Journal of the American College of Cardiology
© 2006 by the American College of Cardiology Foundation
Published by Elsevier Inc.

Vol. 48, No. 8, 2006
ISSN 0735-1097/06/$32.00
doi:10.1016/j.jacc.2006.07.022

PRECLINICAL STUDY

Attenuation by Metallothionein of Early Cardiac Cell Death via Suppression of Mitochondrial Oxidative Stress Results in a Prevention of Diabetic Cardiomyopathy

Lu Cai, MD, PHD,*†‡ Yuehui Wang, MD, PHD,* Guihua Zhou, MD, PHD,* Teresa Chen, PHD,† Ye Song, MD, PHD,* Xiaokun Li, MD, PHD,‡ Y. James Kang, DVM, PHD*†

Louisville, Kentucky; and Wenzhou, China

OBJECTIVES We aimed to test whether attenuation of early-phase cardiac cell death can prevent diabetic cardiomyopathy.

BACKGROUND Our previous study showed that cardiac apoptosis as a major early cellular response to diabetes is induced by hyperglycemia-derived oxidative stress that activates a mitochondrial cytochrome *c*-mediated caspase-3 activation pathway. Metallothionein (MT) as a potent antioxidant prevents the development of diabetic cardiomyopathy.

METHODS Diabetes was induced by a single dose of streptozotocin (STZ) (150 mg/kg) in cardiac-specific, metallothionein-overexpressing transgenic (MT-TG) mice and wild-type (WT) controls. On days 7, 14, and 21 after STZ treatment, cardiac apoptosis was examined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and caspase-3 activation. Cardiomyopathy was evaluated by cardiac ultrastructure and fibrosis in the diabetic mice 6 months after STZ treatment.

RESULTS A significant reduction in diabetes-induced increases in TUNEL-positive cells, caspase-3 activation, and cytochrome *c* release from mitochondria was observed in the MT-TG mice as compared to WT mice. Cardiac protein nitration (3-nitrotyrosine [3-NT]) and lipid peroxidation were significantly increased, and there was an increase in mitochondrial oxidized glutathione and a decrease in mitochondrial reduced glutathione in the WT, but not in the MT-TG, diabetic mice. Double staining for cardiomyocytes with alpha sarcomeric actin and caspase-3 or 3-NT confirmed the cardiomyocyte-specific effects. A significant prevention of diabetic cardiomyopathy and enhanced animal survival were observed in the MT-TG diabetic mice as compared to WT diabetic mice.

CONCLUSIONS These results suggest that attenuation of early-phase cardiac cell death by MT results in a significant prevention of the development of diabetic cardiomyopathy. This process is mediated by MT suppression of mitochondrial oxidative stress. (J Am Coll Cardiol 2006; 48:1688–97) © 2006 by the American College of Cardiology Foundation

Cardiac dysfunction has become the major cause of mortality in diabetic patients (1–3). Diabetic cardiomyopathy was found not directly related to vascular pathogenesis (2,3), but the precise mechanism remains elusive (1–3). Hearts from diabetic patients and streptozotocin (STZ)-induced diabetic animals display a reduction in cardiac mass over time and interstitial and perivascular fibrosis at late phase (2–5). These late-phase changes, however, are believed to result from early cellular responses such as cardiac cell death (2,4,6,7).

Cardiac apoptosis as a comprehensive consequence of cardiac responses to various stresses causes a loss of contractile tissue, which initiates a cardiac remodeling and fibrosis; therefore, the cardiac apoptosis has been identified as a pivotal cause of various cardiomyopathies (8–10). We, as well as others, have shown that cardiac apoptosis increases in diabetic animals and patients (11–14). It remains to be determined whether the increased cardiac apoptotic cell death plays an important role in the development of diabetic cardiomyopathy (7).

Mitochondrial cytochrome *c* release and activation of caspase-3 play an important role in diabetes-induced cardiac cell death (14,15). Among apoptotic stimuli, reactive oxygen species (ROS) or reactive nitrogen species (RNS) play a critical role in the mitochondrial cytochrome *c* release and caspase-3 activation (7,8,14–17). A significant increase in ROS and RNS was found in cardiac cells exposed to high levels of glucose in vitro (14) and in isolated cardiomyocytes from diabetic mice (13,18). We have further identified an increased accumulation of superoxide with an increase in

From the Departments of *Medicine and †Pharmacology and Toxicology, the University of Louisville, Louisville, Kentucky; and the ‡School of Pharmaceutical Sciences, Wenzhou Medical College, Wenzhou, China. This work was supported, in part, by grants PM020187 from Philip Morris USA, Inc., ADA02-07-JF-10 and ADA05-07-CD-02 from the American Diabetes Association (to Dr. Cai), HL63760 and HL59225 from the National Institutes of Health (to Dr. Kang), and by the Wenzhou Medical College Collaborating Fund (5010 Project, to Drs. Cai and Li). Dr. Kang is a Distinguished University Scholar of the University of Louisville.

Manuscript received April 7, 2006; revised manuscript received June 15, 2006, accepted June 19, 2006.

Abbreviations and Acronyms

ANOVA = analysis of variance
Gpx = glutathione peroxidase
GR = glutathione reductase
GSH = glutathione
GSSG = oxidized glutathione
IGF-1 = insulin-like growth factor 1
LVEDP = left ventricular end-diastolic pressure
MT = metallothionein
MT-TG = cardiac-specific, metallothionein-overexpressing transgenic
RNS = reactive nitrogen species
ROS = reactive oxygen species
STZ = streptozotocin
TBARS = thiobarbituric acid-reactive substance
3-NT = 3-nitrotyrosine
TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
WT = wild-type

protein nitration (measured by 3-nitrotyrosine [3-NT]) in the hearts of diabetic mice (19). Therefore, we hypothesize that diabetes-derived oxidative and/or nitrosative damage causes an early-stage cell death that activates a series of cardiac remodeling responses, leading to morphological and functional abnormalities, and eventually to cardiomyopathy at the late stage (7).

Metallothioneins (MTs), a group of intracellular metal-binding proteins with high cysteine contents, are able to efficiently protect cells or tissues from oxidative damage (20–22). By functional examination, the preventive effect of MT against diabetes-induced cardiac injury has been demonstrated in our recent studies (19,23) and also in other studies (18,24). In the present study, we used cardiac-specific, MT-overexpressing transgenic (MT-TG) mice to determine (1) whether MT can prevent diabetes-induced cardiac apoptosis at early stage via suppression of oxidative stress, and (2) whether MT's attenuation of diabetes-induced early-stage cardiac apoptosis can result in a prevention of the development of cardiomyopathy at late stage.

