Overexpression of glutathione peroxidase attenuates myocardial remodeling and preserves diastolic function in diabetic heart

Shouji Matsushima,1 Shintaro Kinugawa,2 Tomomi Ide,1 Hidenori Matsusaka,1 Naoki Inoue,2 Yukihiro Ohta,2 Takashi Yokota,2 Kenji Sunagawa,1 and Hiroyuki Tsutsui2

1Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka; and 2Department of Cardiovascular Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Submitted 28 April 2006; accepted in final form 18 June 2006

The excess production of reactive oxygen species (ROS), resulting in oxidative stress, is widely considered to be a cause of tissue damage associated with diabetes (2, 23). Our previous studies have demonstrated that H2O2 and hydroxyl radical (·OH), generated via superoxide anion (O2·−), play an important role in the development and progression of myocardial remodeling in the pacing-induced heart failure as well as in the postinfarct heart failure model (10, 28). Furthermore, in vitro studies demonstrated that ROS could directly induce hypertrophy and apoptosis in cardiac myocytes (30). Therefore, oxidative stress may contribute to the development of myocardial remodeling seen in diabetes (27). In fact, previous studies reported that antioxidants could attenuate myocardial damage in experimental diabetes (24, 36).

The first line of defense against ROS-mediated cardiac injury comprises several antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx). SOD, including mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD, could provide antioxidant protection by inactivating O2·−, sparing nitric oxide from destruction, and preventing O2·− from forming more destructive ROS such as peroxynitrite and its reaction products, including ·OH. However, greater dismutation of O2·− by SOD results in an increase of H2O2. Therefore, among these antioxidants, GSHPx is an important enzyme that performs several vital functions (28). GSHPx not only functions by removing H2O2 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 or catalase or the combination of SOD and catalase (31). The great efficiency of GSHPx as an antioxidant may be attributable to the fact that it is located in both the cytosol and the mitochondrial matrix and that it can utilize lipid peroxides as well as H2O2 for substrates. These beneficial characteristics make GSHPx an important candidate for therapy against myocardial remodeling and failure due to increased ROS production. Our previous studies have demonstrated that GSHPx overexpression can attenuate oxidative stress as well as postinfarct cardiac remodeling and failure (28). Therefore, the purpose of this study was to determine whether overexpression of GSHPx could attenuate the structural remodeling and func-
tional decline in the hearts with diabetes using GSHPx transgenic (TG) mice (20).

MATERIALS AND METHODS

TG mice. This 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 the C57BL/6 × CBA/J hybrid mice with the overexpression of the human GSHPx1 gene (20). The coding region of the human GSHPx gene was inserted into the unique BamHI site of the pHMG cassette vector. To generate TG mice, NolI fragments of recombinant plasmids were microinjected into the pronuclei of the C57BL/6 × CBA/J hybrid. Mouse strain 23 (containing 200 copies of the human GSHPx gene) was used for further studies. To obtain TG animals and their normal littermates for experiments, TG founders (kindly provided by Drs. Oleg Mirochnitchenko and Masayori Inouye, University of Medicine and Dentistry of New Jersey) were bred with (C57BL/6 × CBA/J)F1 mice. Tail clips and a PCR protocol to confirm the genotype were performed by a group of investigators.

Induction of diabetes. Diabetes was induced in 8-wk-old male mice, weighing 26–32 g, by injection with streptozotocin (STZ; 160 mg/kg ip). Tail vein blood glucose samples were measured 5 days after injection to insure induction of diabetes. As a control, vehicle (0.1 mol/l citrate buffer, pH 4.5) was injected in wild-type (WT) and TG mice. This assignment procedure was performed with the use of numeric codes to identify the animals.

Echocardiographic and hemodynamic measurements. After 8 wk of injection, echocardiographic studies were performed under light anesthesia with tribromoethanol-amylene hydrate (Avertin; 2.5% wt/vol, 8 μl/g ip) and spontaneous respiration. Two-dimensional targeted M-mode tracings were recorded at a paper speed of 50 mm/s (28).

