Repression of Hypoxia-Reoxygenation Injury in the Catalase-Overexpressing Heart of Transgenic Mice (44162B)

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Abstract. **Hypoxia-reoxygenation injury results at least in part from reactive oxygen free radicals. Catalase is a major enzyme involved in detoxification of hydrogen peroxide. The activity of catalase per gram of tissue in the heart is very low, being only about 2% that of liver in rodents and humans, which may be responsible for the high sensitivity of the heart to hypoxia-reoxygenation injury. The present study was undertaken to determine whether elevation of catalase specifically in the heart of transgenic mice could provide protection against hypoxia-reoxygenation injury. Transgenic mice with elevated cardiac catalase 60-fold higher than normal were selected, and the effects of catalase elevation on hypoxia-reoxygenation induced functional and morphological changes in isolated atria were determined. Catalase overexpression ameliorated reductions in contractile force and heart rate caused by hypoxia-reoxygenation, and eliminated reoxygenation-induced arrhythmia. The catalase-overexpressing transgenic atria were also highly resistant to hypoxia-reoxygenation–induced morphological alterations, as examined by electron microscopy. Use of cardiac catalase-overexpressing transgenic mice thus demonstrates that hydrogen peroxide is involved in hypoxia-reoxygenation cardiotoxicity, and that this mouse model provides a useful tool for study of free radical mechanism in the heart damage.**

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Ischemia-reperfusion causes depressed myocardial functional changes associated with deleterious morphological alterations, leading to heart failure and cell death (1). Mechanisms by which this injury occurs are not well defined. Studies using antioxidants such as superoxide dismutase (SOD) and catalase suggest that oxidative stress and burst of free radical production are important mediators of the myocardial damage (2). The available evidence at present indicates that reperfusion arrhythmias and myocardial stunning result at least in part from oxygen radicals (3, 4). Myocardial infarction or cell death may also relate to oxygen radicals (5).

Catalase is a major enzyme involved in the detoxification of hydrogen peroxide (H_2O_2). This enzyme catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water. The primary physiological function of this enzyme is to detoxify the H_2O_2 produced as a result of peroxisomal aerobic dehydrogenase reactions. The importance of catalase in providing protection against extra-peroxisomal H_2O_2 is not clear. Studies with isolated hepatocytes, however, have demonstrated that, under conditions of glutathione (GSH) depletion, catalase functions in metabolism of H_2O_2 produced by the cytochrome P-450–linked monooxygenase system (6). It is thus possible that under conditions of extreme oxidative stress, which in most cases involves GSH depletion, catalase becomes important in providing cytoprotection.

Catalase activities are highest in liver and kidney, relatively high in erythrocytes, intermediate in lung and pancreas, and very low in heart and brain (7). It has been shown

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that catalase activity in the heart is about 2% of that in the liver of humans (7) and rodents (8, 9). Several studies have been undertaken to determine the role of catalase in cardioprotection against oxidative injuries. Supplementation of perfusion medium with catalase significantly reduced ischemia-reperfusion injury to the isolated rat heart by detoxifying H_2O_2 (10). The role of catalase in the metabolism of H_2O_2 in the heart tissue was also tested directly by adding H_2O_2 into the perfusion medium (11). Accumulated evidence shows that catalase, although present at low levels of activity in the heart, functions in detoxification of H_2O_2 in the myocardium.

It is unknown, however, whether the low catalase activity in the heart is responsible for the high sensitivity of this organ to oxidative stress. Current approaches using the isolated heart perfused with catalase have limited application. Exogenously added catalase may not be able to function intracellularly, but react with H_2O_2 directly in the perfusion medium. Furthermore, it is difficult to prolong the catalase perfusion procedure, if that should be an appropriate approach. Thus, the relationship between the low constitutive catalase activity and the high sensitivity to oxidative stress in the heart cannot be defined. A unique *in vivo* model is thus required to overcome these limitations and to determine precisely the role of catalase in protection against oxidative cardiotoxicity. In this context, we have developed a transgenic mouse model in which catalase is overexpressed only in the heart (12). The present study was undertaken to determine the effect of catalase elevation on hypoxia-reoxygenation–induced damage to isolated atria. Functional alterations and morphological changes of the atria under hypoxia-reoxygenation conditions were examined. The results demonstrate that overexpression of catalase in the heart provides protection against hypoxia-reoxygenation–induced cardiac injury.

