Postischemic cardiac recovery in heme oxygenase-1 transgenic ischemic/reperfused mouse myocardium

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ABSTRACT

Heme oxygenase-1 (HO-1) transgenic mice (Tg) were created using a rat HO-1 genomic transgene. Transgene expression was detected by RT-PCR and Western blots in the left ventricle (LV), right ventricle (RV), and septum (S) in mouse hearts, and its function was demonstrated by the elevated HO enzyme activity. Tg and nontransgenic (NTg) mouse hearts were isolated and subjected to ischemia/reperfusion. Significant postischemic recovery in coronary flow (CF), aortic flow (AF), aortic pressure (AOP), and first derivative of AOP (AOPdp/dt) were detected in the HO-1 Tg group compared to the NTg values. In HO-1 Tg hearts treated with 50 μmol/kg of SnPPIX, an HO enzyme inhibitor, abolished the postischemic cardiac recovery. HO-1 related CO production was detected in NTg, HO-1 Tg, and HO-1 Tg + SnPPIX treated groups, and a substantial increase in CO production was observed in the HO-1 Tg hearts subjected to ischemia/reperfusion. Moreover, in ischemia/reperfusion-induced tissue Na^+ and Ca^{2+} gains were reduced in HO-1 Tg group in comparison with the NTg and HO-1 Tg + SnPPIX treated groups, furthermore K^+ loss was reduced in the HO-1 Tg group. The infarct size was markedly reduced from its NTg control value of 37±4% to 20±6% ($p<0.05$) in the HO-1 Tg group, and was increased to 47±5% ($p<0.05$) in the HO-1 KO hearts. Parallel to the infarct size reduction, the incidence of total and sustained VF were also reduced from their NTg control values of 92% and 83% to 25% ($p<0.05$) and 8% ($p<0.05$) in the HO-1 Tg group, and were increased to 100% and 100% in HO-1 knock out (-/-) hearts. Immunohistochemical staining of HO-1 was intensified in HO-1 Tg compared to the NTg myocardium. Thus, the HO-1 Tg mouse model suggests a valuable therapeutic approach in the treatment of ischemic myocardium.

INTRODUCTION

With respect to gene control and regulation, a great number of inducer-responsive elements have been identified and characterized within the 5'-flanking regions of the mouse, rat, and human heme oxygenase-1 (HO-1). In the past decades, substantial progress has been made in our understanding of the regulation and function of HO-1 and its isozymes. Thus, heat shock proteins including HO-1, known as 32-kDa stress-inducible protein (HSP-32), provides significant protection against a variety of tissue injuries [1]. Different isozymes of HO have been identified, cloned, and studies demonstrated that HO-1 is induced in response to various interventions causing oxidative stress including ultraviolet irradiation, hypoxia, and ischemia [2-6]. By degrading oxidant heme and generating antioxidant bilirubin, and a gaseous molecule, endogen carbon monoxide (CO), and HO-1 induction protect tissues from death caused by various pathological processes [7-10]. Thus, in various diseases that are induced by different factors, the lack of HO-1 or its expression substantially modifies tissue and organ functions. Given the cytoprotective role of HO-1, there is a growing interest in the importance of HO-1 and its byproducts e.g., iron and endogenous CO, in the recovery of cardiac injury caused by ischemia [11,12].

In our previous studies, we observed a reduction in HO-1 mRNA and protein expression causing decreased enzyme activity leading ultimately to poor recovery of the ischemic/reperfused fibrillated myocardium [13,14]. To conclusively demonstrate whether HO-1 plays a critical role in the recovery of postischemic myocardium, we generated HO-1 transgenic mice. We hypothesized that the vulnerability of HO-1 transgenic mice to reduce the incidence of VF, attenuation of infarct size, and improved recovery of postischemic function during ischemia/reperfusion injury would be related to HO-1 protein expression and its byproducts including the endogenous CO or iron. We also speculated that HO-1 signaling and protein expression would play an essential role in the improvement postischemic cardiac

aortic flow, coronary flow, aortic pressure, the first derivative of aortic pressure, myocardial ion contents including Na^+, K^+, and Ca^{2+}. Various mechanisms have been proposed to explain the postischemic recovery and the causes of arrhythmias, but relatively little attention has been paid, to our knowledge, in order to clarify the mechanism(s) of VF at a gene expression level in ischemic/reperfused hearts. For instance, the long QT syndrome and idiopathic ventricular fibrillation, as known up to now, are cardiac disorders based on genetic mutation of potassium channels and cause sudden cardiac death from ventricular arrhythmias [15-17]. The aim of our present study was to generate and use HO-1 transgenic mice to directly analyze the impact and role of HO-1 on postischemic recovery in the myocardium. In some additional studies, we made attempt to estimate the incidence of reperfusion-induced VF and infarct size in HO-1 Tg mouse heart in comparison with HO-1 knock out (-/-) and wild type mouse myocardium. We believe that the present study provides novel insights into the function of HO-1 and its contribution to the recovery of postischemic cardiac function and reduction of arrhythmias in the heart.

METHODS

Transgenic mice:

Transgenic mice were generated as described by Araujo et al. [9]. In brief, CFY mouse eggs were injected with 52 kb rat HO-1 construct containing 27.7 kb of the 5' upstream region, 8.3 kb of HO-1 gene, and 16 kb of the 3' downstream region. Cloning was carried out by PCR of rat genomic P1 library with two sets of PCR primers corresponding to the first exon (5'-GCT-TCG-GTG-GGT-TAT-CTG-CCG-TTA-T-3' and 5'-CAG-TCT-TAC-AGG-CGG-GGA-ATG-TGA-G-3'), and the 5th exon of rat HO-1 gene (5'-GAG-ACG-CCC-CGA-GGA-AAA-TCC-CAG-AT-3' and 5'-CCC-AAG-AAA-AGA-GAG-CCA-GGC-AAG-AT-3'). Two clones were identified as positives, and restriction mapping showed that both of them included the same insert. The 52 kb band was equivalent to the HO-1 gene and flanking regions. This 52 kb fragment, including the native HO-1 gene and its promoter, was excised and digested with beta-agarase, and then microinjected. Mice were genotyped by PCR using the set of primers specific for rat HO-1 exon 5.

Animals and heart preparation:

Male wild type (nontransgenic, NTg), HO-1 transgenic, and HO-1 KO (-/-) mice (25-35 g) (Charles Rivers Laboratories), were used for all studies. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health (NIH Publication No. 86-23, revised 1996). Mice were anaesthetized with 60 mg/kg of pentobarbital sodium. After i.p. administration of heparin (1,000 IU/kg) the chest was opened, the heart was rapidly excised and mounted to a "working" perfusion apparatus described by Hewett et al [18]. The perfusion was established with a modified oxygenated Krebs-Henseleit

buffer with the following concentrations (in mM): 118.4 NaCl, 4.1 KCl, 2.5 $CaCl_2$, 25 $NaHCO_3$, 1.17 KH_2PO_4, 1.46 $MgCl_2$, and 11.1 glucose. The perfusion buffer was previously saturated with a mixture of 95% O_2 and 5% CO_2, pH 7.4 at 37 °C. To prevent the myocardium from drying out, the heart chamber, in which hearts were suspended, was covered and the humidity was kept at a constant level (90%-95%). In all experiments global ischemia was induced for 20 min followed by 2 hours of reperfusion. In additional experiments, mice were i.p. injected with 50 μmol/kg of tin protoporphyrin IX (SnPPIX), an HO enzyme inhibitor, one day prior to the isolation of the heart and induction of ischemia and reperfusion. HO enzyme activities and recovery of postischemic cardiac function were measured in hearts obtained from NTg, HO-1 Tg, and HO-1 Tg-SnPPIX treatred mice. The schematic presentation of the experimental time course and parameters measured are shown in Figure 1.