To assess diastolic cardiac function, two-dimensional guided Doppler flow measurements of mitral inflow were obtained. Mitral inflow velocities were recorded only after extensive scanning from multiple vantage points to ensure that the maximal velocity was obtained. In most situations, this was an apical window corresponding to an “off-axis” apical window (displaced toward the parasternal window). Early and late mitral inflow velocity (E wave and A wave, respectively) and E wave deceleration time were measured from the Doppler recordings in the standard fashion, and the E-to-A ratio was calculated (17).

Under the same anesthesia with Avertin, a 1.4-Fr micromanometer-tipped catheter (Millar Instruments) was inserted into the right carotid artery and then advanced into the left ventricle (LV) to measure LV pressures. The following indexes of cardiac performance were measured and averaged from three consecutive beats; LV systolic pressure, LV end-diastolic pressure (EDP), the maximum and minimum values of the first derivative of LV pressure (LVEDP/dtmax and LVdP/ dtmin, respectively), and the time needed for relaxation of 50% maximal LV pressure to baseline value (tau). One subset of investigators, who were not informed of the experimental groups, performed in vivo LV function studies.

Plasma glucose. After completion of the cardiac function measurements performed after 2 h of fasting, blood samples were collected for the determination of plasma glucose.

Myocardial histopathology. After in vivo studies, the heart was excised and dissected into the right and left ventricles, including the septum. The LV was cut into three transverse sections: apex, middle ring, and base. From the middle ring, 5-μm sections were cut and stained with Masson’s trichrome. Myocyte cross-sectional area was determined by quantitative morphometry of tissue sections from the mid-LV (28). Collagen distribution was also determined by using picrosirius red (0.1% Sirius Red F3BA in picric acid)-stained sections. Slides were left in 0.2% phosphomolybdic acid for 5 min, washed, and left in picrosirius red for 90 min and then in 1 mmol/l HCl for 2 min and 70% ethanol for 45 s.

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 10 5 total nuclei identified by hematoxylin-positive staining in the same sections (28).

Antioxidant enzyme activities and lipid peroxidation. The enzymatic activities of GSHPx, catalase, and total SOD were measured in the LV according to the methods described previously (28).

Lipid peroxidation is a major biochemical consequence of ROS attack on biological tissue. We therefore determined the degree of lipid peroxidation in the myocardial tissue through biochemical assay of thiobarbituric acid reactive substances (TBARS) (28). In brief, LV myocardial tissue was homogenized (10% wt/vol) in 1.15% KCl solution (pH 7.4). The homogenate was mixed with 0.4% SDS, 7.5% acetic acid adjusted to pH 3.5 with NaOH, and 0.3% thiobarbituric acid. Butylated hydroxytoluene (0.01%) was added to the assay mixture to prevent autoxidation of the sample. The mixture was kept at 5°C for 60 min and then was heated at 100°C for 60 min. After cooling, the mixture was extracted with distilled water and n-butanol-pyridine (15:1, vol/vol) and centrifuged at 1,600 g for 10 min. The absorbance of the organic phase was measured at 532 nm. The amount of TBARS was determined by absorbance with the molecular extinction coefficient of 156,000 (and expressed as μmol/g wet wt).