Materials and Methods

Animals. Transgenic mice were produced from FVB strain obtained from the University of North Dakota Biomedical Research Center as described previously (12). All animal procedures were approved by the American Association of Accreditation of Laboratory Animal Care–certified Institutional Animal Care Committee. The transgenic founder mice, which had integrated the transgene, were bred with mice of the same strain. The resultant litters were analyzed by polymerase chain reaction (PCR) procedure using the genomic DNA isolated from 1-cm tail clips from 3- to 4-week-old mice. Transgenic positive mice (heterozygotes) and negative littermates were then used for experiments. Catalase activities were measured in atria after each experiment as previously described (12). The level of catalase activities in the transgenic atria was about 60-fold higher than normal.

Determination of Functional Alterations. To isolate atria, the experimental procedure described by de Jong *et al.* (13) was followed with modifications. Both transgenic and control mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body wt; Vet Labs, Lenexa, KS). Atria were separated from ventricles and placed in a 30-ml chamber containing Krebs-Henseleit (KH) buffer of the following composition: NaCl (118 m*M*); KCl (4.7 m*M*); $NaHCO_3$ (3.0 m*M*); $CaCl_2$ (3.0 m*M*); $MgSO_4$ (1.2 m*M*); KH_2PO_4 (1.2 m*M*); glucose (10 m*M*); Na_2EDTA (0.5 m*M*); pH 7.4, gassed with 5% CO_2 in O_2. A preload tension of 0.25 g was applied. Right atria were allowed to spontaneously contract; left atria were paced by a fixed frequency of 4 Hz and a voltage of 1.5 times the threshold value of each tissue preparation. The atrium was allowed to stabilize for 30 min with normally oxygenated KH buffer (5% CO_2 in O_2) before induction of hypoxia. Hypoxia was induced by gassing KH buffer with 95% N_2–5% CO_2 (20 mm Hg) at 37°C for 60 min. Reoxygenation was done with KH buffer gassed by 5% CO_2 in O_2 (20 mm Hg) for an additional 240 min. Muscle contractions were recorded isometrically by using a Kulite Semiconductor BGG 10-g force transducer (Leonia, NJ) and a Gould TA 2000 recorder (Cleveland, OH).

Electron Microscopic Examination of Morphological Changes. The isolated atria subjected to hypoxia and reoxygenation were cut into 1-mm^3 blocks and immediately fixed with Karnovsky's solution at 4°C for 90 min and post-fixed in 1% osmium tetroxide in 0.1 *M* S-collidine buffer at 4°C for 90 min in the dark. Then, the blocks were serially dehydrated in ethanol and propylene oxide, and finally embedded in Epon/Araldite resin. The atrial tissue was selected under light microscope on semithin sections stained with 1% toluidine blue. Subsequently, ultrathin sections obtained with a diamond knife were stained with uranyl acetate and lead citrate, and examined with Joel 100 S transmission electron microscope (EM) at 80 kV. The EM operator was unaware of the transgenic status and the treatment of the atria. For each atrium, three fixed tissue blocks were randomly selected and more than 10 fields of each slide and a total of 10 slides for each block were examined. One EM micrograph that best represents the status of the tissue damage was selected to demonstrate the injury.

Statistical Analysis. Data obtained were initially analyzed by two-way analysis of variance (ANOVA). Differences were further compared by a two-tail *t* test. The *P* value calculated from the *t* test was further corrected with the Bonferroni procedure. Difference were considered to be significant when the Bonferroni corrected *P* value (P') less than 0.05.

Results

Spontaneous contractions of isolated atria are shown in Figure 1. Transgenic atria and normal controls, gassed with 95% O_2–5% CO_2, displayed similar contractions during the 30-min equilibration period as shown at Time 0. The contractile force and frequency of both atria were gradually suppressed by 60 min of hypoxia (95% N_2–5% CO_2). However, the transgenic atrium showed marked resistance to

