

Overexpression of Glutathione Peroxidase Prevents Left Ventricular Remodeling and Failure After Myocardial Infarction in Mice

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Background—Oxidative stress plays an important role in the pathophysiology of heart failure. We determined whether the overexpression of glutathione peroxidase (GSHPx) could attenuate left ventricular (LV) remodeling and failure after myocardial infarction (MI).

Methods and Results—We created MI in 12- to 16-week-old, male GSHPx transgenic mice (TG+MI) and nontransgenic wild-type littermates (WT+MI) by ligating the left coronary artery. GSHPx activity was increased in the hearts of TG mice, with no significant changes in other antioxidant enzymes. LV concentrations of thiobarbituric acid-reactive substances measured in TG+MI at 4 weeks were significantly lower than those in WT+MI. The survival rate during 4 weeks of MI was significantly higher in TG+MI than in WT+MI, although the infarct size was comparable. LV cavity dilatation and dysfunction were significantly attenuated in TG+MI. LV end-diastolic pressure was increased in WT+MI and reduced in TG+MI. Improvement of LV function in TG+MI was accompanied by a decrease in myocyte hypertrophy, apoptosis, and interstitial fibrosis in the noninfarcted LV. Myocardial matrix metalloproteinase-9 zymographic and protein levels were increased in WT+MI after 3 days but were attenuated in TG+MI.

Conclusions—Overexpression of GSHPx inhibited LV remodeling and failure after MI. Therapies designed to interfere with oxidative stress might be beneficial to prevent cardiac failure. (*Circulation*. 2004;109:544-549.)

Key Words: myocardial infarction ■ heart failure ■ remodeling ■ antioxidants ■ free radicals

There is increasing evidence that oxidative stress plays a major role in the development and progression of left ventricular (LV) remodeling and failure that occur after myocardial infarction (MI).¹ The degree of oxidative stress and the severity of subsequent myocardial damage might depend on the imbalance between excess production of reactive oxygen species (ROS) and the antioxidant defense within the heart. ROS include superoxide anion ($\cdot\text{O}_2^-$), H_2O_2 , and hydroxyl radical ($\cdot\text{OH}$). Our previous studies demonstrated that H_2O_2 and $\cdot\text{OH}$ were generated via $\cdot\text{O}_2^-$ within the failing myocardium.² Increased ROS production can result in myocyte hypertrophy, apoptosis, and interstitial fibrosis,^{1,3} which may contribute to the development of depressed cardiac function and progression of cardiac failure.

The first line of defense mechanism against ROS-mediated cardiac injury comprises several antioxidant enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx). Among these antioxidants, GSHPx is an important enzyme that performs several vital functions. GSHPx not only functions by

removing H_2O_2 formed after the SOD-catalyzed dismutation reaction but also detoxifies the lipid hydroperoxides. In several in vitro studies, GSHPx alone was demonstrated to confer greater protection against oxidative damage than either SOD, catalase, or the combination of SOD and catalase.⁴ The great efficiency of GSHPx as an antioxidant may be attributable to the fact that it is located both in the cytosol and in the mitochondrial matrix and that it can utilize lipid peroxides as well as H_2O_2 for substrates. These beneficial characteristics make GSHPx an important candidate for therapy against myocardial failure caused by increased ROS production. Despite several previous reports on the beneficial effects of antioxidants on heart failure,^{1,5} no study has ever been performed to specifically examine the protective role of GSHPx against myocardial remodeling and failure. Therefore, the purpose of the present study was to determine, using GSHPx-transgenic (TG) mice,⁶ whether overexpression of GSHPx could attenuate the progressive post-MI LV structural remodeling and functional decline.

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Methods

Transgenic Mice

The study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society. We used the progeny of heterozygous breeding pairs of C57BL/6×CBA/J hybrid mice with overexpression of the human GSHPx1 gene.⁶ The original breeding pairs used to develop the mice for the present study were obtained from Dr Oleg Mirochnitchenko and Masayori Inouye (University of Medicine and Dentistry of New Jersey).

