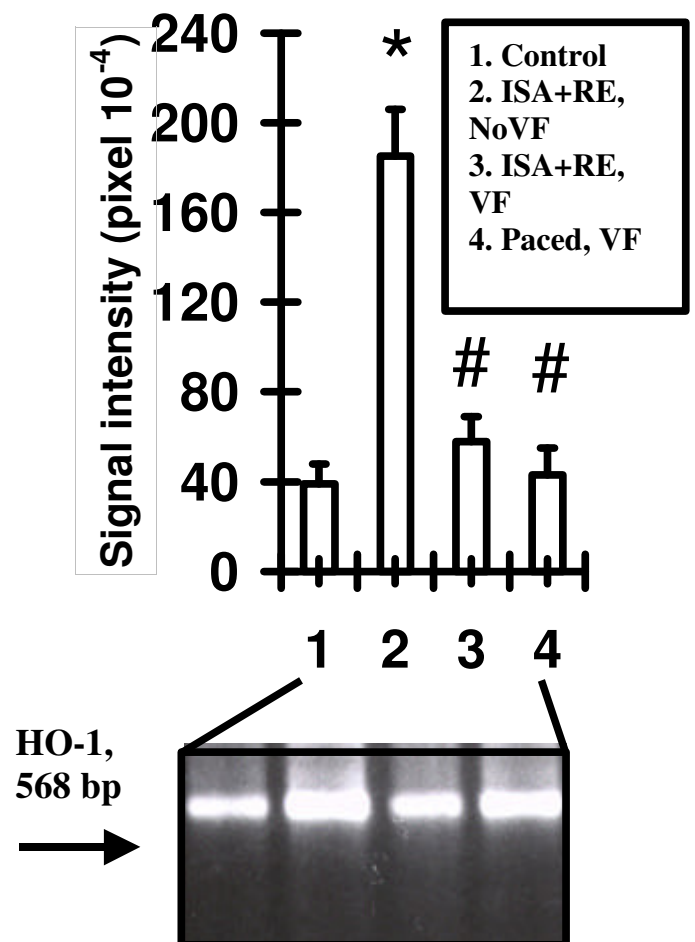
**Fig 1** HO-1 mRNA expression in nonfibrillated and fibrillated hearts. Northern blots (representative blots) were probed with  $^{32}\text{P}$ -HO-1, and then the same blots were stripped and re-probed with  $^{32}\text{P}$ -GAPDH cDNA. In nonischemic control myocardium (lane 1) the signal of HO-1 can be

detected. Hearts subjected to 30 min of ischemia followed by 120 min of reperfusion, and VF was not developed (lane 2) or was developed (lane 3). Hearts were electrically fibrillated (lane 4) for 10 min then perfused for additional 110 min, and HO-1 mRNA was detected. The results show the quantitative values (the ratio between HO-1 and GAPDH) of 6 hearts in each group. \* $p < 0.05$  compared to the nonischemic control group (group 1), # $p < 0.05$  compared to the ischemic/reperfused nonfibrillated group (group 2). Ischemia (ISA), Reperfusion (RE), Ventricular fibrillation (VF), No ventricular fibrillation (NoVF).

showed the same HO-1 mRNA expression than it was observed in ischemic and reperfused fibrillated myocardium (Fig. 1, lane 3) indicating that the mechanism of reperfusion-induced VF and electrically-induced VF could be based on the same mechanism. Furthermore, it is of interest to note that the ischemic and reperfused myocardium which did not show any incidence of VF upon reperfusion, a significant increase in HO-1 mRNA expression was observed, suggesting that the stimulation of HO-1 mRNA expression could prevent the development of reperfusion-induced VF.

Figure 2 shows the results of HO-1 amplification by RT-PCR, and support the data obtained by Northern blotting. Thus, in nonfibrillated ischemic and reperfused myocardium, a significant increase in RT-PCR signal intensity was observed (Fig. 2, lane 2) in comparison with the nonischemic control value (Fig. 2, lane 1). In fibrillated ischemic and reperfused hearts (Fig. 2, lane 3), an increase in RT-PCR signal intensity was not detected, and

no significant change was observed in comparison with the nonischemic control value (Fig. 2, lane 1). In other words, a significant reduction in RT-PCR signal intensity was observed in ischemic and reperfused fibrillated myocardium (Fig. 2, lane 3) compared to the ischemic and reperfused nonfibrillated hearts (Fig. 2, lane 2). Electrically fibrillated and perfused myocardium showed the same signal intensity in HO-1 RT-PCR amplification (Fig. 2, lane 4) as it was detected in ischemic and reperfused fibrillated hearts (Fig. 2, lane 3).



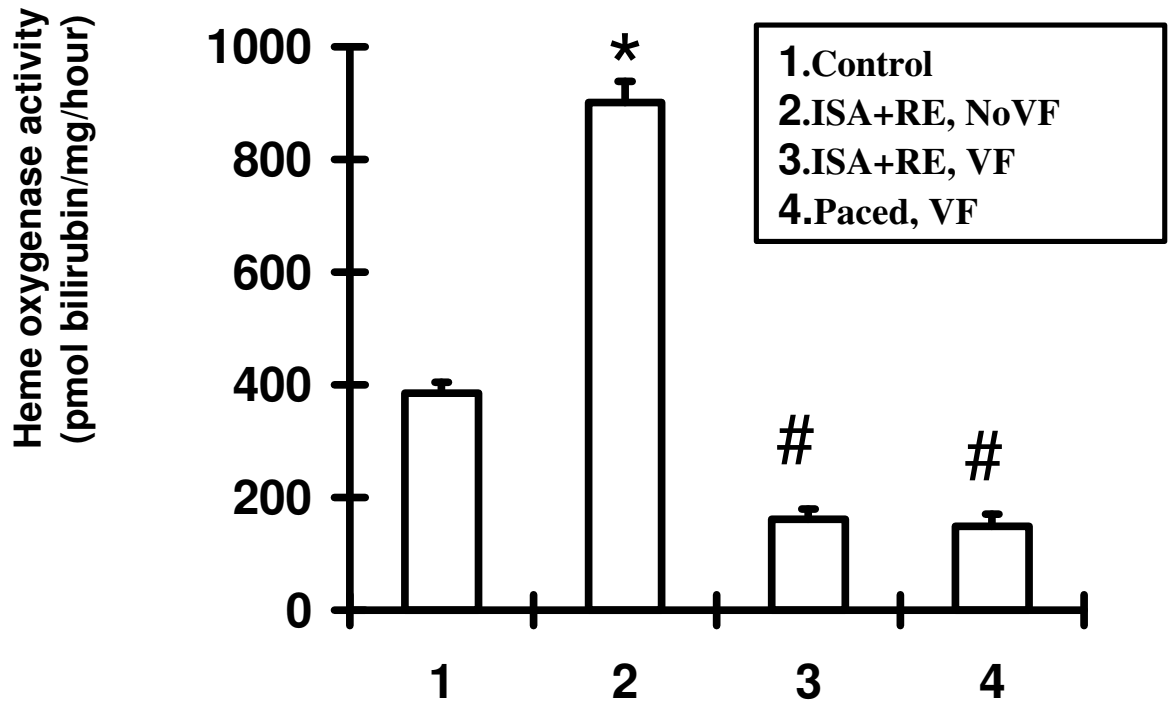
**Figure 2.** RT-PCR amplification for HO-1 mRNA. Aliquots were taken after 28 cycles amplification of cDNA obtained by reverse

transcription. Total RNA was separated on a 2% agarose gel and stained with ethidium bromide. Lanes (representative lanes) show the results of amplification with the primer for HO-1. Columns show the quantitative results of 6 hearts in each group, mean  $\pm$  SEM. \* $p$ <0.05 compared to the nonischemic control group (group 1), # $p$ <0.05 compared to the ischemic/reperfused nonfibrillated group (group 2). Ischemia (ISA), Reperfusion (RE), Ventricular fibrillation (VF), No ventricular fibrillation (NoVF).

