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Heme oxygenase-1 related carbon monoxide production and ventricular fibrillation in isolated ischemic/reperfused mouse myocardium

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ABSTRACT

Heme oxygenase-1 (HO-1)-dependent carbon monoxide (CO) production related to reperfusion-induced ventricular fibrillation (VF) was studied in HO-1 wild-type (+/+), heterozygous (+/–), and homozygous (–/–) isolated ischemic/reperfused mouse heart. In HO-1 homozygous myocardium, under aerobic conditions, HO-1 enzyme activity, HO-1 mRNA, and protein expression were not detected in comparison with aerobically perfused wild-type and heterozygous myocardium. In wild-type, HO-1 hetero- and homozygous hearts subjected to 20 min ischemia followed by 2 h of reperfusion, the expression of HO-1 mRNA, protein, and HO-1 enzyme activity was detected in various degrees. A reduction in the expression of HO-1 mRNA, protein, and enzyme activity in fibrillated wild-type and heterozygous myocardium was observed. In reperfused/nonfibrillated wild-type and heterozygous hearts, a reduction in HO-1 mRNA, protein expression, and HO-1 enzyme activity was not observed, indicating that changes in HO-1 mRNA, protein, and enzyme activity could be related to the development of VF. These changes were reflected in the HO-1-related endogenous CO production measured by gas chromatography. In HO-1 knockout ischemic/reperfused myocardium, all hearts showed VF, and no detection in HO-1 mRNA, protein, and enzyme activity was observed. Thus, interventions that are able to increase endogenous CO may prevent the development of VF.

Key words: heme oxygenase-1 expression • ischemia/reperfusion • HO-1 knockout mouse hearts

It has been proposed that most cases of sudden cardiac death may result from ischemia and/or reperfusion-induced ventricular fibrillation (VF; 1–3). Interest in the development and pharmacological control of reperfusion-induced VF 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 (4). There is considerable controversy over the mechanisms responsible for the induction of these arrhythmias, and a number of

different mechanisms have been suggested (5), but the two major factors proposed and generally accepted to explain reperfusion-induced injury and VF are *1*) calcium overload and *2*) free radical formation. Examples of oxidative stress-related diseases include reperfusion-induced injury occurring after tissue ischemia or stroke and inflammatory processes, such as arthritis (6). Heme oxygenase (HO) catalyzes the rate-limiting step in the oxidative degradation of heme to biliverdin and carbon monoxide (CO; ref 7). Three isozymes of HO have been identified and cloned; HO-1, an inducible form, HO-2, and HO-3 constitutive forms (8). Studies demonstrate that HO-1 is induced in response to various interventions causing oxidative stress, including ultraviolet irradiation, hypoxia, and ischemia (6, 9–12).

In previous studies, we observed a reduction in HO-1 mRNA expression, its protein, and enzyme activity in ischemic/reperfused fibrillated myocardium but not in nonfibrillated ischemic/reperfused hearts (13). Therefore, in the present study, we decided to approach the question from a different angle using wild-type (+/+), HO-1 heterozygous (+/–), and homozygous (–/–) mouse hearts. The aforementioned findings have led us to speculate that the vulnerability of HO-1 knockout subjects' myocardium to VF may be related to HO-1 mRNA, its protein expression, enzyme activity, and HO-1-related endogenous CO production. If this were so, we would stress that the absence of HO-1 mRNA and its protein 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 the absence of HO-1 mRNA, its protein, enzyme activity, and HO-1-mediated endogenous CO production.

Although many mechanisms have been proposed to explain the causes of arrhythmias, relatively little work has been done, to our knowledge, to clarify the mechanism(s) of VF at a gene expression level in ischemic/reperfused myocardium. The long QT disease and idiopathic VF, as known up to now, are the only cardiac disorders based on genetic mutation and cause sudden cardiac death from ventricular arrhythmias (14–16). Thus, our study may offer a further understanding of the arrhythmogenic mechanism(s) at a molecular level and identify the responsibility of HO-1 and HO-1-mediated CO formation for arrhythmogenesis in ischemic/reperfused hearts.

METHODS

Animals and heart preparation

Male mice (25-35 g), wild-type (+/+), heterozygous (+/–), and homozygous mutant (–/–) HO-1 knockout, 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 1985). Mice were anaesthetized with 50 mg/kg of pentobarbital sodium. After intraperitoneal administration of heparin (1,000 IU/kg), the chest was opened and the heart was rapidly excised and mounted to a "working" perfusion apparatus described by Hewett et al. (17). 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%).

Registration of VF and measurement of cardiac function

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

Determination of infarct size

Hearts for infarct size measurement were perfused, at the end of each experiment, with 15 ml of 1% triphenyl tetrazolium (TTC) solution in phosphate buffer (88 mM Na_2HPO_4, 1.8 mM NaH_2PO_4) via the side arm of the aortic cannula and then stored at -70°C for later analysis. Frozen hearts were sliced transversely (18) in a plane perpendicular to the apico-basal axis into 2-3 mm thick sections, weighted, blotted dry, placed in between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single pass flat bed scanner (Hewlett-Packard, Palo Alto, CA). With the use of 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 (19). The areas were measured by computerized planimetry software, these areas were multiplied by the weight of each slice, and then the results were summed up to obtain the weight of the risk zone (total weight of the left ventricle, mg) and the infarct zone (mg). Infarct size was expressed as the ratio, in percent, of the infarct zone to the risk zone.

Determination of CO content

We measured tissue CO content using gas chromatography as described by Cook et al. (20). Briefly, hearts were removed at various time points (before the induction of ischemia and after 5, 15, 30, and 120 min of reperfusion, respectively) from the perfusion set up and homogenized in 4 volumes of 0.1 M phosphate-buffer (pH: 7.4) using an x520 homogenizer (Ingenieurburo CAT, M. Zipperer GmbH, Staufen, Germany). The homogenates were centrifuged at 4°C for 15 min at 12,800 *g*, and the supernatant fractions were used for the determination of tissue CO. The reaction mixtures contain: 150 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. The samples were preincubated at 37°C for 5 min, then the headspace was purged, and the incubation was continued for 1 h in dark at 37°C. The reaction was stopped by placing the samples on ice, and the headspace gas was analyzed. One-thousand microliters of the headspace gas from each vial was injected into the gas chromatograph using a gastight syringe (Hamilton Co.) in hydrogen gas flow with a speed of 30 ml/min. Analysis took place during the next 150 s 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 millivolts, and then the area of the peak was integrated and expressed in arbitrary units. The column was packed with Molselect 5 Å and maintained at 30°C. The temperature of the injector and detector was controlled and kept at 50°C.

