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

THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY

TÜNDE PATAKI

Supervisor: Prof. Dr. Árpád Tósaki

PROGRAM DIRECTOR: PROF. DR. LAJOS GERGELY

University of Debrecen
Medical and Health Science Center
Department of Pharmacology and Pharmacodynamics

Debrecen
2001

INDEX

1. INTRODUCTION 1

2. METHODS 2

3. RESULTS 6

4. DISCUSSION 19

5. CONCLUSION 22

6. REFERENCES 23

7. ANNEX 27

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.

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

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_2O_2 and assessed the expression and activities of SOD, catalase and glutathione peroxidase (GPx). 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.

An inverse relation between moderate alcohol consumption and the risk of cardiac disorders has been emphasized in several studies (17-19). Flavonoids, which increase the antioxidant capacity of cells and tissues (20-22), 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 (23). Thus, the beneficial effect of red wine has been attributed to the antioxidants present in the polyphenol fraction of red wine (24) including resveratrol, catechin, and proanthocyanidins.

2. METHODS

Animals:

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

Isolated working heart preparation:

Rats were anesthetized and 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. The Langendorff preparation was switched to the working mode as previously described by Tosaki and Braquet (25). 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 hemeoxigenase expression:

The ECGs were analyzed to determine the incidence of VF. 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 ($LVdp/dt_{max}$) were also recorded. 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. Then hearts were defibrillated, switched to working mode, and perfused. 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, a guanidium thiocianate method (26).

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 $MgCl_2$, 10 DTT, 1 dNTP's (each), 0.005 random hexamer, 0.5 IU/µl Mo-Mu-LV reverse transcriptase. 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 (27): 5'-CTT CAT ACT CAG GTC CAA GGC-3'; GAPDH sense: 5'-TCC TGC ACC ACC AAC TGC TTA GCC-3'; GAPDH antisense (28): 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 $MgCl_2$, 100 µM dNTP's (each), 100 µM primers and 0.025 U/µl Taq polimerase. 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 according to Sambrook et al (29).

HO activity assay:

One hundred mg of tissue were homogenized in 10 ml of 200 mM phosphate buffer and centrifuged at 19

000*g* at 4 °C for 10 min. The supernatant was removed and re-centrifuged at 100 000*g* 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 (30). HO activity was assayed as described by Yoshida et al (31). Protein content was determined according to Lowry et al (32) 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 μM of H_2O_2, followed by a 30 min washout period. In additional studies, after 15 min equilibration period, hearts were subjected to 30 min global ischemia followed by 30 min reperfusion. Control hearts were subjected to 60 min perfusion without H_2O_2.

Measurement of cardiac function in H_2O_2–free and H_2O_2–treated hearts:

Heart function was recorded after 15 min equilibration period, immediately after H_2O_2 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 ($+LVdp/dt_{max}$, $-LVdp/dt_{max}$) were also recorded.

Polymerase chain reaction for MnSOD and ZnSOD:

For RT-PCR (reverse transcriptase-polymerase chain reaction), total RNA (2 μg) was subjected to random primed first-strand cDNA synthesis in 40 μl reactions composed of (in mM) 50 Tris-HCl, 75 KCl, 3 $MgCl_2$, 10 DTT, 1 dNTPs (each), 50 ng random hexamer, 0.5 IU/μl Mo-Mu-LV reverse transcriptase. 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’ (33); GAPDH sense, 5’-TCC TGC ACC ACC AAC TGC TTA GCC-3’; GAPDH antisense, 5’-TAG CCC AGG ATG CCC TTT AGT

GGG-3' (28). Amplification products were visualized on 2% agarose gels containing ethidium bromide.

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 according to Sambrook et al (29) and U.V. cross linked. The rat catalase cDNA containing plasmid, pRCA38 (34) was from Dr. S. Furuta, Nagano/Japan and the purified GAPDH probe from Dr. E. Zador, Szeged/Hungary.

Catalase, MnSOD, and ZnSOD enzyme activities:

Catalase activity (E.C. 1.11.1.6) was determined and expressed as k in $mg^{-1}min^{-1}$ protein (first-order rate constant of the reaction). 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 measured by a protein assay kit.

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 (35). A commercially available IH636 grape seed proanthocyanidin-extract (ActiVin, InterHealth Nutraceuticals, Benecia, CA, USA) has been used in our study. 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 (25). 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.

The second objective of our work was to study whether proanthocyanidins could attenuate the formation of oxygen free radicals measured by ESR in ischemic and 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 3rd 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 (36).

Electron spin resonance (ESR) studies:

Spin trap studies were performed by infusing the spin trap 5,5-dimethyl-pyrroline-N-oxide (DMPO), through the side arm located just proximal to the end of the heart perfusion cannula. 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.

Statistics:

The data for HR, CF, AF, LVDP, mRNA expressions, enzymes activities, and signal intensity of DMPO-OH adduct were expressed as the mean ± 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 (37). 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 groups was considered to be significant.

3. RESULTS

RESULTS I.