MATERIALS AND METHODS

Diabetic mouse model. Cardiac-specific, MT-TG mice were produced from FVB mice, originally obtained from Harlan Bioproducts for Science (Indianapolis, Indiana) and characterized as described in a previous study (22). Metallothionein is overexpressed only in cardiac myocytes without alterations of other antioxidants, including glutathione (GSH), glutathione peroxidase (Gpx), glutathione reductase (GR), catalase, and superoxide dismutase (22). Both MT-TG and wild-type (WT) mice were housed in the University of Louisville Research Resources Center at 22°C with a 12-h light/dark cycle and were provided free access to standard rodent chow and tap water. All animal procedures were approved by the Institutional Animal Case and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care. Eight-week-old, male mice were intraperitoneally administrated a single dose of STZ (Sigma Co., St. Louis, Missouri) at 150 mg/kg body weight, dissolved in sodium citrate buffer (pH 4.5). On day 3 after STZ treatment, whole-blood glucose obtained from the mouse tail-vein was monitored using the SureStep complete blood glucose monitor (LifeScan Inc., Milpitas, California). Streptozotocin-treated mice with whole-blood glucose higher than 12 mmol/l were considered diabetic. To eliminate possible confounding effects of STZ on the early cell death, insulin-treated diabetic mice were examined 14 days after STZ treatment. When hyperglycemia was diagnosed on day 3 after STZ treatment, insulin was immediately given using a long-term insulin preparation (Humulin U, Eli Lilly and Company, Indianapolis, Indiana) at a concentration of 10 U/mouse twice a day to maintain the blood glucose levels between 5.6 and 11.2 mmol/l, with an average of 7.6 ± 1.1 mmol/l.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Cardiac sections in paraffin were prepared as before (14) and were processed for a TUNEL assay using an ApopTag in situ detection kit from Intergen (Purchase, New York). Mouse testicular tissue was used as positive control. For negative control, terminal deoxynucleotidyltransferase was omitted from the reaction mixture. The apoptotic cell death was quantitatively analyzed by counting the TUNEL-positive cells randomly selected from 5 fields from each of the 3 slides from each mouse.

Detection of cardiac caspase-3. Caspase-3 activation was measured by Western blotting, based on a published method (15). Briefly, cardiac tissue homogenates were lysed and fractionated electrophoretically on sodium dodecyl (lauryl) sulfate polyacrylamide gel electrophoresis (15% gradient gels), and proteins were transferred to a nitrocellulose membrane. The membrane was blocked with a 5% non-fat, dried milk for 1 h and subsequently incubated with the primary antibody against the active form of caspase-3 (Sigma, St. Louis, Missouri) at 1:500 overnight at 4°C. After the unbound antibodies with Tris-buffered saline (pH 7.2) containing 0.05% Tween 20 (Bio-Rad Laboratories, Hercules, California) were removed, the membrane was incubated with the secondary antibody for 2 h at room temperature. Glyceraldehyde-3-phosphate dehydrogenase protein expression was probed as an internal control.

Caspase-3 activation was also examined by caspase-3 activity as described before (14). Briefly, fresh heart tissues were homogenized with Telfon homogenizer in an extract buffer, and the homogenate was centrifuged at 20,000 *g* for 30 min. The supernatant was diluted with an assay buffer and incubated at 37°C with 200 μmol/l caspase-3 substrate I (Ac-DEVD-*p*NA [N-acetyl-asp-glu-val-asp-*p*NA], CalBiochem, La Jolla, California). Cleavage of the substrate was monitored at 405 nm using a microplate reader and recorded in 10-mm intervals for 2 h.

The localization of the activated caspase-3 was determined by double stains for cardiomyocyte and caspase-3, on the basis

of a published study (25). Briefly, cardiac tissues were fixed with 75% alcohol and embedded in paraffin. Tissue sections at 5-μm thickness were deparaffinized and rehydrated in a graded alcohol series. Sections were incubated with cocktail primary antibodies overnight at 4°C. The cocktail primary antibodies included a mouse anti-α-sarcomeric actin monoclonal antibody (Abcom Inc., Cambridge, Massachusetts) and rabbit anti-active caspase-3 polyclonal antibody (Becton Dickinson, Franklin Lakes, New Jersey), both at a final dilution of 1:50. After being washed with phosphate-buffered saline, sections were incubated with cocktail secondary antibodies, fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG + IgM + IgA (Abcam Inc., Cambridge, Massachusetts) at a dilution of 1:40 and Cy3-conjugated goat anti-rabbit IgG H & L (Abcom Inc.) at a dilution of 1:100, for 1 h in darkness at room temperature. Sections were examined using confocal laser scanning microscopy (Zeiss LSM510, Carl Zeiss Meditec Inc., Dublin, California) at emission of 500 to 530 nm and 550 to 560 nm, after excitation at 488 and 543 nm, respectively. Laser power and detection gains were set such that signals from single-stained controls would not appear in adjacent channels. Negative controls were realized on specimens incubated either with nonimmune serum or with the incubation buffer alone, followed by the second conjugated antibody.

Detection of mitochondrial cytochrome *c* release. Cytosolic and mitochondrial proteins were prepared for Western blotting assay to detect cytochrome *c* content, as previously described (14). Densitometry analysis for each band was performed using the computer program as described above, and quantitative data are presented as relative to control.

Measurements of mitochondrial GSH, GSSG, Gpx, and GR. To obtain sub-cellular fractionations (cytosolic and mitochondrial), heart tissue (3 mouse hearts were pooled as 1 sample) was homogenized with cold HMS (220 mmol/l D-Mannitol, 70 mmol/l sucrose, 2 mmol/l K-HEPES, pH 7.4) and centrifuged at 4°C for 5 min at 600 × *g*. The supernatants were further centrifuged at 7,000 × *g* for 10 min. The heavy membrane fraction (mitochondria) was washed once again in HMS by centrifugation at 600 × *g* for 5 min to remove any contaminating particles and then recovered by centrifugation at 7,000 × *g* for 10 min. The isolated mitochondria were resuspended in incubation buffer (250 mmol/l sucrose, 10 mmol/l K-HEPES, pH 7.2, 2 mmol/l KH_2PO_4, 5 mmol/l sodium succinate, 2.5 mmol/l EGTA). The cytosol and mitochondria were then frozen immediately for GSH and oxidized glutathione (GSSG) assay. Glutathione and GSSG contents were simultaneously quantified by high-performance liquid chromatography with dual electrochemical detection (26).

The mitochondrial proteins were also used to measure Gpx and GR using the Glutathione Peroxidase Cellular Activity Assay Kit and the Glutathione Reductase Assay Kit (Sigma, St. Louis, Missouri). Both GR and Gpx activities were calculated using the extinction coefficient of 6.22 $mmol/l^{-1}$ cm^{-1}, and expressed as nmol/l of nicotinamide adenine dinucleodtide phosphate consumed per minute per milligram of sample protein (22).

Assay for lipid peroxidation. Cardiac lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substance (TBARS) as described previously (21). The TBARS concentrations were expressed as nmol/g tissue.

Double staining for cardiomyocytes and protein nitration. To determine whether protein nitration occurs in the cardiomyocytes, double stains for cardiomyocytes by α-sarcomeric actin and protein nitration by 3-NT were performed using confocal laser scanning microscopy (25), as previously described except for the primary antibody (i.e., using a rabbit anti-3-NT polyclonal antibody at 1:50 dilution [Chemicon Inc., Temecula, California]).

Ultrastructural examination by electron microscopy. The hearts subjected to electron microscopic examination were fixed in situ by vascular perfusion with saline for 10 min, followed by a Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer, pH 7.4) for 15 min. The in situ fixative perfusion procedure and semi-quantitative analysis were described in our previous studies (19,22).