To further assess the lipid peroxidation by histochemical analysis, LV myocardial sections were immunolabeled with an antibody raised against 4-hydroxy-2-nonenal (HNE)-modified protein, an aldehydic byproduct of lipid peroxidation (10). In brief, paraffin-embedded tissue sections (3-μm thick) were deparaffinized with xylene and refixed with Bouin’s solution for 20 min and immersed in 70, 90, and 100% ethanol to remove picric acid. To inhibit endogenous peroxidase, the sections were incubated with 0.3% H2O2 in methanol for 30 min. After a rinsing in 0.01 mol/l PBS, the sections were incubated with normal goat serum (diluted to 1:10) to inhibit nonspecific binding of antibodies. The sections were further incubated with polyclonal antiserum raised against an HNE-modified histidyl peptide (Gly3-His-Gly3) conjugated with keyhole-limpet hemocyanin. After a rinsing with 0.01 mol/l PBS, the sections were incubated with biotin-labeled mouse anti-mouse IgG antiserum (diluted 1:100; Nihonyushi N213220) for 60 min and then with avidin-biotin complex (Vectastain ABC kit; 1:100) for 60 min. After a rinsing, the sections were finally incubated with 0.02% 3,3’-diaminobenzidine and 0.03% H2O2 in deionized water for 6–9 min. As a negative control, the sections were also incubated with normal rabbit serum.

A morphometric analysis of HNE-positive myocardial area was performed with tissue sections stained with HNE. Each section was photographed under a microscope and magnified (final magnification, ×200). Three to four fields were randomly selected from one or two coronal sections in each animal. As a result, the HNE-positive areas were measured at approximately five to seven fields for each animal. Within each field, myocardial segments that stained positively with anti-HNE antibody were identified and were manually traced by use of a digitizing pad with a computer to calculate the traced area.

Matrix metalloproteinases, transforming growth factor-β, and connective tissue growth factor. We further determined the alterations of profibrotic mediators including matrix metalloproteinases (MMPs), transforming growth factor (TGF)-β, and connective tissue growth factor (CTGF) in this model.

First, the myocardial MMP levels, including MMP-2 and MMP-9, were determined in the LV using gelatin zymography (10). The LV myocardial samples were homogenized (~30-s bursts) in 1 ml of an ice-cold extraction buffer containing cacodylic acid (10 mmol/l), NaCl (0.15 mol/l), ZnCl2 (20 mmol/l), NaH2PO4 (1.5 mmol/l), and 0.01% Triton X-100 (pH 5.0). The homogenate was then centrifuged (4°C, 10 min, 10,000 g), and the supernatant was decanted and saved on ice.
The pH levels of the samples were adjusted to 7.5 by use of Tris (1 mol/l). The final protein concentration of the myocardial extracts was determined using a standardized colorimetric assay. The extracted samples were then aliquoted and stored at −80°C until the time of assay. The myocardial extracts were then directly loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/ml gelatin under non-reducing conditions. The myocardial extracts at a final protein content of 5 μg were loaded onto the gels using a 3:1 sample buffer (10% SDS, 4% sucrose, 0.25 mol/l Tris-Cl, and 0.1% bromophenol blue, pH 6.8). The gels were run at 15 mA/gel through the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), while the running buffer temperature was maintained at 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each, rinsed in water, and incubated for 24 h in a substrate buffer at 37°C (50 mmol/l Tris-HCl, 5 mmol/l CaCl₂, and 0.02% NaN₃, pH 7.5). After incubation, the gels were stained with Coomassie brilliant blue R-250. The zymograms were digitized, and the size-fractionated bands, which indicated the MMP proteolytic levels, were measured by integrated optical density in a rectangular region of interest.

Next, the mRNA levels of myocardial MMPs including MMP-1, -2, -3, -8, and -9 as well as tissue inhibitors of MMPs (TIMPs) including TIMP-1, -2, -3, and -4 were determined by multiprobe ribonuclease protection assay (RPA Riboflour, QuantPharingen). Each value was normalized to that of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in each template set as an internal control, followed by calculation as a ratio to WT.

The expression level of genes including the TGF-β gene was determined by ribonuclease protection assay. The value of each hybridized probe was normalized to that of GAPDH in each template set as an internal control. CTGF protein levels were quantified by Western blot analysis using a specific antibody against recombinant mouse CTGF. To confirm the amount of loaded proteins, total proteins were also visualized by Coomassie brilliant blue staining. Within a given experiment, the densitometric values were normalized, using standards concurrently run within the same gel, and the value for each WT + diabetes mellitus (DM) or TG + DM was calculated as a ratio of WT + Control.