Creation of MI

We created MI in 12- to 16-week-old, male TG mice (TG+MI) and nontransgenic wild-type littermates (WT+MI) by ligating the left coronary artery.^{1,7} Sham operation without coronary artery ligation was also performed in WT (WT+Sham) and TG (TG+Sham) mice. Tail clips and a polymerase chain reaction protocol to confirm the genotype were performed by a group of investigators (H.T. and J.W.). Next, MI was induced in these mice by another subset of investigators (T.S. and S.H.), who were not informed of the genotyping results. This assignment procedure was performed with numeric codes to identify the animals.

Survival Study

The survival analysis was performed in WT+Sham (n=20), TG+Sham (n=22), WT+MI (n=46), and TG+MI (n=44) mice. During the study period of 4 weeks, cages were inspected daily for animals that had died. All dead mice were examined for the presence of MI as well as pleural effusion and cardiac rupture.

Echocardiographic and Hemodynamic Measurements

After 4 weeks of surgery, echocardiographic studies were performed under light anesthesia with tribromoethanol/amylen hydrate (Avertin; 2.5% wt/vol, 8 μ L/g IP) and spontaneous respiration. 2D targeted M-mode tracings were recorded at a paper speed of 50 mm/s. Under the same anesthesia with Avertin, a 1.4F micromanometer-tipped catheter (Millar Instruments) was inserted into the right carotid artery and then advanced into the left ventricle to measure LV pressures.⁷ One subset of investigators (H.M. and M.I.), who were not informed of the experimental groups, performed in vivo LV function studies.

Infarct Size

Infarct size was determined by methods described previously in rats⁸ and also in mice.⁹ The left ventricles were cut from apex to base into 3 transverse sections. Five-micrometer sections were cut and stained with Masson's trichrome. Infarct length was measured along the endocardial and epicardial surfaces from each of the LV sections, and the values from all specimens were summed. Total LV circumference was calculated as the sum of endocardial and epicardial segment lengths from all LV sections. Infarct size (in percent) was calculated as total infarct circumference divided by total LV circumference.

To evaluate the effects of GSHPx overexpression on the risk area and infarct size, a separate group of animals including WT+MI (n=6) and TG+MI (n=6) was created. After 24 hours of coronary artery ligation, Evans blue dye (1%) was perfused into the aorta and coronary arteries, with distribution throughout the ventricular wall proximal to the site of coronary artery ligation.¹⁰ These sections were weighed and then incubated with a 1% triphenyltetrazolium chloride solution at 37°C for 20 minutes. The infarct area (pale), the area at risk (not blue), and the total LV area from each section were measured, multiplied by the weight of the section, and then totaled from all sections.

Myocardial Histopathology

After in vivo hemodynamic studies, the heart was excised and dissected into the right ventricle and LV, including the septum. The LV was cut into 3 transverse sections: apex, middle ring, and base.

From the middle ring, 5- μ m sections were cut and stained with Masson's trichrome. Myocyte cross-sectional area and collagen volume fraction were determined by quantitative morphometry of tissue sections from the mid-LV.⁷

To detect apoptosis, tissue sections from the mid-LV were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. The number of TUNEL-positive cardiac myocyte nuclei was counted, and the data were normalized per 10⁵ total nuclei identified by hematoxylin-positive staining in the same sections. The proportion of apoptotic cells was counted in the noninfarcted LV. We also examined whether apoptosis was present by the more sensitive ligation-mediated polymerase chain reaction fragmentation assays (Maxim Biotech Inc).

Antioxidant Enzyme Activities and Lipid Peroxidation

For the subsequent biochemical studies, the myocardial tissues with MI were carefully dissected into 3 parts: 1 consisting of the infarcted LV, the border zone LV with the peri-infarct rim (a 1-mm rim of normal-appearing tissue), and the remaining noninfarcted (remote) LV. The enzymatic activities of GSHPx, catalase, and SOD were measured in the noninfarcted LV.¹¹

The degree of lipid peroxidation was determined in the myocardial tissues through biochemical assay of thiobarbituric acid-reactive substances (TBARS).¹²

Matrix Metalloproteinases

Myocardial gelatinolytic levels of matrix metalloproteinase (MMP)-2 and MMP-9 were measured in the noninfarcted LV by gelatin zymography.⁷ Within a given experiment, the densitometric values were normalized by using standards run concurrently within the same gel, and the value for each WT+MI or TG+MI was calculated as a ratio of sham. MMP-9 protein levels were quantified by Western blot analysis using a specific antibody against recombinant mouse MMP-9 (R&D Systems).