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 and 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)

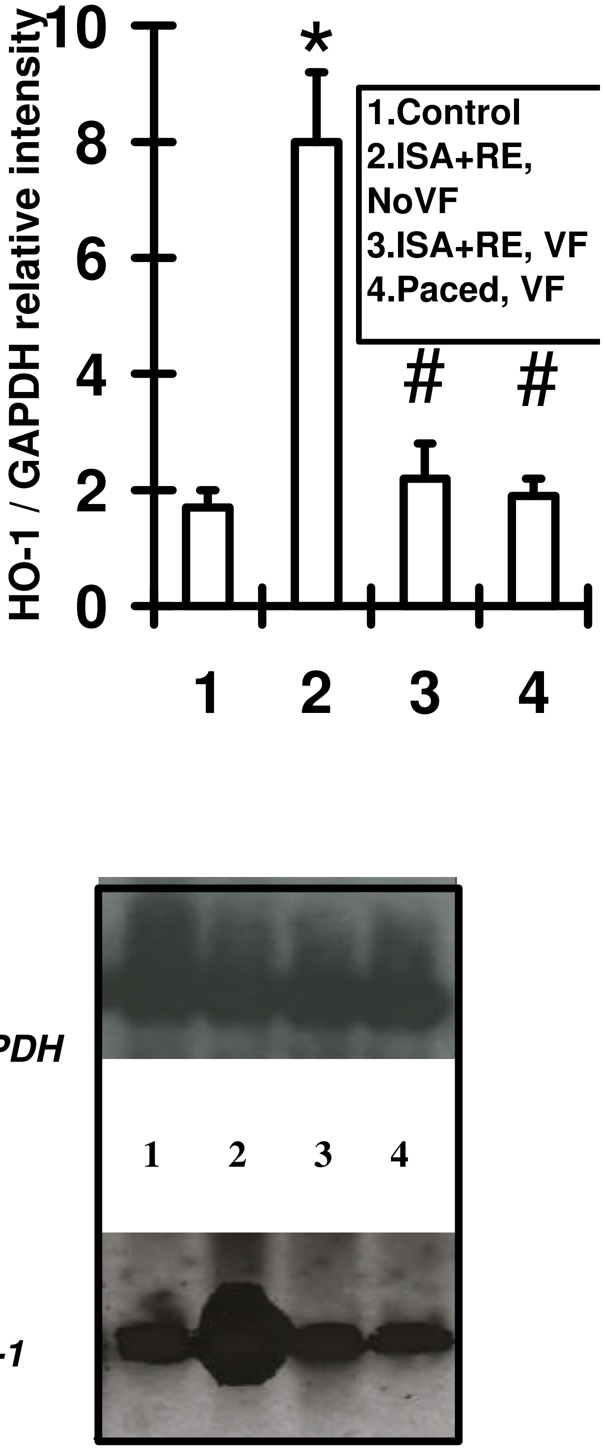

Fig 1 *HO-1 mRNA expression in nonfibrillated and fibrillated hearts. Northern blots (representative blots) were probed with ^{32}P-HO-1, and then the same blots were stripped and re-probed with ^{32}P-GAPDH cDNA. In nonischemic control myocardium (lane 1) the signal of HO-1 can be detected. Hearts subjected to 30 min of ischemia*

lane 4)

*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/eperfused fibrillated myocardium (Fig. 1, lane 3).

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 non-ischemic 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).

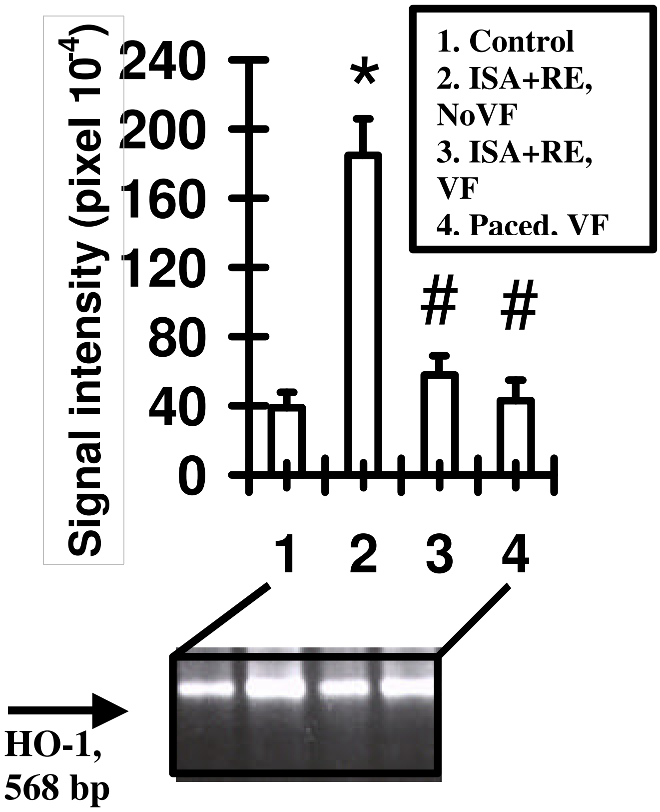

Figure 2. *RT-PCR amplification for HO-1 mRNA. Aliquots were taken after 28 cycles amplification of cDNA obtained by reverse transcription. Total RNA was separated on a 2% agarose gel and stained with ethidium bromide. Lanes (representative lanes) show the results of amplification with the primer for HO-1. Columns show the quantitative results of 6 hearts in each group, mean ± 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 ± 20 pmol bilirubin/mg/hour (Fig. 3, lane 1) to 901 ± 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 ± 20 pmol bilirubin/mg/hour (Fig. 3, lane 1) to 162 ± 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). Without the ischemic/reperfused protocol, in electrically fibrillated hearts, the postfibrillated recovery was significantly improved compared to the ischemic and reperfused fibrillated myocardium (Table 1).