Sirius-red staining of collagen. Tissue sections of 5 μm were used for Sirius-red staining of collagen using 0.1% Sirius-red F3BA and 0.25% fast green FCF. The sections stained for Sirius red then were assessed for the proportion of fibrosis (collagen) using a computer-assisted image-analysis system as described in our previous study (23).

Statistical analysis. Data were collected from repeated experiments and are presented as mean ± SD. Comparisons between 2 groups with normality and homogeneity of variances were performed by 2-tailed unpaired Student *t* test. Alternatively, comparisons between groups with abnormality and heterogeneity of variances were performed by Welch *t* test. One-way analysis of variance (ANOVA) was used, followed by the post-hoc Tukey multiple comparison test to analyze data for WT mice when the insulin-treated diabetic group was included (Figs. 1 and 2A). Disclaimer concerning type 1 error rate is a limitation of this study, since no correction for multiple comparisons was applied. The SPS 9.1 system (SAS Institute Inc., Cary, North Carolina) was used for all statistical tests. Differences were considered to be significant at $p < 0.05$.

RESULTS

Inhibition of apoptosis in the hearts of MT-TG diabetic mice. Both WT and MT-TG diabetic mice showed increased whole-blood glucose levels (Fig. 1A) and glycated hemoglobin (Fig. 1B) on the 14th and 21st day after STZ treatment. These changes were prevented by supplementation with insulin in the WT diabetic mice (Figs. 1A and 1B). Because we have shown that diabetes caused a significant increase in cardiac apoptotic cell death from day 7 to day 21 after STZ treatment (14), TUNEL assay was performed in the cardiac tissues from both WT and MT-TG mice 7, 14, and 21 days after STZ treatment. An increased apoptosis (i.e.,

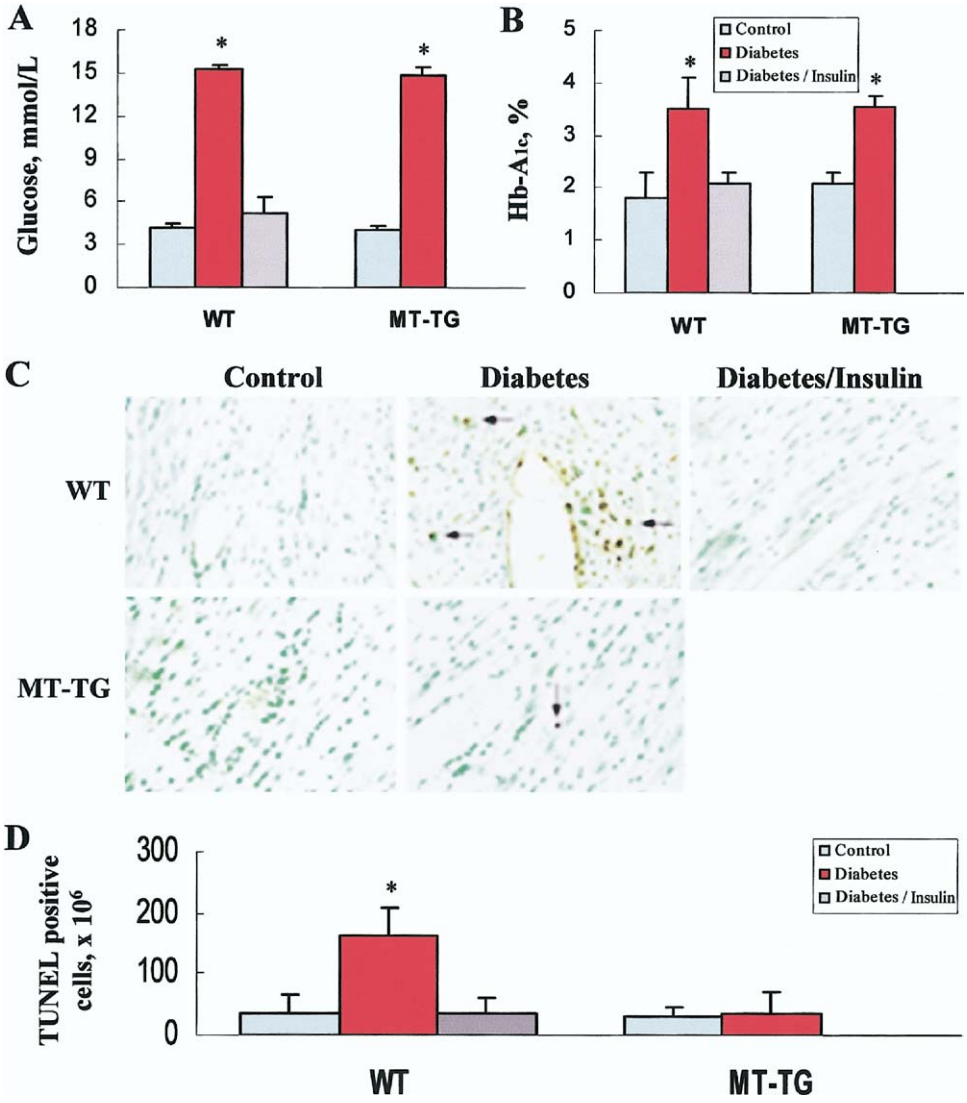

Figure 1. Diabetic model and diabetes-induced cardiac apoptosis. Whole-blood glucose **(A)** and glycated hemoglobin (Hb-A1c) **(B)** were measured for diabetic mice 14 days after streptozotocin (STZ) treatment. Cardiac apoptosis was examined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. **Panel C** presents the representatives of TUNEL staining images in different groups from diabetic mice 14 days after STZ treatment and **panel D** presents combined results for the quantitative analysis of TUNEL-positive cells in the cardiac tissues of the diabetic mice from day 7 to day 21 after STZ treatment. Data were presented as mean values ± SD. For each group, at least 5 mice were used. One-way analysis of variance (ANOVA) was used followed by the post-hoc Tukey multiple comparison test. *$p < 0.05$ versus corresponding control. MT-TG = cardiac-specific, metallothionein-overexpressing transgenic mice, WT = wild-type mice.

TUNEL-positive cells; Fig. 1C) was observed in the hearts of WT diabetic mice, but not in MT-TG diabetic mice. No significant difference was found among the results examined on days 7, 14, and 21 after STZ treatment; therefore, a combined result of TUNEL-positive cells from these 3 time points as the early-phase apoptotic cell death was quantitatively summarized in Figure 1D. Results show that a significant increase in cardiac apoptosis was observed in the WT diabetic mice, but not in the insulin-treated WT diabetic or MT-TG diabetic mice.

To confirm the inhibitory effect of MT on diabetes-induced cardiac apoptosis, caspase-3 activation, another important indication of apoptosis, was measured by its activity and the content of its active form using enzymatic and Western blotting assays (Fig. 2). Enzymatic assay showed a significant increase in caspase-3 cleavage activity in the WT diabetic heart, but not in the insulin-treated WT diabetic or MT-TG diabetic heart (Fig. 2A). Consistent with the activity assay, Western blotting analysis also showed an increase in active isoform of caspase-3 in the hearts of WT diabetic mice, but not in the hearts of MT-TG diabetic mice, as shown in Figure 2B. To define whether apoptosis occurs in cardiomyocytes, double stains for cardiomyocytes using α-sarcomeric actin and active isoform of caspase-3 were examined under confocal laser scanning microscopy (Fig. 2C). Double staining clearly showed that the activated caspase-3 are localized in cardiomyocytes (left panel), whereas such staining was not observed in the hearts of MT-TG mice (right panel) or insulin-treated WT diabetic mice (data not shown).