Statistical Analysis

Data are expressed as mean \pm SEM. Survival analysis was performed by the Kaplan-Meier method, and between-group difference in survival was tested by the log-rank test. Between-group comparison of means was performed by 1-way ANOVA, followed by *t* tests. Bonferroni's correction was performed for multiple comparisons of means. A value of *P*<0.05 was considered to be statistically significant.

Results

Antioxidant Enzymes and TBARS

First, the baseline differences in antioxidant enzyme activities between WT and TG mice were determined (Table). In TG mice, there was a significant increase in GSHPx activities in the LV. Importantly, catalase and SOD activities were not altered in the TG hearts, indicating no effects of GSHPx overexpression on other antioxidant enzymes. Similar results have been reported in the brain, liver, kidney, and lung obtained from the same series of mice.^{13,14} Second, the changes of antioxidant enzyme activities after MI were compared between WT and TG. GSHPx activities were significantly higher in WT+MI than in WT+Sham. More importantly, as expected, GSHPx activities were significantly elevated in TG+MI compared with WT+MI. Catalase activities were also increased after MI, which, however, did not differ between WT+MI and TG+MI. SOD activities were not altered in WT+MI or TG+MI compared with WT+Sham.

Characteristics of Animal Models

	WT+Sham	TG+Sham	WT+MI	TG+MI
Antioxidant enzymes				
n	8	8	8	8
GSHPx, nmol · min ⁻¹ · mg protein ⁻¹	79.7±5.6	98.9±4.3*	106.8±9.3*	148.2±9.2‡§
Catalase, nmol · min ⁻¹ · mg protein ⁻¹	66.4±2.3	69.0±2.3	79.1±3.4	82.6±4.4†
SOD, U/mg protein	29.2±2.6	30.0±2.0	23.6±2.0	25.8±3.0
Echocardiographic data				
n	20	22	25	33
Heart rate, bpm	421±17	463±13	444±15	471±13
LVEDD, mm	3.8±0.04	3.8±0.04	5.3±0.1†	5.0±0.1‡§
LVESD, mm	2.5±0.1	2.5±0.1	4.7±0.1†	4.2±0.1‡§
Fractional shortening, %	33.0±0.9	34.2±0.8	11.6±0.5†	15.4±0.6‡§
Infarct wall thickness, mm	0.46±0.01	0.47±0.01
Noninfarct wall thickness, mm	0.75±0.01	0.74±0.01	1.00±0.02†	0.95±0.01†‡
Hemodynamic and organ weight data				
n	16	21	22	30
Heart rate, bpm	430±12	458±15	457±14	485±11
Mean aortic pressure, mm Hg	84±3	80±3	79±2	80±2
LVEDP, mm Hg	4.0±0.6	4.2±0.5	13.1±1.5†	9.5±0.9‡§
Body wt, g	30.7±0.6	30.6±0.7	30.3±0.7	30.9±0.6
LV wt/body wt, mg/g	3.42±0.08	3.38±0.08	3.79±0.12†	3.78±0.08†
RV wt/body wt, mg/g	0.68±0.03	0.67±0.02	1.06±0.08†	1.11±0.07†
Lung wt/body wt, mg/g	5.2±0.1	5.1±0.2	9.1±0.8†	7.6±0.6†‡
Pleural effusion, %	0	0	45	22

EDD indicates end-diastolic dimension; ESD, end-systolic dimension; EDP, end-diastolic pressure; and RV, right ventricular. Values are mean±SEM.

**P*<0.05, †*P*<0.01 vs WT+Sham; ‡*P*<0.05, §*P*<0.01 vs WT+MI.

TBARS measured in the noninfarcted LV obtained from TG+MI were significantly lower than those from WT+MI (43 ± 4 nmol/g for *n*=8 versus 68 ± 8 nmol/g for *n*=8).