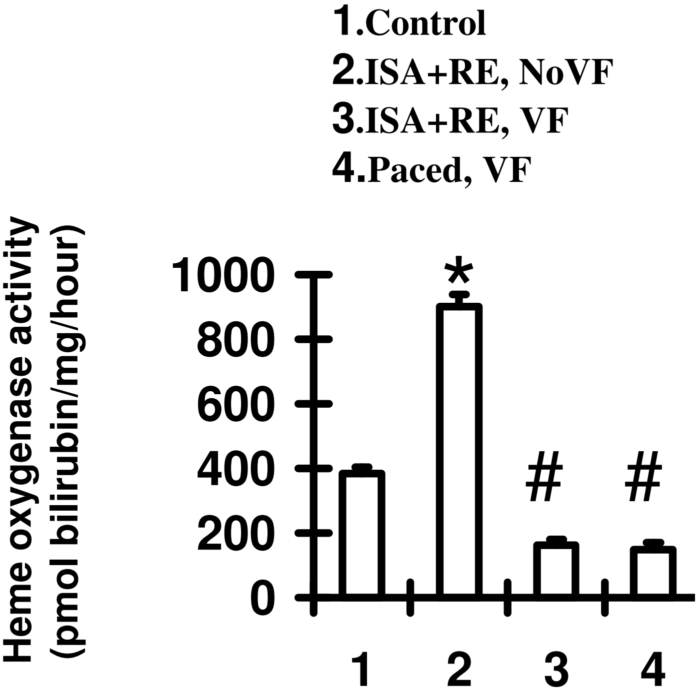

Figure 3. *The effect of reperfusion- and electrically-induced VF on heme oxygenase activity. Group 1: aerobically perfused nonischemic control. Group 2: Hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion, and VF was not developed during reperfusion. Group 3: Hearts were subjected 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 ± 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$^{\#}$	23.1±1.0$^{\#}$	15.2±0.3$^{\#}$	296±8	20.7±1.3$^{\#}$	21.8±2.2$^{\#}$	15.0±0.4$^{\#}$

n=6 in each group, mean ± SEM, *p<0.05 compared to the ISA/RE nonfibrillated group, $^{\#}p<0.05$ 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_2O_2 *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 ± 1.9 vs. 26.2 ± 2.2 ml/min; +LVdp/dt$_{max}$, 375 ± 15 vs. 602 ± 24 kPa/s; -LVdp/dt$_{max}$, 157 ± 12 vs. 267 ± 22 kPa/s; LVDP, 7.4 ± 0.6 vs. 11.8 ± 0.6 kPa; LVEDP, 7.4 ± 05 vs. 6.4 ± 0.4 kPa ($p<0.05$ each). After 30 min of H_2O_2 perfusion, a significant reduction in cardiac function of the WKY group was observed: AF, +LVdp/dt$_{max}$, -LVdp/dt$_{max}$, and LVDP were reduced to 16.0 ± 2.8 ml/min, 521 ± 22 kPa/s, 2011 ± 21 kPa/s, and 10.4 ± 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_2O_2 perfusion: AF, 20.7 ± 2.2 ml/min; +LVdp/dt$_{max}$, 399 ± 14 kPa/s; -LVdp/dt$_{max}$, 169 ± 15 kPa/s; LVDP, 8.4 ± 0.7 kPa; LVEDP, 7.7 ± 0.5 kPa. After the 30 min washout period, heart function remained unchanged in both groups. No other significant differences (except +LVdp/dt$_{max}$, LVDP) were found between the groups during and after the H_2O_2 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$_{max}$, -LVdp/dt$_{max}$, LVDP, CF, and LVEDP) expressed in %, compared to the pre-H_2O_2-perfused values (taking the baseline values 100%).

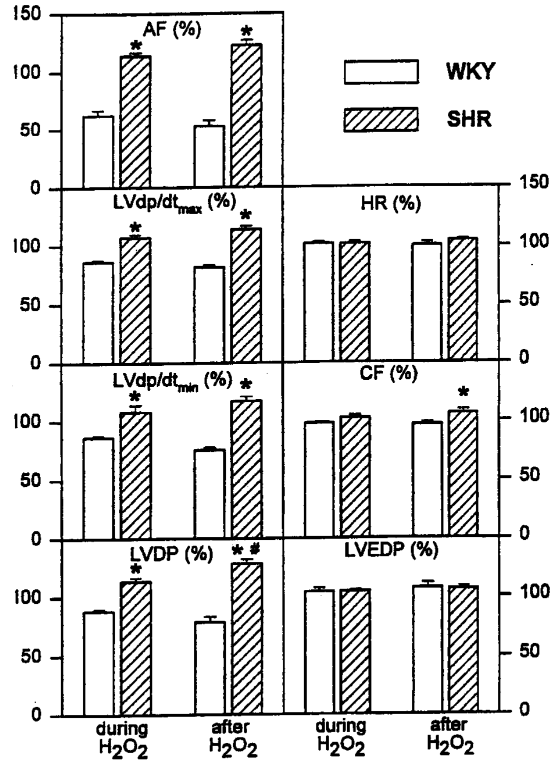

Figure 4. *Function of isolated perfused hearts from 18-month-old SHR (spontaneously hypertensive rats) and WKY (Wistar-Kyoto rats), measured after 30 min perfusion with* H_2O_2*, and after the subsequent 30 min washout period. The data were expressed in %, taking the pre-*H_2O_2 *perfused data as control and 100%. 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 at the end of* H_2O_2 *perfusion. AF, aortic flow; +LVdp/dt*$_{max}$*, maximum of the first derivative of left ventricular (LV) pressure; -LVdp/dt*$_{max}$*, 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_2O_2

SHR hearts exhibited higher activities of antioxidative defense enzymes (GPx, SOD) under control conditions in comparison with WKY hearts. After treatment with H_2O_2, a significant increase in catalase activity was measured (2.46 ± 0.22 *k* min^{-1}mg^{-1} protein) in SHR hearts compared both to the corresponding (treated) WKY group (1.59 ± 0.14 *k* min^{-1}mg^{-1} protein) and to the SHR group before H_2O_2 treatment (1.56 ± 0.29 *k* min^{-1}mg^{-1} protein). A significantly higher myocardial GPx activity was detected in SHR compared to WKY both before and after H_2O_2 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_2O_2. The CuZnSOD activity was actually reduced in the treated SHR group in comparison to the corresponding WKY group (Fig. 5).