Statistical analysis. Data are expressed as means ± SE. Between-group comparison of means was performed by one-way ANOVA, followed by t-tests. The Bonferroni’s correction was done for multiple comparisons of means. P < 0.05 was considered to be statistically significant.

RESULTS

Antioxidant enzymes and TBARS. The baseline differences in antioxidant enzyme activities between WT and TG mice were determined (Table 1). In TG mice, there was a significant increase in GSHPx activities compared with WT mice. GSHPx activities were comparable between WT + Control and WT + DM (Table 1). More importantly, as expected, GSHPx activities were significantly higher in TG + DM compared with WT + DM (Table 1). SOD and catalase activities were not altered in WT + DM or TG + DM compared with WT + Control.

TBARS measured in the LV obtained from WT + DM tended to be greater compared with WT + DM, which, however, did not reach statistical significance (P = 0.05). Importantly, TBARS from TG + DM were significantly lower than those from WT + DM (Fig. 1A). In accordance with TBARS, an immunohistochemical analysis of HNE-modified protein revealed the lipid peroxides to be positively stained in myocytes from WT + DM mice, whereas only faint labeling was observed in the WT + Control, TG + Control, and TG + DM (Fig. 1B). The myocardial area stained positively with HNE was significantly smaller in TG + DM than in WT + DM.

Body weight and plasma glucose. There was no death in any group of mice. DM animals gained less body weight than control mice, which, however, did not differ between WT + DM and TG + DM (Table 2). At 5 days and 8 wk, DM mice had increased plasma glucose levels compared with control animals, which was not altered by GSHPx gene overexpression (Table 2).

Echocardiographic and hemodynamics. The echocardiographic and hemodynamic data of mice are at 8 wk are shown in Table 3. There was no significant difference in heart rate and aortic blood pressure among four groups of mice. Systolic function, as assessed by echocardiographic fractional shortening and LVdP/dtmax, was preserved as normal in both WT + DM and TG + DM mice. In contrast, diastolic function, as assessed by the deceleration time of peak velocity of transmitral diastolic flow (Dec) and the time needed for relaxation of 50% maximal LV pressure to baseline value (tau), was impaired in WT + DM, which was significantly attenuated in TG + DM (Table 3). LVdP/dtmin tended to be decreased in WT + DM compared with WT + Control (P = 0.05) and increased in TG + DM compared with WT + DM (P = 0.09), both of which, however, did not reach statistical significance. LVEDP was slightly, but significantly, elevated in WT + DM, which was attenuated in TG + DM.

Myocardial histomorphometry and apoptosis. Increased cardiac myocyte size, indicating hypertrophy, was evident in WT + DM, and this increase was significantly attenuated in TG + DM (Fig. 2). Collagen volume fraction, indicating interstitial fibrosis, in the WT + DM group was greater than in the control groups (Fig. 2). These fibrotic changes in the heart were significantly reduced in the TG + DM group.

The number of TUNEL-positive cells in the LV from the TG + DM group was significantly decreased compared with the WT + DM group and held constant relative to the control groups (Fig. 3). These fibrotic changes in the heart were significantly reduced in the TG + DM group.

Myocardial MMPs, TGF-β, and CTGF. To further assess the alterations of profibrotic mediators to be involved in interstitial fibrosis, myocardial levels of MMPs, TGF-β, and CTGF were determined.