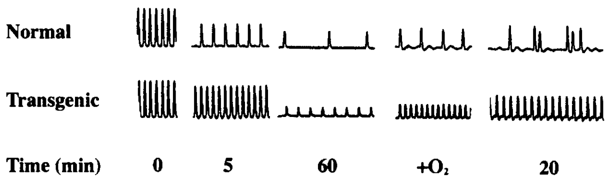

Figure 1. Functional changes induced by hypoxia-reoxygenation in the isolated atria from normal and transgenic mice. The transgenic atrium contains catalase activity about 60-fold higher than normal. The atria were incubated under the conditions described in Materials and Methods. After 30 min of equilibration (Time 0), hypoxia was introduced and lasted for 60 min, then oxygen was reintroduced and spontaneous contraction was recorded for the entire period of hypoxia-reoxygenation. This experiment was repeated five times with the atria isolated from different animals, and similar results were obtained as for the representative atria.

hypoxia-induced functional changes. The transgenic atrium extensively recovered its normal contraction upon reoxygenation with 95% O_2–5% CO_2. The normal atrium displayed abnormal contractions, arrhythmia, a typical reperfusion injury. Catalase activities in the transgenic atria and nontransgenic controls were 1426.5 ± 42.7 and 22.3 ± 2.1 μmol H_2O_2/min · mg protein, respectively.

To determine precisely the effect of elevated catalase on atrial functional alterations induced by hypoxia-reoxygenation, the left atrium was separated from the right one. Cardiac contractile force and contraction rate were recorded from the left and right atria, respectively. The left atrium was paced by a fixed frequency of 4 Hz and a voltage of 1.5 times the threshold value of each tissue preparation. There was no difference in the contractile force between the transgenic atria and the normal controls during the 30-min equilibration period, 0.52 ± 0.02 and 0.51 ± 0.01 g, respectively. As shown in Figure 2A, the contractile force was dramatically depressed under hypoxia in either normal or transgenic mouse atria. Upon reoxygenation, the contractile force was partially recovered in both transgenic and nontransgenic atria. The transgenic atria, however, displayed much better recovery than the normal atria, especially after 150-min (Time 210) reoxygenation ($P' < 0.05$). The transgenic atria also showed significant resistance against hypoxia-reoxygenation induced decrease in heart rate (Fig. 2B). Particularly, a gradual decrease in the heart rate with arrhythmia (as shown in Fig. 1) was observed in the normal atria after reoxygenation, while a stable unchanged chronotropy remained in the transgenic atria.

The myocardial cells of both left and right atria of normal mice subjected to hypoxia and reoxygenation displayed marked pathological changes as examined by EM. These changes as shown in Figure 3 include mitochondrial swelling and the disappearance of cristae, often leaving the remnants of the original structure and myelin figures. The matrix of the mitochondria became translucent. Some myocardial cells showed marked cell swelling; the sarcolemma exhibited large empty bleb-like spaces and small defects in plasma membrane. The basal lamina appeared to be separated from the surface bilayer membrane. Vesiculation and disruption of the sarcoplasmic reticulum were obvious. Hypoxic vacuoles were frequently observed. The myofibril between contraction bands showed partially broken Z line and wisped I band. However, in catalase-transgenic mouse atria these changes were rarely seen.