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

Cellular electrolytes were measured as described previously (21). In brief, hearts were rapidly cooled to 0-5°C being submerged 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 washout ions from the extracellular space and to stop the activity of membrane enzymes responsible for membrane ion transports. Five minutes of cold washing of the heart washes out > 90% of the ions from the extracellular space (22). After the washout, hearts were dried for 48 h at 100°C and made ash at 550°C for 20 h. The ash was dissolved in 5 ml of 3 M nitric acid and diluted 10-fold with deionized water. Myocardial Na^+ was measured at a wavelength of 330.3 nm, K^+ at 404.4 nm, and Ca^{2+} at 422.7 nm in air-acetylene flame using an atomic absorption spectrophotometer (Perkin-Elmer 1100-B). The method for the determination of myocardial ion contents has been described previously (22, 23).

Immunohistochemistry

Paraffin sections (5 μm) of left ventricular tissue were incubated in the presence of polyclonal antibody and purified liver HO-1 obtained from rats (Stress Gen Biotech.). Reactions were visualized by immunoperoxidase color reaction in wild-type (+/+), heterozygous (+/–), and homozygous (–/–) HO-1 mutant mice. Formalin fixed tissues were paraffin embedded, and 5 μm sections were placed on poly-L-lysine coated glass slides (Sigma, St. Louis, MO). After 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 h with HO-1 antibody at a dilution of 1:750 as described in the manufacturer's manual. After being washed in PBS for a few minutes, slides were incubated for additional 30 min in the presence of purified biotinylated anti/rabbit IgG (Vector) at a dilution of 1:200. Slides were rewashed in PBS and incubated with peroxidase conjugated streptavidin (Zymed) 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 s, rinsed with water, and dipped in 1% of lithium carbonate. After the water was drained, slides were placed in oven for 30 min at 80 C^o and then covered with permount coverslips. Sections were photographed using a Zeis (Germany) light microscope.

Total RNA isolation

Total RNA was isolated from heart tissue (100 mg) by homogenization in 1 ml of TRIzol reagent (Gibco-BRL, Life Technologies, Eggenstein, Germany), a guanidium thiocianate method (24), as described in the manual. The RNA pellets were dissolved in 100 μl diethyl pyrocarbonate water, and the concentrations were calculated from the absorbance at 260 nm measured by ultraviolet spectroscopy (Beckman DU 640, Beckman Instruments Inc., Fullerton, CA).

Northern blot

Thirty micrograms of total RNA were transferred to a nylon membrane according to Sambrook et al. (25), and Northern blot was carried out as described previously by Pataki et al. (13). Purified cDNAs were labeled with α–[^{32}P]dCTP by random hexamer priming. Blots were prehybridized for 2 h in 50% formamide, 5x Denhardt's solution, 100 μl herring sperm DNA, and 0.5% SDS, 0.9 M sodium chloride, and 0.09 M sodium citrate at 42°C. Blots were hybridized with cDNAs, labeled to a specific activity of 3 x 10^8 cpm/μg in hybridization fluid at 42°C overnight. Hybridized blots were washed in 0.3, 0.15, and 0.015 M sodium chloride; 0.03, 0.015, and 0.0015 M sodium citrate, respectively, pH 7.0; and 0.1% SDS, for 20 min at 50°C. Kodak Biomed MR-1 films were exposed for 3-4 days with the use of an intensifying screen and kept at -80°C. Blots were washed and reprobed with different cDNAs using the hybridization and washing conditions described above.

Western blot

Myocardial samples were homogenized in Tris-HCl (13.2 mM/l), glycerol (5.5%), SDS (0.44%), and β-mercaptoethanol. The same amount of soluble protein (50 μg) was fractionated by Tris-glycine-SDS-polyacrylamide gel (12%) electrophoresis, and Western blot was carried out as described by Pellacani et al. (26) with the use of an antibody to recombinant rat HO-1 protein.

Experimental time course

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

Statistics

The data for HR, CF, AF, AOP, AOPdp/dt, infarct size, myocardial electrolyte contents, and tissue CO were expressed as means ± SE. One-way ANOVA was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of wild-type groups were compared with those of HO-1 heterozygous and homozygous groups by multiple t test followed by Bonferroni correction. A change of $P < 0.05$ was considered to be statistically significant.

RESULTS

HO-1 knockout mice

The low HO-1 (–/–) mice survival percentage (~18%) was maintained in matings between HO-1 (+/–) and HO-1 (–/–) mice, which yielded 105 HO-1 (+/–) and 13 HO-1 (–/–) mice; however, matings between HO-1 (–/–) males and HO-1 (–/–) females did not yield viable litters. Thus, we got the necessary numbers of HO-1 (–/–) mice, for the present study, in matings between HO-1 (+/–) and HO-1 (–/–) animals. Mice lacking HO-1 gene were slightly lighter (26±3 g) than HO-1 (+/+) (30±4 g) or HO-1 (+/–) (31±3 g) age-matched littermates from birth to early adulthood (12-13 wk) but were otherwise indistinguishable. However, we observed that, as early as 22 wk of age, most HO-1 (–/–) mice became thin, lost weight, were poorly groomed, and appeared less active than HO-1 (+/+) or HO-1 (+/–) animals. Premature mortalities among HO-1 (–/–) mice after 22 wk of age were common. Therefore, in our studies, mice 16-18 wk of age were used in all groups.