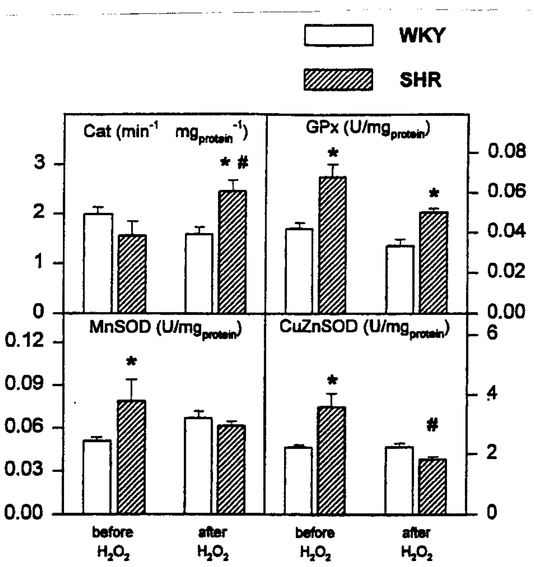

Figure 5. *Activities of catalase (Cat), glutathione peroxidase (GPx), Mn-, and Cu,Zn-superoxide dismutase (MnSOD, CuZnSOD) in the myocardium of 18-month-old SHR and WKY before 30 min H_2O_2 infusion and after the subsequent 30 min washout period. Mean ± SEM, n=6 in each group; * $p<0.05$, significantly different compared to the corresponding age-matched WKY control group; $^{\#}$ $p<0.05$, significantly different compared to the respective group before H_2O_2 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.

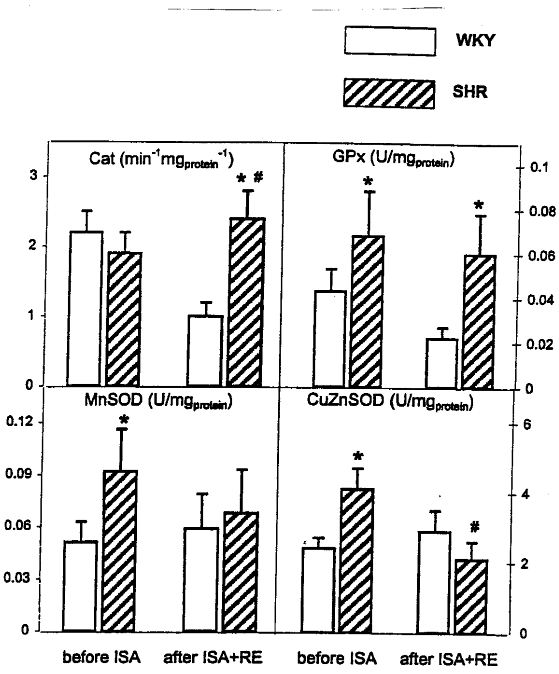

Figure 6. *Catalase (Cat), glutathione peroxidase (GPx), Mn- and Cu,Zn-superoxide dismutase (MnSOD, CuZnSOD) activities in 18-month-old SHR and WKY before the induction of ischemia (ISA), and after 30 min of ISA followed by 30 min of reperfusion (RE). Mean ± SEM, n=6 in each group,* $*p<0.05$ *compared 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_2O_2 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_2O_2 compared to the corresponding WKY group (303.3 ± 17.9 vs. 105.9 ± 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_2O_2 perfusion.

The effect of H_2O_2 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_2O_2 perfusion in SHR hearts compared to the respective values before the H_2O_2 infusion (92.2 ± 3.6 vs. 77.9 ± 3.9 arbitrary units, Fig. 8A and 8B, left panels). In WKY hearts, the MnSOD mRNA concentration remained essentially unchanged (85.3 ± 3.3 vs. 85.9 ± 4.7 arbitrary units; Fig. 8A and 8B, left panels), in agreement with the corresponding enzyme activities (Fig. 5). GADPH did not show any significant changes after the infusion of H_2O_2 in either SHR or WKY hearts (Fig. 8A and 8B, right panels).

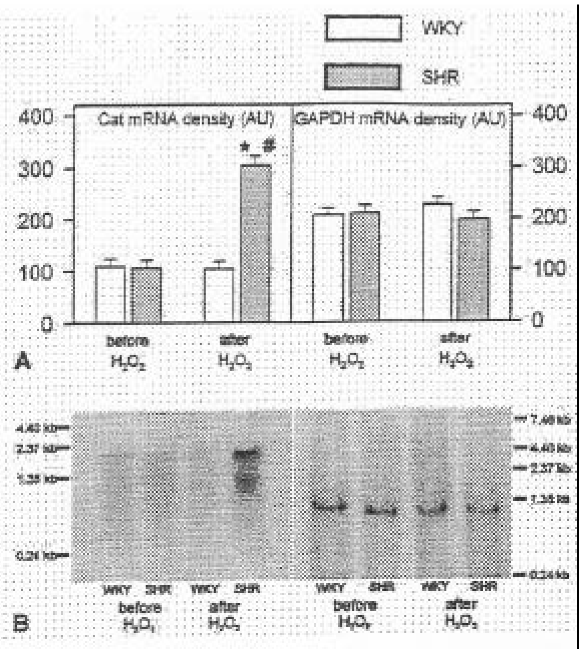

Figure 7. *(A) Dot blot quantification of catalase mRNA (Cat mRNA) in the myocardium of 18-month-old SHR and WKY before infusion with* H_2O_2 *and after the subsequent 30 min washout period, compared to the mRNA levels of the house-keeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Mean ± SEM, n=6 in each group; ** $p<0.05$*, significantly different compared to the WKY group after* H_2O_2*;* $^{\#}$ $p<0.05$*, 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.*