Registration of VF and measurement of cardiac function:

Epicardial electro cardiograms (ECGs) were recorded throughout the experimental period by two silver electrodes attached directly to the heart and connected to a data acquisition system (ADInstruments, Powerlab, Castle Hill, Australia). ECGs were analyzed to determine the presence or absence of ventricular fibrillation (VF). Hearts were considered to be in VF if an irregular undulating baseline was apparent on ECGs. If the duration of VF was longer than 2 min, the VF was defined as sustained VF, otherwise, the VF was nonsustained. If VF developed and the sinus rhythm did not spontaneously return within the first two min of reperfusion, hearts were electrically defibrillated by a defibrillator using two silver electrodes and 15 V square-wave pulse of 1 ms duration and reperfused. Aortic and coronary flow rates were measured by a timed collection of the aortic and coronary effluents that dripped from the heart. Before ischemia and during reperfusion, heart rate (HR), coronary flow (CF) and aortic flow (AF) were registered.

Aortic pressure (AOP), and the first derivative of aortic pressure (AOPdp/dt) were measured by a computer acquisition system (ADInstruments, Powerlab, Castle Hill, Australia).

Measurement of infarct size:

Infarct size was measured, at the end of each experiment, with 10 ml of 1 % triphenyl tetrazolium (TTC) solution in phosphate buffer (Na_2HPO_4 88 mM, NaH_2PO_4 1.8 mM) injected via the side arm of the aortic cannula then stored at -70 ^{o}C for later analysis. Frozen hearts were sliced transversely [19] in a plane perpendicular to the apico-basal axis into 1-2 mm thick sections, weighted, blotted dry, placed in between microscope slides and scanned on a Hewlett-Packard Scanjet 5p single pass flat bed scanner (Hewlett-Packard, Palo Alto, CA). Using the NIH Image 1.61 image processing software, each digitized image was subjected to equivalent degrees of background subtraction, brightness and contrast enhancement for improved clarity and distinctness. Infarct zones of each slice were traced and the respective areas were calculated in terms of pixels [20]. The areas were measured by computerized planimetry software and these areas were multiplied by the weight of each slice, then the results summed up to obtain the weight of the risk zone (total weight of the left ventricle, mg) and the infarct zone (mg). Infarct size was expressed as the ratio, in percent, of the infarct zone to the risk zone.

Northern blots and RT-PCR:

RNA was prepared from the left and right ventricles, and septum (about 50 mg) by guanidine isothiocyanate acid/phenol method [21], and 5 μg total RNA was used to synthesize first strand cDNA by the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, San Diego, California). The product of cDNA was amplified by PCR with specific primers for rat HO-1 exon 5 (CCC-TTC-CTG-TGT-CTT-CCT-TTG and ACA-GCC-GCC-TCT-ACC-

GAC-CAC-A). The product of RT-PCR was separated using 1.5% agarose gel, and visualized by ethidium bromide.

Western blots:

Myocardial samples from LV, RV, and S were homogenized in Tris-HCl (13.2 mM/L), glycerol (5.5%), SDS (0.44%), and β-mercaptoethanol. The same amount of soluble protein (50 μg) was fractionated by Tris-glycine-SDS-polyacrylamide gel (12%) electrophoresis, and Western blot was carried out as described by Pellacani et al [22] with the use of an antibody for recombinant rat HO-1 protein.

Measurement of HO activity:

Fifty milligrams of tissue were homogenized in 10 ml of 200 mM phosphate buffer, and then centrifuged at 19,000 x *g* 4 ^{o}C for 10 min. The supernatant was removed and recentrifuged at 100,000 x *g* 4 ^{o}C for 60 min, and precipitated fractions were suspended in 2 ml of 100 mM K-phosphate buffer. Biliverdin reductase was crudely purified as described by Tenhunen et al.[23], and heme oxygenase activity was assayed as described by Yoshida et al., [24]. Reaction mixtures consisted of (final volume 2 ml) 100 μM K-phosphate (pH 7.4), 15 nM hemin, 300 μM bovine serum albumin, 1 mg biliverdin reductase, and 1 mg microsomal fraction of cardiac tissue. The reaction was allowed to proceed for 1 h at 37 ^{o}C in dark in a shaking water bath and was stopped by placing the test on ice. Incubation mixtures were then scanned using a scanning spectrophotometer, and the amount of bilirubin was calculated as the difference between absorbance at 464 nm and 530 nm [25]. Proteins were determined by the method of Lowry et al.[26], in microsomal fractions.

Measurement of CO:

Tissue CO content using gas chromatography was detected as described by Cook et al [27]. Briefly, hearts were homogenized in 4 volumes of 0.1 M phosphate-buffer (pH: 7.4) using an X520 homogenizer (Ingenieurburo CAT, M. Zipperer GmbH, Staufen, Germany). The homogenates were centrifuged at 4 ^{o}C for 15 min at 12,800 g and the supernatant fractions were used for the determination of tissue CO content. The reaction mixtures contain: 150 µl of supernatant, 60 µl of NADPH (4.5 mM) and 50 µl of 3.5/0.35 mM methemalbumin, and for blank samples 60 µl phosphate buffer was used instead of NADPH. Samples were preincubated at 37 ^{o}C for 5 min, then the headspace was purged and the incubation was continued for 1 hour in dark at 37 ^{o}C. The reaction stopped by placing the samples on ice and the headspace gas was analyzed. One thousand µl of the headspace gas from each vial was injected into the gas chromatograph using a gastight syringe (Hamilton Co., USA) in hydrogen gas flow with a speed of 30 ml/min. Analysis took place during the next 150 seconds on a 200 cm stainless-steel column with a 0.3 cm inner diameter. The detector was a thermal conductivity detector with an AC current of 80 mA. The individual value was expressed in mV. The column was packed with Molselect 5 Å and maintained at 30 ^{o}C. The temperature of the injector and detector was controlled and kept at 50 ^{o}C.

Immunohistochemistry:

Paraffin sections (7 µm) of tissue were incubated in the presence of polyclonal antibody and purified liver HO-1 obtained from rats (Stress Gen Biotech., Canada). Reactions were visualized by immunoperoxidase color reaction in NTg, HO-1 Tg, and HO-1 KO ischemic/reperfused mouse myocardium. Formalin fixed tissues were paraffin embedded and 7 µm sections were placed on poly-l-lysine coated glass slides (Sigma, St.Luis, USA). Following deparaffinization and rehydration, samples were used for quenching endogenous peroxidases and blocking nonspecific binding sites by 3 % H_2O_2 in normal goat serum.