Figure 3 depicts heme oxygenase activities in ischemic and reperfused fibrillated, ischemic and reperfused nonfibrillated, and electrically fibrillated myocardium. Thus, in hearts subjected to 30 min of ischemia followed by 120 min of reperfusion and reperfusion-induced VF was not developed, HO activity was increased from the nonischemic control value of  $385 \pm 20$  pmol bilirubin/mg/hour (Fig. 3, lane 1) to  $901 \pm 38$  pmol bilirubin/mg/hour (Fig. 3, lane 2). Reperfusion-induced VF resulted in a significant reduction ( $p$ <0.05) in HO activity from its nonischemic control value of  $385 \pm 20$  pmol bilirubin/mg/hour (Fig. 3, lane 1) to  $162 \pm 18$  pmol bilirubin/mg/hour (Fig. 3, lane 3). Similar results were obtained in hearts subjected to electrically-induced VF (Fig. 3, lane 4). Thus, in electrically-induced VF resulted in about 60% reduction in heme oxygenase activity (Fig. 3, lane 4) compared to the nonischemic control group (Fig. 3, lane 1).

The ischemia/reperfusion resulted in a significantly lower postischemic recovery

in CF, AF, and LVDP in those hearts developed VF in comparison with the ischemic/reperfused nonfibrillated hearts (Table 1). Thus, the results clearly show that episodes of VF significantly attenuated the postischemic recovery in ischemic and reperfused myocardium compared to the ischemic and reperfused nonfibrillated myocardium. Without the ischemic and reperfused protocol, in electrically fibrillated hearts, the postfibrillated recovery was significantly improved compared to the ischemic and reperfused fibrillated myocardium (Table 1) indicating that an ischemic episode superimposed with VF resulted in a weak postischemic recovery in comparison with the electrically-induced fibrillated group (Table 1).



**Figure 3.** The effect of reperfusion- and electrically-induced VF on heme oxygenase activity. Group 1: aerobically perfused nonischemic control. Group 2: Hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion, and VF was not developed during reperfusion. Group 3: Hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion, and VF was developed during the reperfusion period. Group 4: Hearts were electrically fibrillated (no global

ischemic/reperfusion protocol) for 10 min followed by 110 min of perfusion, and heme oxygenase activity was measured. Data show the quantitative results of 6 hearts in each group, mean  $\pm$  SEM. \* $p < 0.05$  compared to the nonischemic control group (group 1), # $p < 0.05$  compared to the ischemic/reperfused nonfibrillated group (group 2). Ischemia (ISA), Reperfusion (RE), Ventricular fibrillation (VF), No ventricular fibrillation (NoVF).

**Table 1.** Cardiac function in nonfibrillated ischemic/ reperfused, fibrillated ischemic reperfused, and electrically fibrillated myocardium.

Groups	Preischemic values ( prefibrillated )				After 60 min RE				After 120 min RE			
	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP
Time- matched control perfusion	309±7	27.0±1.1	52.0±1.5	17.4±0.4	305±8	5.0±1.3	50.2±2.0	17.0±0.3	302±9	25.5±1.0	48.1±1.5	16.1±0.4
ISA/RE nonfibrillated	316±8	26.8±0.8	51.3±2.0	18.0±0.5	291±9	20.2±1.0	22.3±1.1	14.9±0.4	300±6	19.8±1.1	19.6±2.0	14.4±0.5
ISA/RE fibrillated	312±9	26.3±0.9	50.9±1.4	17.6±0.3	297±7	17.5±0.7*	11.4±0.6*	10.5±0.4*	294±8	16.9±0.8*	10.2±0.7*	9.8±0.6*
Electrically fibrillated	308±9	28.1±1.2	49.8±2.1	17.5±0.4	300±8	19.8±1.1 <sup>#</sup>	23.1±1.0 <sup>#</sup>	15.2±0.3 <sup>#</sup>	296±8	20.7±1.3 <sup>#</sup>	21.8±2.2 <sup>#</sup>	15.0±0.4 <sup>#</sup>

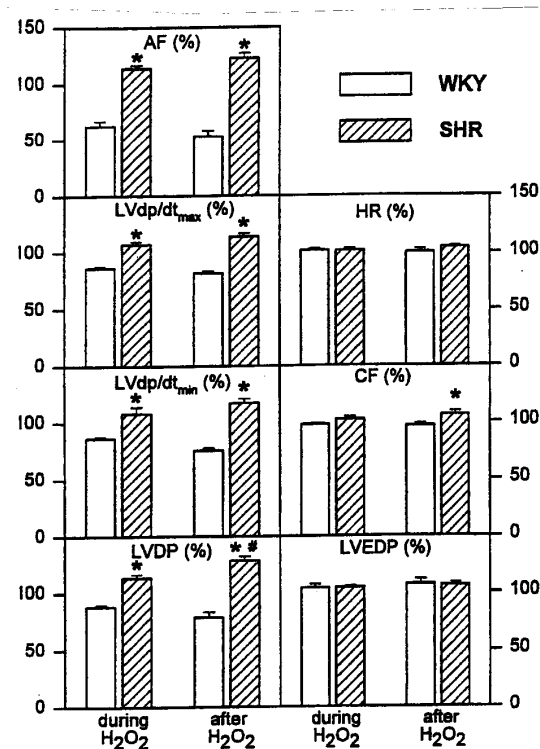
n=6 in each group, mean ± SEM, \*p<0.05 compared to the ISA/RE nonfibrillated group, <sup>#</sup>p<0.05 compared to the ISA/RE fibrillated group. HR: heart rate (beats/min), CF: coronary flow (ml/min), AF: aortic flow (ml/min), LVDP: left ventricular developed pressure, ISA: ischemia, RE: reperfusion.

## RESULTS II.

### *The effect of H<sub>2</sub>O<sub>2</sub> on the function of hearts isolated from WKY and SHR*

In untreated control conditions, SHR showed lower heart function compared to WKY: AF,  $18.2 \pm 1.9$  vs.  $26.2 \pm 2.2$  ml/min; +LVdp/dt<sub>max</sub>,  $375 \pm 15$  vs.  $602 \pm 24$  kPa/s; -LVdp/dt<sub>max</sub>,  $157 \pm 12$  vs.  $267 \pm 22$  kPa/s; LVDP,  $7.4 \pm 0.6$  vs.  $11.8 \pm 0.6$  kPa; LVEDP,  $7.4 \pm 0.5$  vs.  $6.4 \pm 0.4$  kPa ( $p < 0.05$  each). After 30 min of H<sub>2</sub>O<sub>2</sub> perfusion, a significant reduction in cardiac function of the WKY group was observed: AF, +LVdp/dt<sub>max</sub>, -LVdp/dt<sub>max</sub>, and LVDP were reduced to  $16.0 \pm 2.8$  ml/min,  $521 \pm 22$  kPa/s,  $2011 \pm 21$  kPa/s, and  $10.4 \pm 0.6$  kPa respectively ( $p < 0.05$  each). No significant reduction, rather a slight increase, in cardiac function was, however, registered in the SHR group during H<sub>2</sub>O<sub>2</sub> perfusion: AF,  $20.7 \pm 2.2$  ml/min; +LVdp/dt<sub>max</sub>,  $399 \pm 14$  kPa/s; -LVdp/dt<sub>max</sub>,  $169 \pm 15$  kPa/s; LVDP,  $8.4 \pm 0.7$  kPa; LVEDP,  $7.7 \pm 0.5$  kPa. After the 30 min washout period, heart function remained unchanged in both groups. No other significant differences (except +LVdp/dt<sub>max</sub>, LVDP) were found between the groups during and after the H<sub>2</sub>O<sub>2</sub> perfusion. HR and CF did not show any significant differences between both groups throughout the experiment (Fig 4).