Survival

Early operative mortality (within 6 hours) was comparable between groups (15% for WT+MI versus 6% for TG+MI; *P*=NS). There were no deaths in the sham-operated groups. The survival rate up to 4 weeks was significantly higher in TG+MI than WT+MI (84% versus 65%, *P*<0.05; Figure 1). Death was suspected to be attributable to heart failure and/or arrhythmia. Three WT+MI (7%) and 1 TG+MI (2%) mice died of LV rupture (*P*=NS).

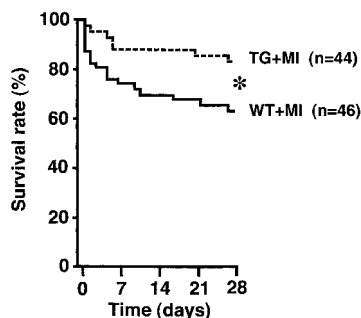


Figure 1. Kaplan-Meier survival analysis. Percentages of surviving WT+MI (*n*=46) and TG+MI (*n*=44) mice were plotted. **P*<0.05 vs WT+MI group.

Subsequent echocardiographic and hemodynamic measurements were performed in the 4-week survivors: 20 WT+Sham, 22 TG+Sham, 30 WT+MI, and 37 TG+MI mice. The measurements could not be performed in 5 WT+MI and 4 TG+MI mice because of technical difficulties. The surviving mice were divided further into 2 groups: those for the subsequent histological analysis, including infarct size and histopathological measurements (10 WT+Sham, 10 TG+Sham, 14 WT+MI, and 15 TG+MI), and those for the biochemical assay, including antioxidant enzyme activity, lipid peroxidation, and gelatin zymography as well as Western blots of MMP (10 WT+Sham, 12 TG+Sham, 16 WT+MI, and 22 TG+MI). Infarct size was not measured in the mice that died. In 1 WT+MI and 3 TG+MI mice, histological analysis could not be performed because of the poor quality of tissue preparation and staining. The amount of tissue was limited; thus, tissue had to be divided so that all of the biochemical analyses could be performed.

Infarct Size

Infarct size determined by morphometric analysis 28 days after ligation was comparable ($57 \pm 2\%$ versus $52 \pm 3\%$; *P*=NS) between WT+MI and TG+MI. In a separate group of additional animals 3 days after ligation, it was also comparable ($49 \pm 4\%$ versus $51 \pm 4\%$; *P*=NS) between WT+MI (*n*=14) and TG+MI (*n*=13).

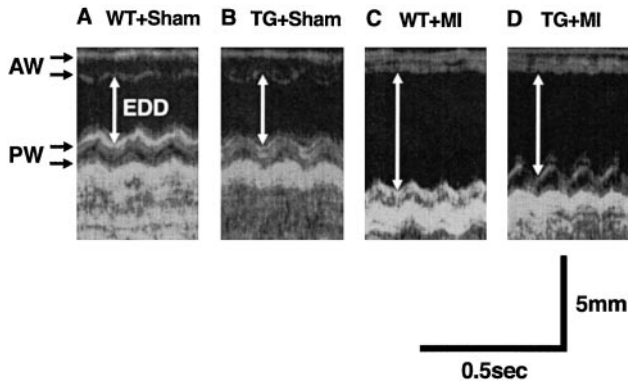


Figure 2. M-mode echocardiograms obtained from WT+Sham (A), TG+Sham (B), WT+MI (C), and TG+MI (D) mice. AW indicates anterior wall; PW, posterior wall; and EDD, end-diastolic diameter.

To further confirm that overexpression of GSHPx did not alter infarct size, area at risk and infarct area were measured in mice 24 hours after coronary artery ligation. Percentages of LV at risk (risk area/LV: $49 \pm 4\%$ versus $48 \pm 5\%$; $P=NS$) and infarct size (infarct/risk area: $80 \pm 2\%$ versus $80 \pm 2\%$; $P=NS$) were comparable between WT+MI ($n=6$) and TG+MI ($n=6$).

Echocardiography and Hemodynamics

The echocardiographic and hemodynamic data of surviving mice at 28 days are shown in Figure 2 and the Table. LV diameters were significantly increased in WT+MI over the values in WT+Sham or TG+Sham. TG+MI exerted less LV cavity dilatation and improved fractional shortening compared with WT+MI. There was no significant difference in heart rate and aortic blood pressure among the 4 groups of mice. LV end-diastolic pressure was increased in WT+MI but was significantly attenuated in TG+MI.