Figure 1 shows the immunohistochemical localization of HO-1 (upper part) in nonischemic mouse heart. Left ventricular cardiac biopsies were obtained from a wild-type (+/+), a heterozygous (+/–), and a homozygous mutant (–/–) mouse heart, respectively, perfused under aerobic conditions and then subjected to immunochemical reaction as we described in Methods. Homogenous cytoplasmic staining (blue) of HO-1 in wild-type (+/+) nonischemic myocardium was observed after 20 min of aerobic perfusion (Fig. 1*a*). Figure 1*b* shows the same cytoplasmic HO-1 staining (blue) after 20 min of aerobic perfusion in the myocardium obtained from (+/–) mouse heart. In the homozygous (–/–) mutant mouse myocardium, cytoplasmic HO-1 staining was not detected under aerobic perfusion (Fig. 1*c*, lack of blue staining).

The middle part of Fig. 1 shows the corresponding Northern blot analysis of total mRNA from a wild-type (+/+), a heterozygous (+/–), and a homozygous mutant (–/–) mouse heart. Blots were hybridized with a rat HO-1 cDNA probe recognizing a major mRNA band of ~1.5 kb in both the (+/+) and (+/–) mouse myocardium, respectively. However, no HO-1 mRNA band was detected in the isolated homozygous mutant (–/–) aerobically perfused mouse heart.

The bottom part of Fig. 1 shows the corresponding Western blot analysis of HO-1 protein expression in wild-type, heterozygous, and homozygous mutant mouse myocardium, respectively. HO-1 protein expression was detected with the use of recombinant rat HO-1 antibody as described in Methods. Data clearly show that neither HO-1 mRNA nor its protein was detected in the homozygous (–/–) myocardium perfused under aerobic conditions.

HO-1 in ischemic/reperfused hearts

Figure 2 shows immunohistochemical detection of HO-1 enzyme (left and middle panels) and HO-1 protein expression (right panel) in hearts subjected to 20 min of ischemia followed by 120 min of reperfusion. Upon reperfusion, in wild-type (+/+) control mice (Fig. 2*A*), HO-1 enzyme localization was detected (blue staining) in nonfibrillated/reperfused myocardium (No-VF). However, in reperfused/fibrillated (VF) wild-type (+/+) myocardium, HO-1 enzyme detection by immunohistochemistry was reduced (less blue area). The same pattern, in HO-1 enzyme

detection, was obtained in the heterozygous (+/–) nonfibrillated (No-VF) and fibrillated (VF) hearts (Fig. 2*B*), indicating that there is no substantial difference between the localization of HO-1 enzyme in wild-type (+/+) and heterozygous (+/–) mouse myocardium. Upon reperfusion, because of the absence of HO-1 enzyme in homozygous (–/–) mice, the localization of HO-1 enzyme (the lack of blue area) was not possible in either nonfibrillated or fibrillated homozygous (–/–) myocardium (Fig. 2*C*). In homozygous (–/–) mouse studies, all hearts developed reperfusion-induced VF; therefore, electric defibrillation (Defib.) was necessary to measure myocardial function during reperfusion.

Figure 2 also shows HO-1 protein expression (right panel) in reperfused/nonfibrillated (No-VF) and fibrillated (VF) wild-type (+/+), HO-1 heterozygous (+/–), and HO-1 homozygous (–/–) mouse myocardium. Thus, corresponding to the immunohistochemical staining (left panel), HO-1 protein expression (right panel) was detected in ischemic/reperfused nonfibrillated (No-VF) wild-type (+/+) and nonfibrillated heterozygous (+/–) hearts. However, in HO-1 homozygous hearts (–/–), HO-1 protein was not detected (Fig. 2*C*) either in ischemic/reperfused nonfibrillated (electrically defibrillated) or fibrillated myocardium. It is important to note that in reperfused/fibrillated wild-type (+/+) mouse heart (Fig. 2*A*), a reduction in HO-1 protein expression was observed in comparison with the nonfibrillated myocardium. HO-1 protein detection was not recorded in heterozygous (+/–) fibrillated myocardium (Fig. 2*B*).

Endogenous CO detection in mouse myocardium

Figure 3 depicts representative curves of endogenous CO production detected by gas chromatography in wild-type (+/+), HO-1 heterozygous (+/–), and HO-1 homozygous (–/–) mouse myocardium subjected to 20 min ischemia followed by 30 and 120 min of reperfusion. It is shown that in wild-type (+/+) and heterozygous (+/–) hearts subjected to 20 min of ischemia followed by 30 min (Fig. 3*b* and *c* chromatograms) and 120 min (Fig. 3*d* and *e* chromatograms) of reperfusion, a substantial increase in CO production was observed in comparison with the nonischemic wild-type (+/+, Fig. 3*a* chromatogram) myocardium. However, in the homozygous (–/–) mouse myocardium (Fig. 3 *f* and g chromatograms), endogenous CO production because of the lack of the function of HO-1 system was detected at a low level. The results depicted in Fig. 3 were obtained from wild-type, heterozygous, and homozygous mice that showed no VF upon reperfusion, or hearts were immediately electrically defibrillated if it was necessary. However, in the presence of VF, endogenous CO production was not detected (data not shown).

Figure 4 shows the time course of endogenous CO production in (+/+) (*A*) and (–/–) (*B*) myocardium subjected to 20 min of ischemia followed by 5, 15, 30, and 120 min of reperfusion. The results clearly show that endogenous CO production was increased in reperfused/nonfibrillated myocardium (hearts were immediately defibrillated if it was necessary). However, a small amount of endogenous CO production was detected in homozygous (–/–) reperfused/nonfibrillated myocardium (Fig. 4*B*), indicating that other pathways may also play some role in endogenous CO production but that these pathways could be relatively unimportant in comparison with the HO-1-dependent pathway.