JACC Vol. 48, No. 8, 2006
October 17, 2006:1688–97

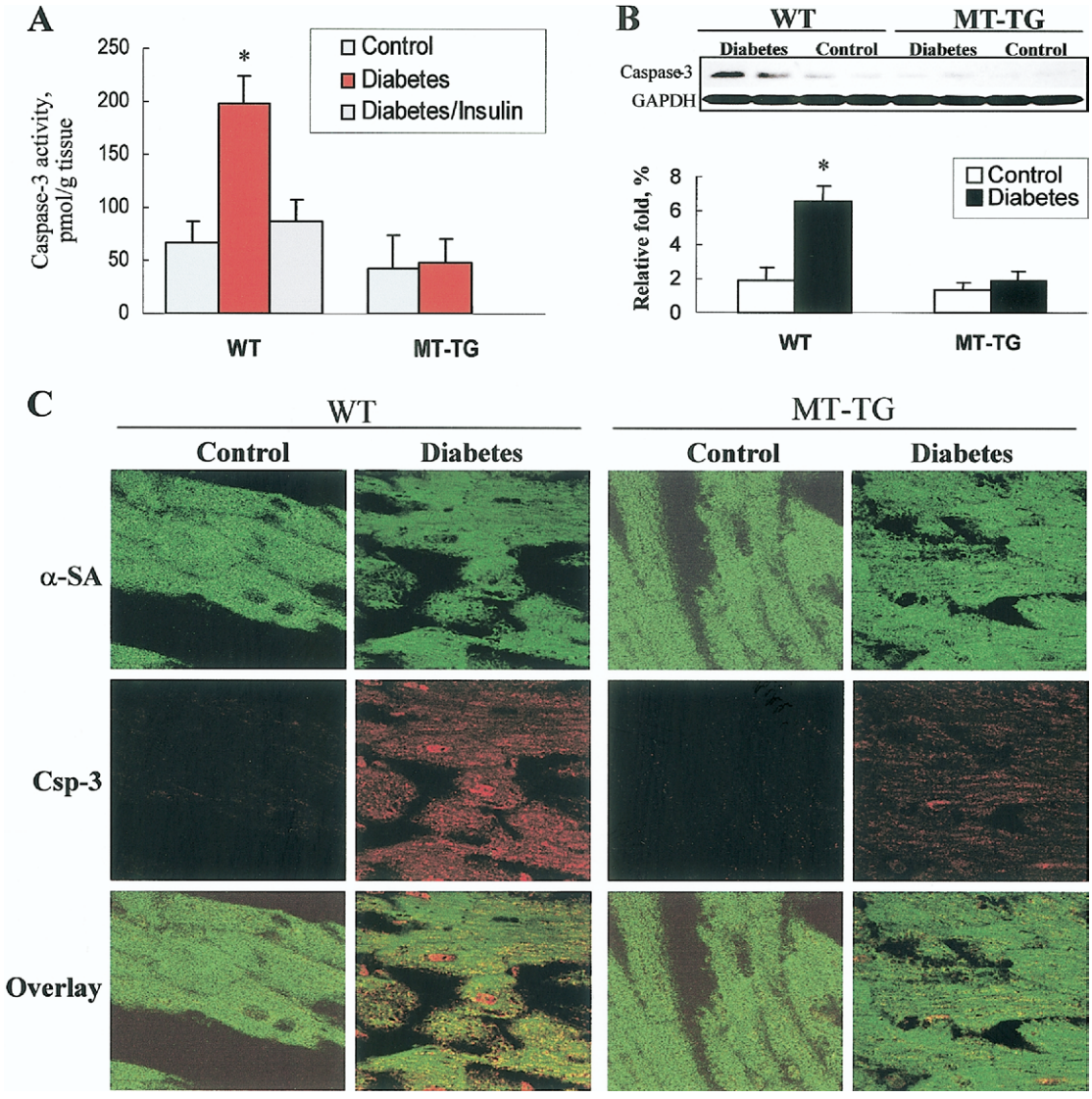

Figure 2. Caspase-3 activation in the diabetic hearts. Activation of caspase-3 was measured by enzymatic assay for its activity **(A)**, Western blotting assay for the protein expression of its active isoform **(B)**, and double staining for its localization **(C)**. Cardiac tissues for both assays were collected from diabetic mice 14 days after STZ treatment. In **panel C**, "α-SA" indicates staining for cardiomyocytes using α-sarcomeric actin; "Csp-3" indicates the specific staining for active isoform of caspase-3; and "overlay" indicates the overlapping of these 2 specific stainings. These images in **panel C** are the representatives of each group including more than 3 mice with 2 sections from each mouse. Data were presented as mean values ± SD. In each group, at least 5 mice were included. One-way ANOVA was used and was followed by the post-hoc Tukey multiple comparison test for the data from WT mice in **A**. A 2-tailed unpaired Student *t* test was used for the rest of the data. *$p < 0.05$ versus corresponding control. Abbreviations as in Figure 1.

In a previous study, we found that mitochondrial cytochrome *c* release is one of the critical upstream events leading to caspase-3 activation and apoptosis in the diabetic heart and cardiac cells exposed to high levels of glucose (14). In the present study, a significant cytochrome *c* release from mitochondria to cytosol in the WT diabetic mice, but not in the MT-TG diabetic mice, was also detected by Western blotting assay (Fig. 3). This indicates that MT inhibits mitochondrial cytochrome *c* release, resulting in a prevention of caspase-3 activation and apoptotic cell death induced by diabetes.

Prevention of diabetes-induced oxidative/nitrosative damage in the hearts of MT-TG mice. We recently demonstrated that MT protection of the heart from diabetes is mainly mediated by suppression of superoxide generation and the associated nitrosative damage (14). To examine whether the prevention of diabetes-induced cardiac apoptosis is also related to the prevention of diabetes-derived nitrosative damage, we stained the cardiac tissue with antibody against 3-NT, which was significantly increased in the WT diabetic mice (Fig. 4A, left panel), but not in the MT-TG diabetic mice (Fig. 4A, right panel). Double staining defines that the increased 3-NT was mainly localized in cardiomyocytes (Fig. 4A). In addition, cardiac lipid peroxidation was determined by TBARS assay. Thiobarbituric acid-reactive substance was significantly increased in the WT diabetic heart, but not in the MT-TG diabetic heart (Fig. 4B).