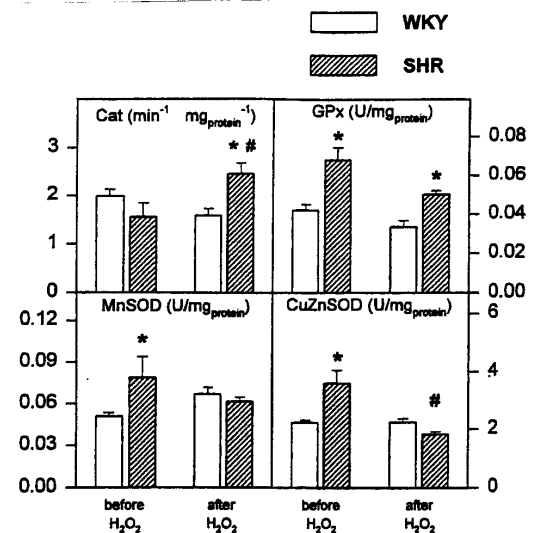
Figure 4 shows the relative changes of cardiac function (AF, +LVdp/dt<sub>max</sub>, -LVdp/dt<sub>max</sub>, LVDP, CF, and LVEDP) expressed in %, compared to the pre-H<sub>2</sub>O<sub>2</sub>-perfused values (taking the baseline values 100%).



**Figure 4.** Function of isolated perfused hearts from 18-month-old SHR (spontaneously hypertensive rats) and WKY (Wistar-Kyoto rats), measured after 30 min perfusion with H<sub>2</sub>O<sub>2</sub>, and after the subsequent 30 min washout period. The data were expressed in %, taking the pre-H<sub>2</sub>O<sub>2</sub> perfused data as control and 100%. Mean  $\pm$  SEM, n=6 in each group; \*  $p < 0.05$ , significantly different compared to the corresponding age-matched WKY control group; #  $p < 0.05$ , significantly different compared to the respective group at the end of H<sub>2</sub>O<sub>2</sub> perfusion. AF, aortic flow; +LVdp/dt<sub>max</sub>, maximum of the first derivative of left ventricular (LV) pressure; -LVdp/dt<sub>max</sub>, minimum of the first derivative of LV pressure; LVDP, LV developed pressure; HR, heart rate; CF, coronary flow; LVEDP, LV end-diastolic pressure.

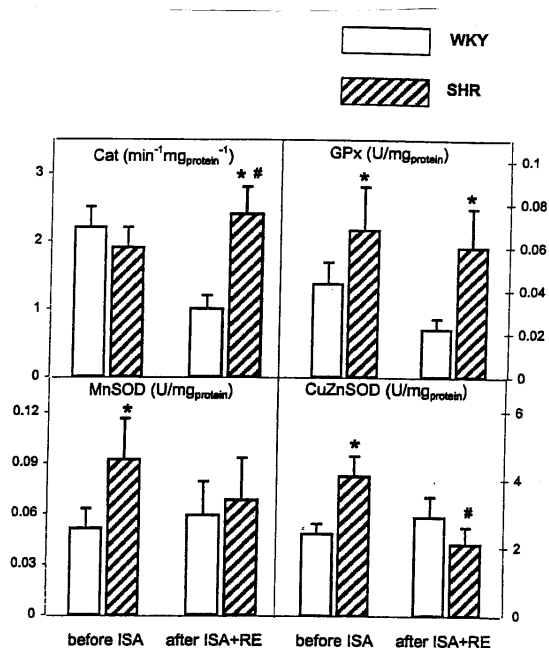
*Antioxidative defense enzymes in the myocardium of WKY and SHR - the effect of H<sub>2</sub>O<sub>2</sub>*

SHR hearts exhibited higher activities of antioxidative defense enzymes (GPx, SOD) under control conditions in comparison with WKY hearts. After treatment with H<sub>2</sub>O<sub>2</sub>, a significant increase in catalase activity was measured ( $2.46 \pm 0.22 \text{ k min}^{-1}\text{mg}^{-1} \text{ protein}$ ) in SHR hearts compared both to the corresponding (treated) WKY group ( $1.59 \pm 0.14 \text{ k min}^{-1}\text{mg}^{-1} \text{ protein}$ ) and to the SHR group before H<sub>2</sub>O<sub>2</sub> treatment ( $1.56 \pm 0.29 \text{ k min}^{-1}\text{mg}^{-1} \text{ protein}$ ). A significantly higher myocardial GPx activity was detected in SHR compared to WKY both before and after H<sub>2</sub>O<sub>2</sub> treatment. On the other hand, the higher activities of MnSOD and CuZnSOD in untreated SHR hearts compared to untreated WKY hearts were not seen after the infusion of H<sub>2</sub>O<sub>2</sub>. The CuZnSOD activity was actually reduced in the treated SHR group in comparison to the corresponding WKY group (Fig. 5).



**Figure 5.** Activities of catalase (Cat), glutathione peroxidase (GPx), Mn-, and Cu,Zn-superoxide dismutase (MnSOD, CuZnSOD) in the myocardium of 18-month-old SHR and WKY before 30 min H<sub>2</sub>O<sub>2</sub> infusion and after the subsequent 30 min washout period. Mean ± SEM, n=6 in each group; \* p<0.05, significantly different compared to the corresponding age-matched WKY control group; # p<0.05, significantly different compared to the respective group before H<sub>2</sub>O<sub>2</sub> infusion.

In hearts subjected to 30 min of ischemia followed by 30 min of reperfusion, the same trends in enzyme activities were observed (Fig. 6) in both WKY and SHR groups. Thus, the free radical-induced oxidative stress and its importance on enzyme activity are proved from a different angle in our studies.



**Figure 6.** Catalase (Cat), glutathione peroxidase (GPx), Mn- and Cu,Zn-superoxide dismutase (MnSOD, CuZnSOD) activities in 18-month-old SHR and WKY before the induction of ischemia (ISA), and after 30 min of ISA followed by 30 min of reperfusion (RE). Mean  $\pm$  SEM, n=6 in each group, \*p<0.05 compared to the corresponding age-matched WKY control group. #p<0.05 compared to the respective group before the induction of ISA.

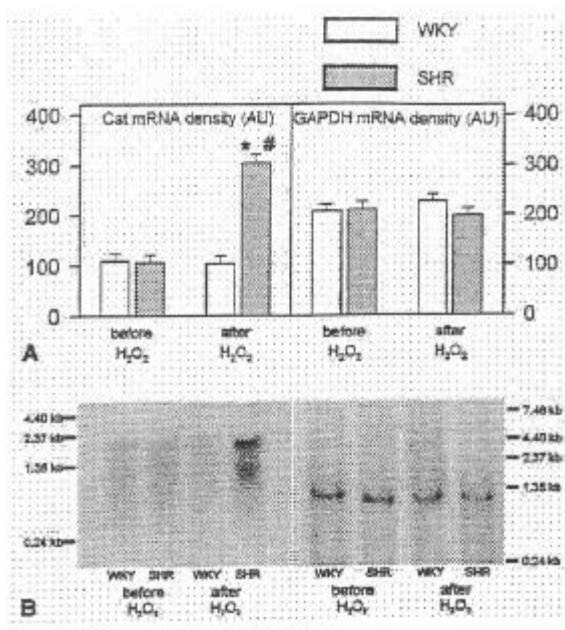
*The effect of H<sub>2</sub>O<sub>2</sub> on the expression of catalase in hearts isolated from WKY and SHR*

The levels of catalase mRNA significantly increased in the SHR group after the infusion of H<sub>2</sub>O<sub>2</sub> compared to the corresponding WKY group ( $303.3 \pm 17.9$  vs.  $105.9 \pm 15.5$  arbitrary units; Fig. 7A and 7B, left panels). This increase in mRNA reflected in the increase of enzyme activity (Fig. 5). The expression in GAPDH, as normalization control and house-keeping gene, showed no significant differences (Fig. 7A and 7B,