Table 1. Antioxidant enzymes

<table>
<thead>
<tr>
<th></th>
<th>WT + Control</th>
<th>TG + Control</th>
<th>WT + DM</th>
<th>TG + DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSHPx, nmol/min·mg protein⁻¹</td>
<td>44.9 ± 2.5</td>
<td>68.1 ± 5.0*‡</td>
<td>45.6 ± 3.1</td>
<td>68.8 ± 8.3*‡</td>
</tr>
<tr>
<td>Catalase, nmol/mg protein</td>
<td>67.8 ± 7.5</td>
<td>89.5 ± 15.4</td>
<td>64.4 ± 7.0</td>
<td>90.0 ± 10.1</td>
</tr>
<tr>
<td>Total SOD, U/mg protein</td>
<td>14.6 ± 1.3</td>
<td>15.7 ± 1.6</td>
<td>19.2 ± 2.4</td>
<td>19.0 ± 1.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = number of mice. GSHPx, glutathione peroxidase; SOD, superoxide dismutase; WT, wildtype; DM, diabetes mellitus. *P < 0.05 vs. WT + Control. †P < 0.05 vs. WT + DM.
Myocardial MMP-2 zymographic levels were increased in WT+DM, and this increase was significantly attenuated in TG+DM (Fig. 4). MMP-9 protein levels, although they were faint, were not altered in WT+DM or TG+DM. Other MMPs including MMP-1, -3, and -8 and TIMPs including TIMP-1, -2, -3, and -4 were not altered in the hearts from four groups of mice (Fig. 5).

Myocardial TGF-β gene expression was increased in WT+DM, which was significantly attenuated in TG+DM (Fig. 6A). In parallel with TGF-β, CTGF protein levels were increased in WT+DM, which, however, was not attenuated in TG+DM (Fig. 6B).

**DISCUSSION**

The present study demonstrated that overexpression of GSHPx attenuated oxidative stress in the myocardial tissue and concurrently improved LV diastolic function as well as reduced myocyte hypertrophy, apoptosis, and interstitial fibrosis. Although the beneficial effects of various antioxidants on the diabetic heart have been demonstrated already by previous studies (6, 24, 36), the present study specifically provided direct evidence for the protective role of GSHPx against myocardial remodeling and dysfunction in this disease state.

Table 2. Body weight and blood glucose data

<table>
<thead>
<tr>
<th></th>
<th>WT+Control (n = 16)</th>
<th>TG+Control (n = 14)</th>
<th>WT+DM (n = 13)</th>
<th>TG+DM (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>33.5 ± 1.1</td>
<td>31.7 ± 1.0</td>
<td>27.2 ± 0.7‡</td>
<td>27.4 ± 0.7‡</td>
</tr>
<tr>
<td>Glucose, mg/dl (5 days after injection)</td>
<td>119 ± 5</td>
<td>114 ± 4</td>
<td>332 ± 24‡</td>
<td>350 ± 36‡</td>
</tr>
<tr>
<td>Glucose, mg/dl (8 wk after injection)</td>
<td>127 ± 8</td>
<td>125 ± 6</td>
<td>431 ± 36‡</td>
<td>420 ± 7‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n is number of mice. ‡P < 0.01 vs. WT+Control.

The most effective way to evaluate the contribution of the specific antioxidant and obtain the direct evidence for a role of oxidative stress is through gene manipulation instead of the administration of antioxidants. Therefore, the novel finding of the present study from the point of view of understanding the pathophysiology and treatment of maladaptive myocardial remodeling in response to diabetes mellitus is that H$_2$O$_2$ is critically involved in this disease process and may be a potential therapeutic target. Although the present study does not support the notion that the alterations in GSHPx are involved in oxidative stress in the diabetic heart, the overexpression of this gene can prevent the increase of oxidative stress as well as cardiac remodeling in diabetes mellitus.

A growing body of evidence suggests that the production of ROS is increased in the diabetic heart (14). Specifically, ROS are produced within the mitochondria of these hearts (15, 23). Furthermore, antioxidants have been shown to prevent the structural and functional alterations of the diabetes heart (6, 24, 36). Therefore, the present study not only extends the previous observation that employed antioxidants but also reveals the major role of ROS in the pathophysiology of cardiac remodeling associated with diabetes.