Sections were incubated for another 2 hours with HO-1 antibody at a dilution of 1:750 as described in the manufacturer's manual. After 10 min of PBS washing, slides were incubated for additional 30 min in the presence of purified biotinylated anti/rabbit IgG (Vector, USA) at a dilution of 1:200. Slides were rewashed in PBS and incubated with peroxidase conjugated streptavidin (Zymed, USA) for 30 min followed by red color development using 3-amino-9-ethylcarbazole in 0.1 M of acetate buffer (pH 5.2). Sections were counter-stained with Gill's hematoxylin for 15-20 sec, rinsed with deionized water, and dipped in 1 % of lithium carbonate. After draining off water, slides were placed in oven for 30 min at 80 C^{o}, and then covered with permount coverslips. Sections were photographed using a Zeiss (Germany) light microscope.

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

Cellular cations were measured as previously described [28]. In brief, hearts were rapidly cooled to 0-5 ^{o}C by submersion in, and then perfused for 5 min with an ice-cold ion-free buffer solution containing 100 mmol/L of trishydroxy-methyl-amino-methane and 220 mmol/L of sucrose to wash out ions from the extracellular space and to stop enzyme activities responsible for membrane ion transport processes. Five minutes of cold washing of the heart washed out > 90 % of the ions from the extracellular space [29]. Following the wash out period, left ventricular tissues were dried for 48 hours at 100 ^{o}C, and made ash at 550 ^{o}C for 20 hours. The ash was dissolved in 5 ml of 3 M nitric acid and diluted 10-fold with ion-free deionized water. Cellular Na^{+} was measured at a wavelength of 330.3 nm, K^{+} was measured at 404.4 nm, and Ca^{2+} at 422.7 nm in air-acetylene flame using an atomic absorption spectrophotometer (Perkin-Elmer 1100-B). This method for the measurement of cellular ion contents has been previously described in the myocardium [29,30] and central nervous system [31].

Statistics:

The data for HR, CF, AF, AOP, AOPdp/dt, cellular Na^{+}, K^{+}, Ca^{2+}, and infarct size were expressed as the MEAN ± SEM. One-way analysis of variance was first carried out to test for any differences between the mean values of groups. If differences were established the values of NTg group were compared to those of HO-1 transgenic, and HO-1 KO (-/-) groups by multiple t-test followed by Bonferroni test. Because of the nonparametric distribution of the incidence of VF (sustained and nonsustained), the chi-square test was used to compare individual groups. A change of $p<0.05$ was considered to be statistically significant.

RESULTS

For the confirmation of rat HO-1 expression at mRNA level, RT-PCR was used and total RNA was isolated from the nonischemic left and right ventricles, and septum of Tg and NTg littermates. The PCR product was rat HO-1 specific and was detected selectively in Tg mouse samples by RT-PCR (Fig. 2.A) and Western blot (Fig. 2.B). Under our experimental conditions, two clones were identified, and the fragment, the promoter, and HO-1 gene were excised by SfiI. Primers were amplified using 166-bp product originated from rat HO-1 exon 5. To avoid genomic DNA-based amplification, before the first stand cDNA synthesis, all biopsies were treated with DNase. The specific band was amplified in tissues obtained from left ventricle (LV), right ventricle (RV) , and septum (S), respectively, by PCR from RNA samples of Tg mouse from different lines (Fig. 2.A, Tg1: upper part, Tg2: middle part), but was absent in RNA samples of NTg (Fig.2.A, lower part). Tg1 and Tg2 proteins were also detected by Western blots in the LV, RV, and S of mouse hearts (Fig. 2.B upper and middle parts), but the expression of rat HO-1 proteins were absent in the NTg mouse myocardium (Fig. 2.B lower part).

HO enzyme activities were also measured in the LV, RV, and S obtained from NTg and Tg mice (Fig. 2.C). The results show that HO activities were significantly increased in samples obtained from the LV, RV, and S of Tg mouse hearts in comparison with NTg values (Fig. 2.C) indicating the function of Tg1 and Tg2 genes in the mouse myocardium. It is important to note that HO-1 mRNA and protein were not detected in NTg mouse myocardium either by PCR (Fig. 2.A lower part) or Western blot (Fig. 2.B lower part), but HO enzyme activities were present in the samples of NTg mouse myocardium including LV, RV, and S, respectively, indicating that measured HO enzyme activity involves all isoforms such as HO-1, HO-2, HO-3, and HO-4 (Fig. 2.C). Thus, the differences in HO enzyme activities (Fig. 2.C) between NTg and Tg in the LV, RV, and S were clearly related to the rat HO-1 transgene in the mouse myocardium.

Table 1 shows the recovery of postischemic cardiac function in NTg and HO-1 Tg hearts subjected to 20 min of normothermic global ischemia followed by 2 hours of reperfusion. Before the induction of ischemia significant changes were not detected between NTg and HO-1 Tg groups in HR, CF, AF, AOP, and AOPdp/dt. Upon reperfusion, the postischemic recovery of CF, AF, AOP, and AOPdp/dt were observed in the HO-1 Tg group in comparison with the NTg values without any significant differences in HR. Thus, for instance, after 30 min and 120 min of reperfusion, AF was significantly increased from its NTg control values of 0.9 ± 0.1 ml/min and 0.8 ± 0.1 ml/min to 2.3 ± 0.2 ml/min ($p<0.05$) and 2.2 ± 0.2 ml/min ($p<0.05$) in the Tg group, respectively (Table 1). The same pattern was observed in the postischemic recovery in CF, AOP, and AOPdp/dt (Table 1). The results also show (Table 1) that 50 μmol/kg of SnPPIX, an HO enzyme inhibitor, abolished the postischemic recovery of cardiac function in comparison with the observed protection in the HO-1 Tg group. Thus, the values obtained in the HO-1 Tg + SnPPIX group were not statistically significant in comparison with the NTg values (Table 1).

Figure 3 depicts representative curves of endogenous CO production detected by GC in NTg, Tg, and Tg SnPPIX treated myocardium subjected to 20 min ischemia followed by 120 min of reperfusion. It is shown that in HO-1 Tg hearts subjected to 20 min of ischemia followed by 120 min of reperfusion (Fig. 3.B chromatogram), a substantial increase in CO production was observed in comparison with the NTg myocardium (Fig. 3. A chromatogram). However, the endogenous production of CO in the HO-1 Tg myocardium treated with SnPPIX was detected at relatively low level. (Fig. 3. C chromatogram)

Figure 4 shows representative pictures of infarct size (Fig. 4. A, lower part, to the right) and immunohistochemical staining of HO-1 (Fig. 4. A, lower part to the left) in isolated mouse hearts subjected to ischemia and reperfusion. The infarct size (Fig. 4. A) was markedly reduced from its NTg control value of 37±4% to 20±6% (*$p<0.05$) in the HO-1 Tg group

(Fig. 4. A). However, in the HO-1 knock out hearts, the infarct size was significantly increased to 47±5% (*$p<0.05$) in comparison with NTg group (Fig. 4. A).

In addition, Figure 4 A shows the representative immunohistochemical localization of HO-1 (Fig. 4. A, lower part, to the left) in NTg, Tg, and HO-1 KO ischemic/reperfused mouse hearts. Left ventricular cardiac samples were obtained from NTg, HO-1 Tg, and HO-1 KO mouse myocardium, respectively. Cytoplasmic protein staining (blue) of HO-1 in NTg myocardium was detected (Fig. 4. A, lower part, to the left) which was more intense in Tg myocardium, and markedly reduced in KO myocardium after 20 min of ischemia followed by 120 min of reperfusion (Fig. 4. A lower part, to the left).