Cardiac function and infarct size

Before the induction of global ischemia (Table 1), a significant reduction in HR, CF, AF, AOP, and AOPdp/dt was observed in HO-1 knockout homozygous (–/–) mouse hearts in comparison with the corresponding values obtained in wild-type (+/+) and heterozygous (+/–) mouse myocardium. These significant changes, the same trend, in cardiac function were also detected between groups after 20 min of ischemia followed by 30 min and 120 min of reperfusion (Table 1). It is important to note that all hearts developed reperfusion-induced VF in the HO-1 knockout homozygous (–/–) group; therefore, all hearts were electrically defibrillated during reperfusion. Infarct size was significantly increased from 37.6 ± 4% (wild type) and 38.5 ± 4% (HO-1 heterozygous) to 49.5 ± 5% ($P<0.05$) in the HO-1 homozygous group.

Myocardial Na^+, K^+, and Ca^{2+} contents

The results (Table 2) show that tissue Na^+ and Ca^{2+} contents are significantly elevated before the induction of ischemia in the (–/–) myocardium in comparison with the (+/+) and (+/–) values. In addition, the cellular K^+ (Table 2) was significantly at a lower level (253±8 μmol/g dry weight) in the (–/–) group compared with the nonischemic (+/+) value (278±7 μmol/g dry weight). Reperfusion of the ischemic myocardium resulted in a same maldistribution of cellular ion contents in all groups that were further aggravated in the (–/–) myocardium (Table 2).

DISCUSSION

The sudden onset of severe ischemia in previously healthy or diseased myocardium sets into motion a series of pathological events that continue until the cardiocytes die. Interest in the complications of myocardial ischemia/reperfusion has resulted in much emphasis being placed upon the characterization and treatment of supraventricular and ventricular arrhythmias (27, 28). However, the realization that the mechanisms of reperfusion-induced arrhythmias are quite distinct from those associated with the mutation in cardiac ion channel genes and the growing awareness of genetically derived arrhythmias as a clinical encountered event have done much to redress the balance of research interest (29). Interestingly, we have known for a longer period of time that there exists another gaseous molecule, beside nitric oxide (NO), and this is CO, which can be generated endogenously in various tissues. Since the isolation of HO enzyme by Tenhunen et al. (30), in 1968, much of the focus of HO and HO-related CO production has been based on the known fact that HO enzyme serves as the rate-limiting enzyme in the degradation of heme. In addition, CO contributes to the regulation of vascular tone and is reported to have antiischemic and immunosuppressive properties, which may contribute to the cytoprotective action of HO-1 in ischemic/reperfused myocardium (12, 31–33). It is of interest to note the findings of Katori et al. (34) and Vulapalli et al. (35) demonstrating that HO-1 overexpression provides protection against ischemia/reperfusion-induced injury in rat hearts, and the protective effect depends on HO-1 modulation of the antiapoptotic pathway. In additional studies, Melo et al. (36) showed that recombinant adeno-associated virus as a vector for direct delivery of the cytoprotective gene HO-1 into the rat heart results in prolonged transgene expression and long-term cardiac protection by reducing the infarct size. We found, in the present study, that infarct size was significantly increased in homozygous (–/–) mouse hearts, and this increase in infarct size related to a significant elevation in myocardial Na^+ and Ca^{2+} accumulation and K^+ loss. We have shown that HO-1 and HO-1-mediated endogenous CO production play an important role in

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

The precise molecular mechanism(s) by which HO-1 and HO-1 related endogenous CO production confers myocardial protection is currently under extensive investigation. For instance, bilirubin protects myocardial cells against oxidative damage and improves the recovery of postischemic function in isolated rat hearts (37). Furthermore, elevated serum bilirubin concentrations are associated with reduced risks for the development of early familial coronary artery diseases (38). Thus, it is reasonable to believe that antioxidant effects of bilirubin contribute to cardiac protection, which has a relatively week endogenous defense in comparison with those of other organs such as the liver and intestines (39). It has been recently suggested that CO could prevent the inflammatory response in hyperoxic injury reducing oxidative damage (40, 41). Another well-known pharmacological property of CO is its vasodilator effect, which could increase the cellular circulation during reperfusion. Thus, it is likely that both the anti-inflammatory and vasodilator effects of CO could contribute to the cardioprotective effect of HO-1 in ischemic/reperfused hearts.

There is a substantial body of evidence supporting the existence of an association in cells between the HO system activity and function that are related to cGMP synthesis. The activation of soluble guanylyl cyclase (sGC) by CO leads to the accumulation of cGMP (42) in cells, indicating a direct relationship between endogenous CO production and cGMP concentration. Enzymatically derived CO, like nitric monoxide, among other physiological activities, causes relaxation of vascular smooth muscle and inhibits platelet aggregation. Studies have in fact established that, under physiological conditions, maintenance of normal vascular tone and blood flow can be attributed in part to enzymatically derived CO (43, 44). Therefore, changes in vascular tone, endothelial permeability, and/or coagulation function observed under hypoxic or ischemic conditions may in part be a consequence of HO-1 induction and localized production of CO.

In the present study, we endeavored to obtain more circumstantial evidence for the involvement of HO-1-related CO production and its direct measurement in the genesis of reperfusion-induced VF in wild-type, heterozygous, and homozygous mice. However, some indication regarding the importance of endogenous CO production in the regulation of reperfusion-induced VF may be perhaps obtained from our previous studies (13), indicating that downregulation of HO-1 mRNA expression could not be observed in ischemic/reperfused and nonfibrillated myocardium. In a

previous study (13) we demonstrated that electrically fibrillated myocardium, without the application of an ischemia/reperfusion induced event, resulted in a significant downregulation in HO-1 gene, which could lead to reduced CO production. This finding was supported in additional studies (45) using zinc-protoporphyrin IX (ZnPPIX), an inhibitor of HO-1 enzyme activity, and N-tert-butyl-α-phenylnitrone (PBN) as an inducer of HO-1 mRNA and protein. Thus, ZnPPIX induced the incidence of reperfusion-induced VF and decreased HO-1 signal intensity leading to a reduced amount of endogenous CO production, whereas PBN significantly increased HO-1 mRNA and protein expression resulting in an increased formation of endogenous CO production in the rat myocardium. We now provide direct evidence, in the present mouse study, by measuring cellular CO production by gas chromatography, suggesting that this is the case and the protective effect against the development of reperfusion-induced VF is attributable to the generation of endogenous CO through HO-1 mRNA induction. Thus, the stimulation of HO-1 mRNA, its protein expression, enzyme activity, and endogenous CO production demonstrate that endogenous CO production is essential for the prevention of reperfusion-induced VF. The HO-1 knockout mouse studies strongly suggest the involvement of endogenous CO production in the mechanism of reperfusion-induced VF.