Organ Weights and Histomorphometry

Both LV weight/body weight and right ventricular weight/body weight were increased in WT+MI. Coinciding with an increased LV end-diastolic pressure, lung weight/body weight was increased in WT+MI and was also attenuated in TG+MI (Table). The prevalence of pleural effusion tended to be lower in TG+MI than in WT+MI (45% versus 22%), which, however, did not reach statistical significance ($P=0.05$).

Figure 3 shows the transverse mid-LV sections stained with Masson's trichrome. The sections obtained from

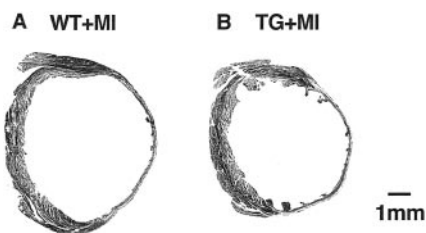


Figure 3. Low-power photomicrographs of Masson's trichrome-stained LV cross section obtained from WT+MI (A) and TG+MI (B) mice. Bar=1 mm.

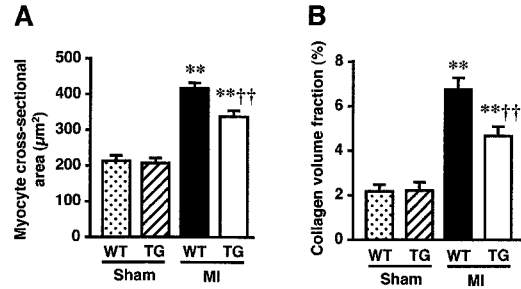


Figure 4. Myocyte cross-sectional area (A) and collagen volume fraction (B) in WT+sham ($n=10$), TG+Sham ($n=10$), WT+MI ($n=13$), and TG+MI ($n=12$) mice. Values are mean \pm SEM. $**P<0.01$ vs WT+Sham value. $\dagger\dagger P<0.01$ vs WT+MI value.

WT+MI revealed an anteroapical infarct extending into the anterolateral wall. Consistent with echocardiographic data (Figure 2), TG+MI had significantly smaller LV chamber diameters and volume than WT+MI.

Myocyte cross-sectional area was increased in WT+MI but was significantly attenuated in TG+MI (Figure 4). These results are concordant with LV wall thickness data obtained from echocardiography (Table). Collagen volume fraction was increased in WT+MI but was significantly inhibited in TG+MI (Figure 4).

Myocardial Apoptosis

There were rare TUNEL-positive nuclei in control mice. The number of TUNEL-positive myocytes in the noninfarcted LV was increased in WT+MI and was significantly decreased in TG+MI (Figure 5A). In addition, the intensity of DNA ladder indicated that the apoptosis from TG+MI was decreased compared with that from WT+MI (Figure 5B).

Myocardial MMPs

MMP-9 zymographic levels were increased at day 3 in WT+MI, but this increase was significantly attenuated in TG+MI (Figure 6A). Similarly, MMP-9 protein levels were increased in WT+MI, but this increase was inhibited in TG+MI (Figure 6B). MMP-2 zymographic level was also

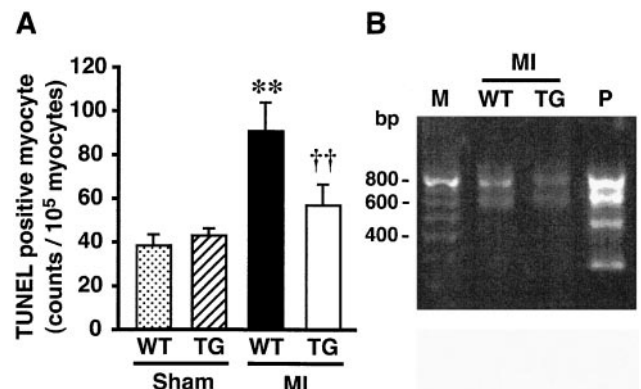


Figure 5. A, number of TUNEL-positive myocytes in noninfarcted LV from WT+Sham, TG+Sham, WT+MI, and TG+MI mice ($n=8$ each). Values are mean \pm SEM. $**P<0.01$ vs WT+Sham value. $\dagger\dagger P<0.01$ vs WT+MI value. B, DNA ladder indicative of apoptosis in genomic DNA from LV. M indicates marker; P, positive control.