A recent study has demonstrated that mitochondrial oxidative stress, i.e., GSH depletion, is the predominant cause for

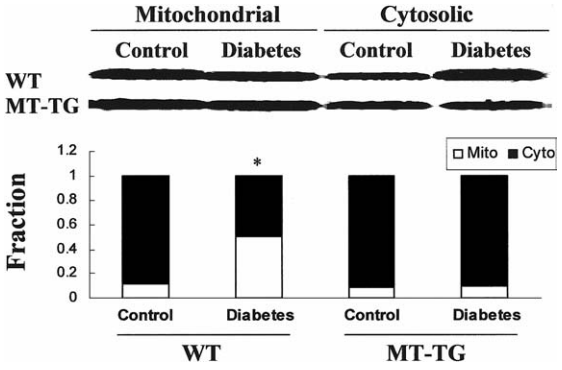

Figure 3. Mitochondrial cytochrome *c* release to cytosol was measured by Western blotting assay in the cytosolic and mitochondrial fractions from the hearts of control and diabetic mice 14 days after STZ treatment. Gel was a representative of 2 experiments using 3 hearts as 1 sample to isolate the cytosolic and mitochondrial proteins. A 2-tailed unpaired Student *t* test was used. *$p < 0.05$ versus corresponding control. Abbreviations as in Figure 1.

cardiac cell death under diabetic condition (17); therefore, in order to examine whether MT's prevention of cardiac cell death under diabetic condition is mediated by suppression of mitochondrial oxidative stress, i.e., GSH depletion, both cytosolic and mitochondrial GSH and its oxidized form (GSSG) were measured in the present study (Fig. 5). No significant change of cytosolic GSH or GSSG was observed either in WT or MT-TG diabetic mice (Fig. 5A); however, a significant decrease in mitochondrial GSH and a significant increase in mitochondrial GSSG were observed in the WT, but not in the MT-TG, diabetic mice (Fig. 5B). Corresponding to the increased conversion of GSH to GSSG, Gpx activity was significantly increased in the cardiac mitochondria of WT diabetic mice, but not in MT-TG diabetic mice (Fig. 5C). In addition, GR activity was also significantly increased in the WT diabetic hearts (Fig. 5C).

Preservation of structural normality of the heart in MT-TG diabetic mice. To test our hypothesis that cell death, as one of the major early cellular events of the heart in response to diabetes, is critically involved in the development of late-stage cardiomyopathy, cardiac ultrastructure and fibrosis were examined in the diabetic mice 6 months after STZ treatment. As shown in Figure 6, the hearts of

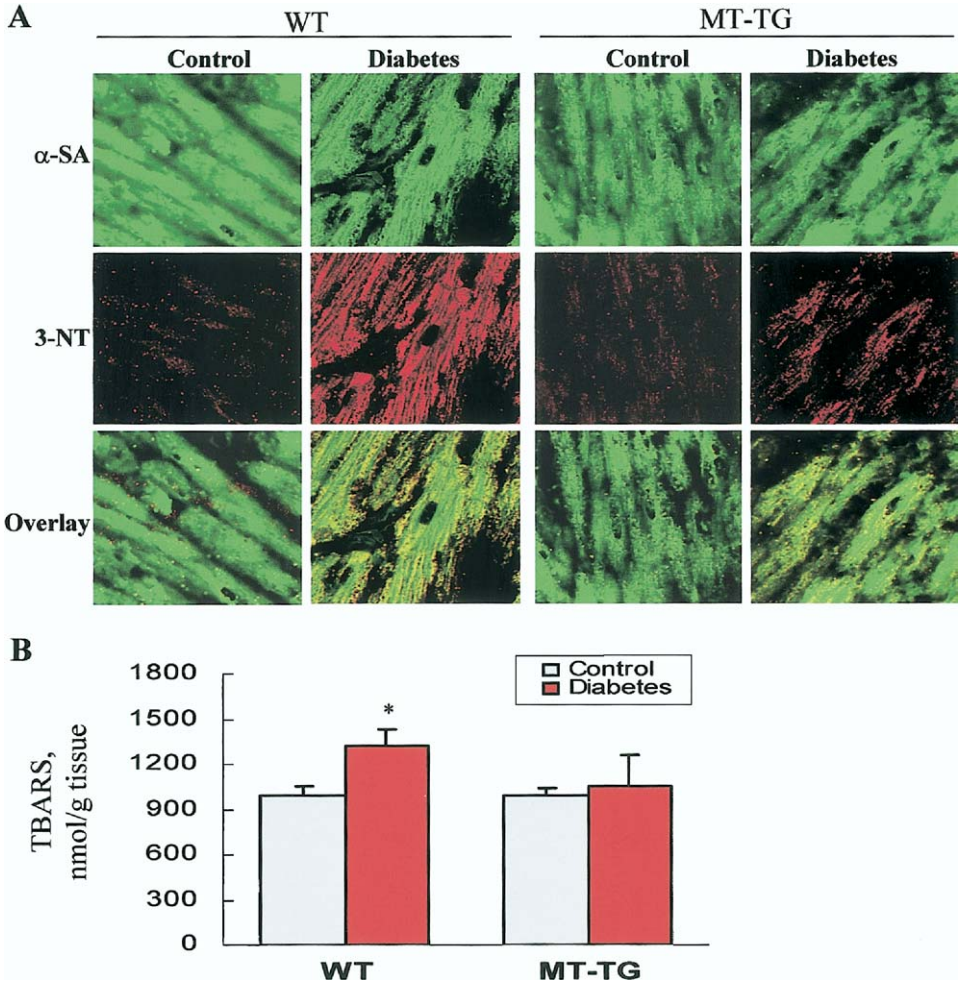

Figure 4. Diabetes-induced cardiac protein nitration and lipid peroxidation. **Panel A** presents the double staining for cardiomyocytes by α-sarcomeric actin (labeled by "α-SA") and protein nitration by 3-NT. These images in **panel A** are the representatives of each group including more than 3 mice with 2 sections from each mouse. For **panel B**, cardiac tissues were collected from control and diabetic mice (at lease 5 mice for each group) 14 days after STZ treatment, and lipid peroxidation was measured by thiobarbituric acid-reactive substance (TBARS) assay. Data were presented as mean values ± SD. A 2-tailed unpaired Student *t* test was used. *$p < 0.05$ versus corresponding control. Abbreviations as in Figure 1.