How can HO expression provide protection against ischemia/reperfusion-induced injury? Although the main subject of the present study is the delineation of HO-CO system in the ischemic/reperfused myocardium, some discussion of the putative mechanism of action of this pathway must also include some mention of nitric oxide synthase (NOS) system. These two pathway systems show many similarities: both HO and NOS have distinct constitutive and inducible isoforms, and both CO and NO stimulate soluble guanylyl cyclase to produce cGMP as the second messenger effector. The relative contribution of CO and NO to the activation of guanylyl cyclase in the cardiovascular system remains unknown, even under physiological conditions. In many pathological conditions such as hypoxia, thermal injury, and ischemia/reperfusion, CO-mediated effects may be predominant. For instance, it was found that an HO-inhibitor blocked cGMP production, whereas a NOS inhibitor had no effect (46). Pannen et al. (47) showed that in a rat model of hemorrhagic shock CO induced sinusoidal dilatation and protection, whereas NO did not appear to play a significant role. However, hyperthermic stress also elevates cGMP via CO but not NO (48). Recent studies have demonstrated that NO is a much more potent stimulator of soluble guanylyl cyclase producing ~300- to 400-fold increase in basal guanylyl cyclase activities in comparison with 5- to 15-fold increase generated by CO (49, 50). It appears likely that, although in general NO is the more dominant activator of guanylyl cyclase, under certain conditions of altered redox state and thermal injury in specific tissues, CO may be physiologically more important. Because both NO and CO mediate their effects through the stimulation of guanylyl cyclase, in future investigation, it is necessary to demonstrate that an increased cardiac cGMP level is at least partly due to CO rather than NO in our model system.

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

reperfusion-induced VF. Thus, it is reasonable to assume that controlled application of exogenous CO, as well as the stimulation of endogenous CO production, may protect the myocardium as it was stressed in the study of Sato et al. (32) using a model of mouse-to-rat cardiac transplant. In addition, the effects of inhaling (exogenous) CO were studied by DeBias et al., (51) under in vivo conditions, and the authors found that the voltage required to induce VF (fibrillation threshold) was higher in control monkeys in comparison with infracted animals inhaling CO. This finding could be related with the presence of blood and its elements and their interaction with CO in their model system. Thus, additional studies are needed to resolve the links in the apparent cascade of the up- and down-regulation of ischemia/reperfusion-induced gene expression and HO enzyme activity in fibrillated and nonfibrillated myocardium.

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Table 1

Effects of 30 min of ischemia followed by 120 min of reperfusion on the recovery of cardiac function in wild-type, HO-1 heterozygous, and HO-1 homozygous mice

	Before ischemia					Reperfusion									
						30 min					120 min				
	HR	CF	AF	AOP	AOPdp/dt	HR	CF	AF	AOP	AOPdp/dt	HR	CF	AF	AOP	AOPdp/dt
Wild type (+/+)	290±10	2.8±0.2	3.1±0.2	161±7	3876±76	260±11	2.1±0.1	1.0±0.1	74±5	990±40	270±10	2.2±0.1	0.8±0.1	69±5	964±55
Heterozygous (+/-)	294±9	2.7±0.2	3.0±0.1	152±8	3924±90	248±8	1.9±0.2	0.9±0.1	68±5	935±38	251±8	2.0±0.1	0.7±0.1	61±4	893±37
Homozygous (-/-) defibrillated	252±7*	2.0±0.1*	1.7±0.1*	127±6*	2851±57*	224±7*	0.8±0.1*	0.4±0.1*	42±4*	641±32*	230±6*	1.1±0.1*	0.6±0.1*	44±5*	627±29*

Values are means ± SE; $n = 6$ in each group. HR, heart rate (beats/min); CF, coronary flow (ml/min); AF, aortic flow (ml/min); AOP, aortic pressure (mmHg); AOPdp/d*t*, first derivative of aortic pressure (mmHg/s). *$P < 0.05$, comparisons were made to the time-matched control values (wild type).

Table 2

Cellular Na^+, K^+, and Ca^{2+} contents (μmol/g dry weight) before ischemia and after reperfusion in HO-1 wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mouse hearts

Group	Before ISA			After 120 min RE		
	Na^+	K^+	Ca^{2+}	Na^+	K^+	Ca^{2+}
+/+	29±4	278±7	1.7±0.2	80±6	233±6	4.3±0.5
+/-	31±3	270±9	1.8±0.3	86±8	233±9	4.3±0.7
-/-	39±4*	253±8*	2.6±0.4*	99±7*	180±9*	5.5±0.6*

Comparisons were made to the wild-type (+/+) values; $n = 6$ in each group. *$P < 0.05$. ISA, ischemia; RE, reperfusion.