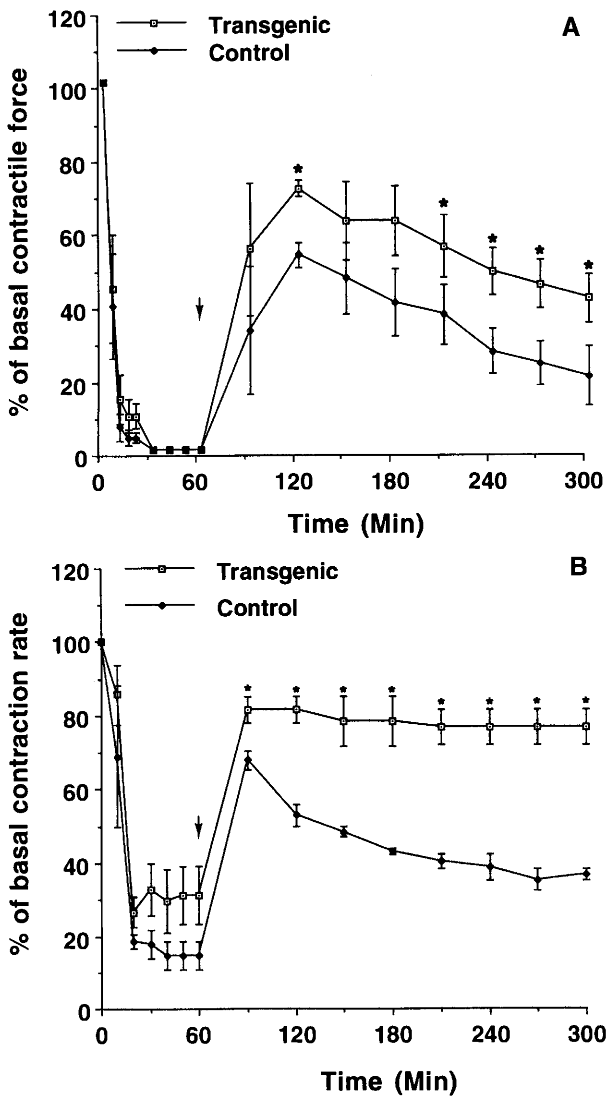

Figure 2. Effect of catalase elevation (60-fold higher than normal) on hypoxia and reoxygenation reduced inotropy (A, left atrium) and chronotropy (B, right atrium). The left atrium was paced by a fixed frequency of 4 Hz and a voltage of 1.5 times the threshold value. Spontaneous contraction was recorded in the right atrium. After 30 min of equilibration (Time 0), hypoxia was introduced and lasted for 60 min, then oxygen was reintroduced as indicated by the arrow. This measurement was repeated six times with atria isolated from different animals, and the data are presented as the mean ± SEM. $^*P' < 0.05$.

Discussion

Myocardial injury caused by ischemia-reperfusion has been extensively studied. This injury results at least in part from reactive oxygen radicals, although the exact mechanism by which the pathogenesis occurs is unknown. Recent studies have shown myocardial protection against ischemia-

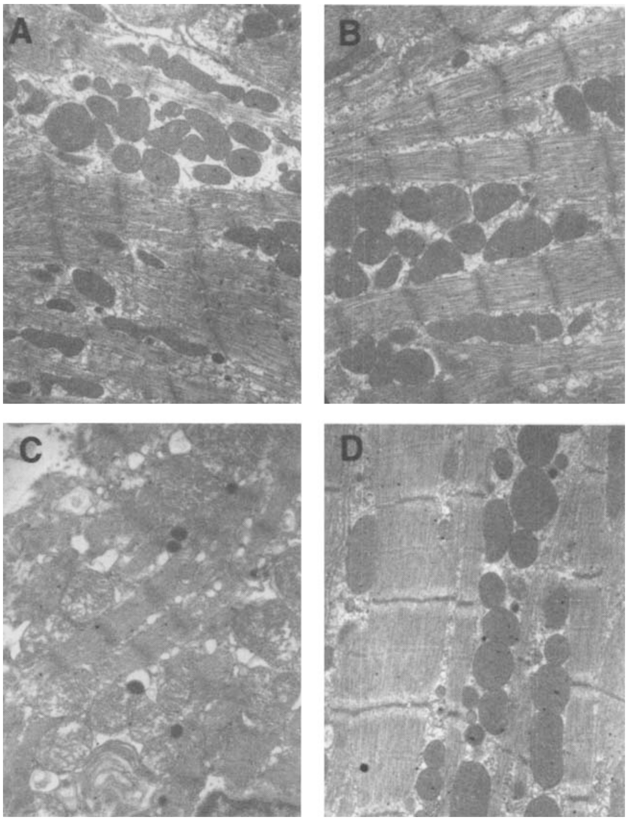

Figure 3. Representative micrographs showing the effect of elevated cardiac catalase on hypoxia-reoxygenation–induced myocardial morphological changes examined by electron microscopy. (A) A micrograph of left atrium of nontransgenic control incubated continuously with KH buffer and 5% CO_2 in O_2. (B) A micrograph of left atrium of transgenic mouse treated as in Panel A. (C) A micrograph of left atrium of nontransgenic control subjected to 60-min hypoxia and 240-min reoxygenation. (D) A micrograph of left atrium of transgenic mouse subjected to 60-min hypoxia and 240-min reoxygenation. Magnification: ×10,000.

reperfusion injury and changes in gene expression occur after whole body heat stress (14–19). For example, reduced infarct size *in vivo* (16, 17) and *in vitro* (18) caused by ischemia-reperfusion has been shown 24 hr after whole-body temperature elevation to 42°C for 15 min. This whole-body heat stress procedure also enhanced postischemic contractile function *in vitro* (14, 15, 19). The exact nature of this induced protection is unclear. A number of studies (10, 14, 15, 20) have suggested that alterations in myocardial antioxidant systems may be involved.