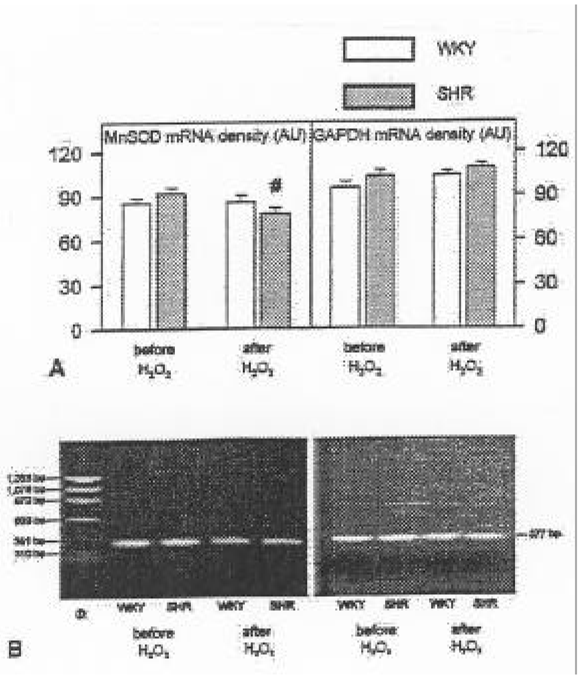

Figure 8. *(A) RT-PCR quantification of manganese superoxide dismutase (MnSOD) mRNA in the myocardium of 18-month-old SHR and WKY before 30 min* H_2O_2 *infusion and after the subsequent 30 min washout period. Mean ± SEM, n=6 in each group;* $^{\#}$ $p<0.05$*, significantly different compared to the 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. Φ=molecular size standard, in base pairs (bp).*

RESULTS III.

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. The reduction in DMPO-OH signal intensity was reflected in a striking reduction in the incidence of reperfusion-induced VF and VT (Fig. 10). 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.

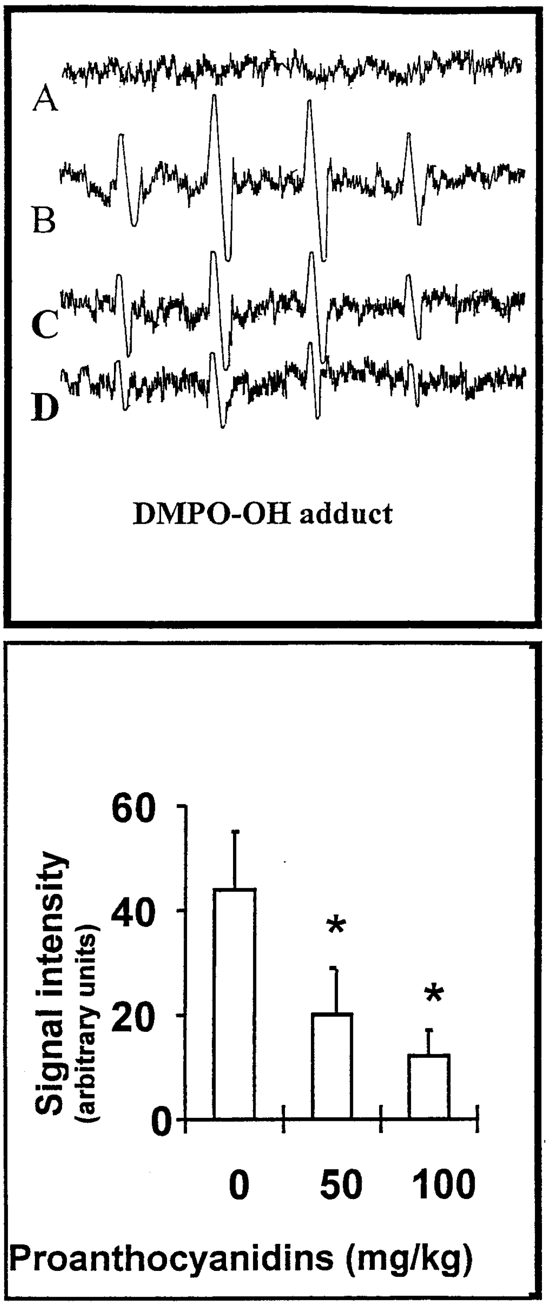

Figure 9. *Representative ESR spectra (upper panel) for the demonstration of $^{\cdot}$OH radical formation in untreated and proanthocyanidins treated ischemic/reperfused hearts. DMPO was infused during the first 3 min of reperfusion then effluent samples were frozen or immediately used for ESR studies. 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 3rd 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).*

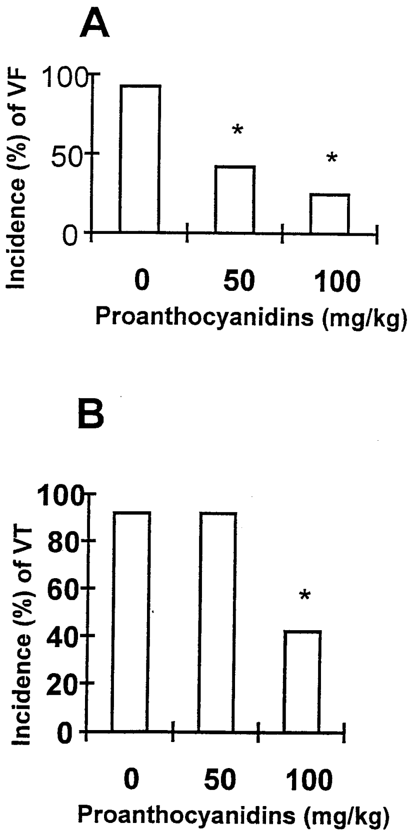

Figure 10. *Dose-response studies for ability of proanthocyanidins to reduce reperfusion-induced arrhythmias. Rats (n=12 in each group) were treated with a daily dose of 0 (vehicle, control), 50 or 100 mg/kg proanthocyanidins for 3 weeks, then hearts were isolated and subjected to 30 min of global ischemia 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 grou*