JACC Vol. 48, No. 8, 2006
October 17, 2006:1688–97

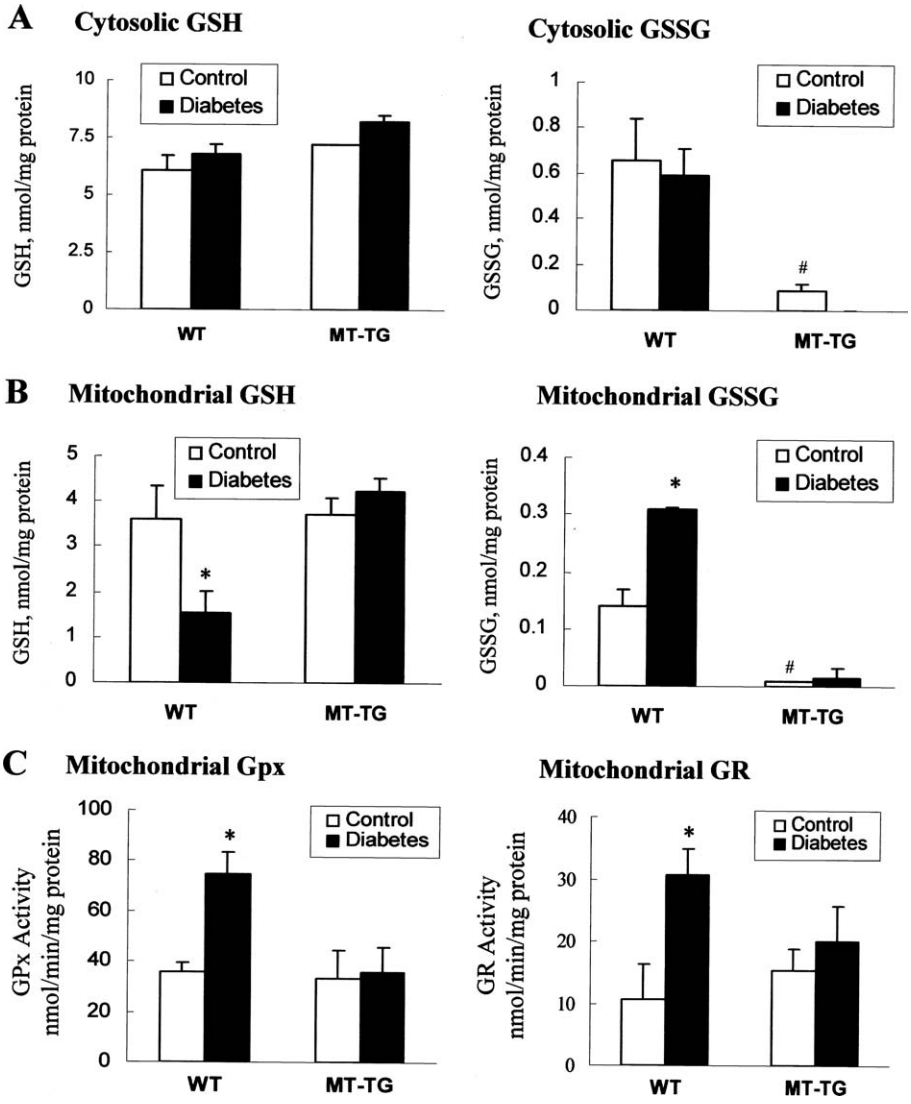

Figure 5. Changes of glutathione (GSH), oxidized glutathione (GSSG), glutathione peroxidase (Gpx), and glutathione reductase (GR) contents or activity in the diabetic hearts and MT's effect. The GSH and GSSG contents were measured by high-performance liquid chromatography with a dual electrochemical method from the cytosolic **(A)** and mitochondrial **(B)** fractions of the control and diabetic tissues from WT and MT-TG diabetic mice. The Gpx and GR activities **(C)** were measured by biochemical assay using commercially available kits and visible spectrometer. Data were presented as mean values ± SD. Animal numbers are 4 to 5 in each group. A 2-tailed unpaired Student *t* test was used except for the **right panel of B**, for which, because the variables seriously violated the assumptions for normality and homogeneity of variances, a Welch *t* test was used. *$p < 0.05$ versus corresponding control; #$p < 0.05$ versus WT control. Other abbreviations as in Figure 1.

WT diabetic mice showed extensive structural abnormalities, including 1) swelled and disrupted mitochondria; 2) reduced and disarranged muscular fibroses with fewer or irregular Z-lines; 3) chromatin condensation of the nucleus with leaked nuclear content (i.e., micronuclear formations); and 4) extensively lipid droplets. These changes were rarely observed in the hearts of MT-TG diabetic mice (Fig. 6A).

In a previous study, we demonstrated that left ventricular end diastolic pressure (LVEDP) was significantly increased in the WT, but not in the MT-TG, diabetic mice 6 months after STZ treatment (19). We assumed that the significant prevention of the increased LVEDP in MT-TG diabetic mice may result from the MT's prevention from diabetes-induced cardiac fibrosis in the MT-TG diabetic mice. To examine the effect of MT on diabetes-induced fibrosis in the heart, Sirius-red staining of collagen was performed. A strong staining of collagen in the hearts of WT diabetic mice, but not in the hearts of MT-TG diabetic mice, was observed (Fig. 6B).

Attenuation of animal mortality in the MT-TG diabetic mice. Diabetes causes an increase in mortality at late stage in diabetic patients (1–3), probably because of multiple organ dysfunctions including cardiomyopathy. As shown in Figure 7, WT diabetic mice showed a significant decrease in the survival rate, an effect significantly prevented in the MT-TG diabetic mice. Although the mortality may not directly indicate the cardiac effect, the improved animal survival rate in MT-TG diabetic