Figure 4 also shows the incidence (%) of reperfusion-induced VF (Fig. 4 B) as total (sustained and nonsustained) and sustained VF in nontransgenic, transgenic, and knock out mouse groups. The incidence of reperfusion-induced total and sustained VF (Fig. 4 B) was significantly reduced from their nontransgenic (NTg) control values of 92% and 83% to 25% (*$p<0.05$) and 8% (*$p<0.05$), respectively, in the HO-1 Tg group. In the knock out group (Fig. 4 B), the incidence of reperfusion-induced total and sustained VF were 100% and 100%, respectively, showing the important role of HO-1 in arrhythmogenesis.

Table 2 clearly shows that left ventricular tissue Na^+, K^+, and Ca^{2+} contents were not significantly varied between NTg, HO-1 Tg, and HO-1 Tg + SnPPIX groups before the induction of ischemia. However, the results (Table 2) also depict that left ventricular tissue Na^+ and Ca^{2+} contents were significantly reduced after 20 min of ischemia followed by 120 min of reperfusion in the HO-1 Tg group in comparison with the NTg and HO-1 Tg + SnPPIX values. In addition, the left ventricular tissue K^+ content (Table 2) was significantly elevated (261±8 μmol/g dry weight, $p<0.05$) in the HO-1 Tg group compared to the K^+ loss measured in the NTg group (229±5 μmol/g dry weight).

In hearts treated with SnPPIX, the ischemia/reperfusion resulted in the same maldistribution in cellular Na^+, K^+, and Ca^{2+} contents to those of the NTg group (Table 2). In other worlds, SnPPIX completely abolished the cardiac protection detected concerning the cellular Na^+ and Ca^{2+} gains, and K^+ loss in the HO-1 Tg group in the ischemic/reperfused myocardium. The values measured in the SnPPIX group were the same to the NTg mouse hearts (Table 2) after 20 min of ischemia followed by 120 min of reperfusion.

DISCUSSION

Myocardial ischemia/reperfusion-induced damage is a major cause of morbidity and mortality in the Industrialized Society, and considerable efforts have been made to target interventional therapies for this condition. In recent years, the concept of the modification in various gene expressions or repressions has emerged as new mechanisms and therapeutic tools for the induction of protection in ischemic/reperfused tissues. It is now also relatively well accepted that many human vascular diseases such as hypertension [32,33], heart failure [34-36], organ transplantation [9,37], and arrhythmias [38,39] can be treated with various interventions at a level of underlying genetic mechanisms. Several approaches have been made to manipulate cell phenotypes including the inhibition of genes associated with cell proliferation by preventing the development of atherosclerosis and restenosis [40-42] and the production of growth factors to promote angiogenesis [43-47] for the treatment of peripheral vascular disease and myocardial ischemia.

In our previous studies, we found a substantial reduction in the expression of HO-1 mRNA and its protein with a causative reduction in the HO-1 enzyme activity in fibrillated ischemic/reperfused rat myocardium [13]. Moreover, we have shown that, in this respect, no difference exists between nondiabetic and diabetic heart [48]. Furthermore, HO-1 knock out mouse hearts displayed a reduced left ventricular function after ischemia/reperfusion [14]. If HO-1 system has a crucial role in the protection against ischemic/reperfusion-induced injury in the myocardium, with the enhanced expression of the HO-1, the more ischemic tissue can be salvaged. Therefore, in the present study, because of the HO-1 system can play a crucial role in the pathology of the ischemic/reperfused myocardium, we decided to approach the question from a different angle using HO-1 transgenic mouse hearts.

We generated HO-1 overexpressing Tg mice with an elevation in baseline HO enzyme activity by about 50% in the LV, RV, and S, respectively. Thus, the application of a genome-

based transgene resulted in expression levels in relation with increased HO enzyme activities in comparison with those of HO activities levels detected in the LV, RV, and S obtained from NTg mice. Although, in our studies, the very similar homology between the mouse and rat HO-1 made it impossible to differentiate the HO-1 and Tg-induced enzyme activities, but we were able to distinguish them at mRNA level. In addition, our results obtained in NTg, HO-1 Tg, and Tg-SnPPIX treated mouse hearts, we favor the idea that increased endogenous CO production levels in the myocardium represents the major signal transduction responsible for HO-1 related protection reflected in the improvement of the recovery in postischemic cardiac function, reduction of the incidence of reperfusion-induced VF and infarct size connected to the recovery of cellular ion contents. Our results further supported by the complete loss of cardiac protection in the Tg ischemic/reperfused mice treated with SnPPIX, the inhibitor of HO enzyme.

The exact mechanism by which HO-1 induces cardiac protection remains to be elucidated, however, one of the most significant protective mechanisms of the HO system can be explained by the generation of endogenous CO and its signaling mechanism. CO could suppress cell apoptosis [37] suggesting that the antiapoptotic effect of HO-1 is mediated via the generation of CO. In addition, anti-inflammatory property of CO suppresses the expression of proinflammatory genes associated with the activation of monocyte/Mϕ [49].

Another possible cellular signaling mechanism of CO is associated with the guanylyl cyclase activation and tissue levels of cGMP and cAMP in isolated ischemic/reperfused myocardium [50]. Very low concentrations of CO, in the perfusion buffer, afford a significant protection against the ischemia/reperfusion-induced damage in isolated buffer perfused hearts. The isolated buffer perfused heart could be an ideal model to study the direct effect of exogenous CO on cardiac function and molecular signaling because the blood and its elements are excluded from the model, thus the oxygen transport to cells and tissues is not

directly damaged via the CO/blood/haemoglobin system. Previous results have shown that low concentrations of exogenous CO protect the isolated ischemic/reperfused myocardium and act by increasing tissue cAMP and cGMP levels [50]. Indeed, a moderate increase in cAMP content could lead to arrhythmogenesis and development of various arrhythmias by elevating cytosolic calcium levels [51] in the ischemic and postischemic myocardium. It is of interest to note that multiple increases in cGMP levels could mask and interfere with the arrhythmogenic effects of cAMP leading to the suppression of reperfusion-induced VF in the myocardium [50]. The significant increase in cGMP levels can be related to guanylate cyclase activities in CO-treated myocardium, and suggests that induction of guanylate cyclase-cGMP system via CO signaling is essential for cardioprotection in ischemic/reperfused hearts. However, not only the absolute levels of cGMP and cAMP are the critical factors after ischemia and reperfusion that determine the recovery of postischemic cardiac function, but also the ratio of these two nucleotides [52]. Thus, the lower cAMP/cGMP ratio during ischemia, the better the postischemic cardiac recovery is achieved in isolated ischemic/reperfused hearts.

"It has to be noted, that Lakkisto et al., have recently found that cadioprotection induced by hemin, a well known inducer of hemoxigenase 1, is independent of cGMP levels and, at least by part, being mediated via the preservation of Cx43 [53]. They found that elevated HO-1 levels, induced by hemin, are not accompanied by elevated cGMP levels. In our previous study [50], we have found that cGMP levels are elevated after exogenous CO application, moreover, after exogenous CO administration the ratio of cAMP/cGMP is reduced. In their study [53] the authors measured only the level of cGMP and not the ratio of cAMP/cGMP. The difference between their and our results is probably due to the higher CO concentration after the exogenous administration.