GSHPx is a key antioxidant that catalyzes the reduction of H$_2$O$_2$ and hydroperoxides. It not only scavenges H$_2$O$_2$ but also prevents the formation of other more toxic radicals such as •OH. GSHPx possesses a higher affinity for H$_2$O$_2$ than catalase. Furthermore, it is present in relatively high amounts within the heart, especially in the cytosolic and mitochondrial compartments (18). 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 O$_2^-$ by SOD may result in an increase of H$_2$O$_2$. Therefore, compared with SOD or catalase,
Fig. 2. A: representative photomicrographs of Masson’s trichrome-stained left ventricular (LV) cross section obtained from 4 groups of mice. B: summary data for myocyte cross-sectional area and collagen volume fraction in WT + Control (n = 11), TG + Control (n = 8), WT + DM (n = 12), and TG + DM (n = 11) mice. Values are means ± SE. *P < 0.05, difference from the WT + Control value. †P < 0.05, difference from the WT + DM value.

Table 3. Echocardiographic and hemodynamic data

<table>
<thead>
<tr>
<th></th>
<th>WT + Control</th>
<th>TG + Control</th>
<th>WT + DM</th>
<th>TG + DM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Echocardiographic data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>16</td>
<td>14</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>466 ± 12</td>
<td>448 ± 7</td>
<td>417 ± 6</td>
<td>420 ± 7</td>
</tr>
<tr>
<td>LV EDD, mm</td>
<td>3.7 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>LV ESD, mm</td>
<td>2.4 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>35.1 ± 0.5</td>
<td>35.5 ± 0.8</td>
<td>34.1 ± 0.7</td>
<td>36.1 ± 0.6</td>
</tr>
<tr>
<td>IVS thickness, mm</td>
<td>0.71 ± 0.02</td>
<td>0.70 ± 0.02</td>
<td>0.68 ± 0.02</td>
<td>0.68 ± 0.02</td>
</tr>
<tr>
<td>PW thickness, mm</td>
<td>0.74 ± 0.02</td>
<td>0.74 ± 0.03</td>
<td>0.72 ± 0.02</td>
<td>0.73 ± 0.02</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.49 ± 0.06</td>
<td>1.60 ± 0.12</td>
<td>1.28 ± 0.06</td>
<td>1.31 ± 0.07</td>
</tr>
<tr>
<td>Dct, ms</td>
<td>33.6 ± 0.6</td>
<td>34.1 ± 1.3</td>
<td>45.3 ± 1.6‡</td>
<td>37.5 ± 0.9§</td>
</tr>
</tbody>
</table>

|                     |            |              |         |         |
| **Hemodynamic data** |            |              |         |         |
| n                   | 13          | 13           | 13      | 13      |
| Heart rate, beats/min | 438 ± 12   | 439 ± 13     | 413 ± 4 | 431 ± 17 |
| Systolic aortic pressure, mmHg | 98 ± 3   | 101 ± 4      | 102 ± 5 | 101 ± 6 |
| Diastolic aortic pressure, mmHg | 66 ± 3  | 72 ± 4       | 72 ± 4  | 71 ± 5  |
| Mean aortic pressure, mmHg | 76 ± 3   | 82 ± 4       | 83 ± 5  | 82 ± 6  |
| LVEDP, mmHg         | 7376 ± 912  | 9816 ± 758   | 5742 ± 665 | 8646 ± 649 |
| LVdP/dtₘₐₓ, mmHg/s  | 6996 ± 822  | 7052 ± 700   | 4458 ± 330 | 6036 ± 547 |
| Tau, ms             | 7.8 ± 0.2   | 8.2 ± 0.4    | 13.5 ± 1.2‡ | 8.9 ± 0.7§ |

Values are means ± SE; n, number of mice. LV, left ventricular; EDD, end-diastolic diameter; ESD, end-systolic diameter; IVS, interventricular septal; PW, posterior wall; E, peak velocity of early mitral flow; A, peak velocity of late mitral flow; Dct, deceleration time; EDP, end-diastolic pressure; dP/dt, change in pressure over time; tau, time needed for relaxation of 50% maximal LV pressure to baseline value. ‡P < 0.01 vs. WT + Control. §P < 0.01 vs. WT + DM.
GSHPx is thought to be more effective in protecting cells, tissues, and organs against oxidative damage (5, 31). In fact, our previous studies demonstrated that the mice with GSHPx gene overexpression were more resistant to myocardial oxidative stress as well as remodeling and failure after myocardial infarction (28). Thus the present study extends the previous observation by demonstrating that they can attenuate not only post-myocardial infarction failure but also diabetes-associated cardiac dysfunction.