Catalase is increased within the myocardium 24 hr after whole-body heat stress (15). This enzyme is highly relevant to the protection because of its role in detoxification of hydrogen peroxide. Supplementation of perfusion medium with catalase significantly reduced ischemia-reperfusion injury to the isolated heart (15), and inhibition of catalase can at least partially abolish post–heat stress protection when contractility is used as the end point of injury (20).

Catalase is, however, not the only protein that is elevated after heat stress. An inducible member of the *hsp*70 family shows marked changes 24 hr after heat stress (14–19). Numerous studies have suggested the possible involvement of HSP70 in myocardial protection. For example, elevated levels of the highly inducible member of the HSP70 family were associated with improved postischemia recovery (21–23) and with reduction in infarct size in hearts (20, 24). There appears to be a correlation between the amount of inducible HSP70 and the extent of myocardial protection (25). A more direct demonstration of the involvement of inducible HSP70 in myocardial protection comes from two recent studies that demonstrated a marked resistance to ischemia-reperfusion injury in isolated hearts from transgenic mice that overexpress inducible HSP70 (26, 27).

To study precisely the role of catalase in detoxification of reactive oxygen radicals, a transgenic mouse model in which catalase is specifically overexpressed in the heart was produced (12). Fifteen healthy transgenic mouse lines were produced. Cardiac catalase activity was constitutively overexpressed in both atrium and ventricle, ranging from 2- to 630-fold higher than normal. This enzyme activity was not altered in liver, kidneys, lungs, and skeletal muscles. Other antioxidant components including glutathione, glutathione peroxidase, glutathione reductase, metallothionein, and superoxide dismutase were not altered in the catalase-overexpressing heart. Compared with normal controls, transgenic lines expressing catalase activity 60- or 100-fold higher than normal exhibited a significant resistance to doxorubicin-induced cardiac lipid peroxidation, elevation of serum creatine phosphokinase, and functional changes in isolated atria. This transgenic mouse model therefore provides a unique experimental tool to study the role of catalase in hypoxia-reoxygenation injury to the heart.

In the present study, direct evidence regarding the importance of catalase in myocardial protection against hypoxia-reoxygenation was obtained. We took advantage of elevation of catalase specifically in both atria and ventricles of the transgenic mice and directly monitored atrial functional alteration under hypoxia-reoxygenation. Importantly, the isolated atria allowed precise determination of the effects of hypoxia and reoxygenation on heart rate and contractile force. The results demonstrated that hypoxia-reoxygenation reduces contractile force and frequency in the isolated atria. Reoxygenation resulted in arrhythmia, a typical reperfusion injury observed in the heart both *in vitro* and *in vivo.* The catalase-overexpressing atrium displayed a marked attenuation in the hypoxia-reoxygenation–induced reduction of contractile force and contraction frequency, and abolished the reoxygenation-induced arrhythmia. Corresponding to these protective effects on functional alterations induced by hypoxia-reoxygenation, catalase overexpression in the heart also markedly suppressed the morphological changes, as examined by electron microscopy.

Other experimental approaches have used exogenously added catalase to study its role in cardiac protection against ischemia-reperfusion injury. Most of these studies either supplemented catalase *in vivo* or added catalase to the perfusion buffer *in vitro.* Three major problems complicate data interpretation from these studies: (i) it is impossible to maintain constant plasma antioxidant concentrations and to accurately predict the target tissue concentrations; (ii) metabolic activation and inactivation by multiple metabolic organs such as liver and kidney would greatly affect the efficacy of the antioxidant; and (iii) high–molecular weight catalase is unlikely to be transported into intracellular compartments. To overcome the shortcomings of these earlier studies, we produced the unique transgenic model used in the present study. In this model, catalase activities are maintained at permanently elevated levels specifically in the cardiac tissue without alterations in any other antioxidant systems. The experiments with the catalase-overexpressing transgenic mouse heart thus strongly suggest that an elevated level of catalase can play a role in cell protection against reactive oxygen–induced damage. This study also suggests that low catalase activity in the heart is a major factor responsible for the high sensitivity of the heart to ischemia-reperfusion injury. In addition, because catalase is a major enzyme that metabolizes H_2O_2 in the cell and this catalase activity is quite specific, the results thus provide direct evidence to support the oxidative injury hypothesis for hypoxia-reoxygenation injury.

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