Although not specifically studied in the present investigation at molecular level, it is of interest to note the findings of Piantadosi et al. [54] with myocardial cells. In their studies, Piantadosi et al., implicated the role of HO-1/CO pathway in mitochondrial biogenesis through Akt1 activation [55] involving Nrf2 expression, downstream GSK3β blockade, and Nrf2 nuclear translocation leading to Nrf2-dependent activation of NRF-1 transcription. Their finding suggest HO-1/CO , by sequentially activating the aforementioned two transcription factors, as a remarkable component of a prosurvival program of mitochondrial biogenesis connected to cellular antioxidant defense mechanisms [56,57].

Today, when so many advances and efforts are being made in molecular biology and genetics, we tend to lose sight of the importance of basic ions such as Na^+, K^+, and Ca^{2+} in both clinical and experimental studies. As a gap, in changes between various gene expression and/or repression and the function of myocardial tissue, could closely be connected with changes in myocardial tissue ion contents. An important finding such a final endpoint of our present study was the myocardial "K^+ – conserving effect" related to the prevention of cellular Na^+ and Ca^{2+} gains in Tg myocardium, and this cardiac protection can be reversed by SnPPIX, an HO-1 inhibitor. HO-1 – bilirubin system and one of its byproduct, CO, are essential for regulation of cardiac function and ion transport across cell membranes via the stabilization of various Na, K, and Ca ion channels possibly by an effect exerted on Na-K-ATPase, and these transient outward and inward currents are thought to underline the delayed after depolarization that can be observed after the action potential in Ca-loaded cardiac tissue propagating rhythm disturbances [58]. There are significant differences in the cellular signaling mechanisms induced by CO when they are compared with the actions of other interventions that elevate cyclic nucleotides in tissues. CO has been proposed to increase K_{Ca} channel Ca^{2+} sensitivity in arterial smooth muscle cells [59], and the data of Perez et al., [60] suggest that CO increases Ca^{2+} spark coupling by shifting the Ca^{2+} sensitivity of K_{Ca} channels

produced by Ca^{2+} sparks. Thus, CO may act K_{Ca} channel beta subunits to enhance the coupling relationship leading to cellular K^+ loss, and Ca^{2+} and Na^+ gains as we observed in our present studies.

In our study we have shown that CO level was significantly increased in the HO-1 Tg mouse myocardium. However, we did not measure CO levels during reperfusion, therefore, we believe that the elevated CO production can be responsible for the protection during reperfusion in Tg myocardium. Beside this, we cannot rule out the contribution of other HO-1 related molecules such as biliverdin and bilirubin to the observed cardiac protection.

In conclusion, using HO-1 transgenic mice, we have demonstrated the direct causative role of HO-1 in protection against ischemia/reperfusion-induced injury. Specifically, our results show a significant recovery of postischemic cardiac function, prevention of the development of reperfusion-induced VF, and reduction in infarct size. In addition, we have provided new insights into the ionic mechanisms by which HO-1 may contribute to the attenuation of reperfusion-induced arrhythmias in HO-1 transgenic mice. We believe that these studies would help in developing novel therapies based on targeting HO-1 for the treatment of ischemia/reperfusion injury in patients with coronary artery disease.

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The authors confirm that there are no conflicts of interest.

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LEGENDS FOR FIGURES:

Fig. 1. Schematic presentation of the experimental time course.

Fig. 2. The detection of rat HO-1 transgene by PCR **(A)** and Western blot **(B),** and HO enzyme activities (**C**) in mouse hearts. Total RNA obtained from mouse left ventricle (LV), right ventricle (RV), and septum (S), respectively, was used to synthesize first strand cDNA. HO-1 originated from rat was amplified by PCR exon 5-specific primer and visualized with ethidium bromide as a 166-bp band by agarose gel electrophoresis. In two clones, the specific band was amplified from RNA samples of RV, LV, and S, respectively, in transgene (transgene 1 and transgene 2) mice (**A**, upper and middle parts), but it was absent in the RNA sample of NTg littermate (**A**, lower part). Western blots (**B**) also depict the expression of rat transgene 1, transgene 2, and NTg in the LV, RV, and S of mouse hearts. HO enzyme activities are also shown in the LV, RV, and S in NTg and Tg myocardium (**C**). n=6 in each group, MEAN ± SEM, *$p<0.05$, comparisons were made to the NTg values in each group.

Fig. 3. Representative GC chromatograms for the demonstration of endogenous CO production in nontransgenic (NTg, chromatogram **a)**, HO-1 transgenic (HO-1 Tg, chromatogram **b)**, and HO-1 Tg mouse myocardium treated with SnPPIX (HO-1 Tg + SnPPIX, chromatogram **c**). Hearts were subjected to 20 min of normothermic global ischemia followed by 120 min of reperfusion. The results show, in NTg myocardium (chromatogram **a**), that there is a well detectable endogenous CO production after 20 min of ischemia followed by 120 min of reperfusion. The endogenous CO production was substantially increased in HO-1 Tg myocardium (chromatogram **b**), and a reduction of endogenous CO production was recorded in HO-1 myocardium treated with SnPPIX (chromatogram **c**).

Fig. 4. Infarct size and HO-1 staining in isolated nontransgenic (NTg), transgenic (Tg), and HO-1 knock out (KO) mouse hearts subjected to 20 min of normotermic global ischemia followed by 120 min of reperfusion. * $p<0.05$ compared to the NTg control values. n=6 in each group. Infarct is represented by white area surrounding by the living red tissues (**A**, to the right lower part). Immunochemical localization of HO-1 is stained by blue in NTg, Tg and KO myocardium (**A**, to the left lower part). **B** shows the incidence (%) of total (open bars) and sustained (hatched bars) reperfusion-induced VF in isolated NTg, Tg, and KO mouse hearts. The incidence of reperfusion-induced VF was registered, and comparisons were made to the values of NTg group. n=12 in each group, *$p<0.05$. Because of the nonparametric distribution in the incidence of total and sustained VF, the chi-square nonparametric test was used to compare individual groups. *This Figure was presented at the FEAM Meeting held in Bucharest, Rumania, in March, 2010, and published in the May issue of the JCMM, 2010, as the Proceedings of the Meeting.*

Table 1. Effects of 20 min of ischemia followed by 30 min and 120 min of reperfusion on the recovery of cardiac function in none transgenic (NTg) and HO-1 transgenic mice (Tg), and the effect of SnPPIX.