The beneficial effects of GSHPx overexpression shown in the present study were primarily due to its scavenging action on H$_2$O$_2$ (Table 1). However, we could not completely exclude the possibility that altered expression of other antioxidant enzymes is involved. LV total SOD activities tended to be higher in WT and TG mice with diabetes; however, they did not reach statistical significance. Total SOD may be induced in the presence of DM, even though its pathophysiological significance remains uncertain. In addition, GSHPx TG mice tended to have higher levels of catalase (Table 1), which did not reach statistical significance. Nevertheless, we could not exclude the possibility that there were synergistic antioxidant effects between GSHPx and catalase against H$_2$O$_2$, because both possess a high affinity for H$_2$O$_2$. Furthermore, the beneficial effects of GSHPx overexpression were not due to the effects on diabetes itself, because body weight and plasma glucose levels were comparable between WT+DM and TG+DM mice (Table 2). Importantly, the effects were not attributable to those of GSHPx overexpression on hemodynamics, because blood pressure and heart rate were not altered (Table 3).

Diabetes mellitus causes both diastolic and systolic cardiac dysfunction (27, 37), but an impairment of diastolic function usually occurs before systolic dysfunction develops. The impairment of diastolic function despite normal systolic function is thought to result from an increased myocardial stiffness. The present study has clearly demonstrated that the attenuation of myocardial oxidative stress by GSHPx overexpression (Fig. 1) is associated with the attenuation of diastolic dysfunction (Table 3), myocyte hypertrophy, and interstitial fibrosis (Fig. 2) in diabetes. Previous studies have demonstrated that diabetest induced by STZ leads to an increased collagen deposition (25), resulting in an increased myocardial stiffness and a decrease in LV compliance (19, 24). Although we did not directly assess the diastolic function by using the stress-
strain relations and therefore could not comment on the contribution of myocardial stiffness to LV function in the present study, the decline in myocardial fibrosis in TG/DM mice can well be considered to contribute to the physiological improvement of diastolic properties in these mice. In fact, prior studies have generally shown an association between increased cardiac fibrosis and diastolic chamber stiffening (35).

Fig. 5. A: representative image of myocardial gene expression of MMPs/tissue inhibitors of MMPs (TIMP). B: densitometric analysis of MMP and TIMP gene expression from WT+DM (n = 4) and TG+DM mice (n = 4). Each value was normalized to that of GAPDH in each template set as an internal control and expressed as the ratio to WT+Control (n = 4). Values are means ± SE.

Fig. 6. Myocardial transforming growth factor (TGF)-β (A) and connective tissue growth factor (CTGF; B) levels in WT+Control, TG+Control, WT+DM, and TG+DM mice (n = 6 each). Data were expressed as the ratio to WT+Control values concurrently run on the same gel. Values are means ± SE. *P < 0.05, difference from the WT+Control value. †P < 0.05, difference from the WT+DM value.
The present results are consistent with previous studies demonstrating that ROS are involved in the structural alterations of the extracellular matrix collagens. The increase in collagens and myocardial stiffness in STZ-induced diabetic rats was prevented by treatment with aminoguanidine (24) and antioxidants (26, 34).