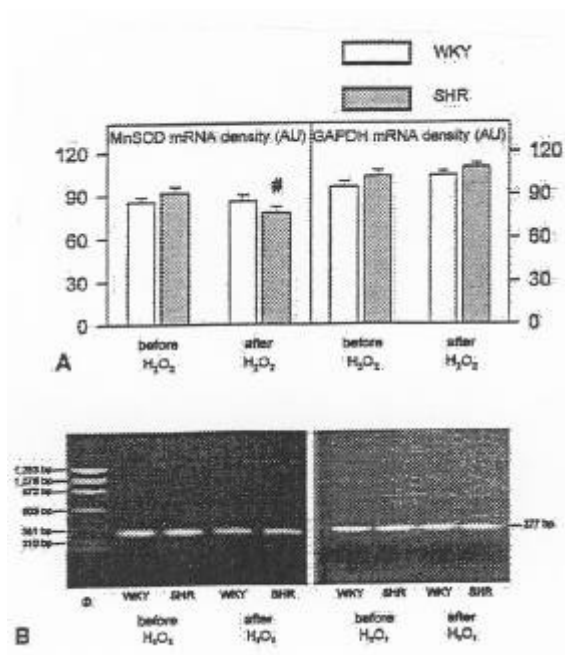
right panels) before and after H<sub>2</sub>O<sub>2</sub> perfusion.

*The effect of H<sub>2</sub>O<sub>2</sub> on the expression of MnSOD in hearts isolated from WKY and SHR*

The expression of MnSOD mRNA was slightly but statistically significantly decreased after H<sub>2</sub>O<sub>2</sub> perfusion in SHR hearts compared to the respective values before the H<sub>2</sub>O<sub>2</sub> infusion ( $92.2 \pm 3.6$  vs.  $77.9 \pm 3.9$  arbitrary units, Fig. 8A and 8B, left panels). In WKY hearts, the MnSOD mRNA concentration remained essentially unchanged ( $85.3 \pm 3.3$  vs.  $85.9 \pm 4.7$  arbitrary units; Fig. 8A and 8B, left panels), in agreement with the corresponding enzyme activities (Fig. 5). GAPDH did not show any significant changes after the infusion of H<sub>2</sub>O<sub>2</sub> in either SHR or WKY hearts (Fig. 8A and 8B, right panels).



**Figure 7.** (A) Dot blot quantification of catalase mRNA (Cat mRNA) in the myocardium of 18-month-old SHR and WKY before infusion with H<sub>2</sub>O<sub>2</sub> and after the subsequent 30 min washout period, compared to the mRNA levels of the house-keeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Mean  $\pm$  SEM, n=6 in each group; \* p<0.05, significantly different compared to the WKY group after H<sub>2</sub>O<sub>2</sub>; # p<0.05, significantly different compared to the SHR group before infusion. (B) Northern blot of Cat mRNA (left panel) and GAPDH mRNA (right panel) from the myocardium before the 30 min infusion period and after the subsequent 30 min washout period. Molecular size standards were run in parallel (indicated in kilobases); Cat mRNA is 1.2 kb, GAPDH mRNA is 2.3 kb.



**Figure 8.** (A) RT-PCR quantification of manganese superoxide dismutase (MnSOD) mRNA in the myocardium of 18-month-old SHR and WKY before 30 min H<sub>2</sub>O<sub>2</sub> infusion and after the subsequent 30 min washout period. Mean  $\pm$  SEM, n=6 in each group; # p<0.05, significantly different compared to the SHR group before the infusion. (B) RT-PCR analysis of the expression of MnSOD mRNA (left panel; 361 bp) and of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA (right panel; 377 bp) before infusion and after the subsequent 30 min washout period.  $\Phi$ =molecular size standard, in base pairs (bp).

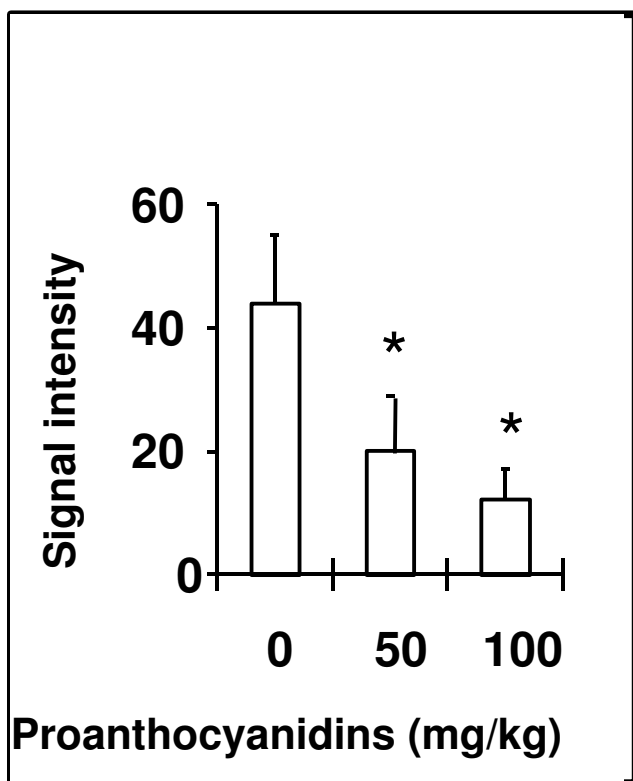
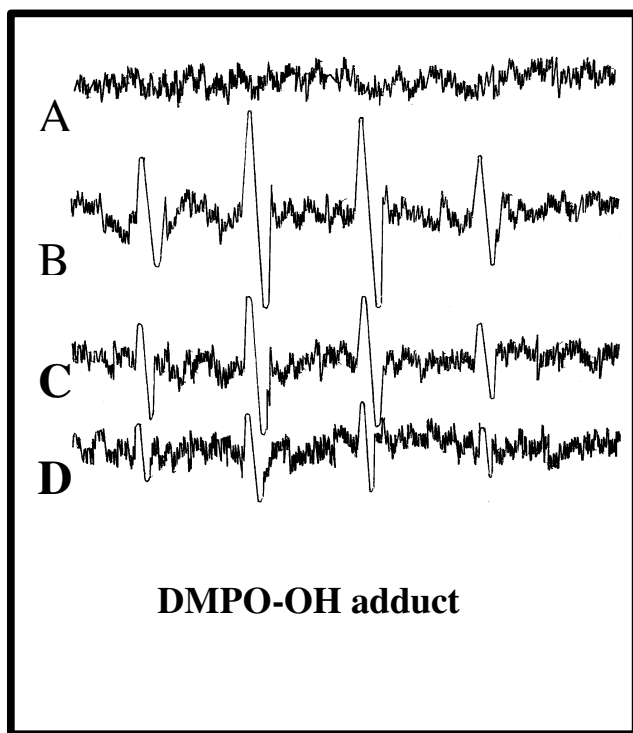
### RESULTS III.

On examination of the time course for the appearance of oxygen free radical production in isolated ischemic/reperfused hearts, we previously reported (59) that after 3 min of reperfusion a prominent ESR spectrum was observed consisting of 1:2:2:1 signal quartet (DMPO-OH adduct). Therefore, this time point (3<sup>rd</sup> min of reperfusion) was chosen to measure oxygen free radical formation in the proanthocyanidin treated groups. Rats treated with 50 and 100 mg/kg doses of proanthocyanidins (Fig. 9, upper panel) resulted in a stepwise reduction in oxygen free radical production, and in the 100 mg/kg proanthocyanidins treated group (Fig. 9, spectrum D), the production of oxygen free radicals, calculated from the ESR signal intensity of the DMPO-OH adduct, was reduced by approximately 75% in comparison with the drug-free ischemic/reperfused control group (Fig. 9, spectrum B).