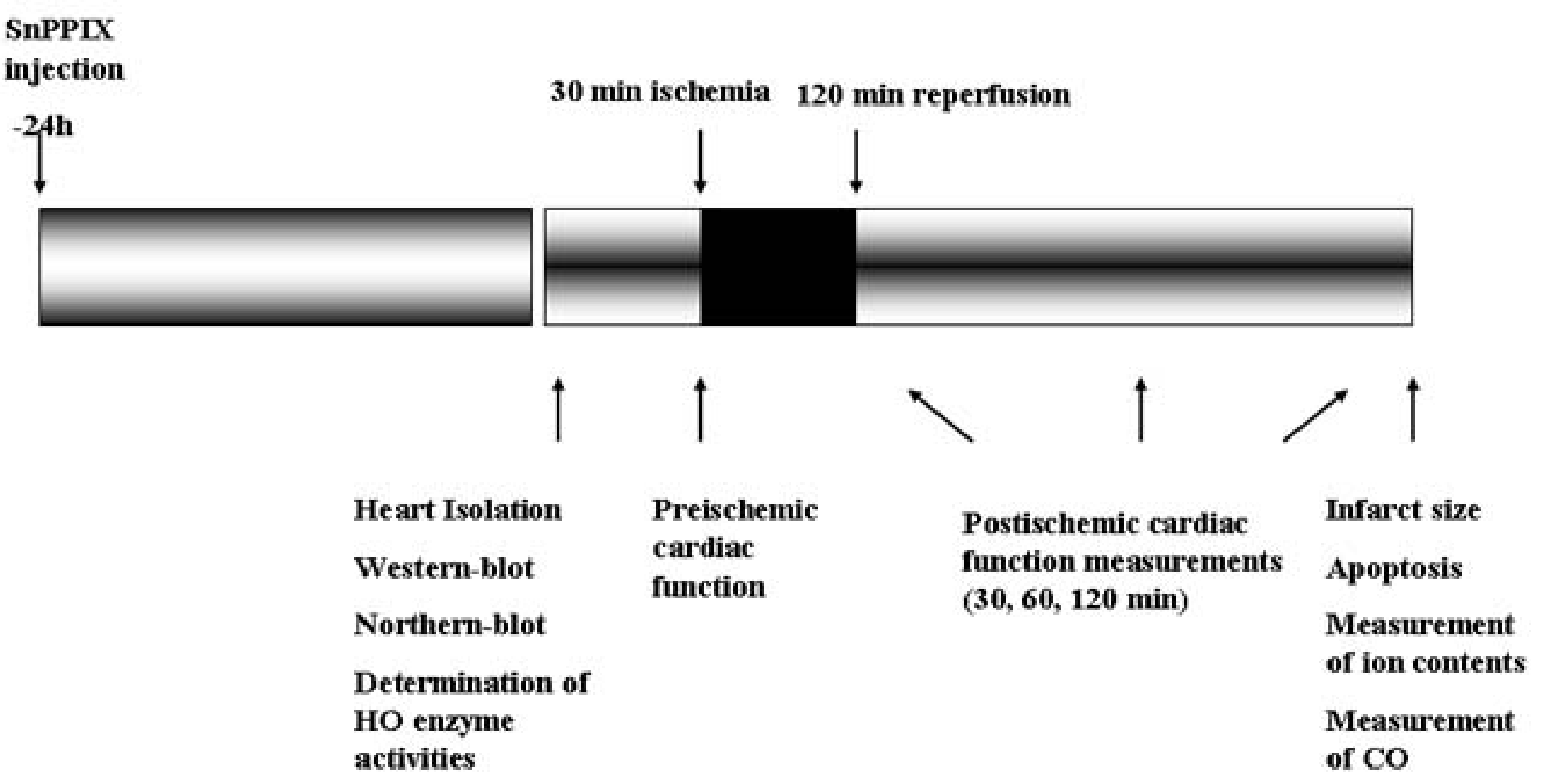
	Before Ischemia					After reperfusion (RE) 30 min					120 min				
	HR	**CF**	**AF**	**AOP**	**AOPdp/dt**	**HR**	**CF**	**AF**	**AOP**	**AOPdp/dt**	**HR**	**CF**	**AF**	**AOP**	**AOPdp/dt**
NTg	288±9	3.0±0.2	3.5±0.2	157±7	3794±73	255±9	2.0±0.1	0.9±0.1	72±6	945±44	262±9	2.1±0.1	0.8±0.1	67±4	930±51
HO-1 Tg	292±8	3.2±0.3	3.4±0.2	149±6	3834±85	261±7	2.8±0.1*	2.3±0.2*	95±7*	2000±63*	269±8	2.9±0.2*	2.2±0.2*	91±6*	1880±80*
HO-1 Tg + SnPPIX	294±9	3.1±0.2	3.2±0.3	152±9	3815±94	265±8	2.2±0.2	1.1±0.2	78±7	1018±80	270±9	2.0±0.3	1.1±0.2	72±8	1000±77

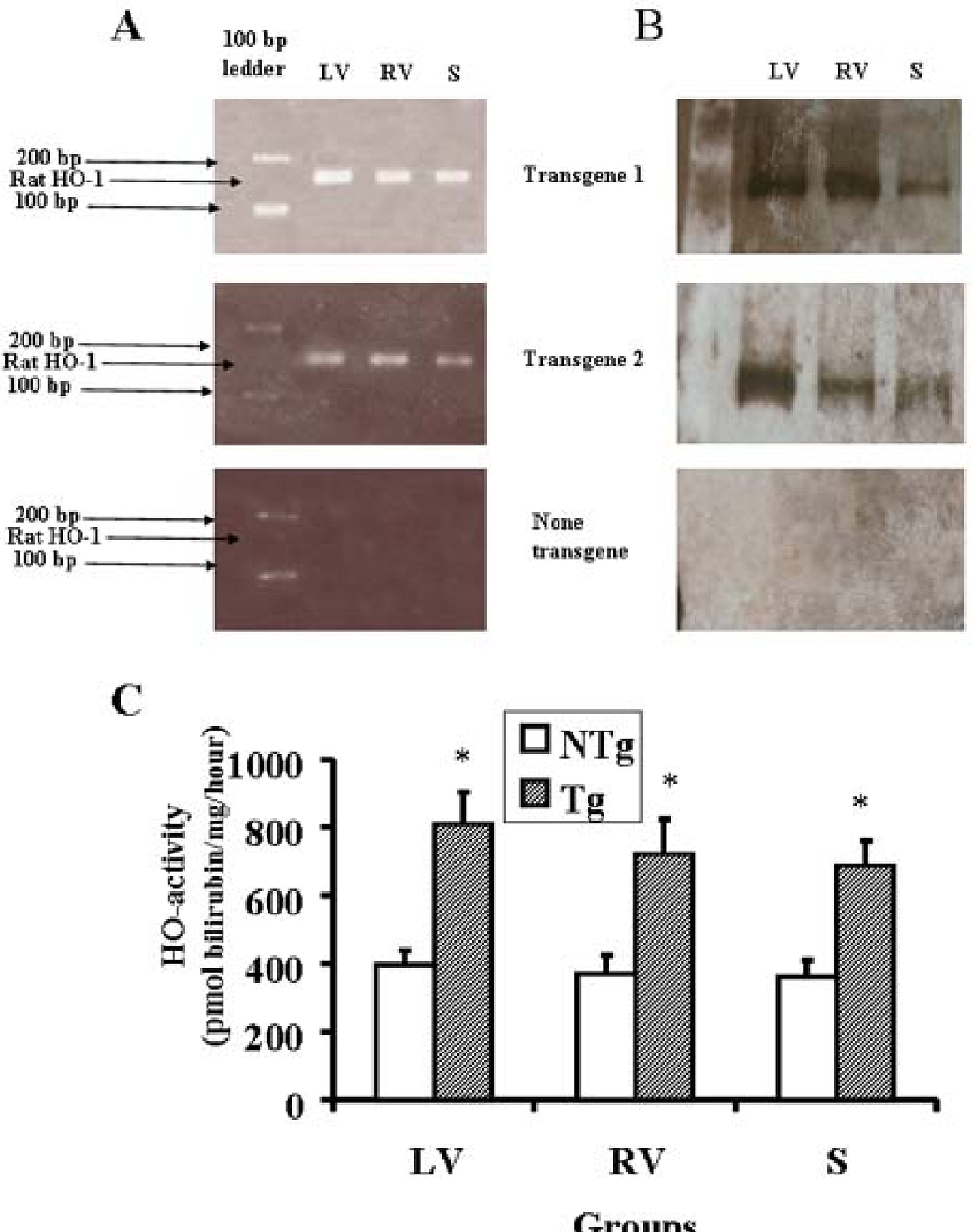
n= 6 in each group, MEAN ± SEM, Heart rate (HR, beats/min), Coronary flow (CF, ml/min), Aortic flow (AF, ml/min), Aortic pressure (AOP, mmHg), First derivative of aortic pressure (AOPdp/dt, mmHg/s). Tin protoporphyrin IX (SnPPIX). *p< 0.05, comparisons were made to the time-matched none transgenic NTg values.