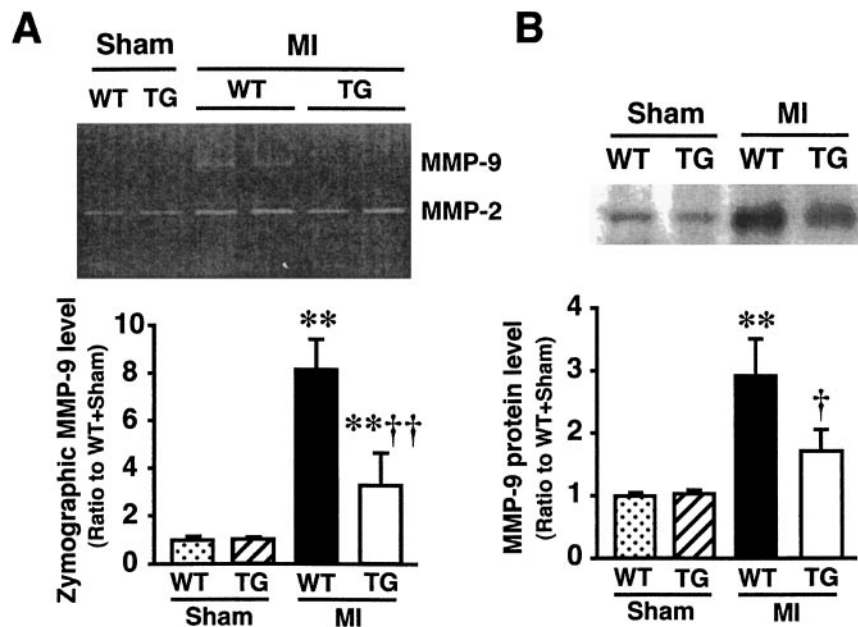


Figure 6. A, Myocardial MMP-9 zymography in WT+Sham, TG+Sham, WT+MI, and TG+MI mice 3 days after operation. B, Western blot analysis comparing MMP-9 protein expression in WT+Sham, TG+Sham, WT+MI, and TG+MI mice ($n=6$ each). Data are expressed as ratio to WT+Sham values run concurrently on same gel. Values are mean \pm SEM. ** $P<0.01$ vs WT+Sham value. * $P<0.05$, †† $P<0.01$ vs WT+MI value.

increased in WT+MI at days 3 and 28 compared with the sham but was not altered in TG+MI.

Discussion

The present study provides direct evidence that the overexpression of GSHPx protects the heart against post-MI remodeling and heart failure in mice. It reduced chamber dilatation and dysfunction as well as myocyte hypertrophy, apoptosis, and interstitial fibrosis of the noninfarcted myocardium. All of these beneficial effects could contribute to the improved survival of TG mice after MI.

A growing body of evidence suggests that ROS play a major role in the development and progression of LV remodeling and failure. In addition, antioxidants have been shown to exert protective and beneficial effects in experimental heart failure.^{1,5,15–17} An antioxidant, vitamin E, prevented the transition from hypertrophy to failure in the guinea pig model of ascending aortic constriction.¹⁵ In addition, probucol had protective effects against pacing-induced heart failure¹⁶ and adriamycin-induced cardiomyopathy.¹⁷ A recent study from our laboratory demonstrated that dimethylthiourea improved survival and prevented LV remodeling and failure after MI.¹ The most effective way to evaluate the contribution of the specific antioxidant enzyme and obtain direct evidence for an adverse role of ROS in heart failure is through gene manipulation. Therefore, the present study not only extends the previous observation that used antioxidants^{1,5,15–17} but also reveals the major role of ROS in the pathophysiology of post-MI remodeling. On the basis of the present results, ebselen, a GSHPx mimetic, could be also effective in preventing LV remodeling after MI. In fact, this compound has been shown to protect against ischemia-reperfusion injury in a canine model of MI.¹⁸