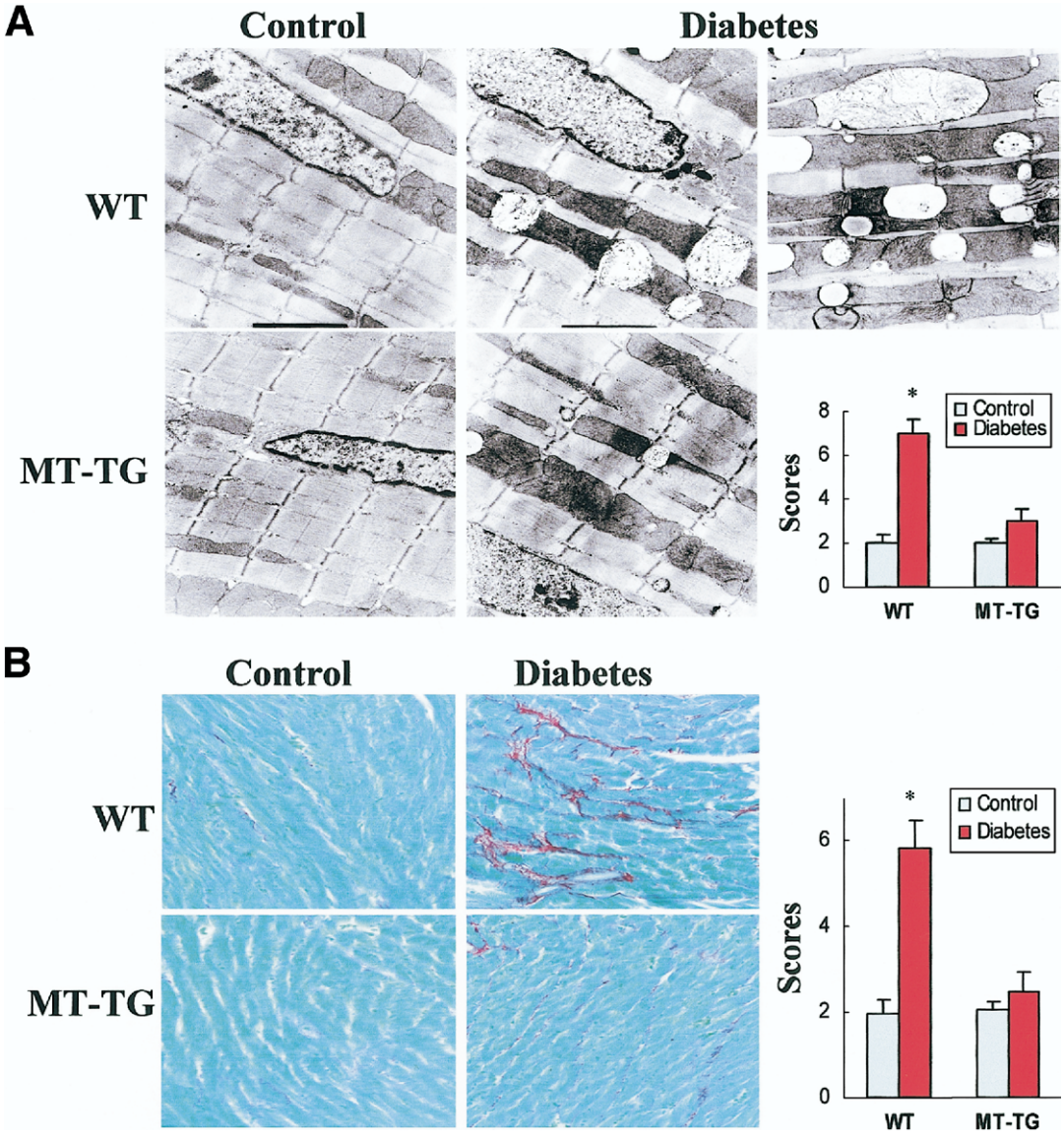

Figure 6. Diabetes-induced ultrastructural changes and fibrotic effect of the heart. Ultrastructural evaluation was performed by electron microscopic examination **(A)** for the hearts of diabetic mice 6 months after STZ treatment. Inset in **panel A** are the scores for the semi-quantitative analysis as described in the Materials and Methods section. Fibrosis was evaluated by Sirius-red staining of collagen in the hearts of diabetic mice 6 months after STZ treatment **(B)**. The semi-quantitative analysis was performed by a computer-image analysis system as described in Materials and Methods. Data were presented as mean values ± SD. In each group, at least 5 mice were included. A 2-tailed unpaired Student *t* test was used. *$p < 0.05$ versus corresponding control. Abbreviations as in Figure 1.

mice would relate to MT prevention of diabetic cardiomyopathy under the conditions of this study, because cardiac MT overexpression is the only difference between WT and MT-TG mice.

DISCUSSION

The results obtained from the present study demonstrate that MT inhibits apoptosis in the diabetic heart of the mouse model. The inhibition of apoptosis is associated with suppression of diabetes-caused mitochondrial oxidative stress, thus a significant suppression of mitochondrial cytochrome *c* release and caspase-3 activation. Importantly, MT inhibition of the early-stage apoptosis in the heart of diabetic mice results in a prevention of cardiomyopathy at the late stage. In addition, animal survival rate was also enhanced in the MT-TG diabetic mice as compared to the WT diabetic mice. Therefore, the present study suggests that MT prevents the development of diabetic cardiomyopathy, at least in part through the attenuation of the early-phase cardiac cell death.

The loss of cardiac myocytes is a major cause of the compromised cardiac function (8–10). Apoptosis of cardiac myocytes and endothelial cells occurs in the hearts of diabetic patients and STZ-induced diabetic animals (11–17). The present study showed that an average incidence of apoptotic cell death, detected by TUNEL-positive cells, in the WT diabetic heart from days 7, 14, and 21 after STZ treatment is 163 of 10^6 cells (Fig. 1D). This incidence

JACC Vol. 48, No. 8, 2006
October 17, 2006:1688–97

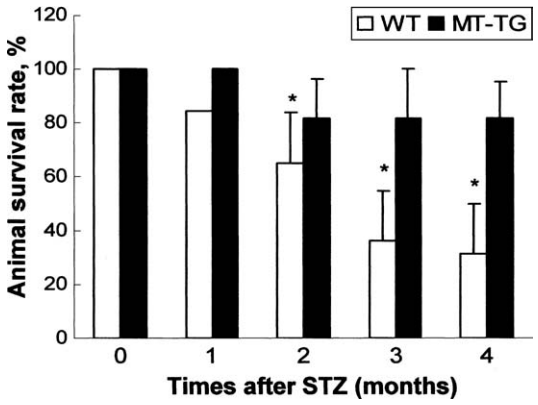

Figure 7. Animal survival rate. Animal survival rate until 4 months was observed by 3 separate experiments with at least 10 mice for each group. Data were presented as mean values ± SD. A 2-tailed unpaired Student *t* test was used. *$p < 0.05$ versus corresponding control (time 0). Abbreviations as in Figure 1.

is in agreement with our previous study using the same mouse model (14). Kajstura et al. (13), using mice, showed that diabetes induced an apoptotic cell death about 4 times as currently observed. Fiordaliso et al. (12), using rats, showed a similar incidence of cardiac apoptosis (about 160 of 10^6 cells at day 10 after STZ treatment) to the current observation. The difference for the incidence of cardiac apoptosis in our own studies and those of Kajstura et al. (13) is unknown, but we used a strain of mice (FVB) which may be different from mice used by Kajstura et al.

Cell death as a comprehensive consequence of abnormal cell metabolisms and gene expressions at an early stage in the heart in response to diabetes may thus be a critical cause of diabetic cardiomyopathy (4,7,15). This assumption was supported by a previous study showing that insulin-like growth factor 1 (IGF-1) overexpression inhibits cardiac cell death in diabetic mice along with a preservation of cardiac function (13), and by the present study, which shows that overexpressing MT in the heart prevents diabetes-induced early cell death, leading to a prevention of late cardiomyopathy.

In the study with IGF-1 overexpression (13), however, the cardiomyopathy was examined by cardiac function only at 7 and 30 days after STZ treatment, and there was also no information about cardiac structural changes. Diabetic cardiomyopathy is a chronic manifestation at late stage, which should be diagnosed by morphological and functional observation in a relative long time period (2,3,7). In a recent study we demonstrated the preservation of cardiac function in MT-TG diabetic mice, whereas WT-diabetic mice showed a significant cardiac dysfunction at 6 months after diabetic onset (19). In the present study, under the same conditions as the previous study (19), we provided further evidence that cardiac cell death occurred at early stage from day 7 to day 21 after STZ treatment. Inhibition of the early-phase cell death in the heart of MT-TG diabetic mice correlates with a significant prevention of ultrastructural abnormalities and fibrosis in the hearts of diabetic mice 6 months after STZ treatment. This finding is consistent with our recent study using a different transgenic mouse model that systemically overexpresses the MT gene to demonstrate the prevention of MT from diabetes-induced cardiac fibrosis (23). The preservation of structural normality and prevention of fibrosis in the heart by MT, observed in the present study, may be the basis of cardiac functional normality of MT-TG diabetic mice 6 months after STZ treatment, observed in our previous study (19). Furthermore, the preservation of the cardiac structure and function in the MT-TG diabetic mice may be responsible for the high animal survival rate (Fig. 7).