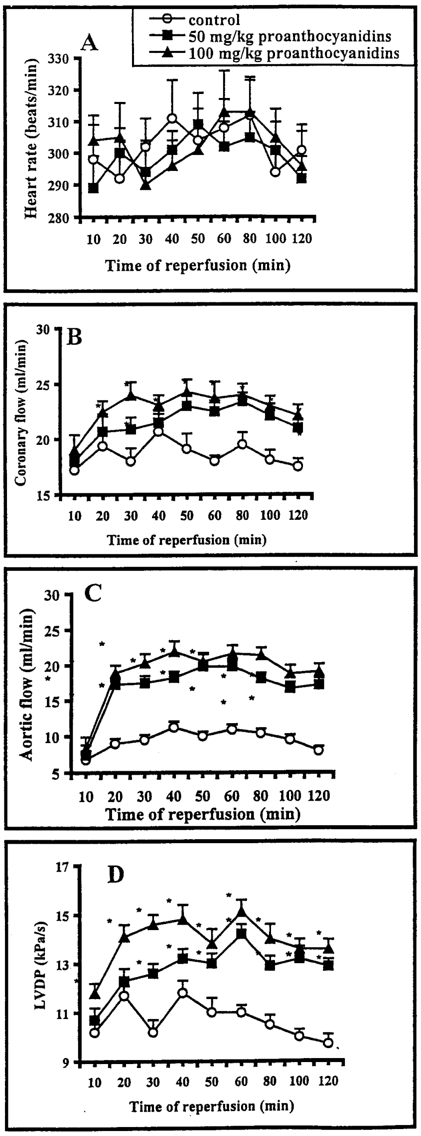

Figure 11. *Cardiac function in untreated and proanthocyanidins treated hearts subjected to 30 min ischemia followed by 120 min reperfusion. Panels A, B, C, and D show the postischemic recovery of heart rate (A), coronary flow (B), aortic flow (C), and LVDP (D), respectively. N = 12 in each group, mean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

4. DISCUSSION

DISCUSSION I .

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 (38-39), 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 (40). 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 abnormalities 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 (40) 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 (40).

The distinct transcription pathway for HO-1 mRNA activation by ischemia and 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 (42).

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.

DISCUSSION II.

The administration of antioxidant enzymes may protect the heart against free radical mediated damage (43-44). The activity of antioxidant enzymes may be decreased, especially when the oxidative load overcomes the defense potential (45). On the other hand, moderate episode(s) of radical stress may potentiate the defense system by stimulating the expression of the respective enzymes (46).

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 (47) 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 without hypertrophy. The expression of GPx has been reported to be induced after the exposure to excessive levels of H_2O_2 (48) which can be produced either by SOD activity or by oxidative stress of different origin (49). 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.

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 (50) 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.

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

(51). On the other hand, our findings are consistent with other's (52-53) who found that preconditioning of hypertrophied SHR hearts improved the postischemic recovery of high-energy phosphates and function in the myocardium.

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.

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 (54). 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 (43, 55), (ii) chelators of iron and copper (56-57), (iii) antioxidant enzymes such as superoxide dismutase and catalase (43), and (iv) redox-metal displacement by zinc (58). Because epidemiological evidence indicates that the consumption of red wine is beneficial in the prevention of coronary artery diseases (59), and this beneficial effect could be attributed to antioxidants present in the polyphenol fraction of red wine (24), 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.

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 (60) providing a further protection against reperfusion-induced calcium overload. Feng et al., (61) indicated 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.

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/reperfused fibrillated hearts. The results 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 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 µM H_2O_2. The results obtained in ischemic and reperfused hearts show the same changes in enzyme activities measured as it was observed in H_2O_2 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.

In the last part of our studies, 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. 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% ± 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.

6. REFERENCES

1. Gray RA, Pertsov AM, and Jalife J. Spatial and temporal organization during cardiac fibrillation. Nature 392: 75-78, 1998.

2. Witkowski FX, Leon LJ, Penkoske PA, Giles WR, Spano ML, Ditto WL, and Winfree AT. Spatiotemporal evolution of ventricular fibrillation. Nature 392: 78-82, 1998.

3. Tzivoni D, Keren A, Granot H, Gottlieb S, Benhorin J, and Stern S. Ventricular fibrillation caused by myocardial reperfusion in Prinzmetal's angina. Am Heart J 105: 323-325, 1983.

4. Curtis MJ, Pugsley MK, and Walker MJA. Endogenous chemical mediators of ventricular arrhythmias in ischemic heart diseases. Cardiovasc Res 27: 703-719, 1993.

5. Hearse DJ. Reperfusion-induced injury: a possible role for oxidant stress and its manipulation. Cardiovasc Drugs Ther 5: 225-236, 1991.

6. Opie LH. Role of calcium and other ions in reperfusion injury. Cardiovasc Drugs Ther 5: 237-248, 1991.

7. Maines MD, Raju VS, and Panahian N. Spin trap (N-t-butyl-α-phenylnitrone)-mediated suprainduction of heme oxygenase-1 in kidney ischemia/reperfusion model: role of the oxygenase in protection against oxydative injury. J Pharmacol Exp Ther 291: 911-919, 1999.

8. Salvemini D, Wang ZQ, Zweier JL, Samouilov A, Macarthur H, Misko TP, Currie MG, Cuzzocrea S, Sikorski JA, and Riley DP. A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats. Science 286: 304-306, 1999.