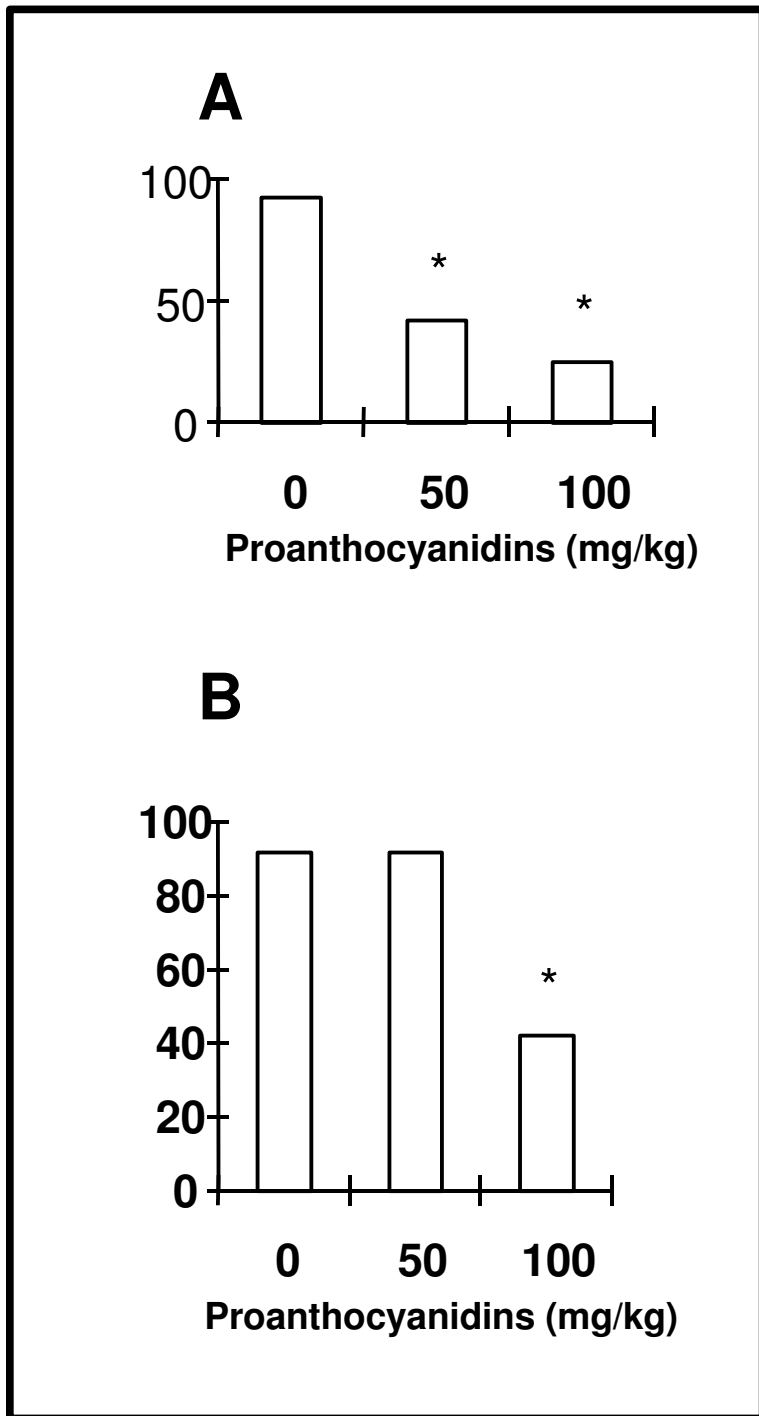
Figure 9 (lower panel) shows the quantitative ESR results of 6 hearts in each group. Thus, in both proanthocyanidins treated groups (50 and 100 mg/kg), a significant reduction in the intensity of DMPO-OH adduct was observed (Fig. 9, lower panel, and upper panel spectra C, D). This reduction in DMPO-OH signal intensity was reflected in a striking

reduction in the incidence of reperfusion-induced VF and VT (Fig. 10). Thus, in rats treated with 50 and 100 mg/kg of proanthocyanidins, the incidence of reperfusion-induced VF was reduced (Fig. 10A) from its control value of 92% to 42% ( $p<0.05$ ) and 25% ( $p<0.05$ ), respectively. However, the incidence of reperfusion-induced VT was reduced by 68% ( $p<0.05$ ) in the myocardium treated with the higher dose (100 mg/kg) of proanthocyanidins (Fig. 10B). It is reasonable to suppose that the reduction in the incidence of reperfusion-induced VF and VT resulted in a significant recovery in postischemic cardiac function (Fig. 11) including CF, AF, and LVDP in the proanthocyanidins treated groups in comparison with the untreated drug-free controls. The results show (Fig. 11) that proanthocyanidins did not significantly change heart rate (Fig. 11A) in comparison with the drug-free control values during reperfusion. However, in hearts obtained from proanthocyanidins treated rats, a significant recovery was observed in CF (Fig. 11B), AF (Fig. 11C), and LVDP (Fig. 11D) compared to the corresponding values of the untreated group. It is of interest to note that the treatment of rats with 100 mg/kg proanthocyanidins resulted in pronounced recovery in cardiac function compared to those of 50 mg/kg proanthocyanidins values.

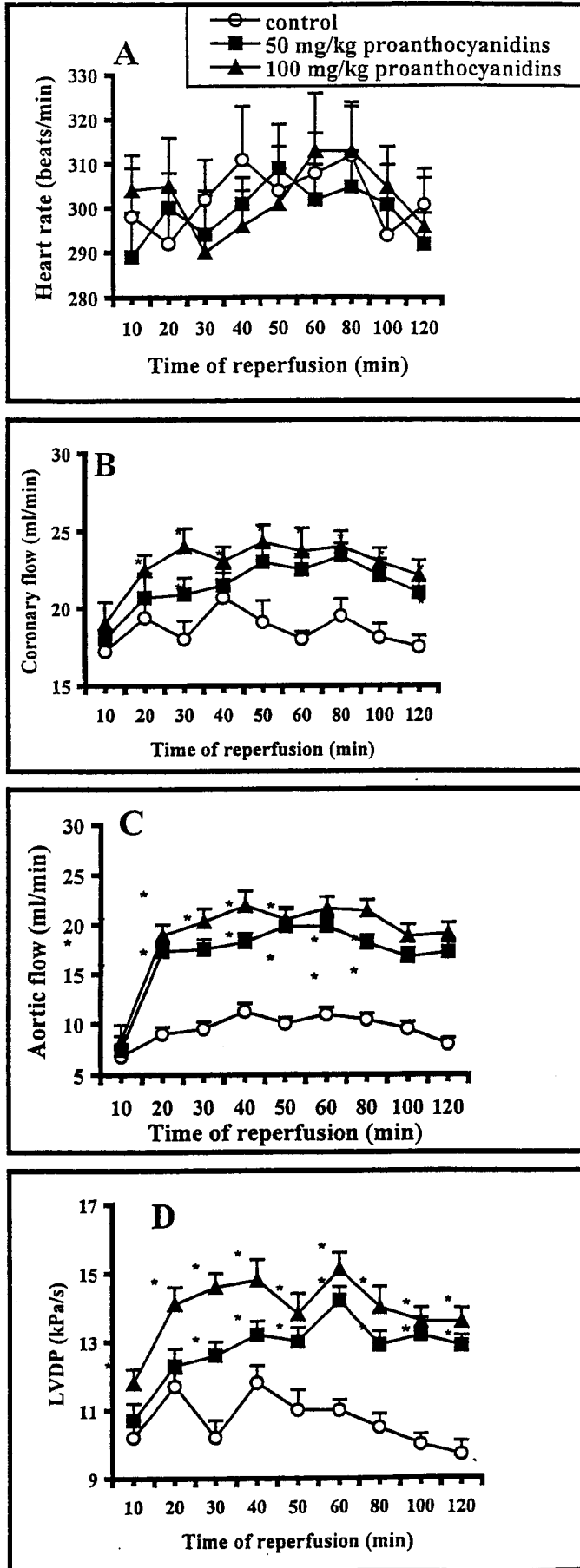
There was no significant difference, after 3-week period, in body weight and food intake between the drug-free control (bw:  $332 \pm 5$  g, food intake at the last day of treatment:  $21.1 \pm 0.9$  g/day), and 50 mg/kg (bw:  $328 \pm 6$  g, food intake:  $19,8 \pm 1.0$  g/day), and 100 mg/kg (bw:  $331 \pm 4$  g, food intake:  $22.1 \pm 1.8$  g/day) grape seed proanthocyanidins treated groups.