Fig. 1

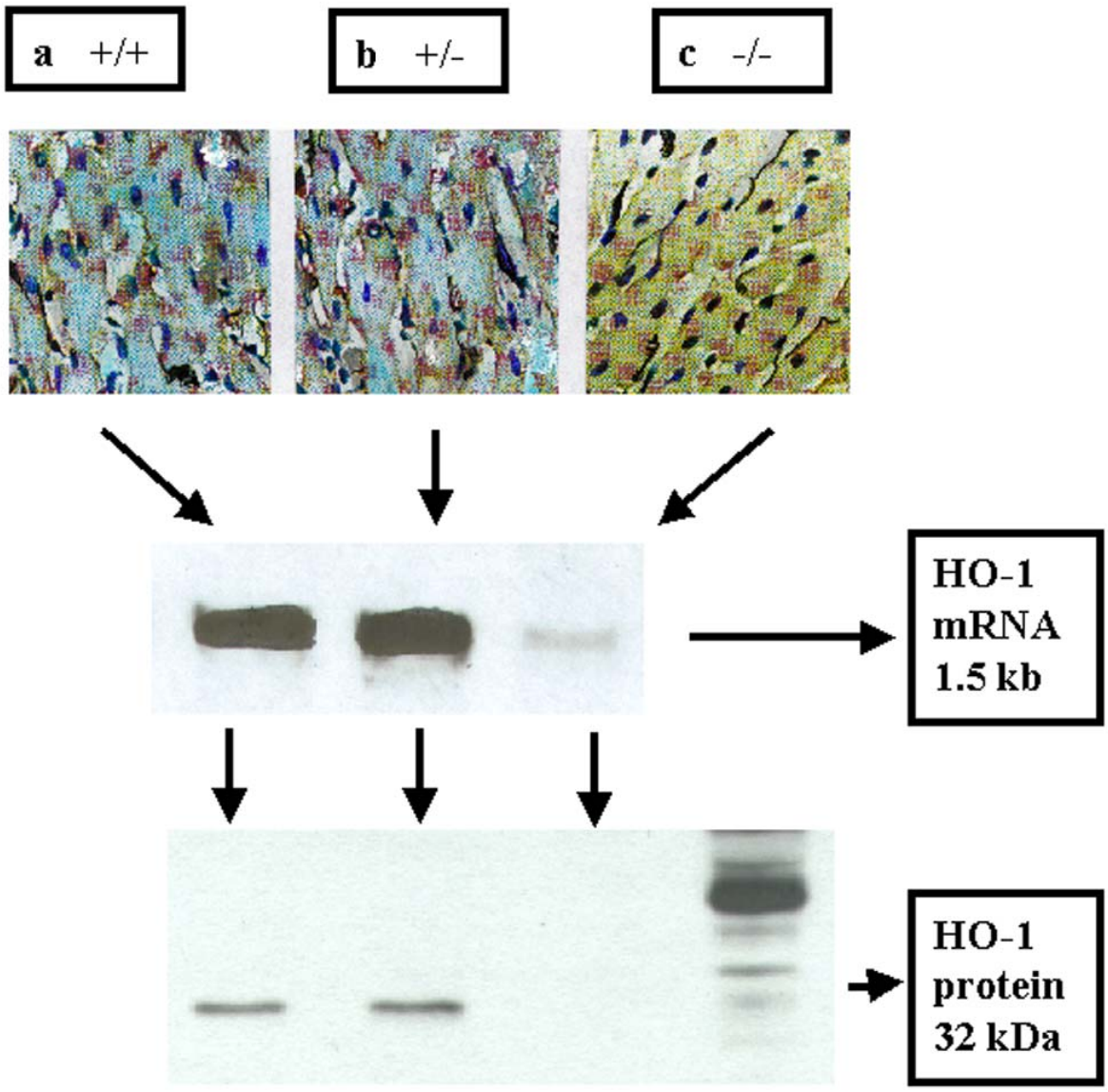

Figure 1. Localization of HO-1 by immunohistochemistry in the mouse heart. A representative picture of left cardiac biopsy obtained from a wild-type (+/+), a heterozygous (+/–), and a homozygous mutant (–/–) mouse heart, respectively, perfused under aerobic conditions. Upper part shows homogenous cytoplasmic staining (blue) of HO-1 in wild-type (+/+) myocardium (***a***) after 20 min of aerobic perfusion. ***b)*** Same cytoplasmic HO-1 staining (blue) after 20 min aerobic perfusion in the myocardium obtained from heterozygous (+/–) mouse. In the homozygous (–/–) mutant mouse heart (***c***), cytoplasmic HO-1 staining was not observed after 20 min of aerobic perfusion. The middle part shows Northern blot analysis of total mRNA from a wild-type (+/+), a heterozygous (+/–), and a homozygous mutant (–/–) mouse heart. Blots were hybridized with a rat HO-1 cDNA probe recognizing a major mRNA band of ~1.5 kb in both the (+/+) and (+/–) mouse myocardium, respectively. The lower part shows Western blot analysis of HO-1 protein expression in wild-type, heterozygous, and homozygous mutant mouse heart, respectively. HO-1 protein expression was detected with the use of recombinant rat HO-1 antibody as described in the Methods.