9. Tyrrell R. Redox regulation and oxidant activation of heme oxygenase-1. Free Rad Res 31: 335-340, 1999.

10. Caudill TK, Resta TC, Kanagy NL, and Walker BR. Role of endothelial carbon monoxide in attenuated vasoreactivity following chronic hypoxia. Am J Physiol 275: R1025-R1030, 1998.

11. Trakshel GM, and Maines MD. Multiplicity of heme oxygenase isozymes: HO-1 and HO-2 are different molecular species in rat and rabbit. J Biol Chem 264: 1323-1328, 1989.

12. Katayose D, Isoyama S, Fujita H, and Shibahara S. Separate regulation of heme oxygenase and heat shock protein 70 mRNA expression in the rat heart by hemodynamic stress. Biochem Biophys Res Commun 191: 587-594, 1993.

13. Lee PJ, Jiang BH, Chin BY, Iyer NV, Alam J, Semenza GL, and Choi AMK. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J Biol Chem 272: 5375-5381, 1997.

14. Otterbein LE, Lee PJ, Chin BY, Petrache I, Camhi SL, Alam J, and Choi AM. Protective effects of heme oxygenase-1 in acute lung injury. Chest 116: S61-S63, 1999.

15. Vile GF, and Tyrell RM. Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase of ferritin. J Biol Chem 268:14678-14681, 1993.

16. Csonka C, Varga E, Kovacs P, Ferdinandy P, Blasig IE, Szilvassy Z, and Tosaki A. Heme oxygenase and cardiac function in ischemic/reperfused rat hearts. Free Rad Biol Med 27: 119-126, 1999.

17. Renaud S, de Rolgeril M. Wine, alcohol, platelets, and the Franch paradox for coronary heart disease. Lancet 1992; 339: 1523-1526.

18. Gaziano JM, Buring JE, Breslow JL, Goldhaber SZ, Rosner B, Van Denburgh M et al. Moderate alcohol intake, increased levels of high density lipoprotein and its subfractions, and decreased risk of myocardial infarction. N Eng J Med 1993; 329: 1829-1834.

19. Camargo CA, Hennekens CH, Gaziano M, Glynn RJ, Manson JE, Stampfer MJ. Prospective study of moderate alcohol consumption and mortality in US male physicians. Arch Intern Med 1997; 157: 79-85.

20. Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, Pruess HG. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology 2000; 148: 187-197.

21. Hollman PC, Hertog MG, Katan MB. Role of dietary flavonoids in protection against malignancy and coronary heart disease. Biochem Soc Trans 1996; 24: 785-789.

22. Lairon D, Amiot MJ. Flavonoids in food and natural antioxidants in wine. Curr Opin Lipidol 1999; 10: 23-28.

23. Klatsky AL, Armstrong MA. Alcoholic beverage choice and risk of coronary artery disease mortality: do red wine drinkers fare best? Am J Cardiol 1993; 71: 467-469.

24. Hertog MGL, Feskens EJM, Kromhout D. Antioxidant flavonols and coronary heart disease risk. Lancet 1997; 349: 699.

25. Tosaki A, and Braquet P. DMPO and reperfusion injury: arrhythmia, heart function, electron spin resonance, and nuclear magnetic resonance studies in isolated working guinea pig hearts. Am Heart J 120: 819-830, 1990.

26. Chomczynski P, and Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chlorophorm extraction. Anal Biochem 162: 156-159, 1987.

27. Kutty RK, Kutty G, Wiggert B, Chader GJ, Darrow RM, and Organisciak DT. Induction of heme oxygenase 1 in retina by intense visible light: Suppression by the antioxidant dimethylthiourea. Proc Natl Acad Sci USA 92: 1177-1181, 1995.

28. Zador E, Mendler L, Ver-Heyen M, Dux L, and Wuytack F. Changes in mRNA levels of the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase isoforms in the rat soleus muscle regenerating from notexin-induced necrosis. Biochem J 320: 107-113, 1996.

29. Sambrook J, Fritsch EF, and Maniatis T. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, second edition, Plainview, New York, 1989.

30. Tenhunen R, Ross ME, Harver HS, and SchmidR. Reduced nicotinamide-adenine dinucleotide phosphate dependent biliverdin reductase: partial purification and characterization. Biochemistry 9: 298-308, 1970.

31. Yoshida T, Takahashi S, and Kikuchi G. Partial purification and reconstitution of the heme oxygenase system from pig spleen microsomes. J Biochem 75: 1187-1191, 1974.

32. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275, 1951.

33. Ho, Y.S.; Howard, A.J.; Crapo, J.D. Molecular structure of a functional rat gene for manganese-containing superoxide dismutase. Am. J. Respir. Cell. Mol. Biol. 4:278-286; 1991.

34. Furuta, S.; Hayashi, H. Purification and properties of recombinant rat catalase produced in Escherichia coli. J. Biochem. 107:708-713; 1990.

35. Sato M, Maulik G, Ray PS, Bagchi D, Das DK. Cardioprotective effects of grape seed proanthocyanidin against ischemic reperfusion injury. J Mol Cell Cardiol 1999; 31: 1289-1297.

36. Tosaki A, Pali T, Droy-Lefix MT. Effects of Ginkgo biloba extract and preconditioning on the diabetic rat myocardium. Diabetologia 1996; 39: 1255-1262.

37. Wallenstein S, Zucker CL, and Fleiss JL. Some statistical methods useful in circulation research. Circ Res 47; 1-9, 1980.

38. Goodman AI, Choudhury M, da Silva JL, Schwartzman ML, and Abraham NG. Overexpression of heme oxygenase gene in renal cell carcinoma. Proc Soc Exp Biol Med 214, 54-61, 1997.