GSHPx is a key antioxidant that catalyzes the reduction of H_2O_2 and hydroperoxides. It not only scavenges H_2O_2 but also prevents the formation of other more toxic radicals, such as $\cdot OH$. GSHPx possesses a higher affinity for H_2O_2 than catalase. Furthermore, it is present in relatively high amounts within the

heart, especially in the cytosolic and mitochondrial compartments.¹⁹ These lines of evidence imply the primary importance of GSHPx as a defense mechanism within the heart compared with catalase. Moreover, GSHPx is expected to exert greater protective effects against oxidative damage than SOD, because greater dismutation of $\cdot O_2^-$ by SOD may result in an increase of H_2O_2 . Therefore, compared with SOD or catalase, GSHPx is thought to be more effective in protecting cells, tissues, and organs against oxidative damage.^{4,20}

Previous studies demonstrated that GSHPx-knockout mice were more susceptible to and TG mice were more resistant to myocardial ischemia/reperfusion injury.^{21,22} The present study extends the previous observation by demonstrating that they can attenuate not only ischemia-reperfusion injury but also post-MI cardiac failure. Our results are also consistent with a study showing that circulatory failure induced by a large dose of lipopolysaccharides was inhibited in GSHPx TG mice.²³ The beneficial effects of GSHPx overexpression shown in the present study were not a result of its MI size-sparing effect, because the infarct size was comparable between WT+MI and TG+MI mice. Furthermore, its effects might not be attributable to the effects of GSHPx overexpression on hemodynamics, because blood pressure and heart rate were not altered (Table). Importantly, it is also unlikely that the protective effects of GSHPx overexpression are a result of altered expression of other antioxidant enzymes.

GSHPx is a selenium-containing protein present in both cytosol and mitochondria of eukaryotic cells. At least 5 isoforms are found in mammals. The major cellular GSHPx (GSHPx-1) is expressed in all tissues and contributes to most of its activity.²⁴ However, the phospholipid-hydroperoxide GSHPx and plasma GSHPx may also protect the heart from oxidative damage. Recently, GSHPx-5 has been added to the family, which is selenium-independent. In addition, the peroxiredoxin (Prx) family can also scavenge H_2O_2 . Among the 6 distinct Prx family members identified in mammals, Prx-3 may exert protective effects against myocardial oxidative damage, because it is

specifically located in the mitochondria.²⁵ However, no previous studies examined the changes of these antioxidant enzymes in failing hearts, and further studies are clearly needed.

There may be several factors attributable to the protective effects conferred by GSHPx overexpression on postinfarct myocardial failure. First, GSHPx overexpression reduced cardiac hypertrophy and apoptosis. Siwik et al³ demonstrated that a subtle increase in ROS caused by partial inhibition of SOD results in hypertrophy and apoptosis in isolated cardiac myocytes, both of which are present in the noninfarcted myocardium. Apoptosis is thought to contribute to the progressive deterioration of LV function after MI. LV weight data were not in parallel to echocardiographic and myocyte size data in this study. If the TG+MI mice had similar infarct size, less myocyte cross-sectional area, and less interstitial collagen content, LV weight should also be lower. The reasons for this apparent inconsistency are not clear. It is possible that the TG mice had a greater number of smaller myocytes, which, however, is unlikely, because the sham myocyte size is similar between WT and TG. Second, GSHPx overexpression decreased the interstitial fibrosis. Importantly, the present study demonstrated that the activation of MMP-9 was inhibited in TG+MI mice. It has been reported that myocardial MMPs are increased in MI.²⁶ In addition, an MMP inhibitor has been shown to limit the chamber remodeling after MI.²⁷ Therefore, MMP activation might play an important role in the pathophysiology of LV remodeling. It has been shown that MMPs can be activated by ROS in cardiac fibroblasts.²⁸ On the basis of these findings, it is conceivable to hypothesize that increased myocardial ROS could contribute to the activation of MMP and thus to the development of LV remodeling.

In conclusion, GSHPx overexpression inhibited the development of LV remodeling and failure after MI, which might contribute to the improved survival. These effects were associated with the attenuation of myocyte hypertrophy, apoptosis, and interstitial fibrosis. Therapies designed to interfere with oxidative stress could be beneficial to prevent heart failure after MI.

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