Fig. 2

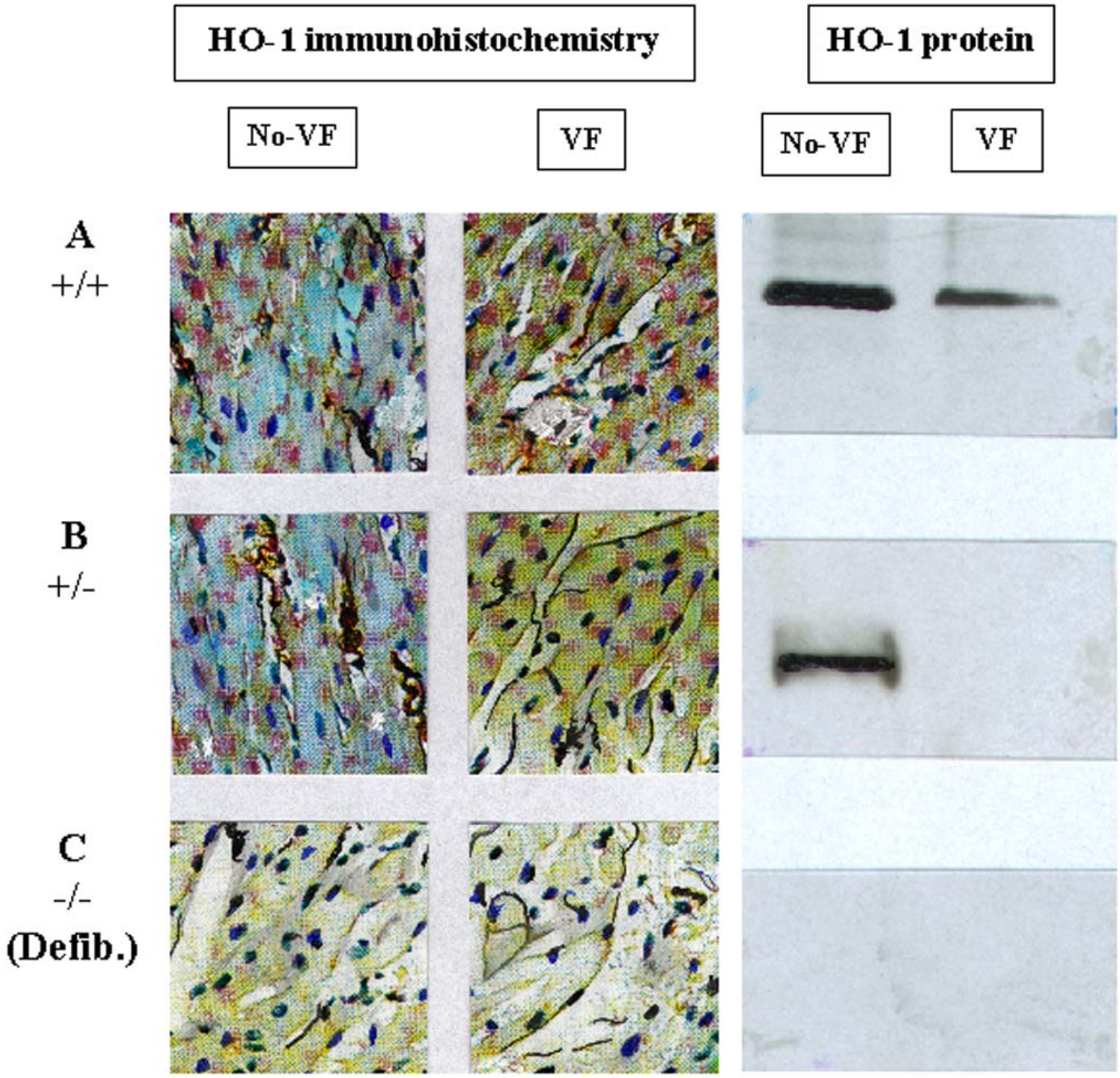

Figure 2. Detection of HO-1 enzyme by immunohistochemistry (left and middle panels) and HO-1 protein expression (right panels) by Western blot in hearts subjected to 20 min of ischemia followed by 120 min of reperfusion in wild-type (+/+; *A*), heterozygous (+/–; *B*), and homozygous (–/–; *C*) nonfibrillated (No-VF) and fibrillated (VF) mouse heart. The presence of HO-1 enzyme is shown in blue (left and middle panels), and HO-1 protein expression (right panels) is represented by lanes, in wild-type, heterozygous, and homozygous nonfibrillated or fibrillated myocardium. Because of the lack of HO-1 enzyme, blue cytoplasmic staining cannot be detected either in No-VF or VF homozygous (–/–) myocardium (***C***). All hearts showed VF upon reperfusion in the homozygous group; therefore, all preparations were defibrillated (Defib.).