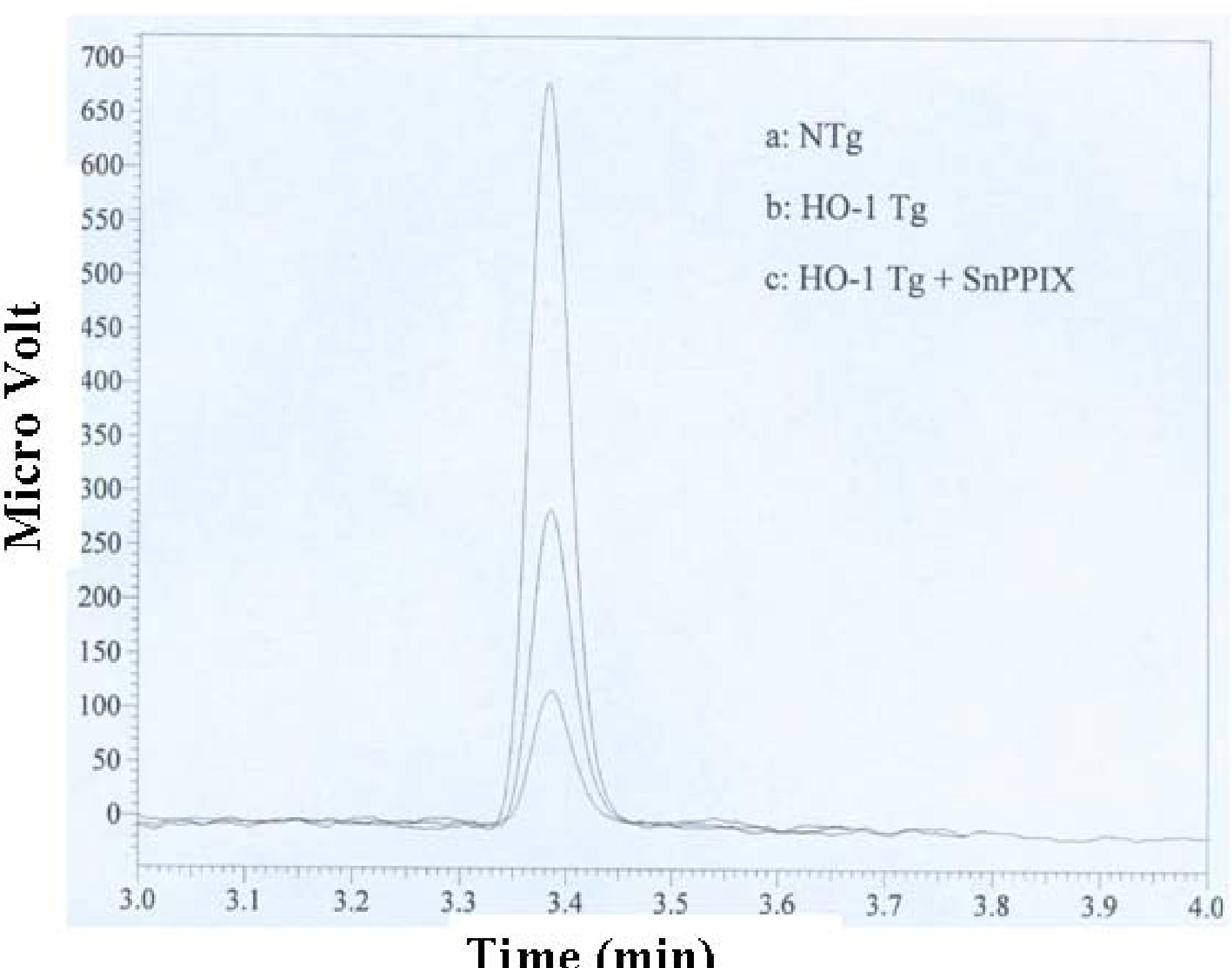
Table 2. Cellular Na^+, K^+, and Ca^{2+} contents (μmol/g dry weight) in the left ventricular tissue before ischemia and after 20 min of ischemia followed by 120 min of reperfusion in none transgenic (NTg) and HO-1 transgenic mice (Tg), and the effect of SnPPIX.

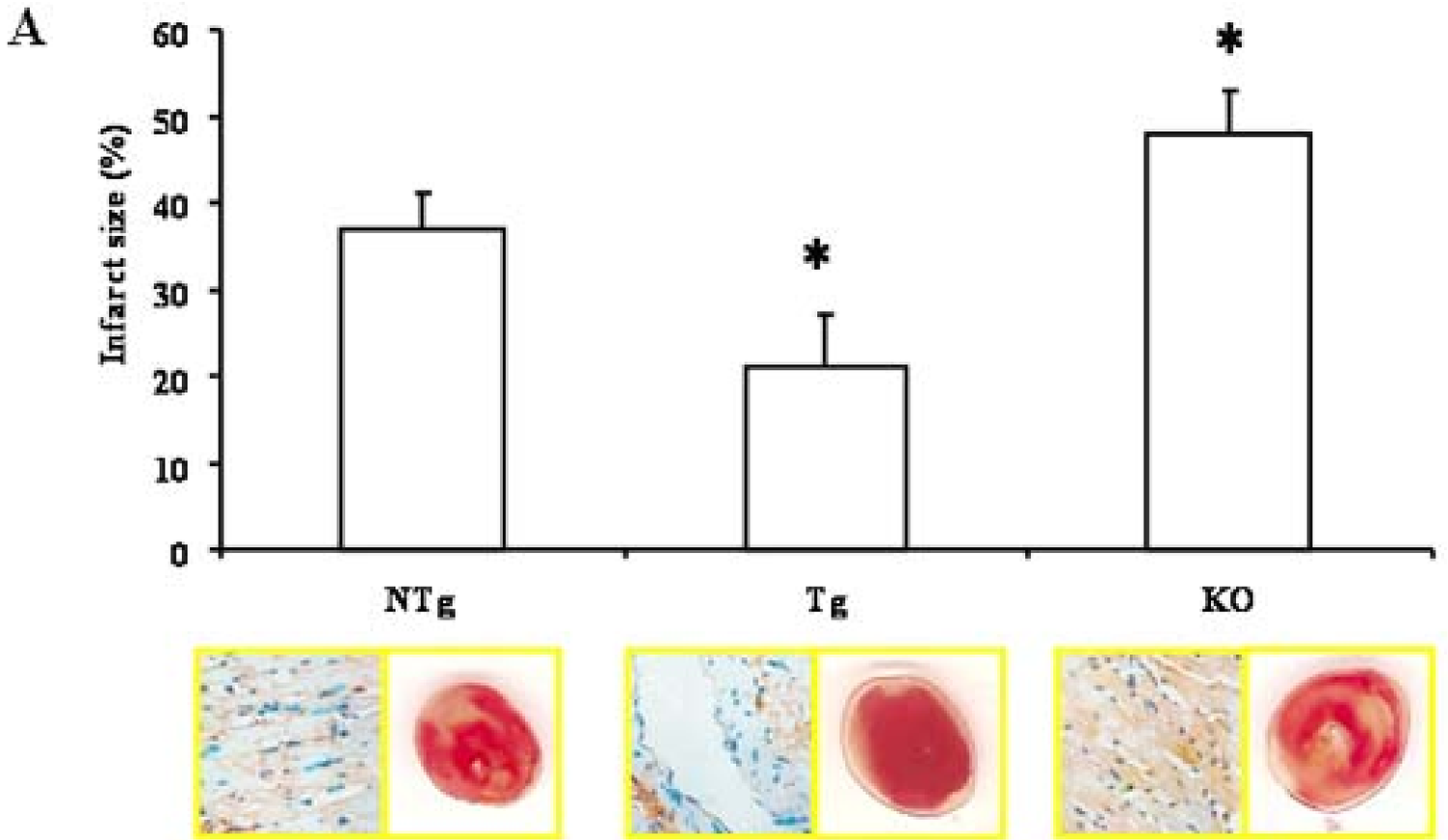
Group	Before ischemia			After 20 min ISA followed by 120 min RE		
	Na^+	K^+	Ca^{2+}	Na^+	K^+	Ca^{2+}
NTg	31±3	282±6	1.6±0.1	84±5	229±5	4.1±0.3
HO-1 Tg	27±4	277±7	1.7±0.2	58±7*	261±8*	2.5±0.5*
HO-1 Tg + SnPPIX	35±5	286±9	1.9±0.3	90±9	219±9	4.5±0.7