**Figure 9.** Representative ESR spectra (upper panel) for the demonstration of  $\cdot\text{OH}$  radical formation in untreated and proanthocyanidins treated ischemic/reperfused hearts. DMPO was infused during the first 3 min of reperfusion then effluent samples were frozen or immediately used for ESR studies. ESR spectra were recorded in cells operating at 9.3 MHz with a 100 kHz modulation frequency. Scans were traced with 0.2 mT of modulation amplitude with 2 min of scan time and with 300 ms of response time. A: typical spectrum recorded from buffer after passage through nonischemic heart; B: Coronary effluent collected after 30 min ischemia followed by 3 min reperfusion in the drug-free control heart; C and D: Coronary effluents were collected after 30 min ischemia followed by 3 min reperfusion in isolated hearts obtained from rats treated with 50 and 100 mg/kg of proanthocyanidins, respectively. The lower panel shows the effect of proanthocyanidins on the signal intensity, expressed in arbitrary units, of DMPO-OH adduct at the 3<sup>rd</sup> min of reperfusion. DMPO was infused at the onset of reperfusion and ESR measurement was done. \* $p < 0.05$ ,  $n = 6$  in each group, comparisons were made to the drug-free control group (0 mg/kg proanthocyanidins).



**Figure 10.** Dose-response studies for ability of proanthocyanidins to reduce reperfusion-induced arrhythmias. Rats (n=12 in each group) were treated with a daily dose of 0 (vehicle, control), 50 or 100 mg/kg proanthocyanidins for 3 weeks, then hearts were isolated and subjected to 30 min of global ischemia followed by 2 hours of reperfusion. The incidence of ventricular fibrillation (A) and ventricular tachycardia (B) was measured and expressed in %. \*p<0.05, comparisons were made to the drug-free control group.



**Figure 11.** Cardiac function in untreated and proanthocyanidins treated hearts subjected to 30 min ischemia followed by 120 min reperfusion. Panels A, B, C, and D show the postischemic recovery of heart rate (A), coronary flow (B), aortic flow (C), and LVDP (D), respectively. N = 12 in each group, mean  $\pm$  SEM, \* $p < 0.05$ .

## 4. DISCUSSION

## DISCUSSION I .

Whereas disturbances of ionic homeostasis, particularly that of sodium, potassium, and calcium, are undoubtedly responsible for the aspects of ventricular fibrillation, there is still considerable doubt as to the primary mechanism responsible for membrane injury which allows ionic imbalance to develop. It is important to note and ascertain that changes in protein activities, arising as a consequence of alterations in the redox state of controlling groups (e.g., disulfide, hydroxyl, or thiol bonds), are more likely to bring about major and sudden changes in the permeability of cell membranes to a variety of ions through the activation or inactivation of carrier proteins regulated by encoded genes and ion channels.

Under physiological conditions HO-1 is present at low levels in all organs, and recently, the induction of HO-1 mRNA has been implicated in the ischemia and reperfusion injury suggesting that this enzyme is induced by a host of stimuli that have in common the ability to produce oxidative stress. HO-1 expression is rapidly accelerated not only in response to pathophysiological conditions such as those of ischemia/reperfusion and cellular transformation (60-61), but also in response to different exogenous molecules (7). We have recently suggested (16) that

heme oxygenase mRNA expression, in particular the heme oxygenase-1, may play an important role in the control of reperfusion-induced ventricular fibrillation. Heme oxygenase enzymes catalyze the rate-limiting step in heme catabolism, the oxidative cleavage of b-type heme to yield equimolar quantities of iron, CO, and biliverdin (62). In addition, the realization that most HO-1 inducers stimulate the production of oxygen radicals or depletion of glutathione, and the fact that heme is a potent pro-oxidant, has led us to speculate that HO-1 activity is a component of the cellular defense mechanism against oxidant stress including reperfusion-induced ventricular fibrillation. The importance of HO-1 activity has been verified and emphasized by numerous studies under *in vivo* and *in vitro* models of oxidative injury (63). While the induction of HO-1 has been recently extensively studied at the level of gene transcription, the physiological function is poorly understood in the mechanism and controlling of reperfusion-induced arrhythmias including VF. In the present study, we have endeavored to obtain more circumstantial evidence for the involvement of HO-1 in the genesis of reperfusion-induced ventricular fibrillation.

If HO-1 is involved in cellular injury leading to electrophysiological abnormal-

lities and VF it is important to question where and how HO-1 gene is mediated, and where it exerts its action. Some indication may perhaps be obtained from our previous (16) and present studies. In support of an association between HO-1 mRNA expression or downregulation and different transcription factors, Alam et al (62) have provided substantial evidence that overexpression of Nrf2M (nuclear factor-erythroid related factor) inhibits HO-1 mRNA accumulation in response to heme, zinc, cadmium, and arsenit. A possible explanation for the situation may be derived from these results that Nrf2M-containing dimeric factor is responsible for induction by heme, zinc, cadmium, and arsenit (62). Another explanation for the inhibition of HO-1 gene induction by the aforementioned agents is that Nrf2M binds to the stress response element interfering with the binding of the actual positive activators. These findings strongly suggest that Nrf2 could be a positive regulator of HO-1 gene induction (62).

The importance and role of increased HO-1 expression under ischemic conditions is not clearly understood. The distinct transcription pathway for HO-1 mRNA activation by ischemia/reperfusion and various pro-oxidants suggests that this significance may be related, at least in part, to the antioxidant activity of HO-1 (e.g. bilirubin production) and the ability

of this enzyme to generate CO. Studies show that under physiological conditions maintenance of physiologic vascular tone and blood flow can be attributed in part to enzymatically derived CO (64). Thus, changes in coronary and vascular tone under ischemic condition may, in part, be a consequence of HO-1 induction and localized production of CO (65). Consistent with this role of HO-1, ischemic tissue exhibits elevated formation of CO, derived from induced HO-1 activity, and increased levels of cGMP leading (52) to the antiarrhythmic action in the myocardium.

Although electrical fibrillation-induced coronary vasoconstriction is unlikely to account for the initiation of HO-1 regulation, there seems little doubt that, after 10 min of electrical fibrillation, this factor can modify the postfibrillated recovery of hearts or support their maintenance compared to the recovery of ischemic and reperfused fibrillated or nonfibrillated myocardium. Thus, in comparison to hearts that were subjected to ischemia/reperfusion protocol and fibrillated or not, coronary flow failed to recover to its preischemic control value, while in electrically fibrillated hearts, coronary flow recovered to 80% of the prefibrillated value. Aortic flow and left ventricular developed pressure showed the same postfibrillated recovery in

electrically fibrillated myocardium. As speculated above, the mechanism underlying the role of HO-1 mRNA expression in arrhythmogenesis involves the encoded proteins which may be responsible for the regulation of ion control mechanisms. Our studies might indicate that the heart appears to be able to recover in terms of cardiac function, and heme oxygenase expression and activity could be originated from ventricular fibrillation. 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 reversibly damaged cardiac cells become functionally close to the normal ones but continue to deteriorate in terms of HO-1 mRNA expression and enzyme activity. Such a proposition gains some support from our observations of HO-1 mRNA expression and enzyme activity in isolated ischemic/reperfused fibrillated and nonfibrillated, and electrically fibrillated hearts indicating that ischemia/reperfusion causes additional injury which is superimposed by VF in ischemic-reperfused myocardium.