There may be several factors whereby oxidative stress contributes to myocardial remodeling in diabetes. First, oxidative stress is involved in cardiac myocyte hypertrophy and apoptosis (Figs. 2 and 3). It has been demonstrated that a subtle increase in ROS caused by partial inhibition of SOD results in hypertrophy and apoptosis in isolated cardiac myocytes (30), both of which are thought to contribute to diabetic myocardial damage. Specifically, recent studies show that the incidence of apoptosis is increased in the diabetic heart (4, 7), which may cause loss of contractile myocytes, compensatory hypertrophy of myocytes, and interstitial fibrosis (6). Myocyte necrosis may also be involved in the increased fibrosis from the diabetic heart. Second, oxidative stress induces the activation of MMP-2 seen in diabetes (Fig. 4). Our previous studies have demonstrated that MMP-2 activation plays an important role in the pathophysiology of cardiac remodeling (16). Moreover, MMPs have been shown to be activated by ROS in cardiac fibroblasts (29). On the basis of these findings, it is conceivable to hypothesize that increased ROS contribute to the activation of MMP and thus to the development of interstitial fibrosis in diabetes. Third, a possible role for growth factors in diabetes-related end-organ complications is increasingly being recognized. TGF-β and CTGF can induce the production of collagen and fibronectin from cardiac fibroblasts and myocytes, and increased expression of both factors has been documented in the diabetic heart (12, 32). CTGF has a unique TGF-β response element in its promoter region (8) and acts downstream of TGF-β. The present study demonstrated that the attenuation of cardiac fibrosis by GSHPx overexpression was associated with the decrease in TGF-β expression, but not with CTGF, suggesting that TGF-β mainly contributed to cardiac fibrosis in this model. Nevertheless, we could not completely exclude the contribution of other growth factors such as VEGF, FGF, or PDGF in our model.

Approximately 20–40% of patients with heart failure have preserved systolic function and are thought to have an impairment of diastolic function as the primary mechanism leading to symptomatic heart failure (33). Diabetes is recognized as one of the major risk factors associated with diastolic heart failure (13). Despite the high prevalence of diabetes among patients with this type of heart failure, the treatment of diastolic heart failure remains empirical (9). There are currently few clinical data to support the efficacy of any particular class of drugs for diabetic heart failure. Therefore, the present study should help clarify the potential that antioxidants may play in the treatment of diastolic heart failure.

There are several limitations to be acknowledged in this study. First, the echocardiographic assessment of LV diastolic function in mice is somewhat difficult. However, intra- and interobserver variabilities of our echocardiographic measurements were small, and measurements were highly reproducible. Therefore, our technique was capable of noninvasively assessing the LV structure and function in mice. Second, the relationships between cardiac function and structural alterations were analyzed only at 8 wk after the induction of diabetes. Longer follow-up must be performed to establish whether cardiac remodeling including myocyte hypertrophy, interstitial fibrosis, and apoptosis due to hyperglycemia may eventually lead to clinical heart failure in diabetes. Third, we employed myocyte cross-sectional area as an index of myocyte hypertrophy. Although this has been commonly used, we need to be cautious in interpreting these data, because increased myocyte cross-sectional area is not equivalent to myocyte hypertrophy, and the length of the cell is an equally important determinant of myocyte volume.

In conclusion, GSHPx overexpression inhibited the development of LV remodeling and diastolic dysfunction associated with diabetes. These beneficial effects of GSHPx were associated with the attenuation of myocyte hypertrophy, apoptosis, and interstitial fibrosis. Therapies designed to interfere with oxidative stress could be beneficial to prevent diabetic heart disease.

ACKNOWLEDGMENTS

A part of this study was conducted in Kyushu University Station for Collaborative Research I and II. We thank Drs. Oleg Mirochnitchenko and Masayori Inouye of University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School for providing us the original breeding pairs of GSHPx TG mice.

GRANTS

This study was supported in part by grants from the Ministry of Education, Science and Culture, Japan (nos. 12670676, 14370230, 17390223, and 17692233).

REFERENCES

11. Ishibashi N, Weisbrot-Lefkowitz M, Reuhl K, Inouye M, and Mirochnitchenko O. Modulation of chemokine expression during ischemia/reperfusion in transgenic mice overproducing human glutathione peroxi-