Fig. 3

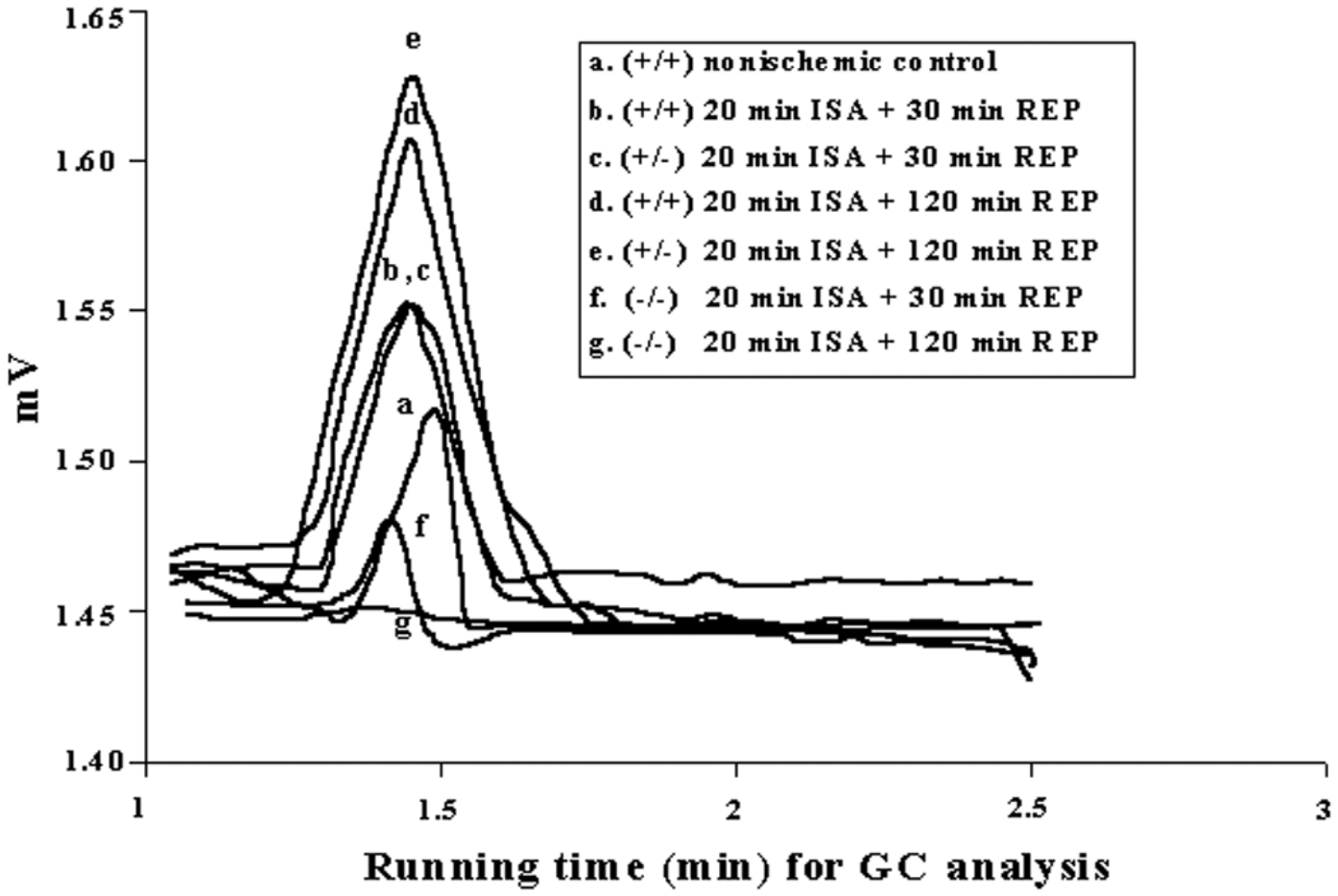

Figure 3. Representative GC chromatograms for the demonstration of endogenous CO production in wild-type (+/+), heterozygous (+/–), and homozygous (–/–) mouse myocardium subjected to 20 min of ischemia followed by 30 min and 120 min of reperfusion, respectively. It is clearly shown that in homozygous (–/–) mutant hearts (***f*** and ***g*** chromatogram), there is very little detectable endogenous CO production after 20 min of ischemia followed by 30 min or 120 min of reperfusion. ISA, ischemia; REP, reperfusions. Note: hearts did not show VF upon reperfusion, or if VF was detected, defibrillation was immediately used.

Fig. 4

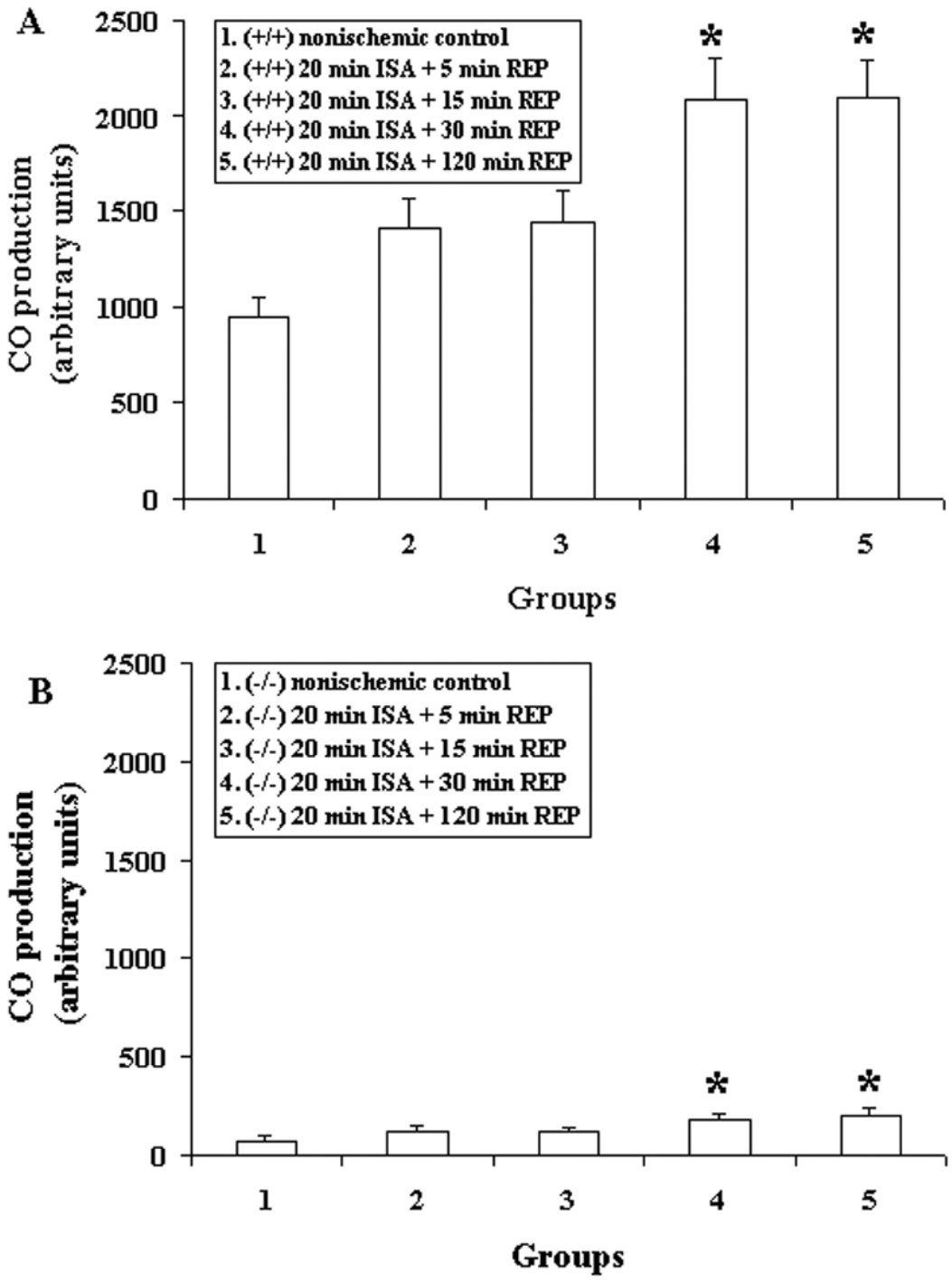

Figure 4. Quantitative and time-course studies for endogenous CO production in ischemic/reperfused wild-type (+/+; *A*) and homozygous (-\-) HO-1 knockout (*B*) hearts. Hearts (*n*=6 in each group) were isolated and subjected to 20 min of global ischemia followed by 2 h of reperfusion. Endogenous tissue CO levels were measured in aerobically perfused myocardium (control) in hearts subjected to 20 min ischemia followed by 5, 15, 30, and 120 min of reperfusion, respectively. *$P < 0.05$, comparisons were made to the control (group 1) nonischemic myocardium.