In conclusion, our results provide evidence that HO-1 mRNA expression and enzyme activity could be inhibited by

ventricular fibrillation in electrically fibrillated myocardium as well as in ischemic/reperfused hearts. Furthermore, our studies clearly show that HO-1 mRNA expression and enzyme activity were increased in ischemic/reperfused nonfibrillated myocardium suggesting that interventions which are able to increase HO-1 mRNA expression and enzyme activity may prevent the development of reperfusion-induced VF. The VF-induced injury can develop with great rapidity, and the mechanism(s) by which ventricular fibrillation exerts its damaging effects remains to be resolved, but damages in mRNA expression, enzymes and proteins structures, and other macromolecules may be involved.

## DISCUSSION II.

Reactive oxygen species are usually derived from an abnormally interrupted oxygen metabolism and play an important role in the oxidative stress of cells and tissues. Antioxidant enzymes are a primary defense, that help prevent oxidative damage to biological molecules. SOD rapidly converts  $O_2^{\cdot-}$  to the less reactive  $H_2O_2$ , which is further degraded by catalase and GPx. The administration of antioxidant enzymes may thus protect the heart against free radical mediated damage (66-67). Exposure to oxidative stress may exhaust the antioxidative capacity of the heart. The activity of antioxidant enzymes may be decreased, especially when the oxidative load overcomes the defense potential (68). On the other hand, moderate episode(s) of radical stress may potentiate the defense system by stimulating the expression of the respective enzymes (69).

In the present study, the influence of oxidative stress caused by  $H_2O_2$  on the expression of antioxidant defense enzymes in the decompensated hearts (24) of 18 month-old SHR has been investigated. SHR exhibited a higher antioxidative potential than WKY, having elevated activities of GPx and SOD. The function of the SHR hearts was not affected by  $H_2O_2$ , probably due the elevated activity of

GPx. Thus, the capacity of these hearts to metabolize  $H_2O_2$  by GPx is higher as compared to the WKY hearts undergoing a reduction in cardiac function (aortic flow, contractility, relaxation, developed pressure) without hypertrophy. The expression of GPx has been reported to be induced after the exposure to excessive levels of  $H_2O_2$  (70) which can be produced either by SOD activity or by oxidative stress of different origin (71). The activities of both Mn- and CuZnSOD were increased in the SHR hearts only. However,  $H_2O_2$  production by these SOD's did not overcome the capacity of the endogenous catalase or GPx, i.e. the  $H_2O_2$  infusion, at the dose used, did not cause functional deterioration in SHR hearts.

It is of interest to note, although it was not the main subject of the present investigation, that we found the same changes in catalase, GPx, MnSOD, and CuZnSOD enzyme activities in hearts subjected to ischemia and reperfusion. This could suggest that oxidative stress is independent of the way it was induced in both SHR and WKY. Our finding indicates the different response of the SHR and WKY myocardium to oxidative stress, but it does not mean that elevated SOD levels in SHR hearts cause a pronounced accumulation of  $H_2O_2$  because the rate of superoxide production

determines the rate of  $H_2O_2$  level via dismutation.

During and after the infusion of  $H_2O_2$ , the activity of GPx remained higher in SHR hearts in comparison with WKY showing a higher antioxidative defense mechanism. In contrast, total SOD activity decreased in the SHR group after the infusion, mainly due to diminished CuZnSOD activity. CuZnSOD is inactivated by  $H_2O_2$  (72) and hence is considered to be exhausted under our experimental conditions; the MnSOD mRNA level decreased slightly, reflecting the slightly reduced expression of this enzyme. Additional to the enhanced GPx activity, the induction of catalase is remarkable in the SHR hearts. Both catalase activity and mRNA expression increased profoundly after the infusion of  $H_2O_2$ . This effect has also been observed in other tissues and cells (73). Furthermore, other studies demonstrated that  $H_2O_2$  is able to activate the transcription factor NF $\kappa$ B which may contribute to the induction of myocardial enzymes (74).

Elucidation of the exact mechanism whereby SHR hearts adapt to oxidative stress warrants further study. Treatment of SHR and WKY hearts with a low concentration of  $H_2O_2$  (25  $\mu$ M) or hearts subjected to ischemia/reperfusion led to oxidative stress in our experimental

conditions. In other studies, similar amounts of  $H_2O_2$  induced oxidative stress in different tissues (75). Systemic arterial hypertension is a recognized risk factor for the development of ischemic heart disease. The hearts of old SHR show typical signs of dilated cardiomyopathy, including hypertrophy (76) and dilation of the left ventricle (22, 24). Thus, SHR at the age of 18 months or older are a suitable animal model to study certain aspects of the pathomechanisms of congestive heart failure in humans (22). It has been established that hypertension is responsible for an enhanced oxidative stress (77, 78). Our results show that this elevated oxidative stress is followed by an increase in the antioxidative activity in SHR hearts.

It was found that hearts from SHR under hypertonic perfusion pressure were more sensitive to ischemia/reperfusion and generated more reactive species during the reperfusion than did those from WKY with normotonic perfusion (22). This implies that the radical defense induced in the failing hearts can compensate only mild oxidative stress. Moreover, despite of differences in the experimental set-up, we have to consider that ischemia and reperfusion includes not only reactive oxygen species, as applied in our model, but also other pathogenic factors, such as disturbances

in calcium homeostasis etc. On the other hand, our findings are consistent with other's (79-80) who found that preconditioning of hypertrophied SHR hearts improved the postischemic recovery of high-energy phosphates and function in the myocardium. Therefore, preconditioning induced by reactive oxygen species known to be released during oxidative stress could also involved to enhance postischemic contractile recovery in the hypertrophied hearts.

It is generally accepted that hypertension, due to atherosclerosis, arterial thrombosis and defective endothelium-dependent vascular relaxation (78), carries a substantially increased risk of morbidity and mortality from all cardiovascular disorders including ischemic heart disease (81). Our data indicate that oxidative stress appears to be a minor but important component in the multifactorial genesis of cardiac hypertrophy and that induction of the radical defense is of a certain impact in protecting the failing myocardium.

### DISCUSSION III.

Ischemic heart disease continues to be the major cause of cardiac death, and ventricular fibrillation is responsible for the development of sudden cardiac death. Considerable evidence suggests that oxygen-derived free radicals are involved in ischemia/reperfusion-induced cardiac injury. Increased postischemic susceptibility to oxygen radical damage results from the build-up of a strongly reducing environment during ischemia along with a decreased antioxidant defense capacity. The mechanism of cellular damage involves the stepwise reduction of molecular oxygen to the relative inactive superoxide and hydrogen peroxide, with subsequent metal ion-catalyzed formation of the highly reactive hydroxyl radical, the ultimate tissue toxicant (82). Recently many studies have been directed at elucidating the role of free radicals in the pathophysiology of injury during myocardial ischemia and reperfusion. Indirect evidence for an involvement of radicals in ischemia- and reperfusion-induced injury derives from studies in which antioxidant enzymes, organic antioxidants, or agents that inhibit the production of oxygen free radicals have been suggested to (i) reduce vulnerability of the myocardium to reperfusion-induced arrhythmias, (ii) limit infarct size, (iii)

attenuate myocardial stunning, and (iv) enhance postischemic recovery in models of surgical global ischemia (83). Therapeutic strategies have attempted to reduce free radical-induced damage either by intervening in their formation process or by scavenging the free radicals that are already formed. Different degrees of protection have been obtained with (i) hydroxyl radical scavengers (66, 84), (ii) chelators of iron and copper (85-86), (iii) antioxidant enzymes such as superoxide dismutase and catalase (66), and (iv) redox-metal displacement by zinc (87). The clinical application of these compounds is limited, however, by their toxicity and side effects, their solubility, persistence and membrane penetration, the requirement for pre-ischemic loading of the tissue, and the incomplete protection they provide even in the best cases. Because epidemiological evidence indicates that the consumption of red wine is beneficial in the prevention of coronary artery diseases (88), and this beneficial effect could be attributed to antioxidants present in the polyphenol fraction of red wine (44), we investigated the effect of grape seed proanthocyanidins on the hydroxyl radical formation measured by ESR, incidence of arrhythmias, and cardiac function in ischemic/reperfused myocardium. In the present report, we