39. Maines MD, and Abrahamson PA. Expression of heme oxygenase-1 (HSP32) in human prostate: normal, hyperplastic and tumor tissue distribution. Urology 47: 727-733, 1996.

40. Alam J, Stewart D, Touchard C, Boinapally S, Choi AMK, and Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of heme oxygenase-1 gene. J Biol Chem 274: 26071-26078, 1999.

41. Choi AMK, and Alam J. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol 15: 9-19, 1996.

42. Christodoulides N, Durante W, Kroll MH, and Schafer AI. Vascular smooth muscle cell heme oxygenase generate guanylyl cyclase-stimulatory carbon monoxide. Circulation 91: 2306-2309, 1995.

43. Bernier, M.; Hearse, D.J.; Manning, A.S. Reperfusion-induced arrhythmias and oxygen-derived free radicals: Studies with ”anti-free radical” interventions and a free radical generating system in the isolated perfused rat heart. Circ. Res. 58:331-349; 1986.

44. Ek, B.; Hallberg, C.; Sjogren, K.G.; Hjalmarson, A. Reoxygenation-induced cell damage of isolated neonatal rat ventricular myocytes can be reduced by chain-breaking antioxidants. Free Rad. Biol. Med. 16:117-121; 1994.

45. Maier, K.L.; Hinze, H.; Meyer, B.; Lenz, A.G. Metal-catalysed inactivation of bovine glucose-6-phosphate dehydrogenase - role of thiols. FEBS Lett. 396:95-98; 1996.

46. Vogt, M.; Bauer, M.K.; Ferrari, D.; Schulze-Osthoff, K. Oxidative stress and hypoxia/reoxygenation trigger CD95 (APO-1/Fas) ligand expression in microglial cells FEBS Lett. 429:67-72; 1998.

47. Wallukat, G.; Blasig, I.E.; Morwinski, R.; Herrmann, H.J.; Rohde, E. The sera of spontaneously hypertensive rats contain agonistic autoantibodies against the $ß_1$-adrenoceptor. J. Hypertension 13:1031-1036; 1995.

48. Erden, M.; Bor, N.M. Changes of reduced glutathion, glutathion reductase, and glutathion peroxidase after radiation in guinea pigs. Biochem. Med. 31:217-227; 1984.

49. Arduini, A.; Mezzetti, A.; Porreca, E.; Lapenna, D.; DeJulia, J.; Marzio, L.; Polidoro, G.; Cucurullo, F. Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane proteins in perfused rat heart. Biochem. Biophys. Acta 970:113-121; 1988.

50. Sinet, P.M.; Garber, P. Inactivation of the human CuZn superoxide dismutase during exposure to O_2^- and H_2O_2. Arch. Biochem. Biophys. 212:411-416; 1981.

51. Bing, O.H.; Brooks, W.W.; Robinson, K.G.; Slawsky, M.T.; Hayes, J.A.; Litwin, S.E.; Sen, S.; Conrad, C.H. The spontaneously hypertensive rat as a model of the transition from compensated left ventricular hypertrophy to failure. J. Mol. Cell. Cardiol. 27:383-396; 1995.

52. Boutros, A.; Wang, J. Ischemic preconditioning, adenosine and bethanechol protect spontaneously hypertensive isolated rat hearts. J. Pharmacol. Exp. Ther. 275:1148-1156; 1995.

53. Nakagawa, C.; Asayama, J.; Katamura, M.; Matoba, S.; Keira, N.; Kawahara, A.; Tsuruyama, K.; Tanaka, T.; Kobara, M.; Akashi, K.; Ohta, B.; Tatsumi, T.; Nakagawa, M. Myocardial stretch induced by increased left ventricular diastolic pressure preconditions isolated perfused hearts of normotensive and spontaneously hypertensive rats. Basic Res. Cardiol. 92:410-416; 1997.

54. Gelvan D, Saltman P, Powell SR. Cardiac reperfusion damage prevented by a nitroxide free radical. Proc Natl Acad Sci 1991; 88: 4680-4684.

55. Woodward B, Zakaria MNM. Effects of some free radical scavengers on reperfusion-induced arrhythmias in the isolated rat heart. J Mol Cell Cardiol 1985; 17: 485-493.

56. Ambrosio G, Zweier JL, Jacobus WE, Weisfeldt ML, Flaherty JT. Improvement of postischemic myocardial function and metabolism induced by administration of deferoxamine at the time of reflow: the role of iron in the pathogenesis of reperfusion injury. Circulation 1987; 76: 906-915.

57. Ferdinandy P, Appelbaum Y, Tosaki A. Role of nitric oxide and TPEN, a potent metal chelator, in ischemic and reperfused rat isolated hearts. Clin Exp Pharmacol Physiol 1998; 25: 496-502.

58. Powell SR, Saltman P, Uretzky G, Chevion M. The effect of zinc on reperfusion arrhythmias in the isolated perfused rat heart. Free Rad Biol Med 1990; 8: 33-46.

59. Rimm EB, Giovannucci EL, Willett WC, Colditz GA, Archerio A, Rosner B et al. Prospective study of alcohol consumption and risk of coronary disease in men. Lancet 1991; 338: 464-486.

60. Johnson EF, Schwab GE, Vickery LE. Positive effectors of the binding of an active site-directed amino steroid to rabbit cytochrome P-450 3c. J Biol Chem 1988; 263: 17672-17677.

61. Feng AN, Chen YL, Chen YT, Ding YZ, Lin SJ. Red wine inhibits monocyte chemotactic protein-1 expression and modestly reduces neointimal hyperplasia after balloon injury in cholesterol-fed rabbits. Circulation 1999; 100: 2254-2259.

7. 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. Antioxidats 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