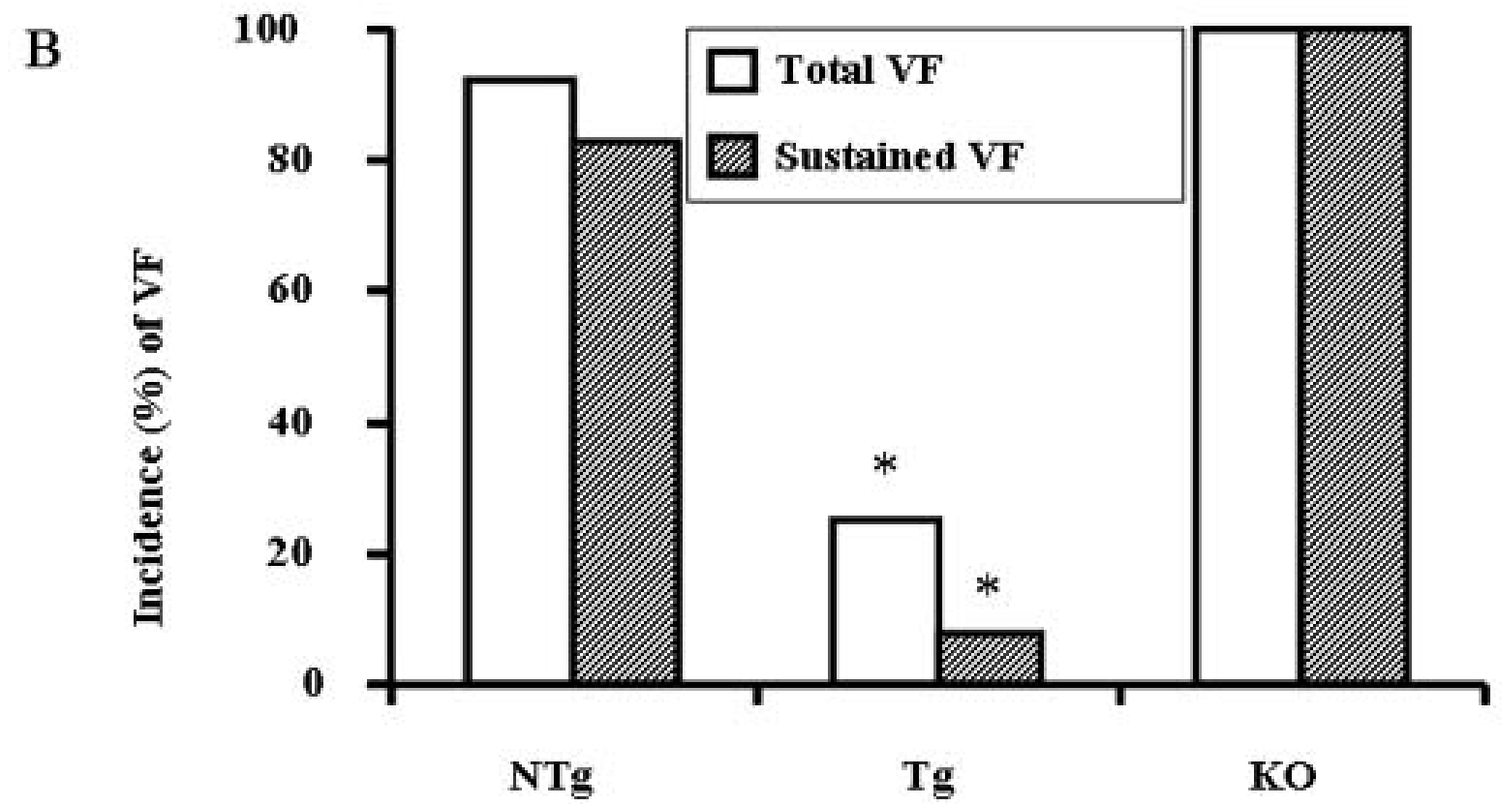
n= 6 in each group, MEAN ± SEM, *p< 0.05, comparisons were made to the time-matched none transgenic (NTg) values. Tin protoporphyrin IX (SnPPIX). Ischemia (ISA), Reperfusion (RE).


SnPPIX
injection
-24h
30 min ischemia
120 min reperfusion
Heart Isolation
Western-blot
Northern-blot
Determination of
HO enzyme
activities
Preischemic
cardiac
function
Postischemic cardiac
function measurements
(30, 60, 120 min)
Infarct size
Apoptosis
Measurement
of ion contents
Measurement
of CO


A
100 bp ledder
LV
RV
S
200 bp
Rat HO-1
100 bp
200 bp
Rat HO-1
100 bp
200 bp
Rat HO-1
100 bp
B
LV
RV
S
Transgene 1
Transgene 2
None transgene
C
HO-activity (pmol bilirubin/mg/hour)
1000
800
600
400
200
0
NTg
Tg
*
*
*
LV
RV
S
Groups


a: NTg
b: HO-1 Tg
c: HO-1 Tg + SnPPIX
Micro Volt
Time (min)
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
3.0
3.1
3.2
3.3
3.4
3.5
3.6
3.7
3.8
3.9
4.0


A
Infarct size (%)
60
50
40
30
20
10
0
*
*
NTg
Tg
KO


B
Incidence (%) of VF
100
80
60
40
20
0
Total VF
Sustained VF
*
*
NTg
Tg
KO