demonstrated that proanthocyanidin-fed rat myocardium was more resistant to ischemia and reperfusion-induced injury in comparison with the drug-free controls. The proanthocyanidin-fed group consistently showed a better postischemic ventricular recovery and a reduced incidence of reperfusion-induced VF and VT compared to the drug-free control group. Our study demonstrates that the development of reperfusion-induced arrhythmias and cardiac cell damage, at least in part, could contribute to the reactive hydroxyl radical formation in the reperfused isolated myocardium. One of the major functions of antioxidants is to interfere with free radical formation. It is well known that antioxidant reserve and antioxidant enzyme capacity are significantly reduced after ischemia and reperfusion. The loss of the key antioxidant enzymes and antioxidants reduces the overall antioxidant reserve of the myocardium, and makes the heart susceptible to ischemia and reperfusion-induced damage. The reduced antioxidative defense is likely to be incapable of providing a complete protection against increased activities of the reactive oxygen species.

Although the results of our study attribute the protective effects of proanthocyanidins to their ability to eliminate hydroxyl radicals, other

possibilities should not be ignored. Proanthocyanidins comprise a group of polyphenolic bioflavonoids ubiquitously found in fruits and vegetables. In this connection, the effect of proanthocyanidins on intracellular calcium levels and the possible biological effects of other components of the extract merit discussion. Flavonoids may interact with intracellular calcium ions leading to a reduction in the ionized calcium content, and increase the binding affinity of a substrate or to improve the electron transfer efficacy between NADPH-cytochrome P-450 reductase and the P-450 enzyme (89) providing a further protection against reperfusion-induced calcium overload. Thus, we believe that any simple effect on intracellular calcium is likely to have also an important relevance to the protection observed in our model system. The observation that proanthocyanidins are active as a 3-week pretreatment is of particular interest. It is of interest to note that antioxidants, e.g., through glutathione peroxidase system, could reduce hydroxyl radicals to H<sub>2</sub>O. Thus, proanthocyanidins may not bind to the myocardium and remain active for several days or weeks and act as a “sink” for hydroxyl radicals. Furthermore, proanthocyanidins may act as a regenerator of other antioxidants keeping the levels of other antioxidants high enough to

affect the formation of hydroxyl radicals. Thus, it has been shown by Cestaro et al (90) that red wine increases ascorbic acid and  $\alpha$ -tocopherol levels, two major antioxidants, in the body. In addition, Roig et al., (91) have shown that red wine consumption by rats resulted in enhanced catalase glutathione peroxidase activities, and reduced/oxidized glutathione ratios.

The ability of proanthocyanidins to improve the functional recovery of the heart and reduce the incidence of arrhythmias after a period of global ischemia could find valuable applications during routine cardiac surgery, and it might also be of value in cardiac transplantation. This statement is supported by Feng et al., (36) indicating that long-term consumption of red and white wine decreased intimal thickening after balloon injury in cholesterol-fed rabbits, and ethanol content as well as the phenolic antioxidants in red wine might be responsible for these favorable effects. Because antioxidants have been shown to effectively inhibit neointimal thickening and macrophage accumulation in animals (92) and to reduce the restenosis after balloon angioplasty in humans (93), it would be worthwhile to determine whether red wine might alter restenosis after coronary angioplasty. The 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.

## 5. CONCLUSION

In summary, our data provide evidence that the development of reperfusion-induced VF inhibits HO-1 mRNA expression and enzyme activity in both electrically fibrillated myocardium and ischemic and reperfused fibrillated hearts. The results clearly show that HO-1 mRNA expression and enzyme activity were increased in ischemic and reperfused nonfibrillated myocardium suggesting that interventions which are able to increase HO-1 mRNA expression and enzyme activity may prevent the development of VF.

Furthermore in the studies presented in this part of my thesis, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were characterized in isolated perfused hearts of 18-month-old SHR and the age-matched normotensive control Wistar-Kyoto rats (WKY), before and after 30 min infusion of 25  $\mu$ M H<sub>2</sub>O<sub>2</sub>. The results obtained in ischemic and reperfused hearts show the same changes in enzyme activities measured as it was observed in H<sub>2</sub>O<sub>2</sub> perfused hearts, indicating that oxidative stress is independent of the way it was induced. The higher catalase activity derived from elevated mRNA synthesis. The antioxidative system in dilative cardiomyopathic hearts of SHR is induced, probably due to episodes of oxidative stress, during the process of decompensation. This conditioning of the antioxidative potential

may help overcome acute stress situations caused by reactive oxygen species in the failing myocardium.

In the last part of my studies the effects of red grape seed proanthocyanidins were studied on the recovery of postischemic function in isolated rat hearts. In rats treated with 50 and 100 mg/kg of grape seed proanthocyanidins, the incidence of reperfusion-induced VF was reduced from its control value of 92% to 42% ( $p < 0.05$ ) and 25% ( $p < 0.05$ ), respectively. The incidence of ventricular tachycardia (VT) showed the same pattern. In rats treated with 100 mg/kg proanthocyanidins, after 60 min reperfusion, the recovery of coronary flow, aortic flow, and developed pressure was improved by 32%  $\pm$  8% ( $p < 0.05$ ), 98%  $\pm$  8% ( $p < 0.05$ ), 37%  $\pm$  3% ( $p < 0.05$ ), respectively, compared to the drug-free controls. ESR studies indicate that proanthocyanidins significantly inhibited the formation of oxygen free radicals. In rats treated with 100 mg/kg of proanthocyanidins, free radical intensity was reduced by 75%  $\pm$  7% ( $p < 0.05$ ) compared to the drug-free value. Grape seed proanthocyanidins possess cardioprotective effect against reperfusion-induced injury through their abilities to reduce or remove, directly or indirectly, free radicals in the ischemic/reperfused myocardium.

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## 8. ANNEX

### LIST OF PUBLICATIONS

#### Abstracts

1. T. Pataki, P. Kovács, P. Ferdinándy, I. E. Blasig.

The control of cardiac function and nitric oxide production by the extract of Ginkgo biloba in ischemic/reperfused rat hearts European society of Cardiology, Barcelona, Spain 1999.

2. T. Pataki, P. Kovács, Z. Szilvássy, I. E. Blasig, A Tosaki.

Gene expression related to fibrillation in ischemic-reperfused isolated rat hearts Fundamental and Clinical Pharmacol, 13: 224s 1999.

3. T. Pataki, P. Kovács, Z. Szilvássy, D. K. Das, A Tosaki.

Expression of cytochrome oxidase B subunit III. Related to ventricular fibrillation in ischaemic-reperfused hearts.

Journal of Physiology, London, p 526, 2000

## Publications

- 1.** Cs. Csonka, T. Pataki, P. Kovacs, Sebastian L. Müller, Matthias L. Schroeter, A. Tosaki and I. E. Blasig. Effect of Oxidative stress on the expression of antioxidative defense enzymes in spontaneously hypertensive rat hearts  
Free Radical Biology & Medicine, 29: 612-619, 2000. IF: 4,079
- 2.** T. Pataki, I. Bak, Cs. Csonka, P. Kovacs, E. Varga, Ingolf E. Blasig, A. Tosaki. Regulation of ventricular fibrillation by heme oxygenase in ischemic/reperfused hearts. Antioxidants and Redox Signaling 3:125-134, 2001. This is a new journal, the IF is due in the year of 2002.
- 3.** T. Pataki, I. Bak, P. Kovacs, D. Bagchi, D.K. Das, A Tosaki. Grape seed proanthocyanidins reduced ischemia/reperfusion-induced injury in isolated rat hearts. Am J Clin Nutrition, 2001 in press, IF: 3,958