Oxidative damage caused by ROS and RNS is related directly to multiple complications of diabetes (27), including cardiomyopathy (11–15). A recent study (17) further indicated that mitochondrial oxidative stress such as GSH depletion is the direct cause for cardiac cell death under the diabetic condition. We show that MT significantly inhibits mitochondrial GSH depletion, cardiac protein nitration, and lipid peroxidation, along with a significant prevention of cardiac cell death in the hearts of diabetic mice, thus suggesting the cause–effect relationship of the mitochondrial oxidative stress and cardiac cell death. Combined with our recent study (19) showing that attenuation of early oxidative damage such as protein nitration (likely belonging to mitochondrial proteins according to the molecular size) in the MT-TG diabetic heart resulted in a significant prevention of diabetes-caused cardiac dysfunction observed 6 months after STZ treatment, it strongly suggests that oxidative stress is a critical cause of early-phase cardiac cell death.

Metallothionein cooperation with GSH to maintain cellular redox status has been implicated (28,29). We have shown that there is no significant change in the total GSH, Gpx, and GR levels in the hearts of MT-TG mice compared to WT mice under physiological conditions (22). However, GSSG contents in both cytosolic and mitochondrial portions were significantly lower in MT-TG hearts than those in WT hearts even under physiological conditions (i.e., in control groups; Figs. 5A and 5B), suggesting the prevention of oxidization of GSH under physiological conditions. We recently confirmed the existence of MT disulfide bonds in the MT-TG hearts under physiological conditions and further increased formation of MT disulfide bonds under oxidative stress conditions (30), suggesting the interaction of MT with ROSs or RNSs. In our previous study (28), it was shown that in the presence of a high concentration of MT, the primary antioxidant defense against ROS and/or RNS would shift from GSH to MT; therefore, there was no increase in mitochondrial GSSG in the hearts of MT-TG diabetic mice even though there was a significant increase in GSSG in the cardiac mitochondria of WT diabetic mice. In the hearts of WT diabetic mice, the increased GSSG that was associated with increased Gpx activity (Fig. 5) may cause the adaptive increase in mitochondrial GR activity in the hearts of WT diabetic mice

(Fig. 5). However, how diabetes causes Gpx activation remains unclear on the basis of the present study. We have demonstrated the overproduction of superoxide in the hearts of WT diabetic mice 2 weeks after STZ treatment (19). The overproduced superoxide may be converted to hydrogen peroxide, which may in turn stimulate the cells to increase Gpx activity in order to compensate.

In summary, the present study, in combination with our recent studies (14,19,23), demonstrates that diabetes causes cardiac mitochondria cytochrome *c* release-dependent apoptosis at early stage. Metallothionein significantly inhibits diabetes-caused early cardiac apoptosis through suppression of mitochondrial oxidative stress (i.e., GSH depletion) leading to a significant prevention of the development of diabetic cardiomyopathy. Although the precise mechanisms underlying how MT protects from diabetes-caused mitochondrial GSH depletion remain to be explored, the clinical implication of MT's cardiac prevention from diabetes is critically important, since zinc supplementation given to diabetic mice significantly prevented the development of diabetic cardiomyopathy through cardiac MT induction (31).

Acknowledgments

The authors are grateful to Mr. Yibo Du and Ms. Marcia C. Liu for their technological assistance.

Reprint requests and correspondence: Dr. Lu Cai or Dr. Xiaokun Li, 511 South Floyd Street, MDR 533, Louisville, Kentucky 40202. E-mail: L0cai001@louisville.edu (Dr. Cai) or xiaokunli@163.net (Dr. Li).

REFERENCES

1. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. Nature 2001;414:782–7.
2. Trost S, LeWinter M. Diabetic cardiomyopathy. Curr Treat Options Cardiovasc Med 2001;3:481–92.
3. Francis GS. Diabetic cardiomyopathy: fact or fiction? Heart 2001;85: 247–8.
4. Depre C, Young ME, Ying J, et al. Streptozotocin-induced changes in cardiac gene expression in the absence of severe contractile dysfunction. J Mol Cell Cardiol 2000;32:985–96.
5. Singh JP, Larson MG, O'Donnell CJ, et al. Association of hyperglycemia with reduced heart rate variability (The Framingham Heart Study). Am J Cardiol 2000;86:309–12.
6. Swynghedauw B. Molecular mechanisms of myocardial remodeling. Physiol Rev 1999;79:215–62.
7. Cai L, Kang YJ. Cell death and diabetic cardiomyopathy. Cardiovasc Toxicol 2003;3:219–28.
8. Narula J, Kolodgie FD, Virmani R. Apoptosis and cardiomyopathy. Curr Opin Cardiol 2000;15:183–8.
9. Bishopric NH, Andreka P, Slepak T, et al. Molecular mechanisms of apoptosis in the cardiac myocyte. Curr Opin Pharmacol 2001; 1:141–50.
10. Garg S, Narula J, Chandrashekhar Y. Apoptosis and heart failure: clinical relevance and therapeutic target. J Mol Cell Cardiol 2005;38: 73–9.
11. Frustaci A, Kajstura J, Chimenti C, et al. Myocardial cell death in human diabetes. Circ Res 2000;87:1123–32.
12. Fiordaliso F, Li B, Latini R, et al. Myocyte death in streptozotocin-induced diabetes in rats is angiotensin II-dependent. Lab Invest 2000;80:513–27.
13. Kajstura J, Fiordaliso F, Andreoli AM, et al. IGF-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II-mediated oxidative stress. Diabetes 2001;50:1414–24.
14. Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. Diabetes 2002; 51:1938–48.
15. Bojunga J, Nowak D, Mitrou PS, Hoelzer D, Zeuzem S, Chow KU. Antioxidative treatment prevents activation of death-receptor- and mitochondrion-dependent apoptosis in the hearts of diabetic rats. Diabetologia 2004;47:2072–80.
16. Fiordaliso F, Bianchi R, Staszewsky L, et al. Antioxidant treatment attenuates hyperglycemia-induced cardiomyocyte death in rats. J Mol Cell Cardiol 2004;37:959–68.
17. Ghosh S, Pulinilkunnil T, Yuen G, et al. Cardiomyocyte apoptosis induced by short-term diabetes requires mitochondrial GSH depletion. Am J Physiol Heart Circ Physiol 2005;289:H768–76.
18. Ye G, Metreveli NS, Ren J, Epstein PN. Metallothionein prevents diabetes-induced deficits in cardiomyocytes by inhibiting reactive oxygen species production. Diabetes 2003;52:777–83.
19. Cai L, Wang J, Li Y, et al. Inhibition of superoxide generation and associated nitrosative damage is involved in metallothionein prevention of diabetic cardiomyopathy. Diabetes 2005;54:1829–37.
20. Kang YJ. The antioxidant function of metallothionein in the heart. Proc Soc Exp Biol Med 1999;222:263–73.
21. Cai L, Klein JB, Kang YJ. Metallothionein inhibits peroxynitrite-induced DNA and lipoprotein damage. J Biol Chem 2000;275: 38957–60.
22. Kang YJ, Chen Y, Yu A, Voss-McCowan M, Epstein PN. Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. J Clin Invest 1997;100:1501–6.
23. Song Y, Wang J, Li Y, et al. Cardiac metallothionein synthesis in streptozotocin-induced diabetic mice, and its protection against diabetes-induced cardiac injury. Am J Pathol 2005;167:17–26.
24. Liang Q, Carlson EC, Donthi RV, Kralik PM, Shen X, Epstein PN. Overexpression of metallothionein reduces diabetic cardiomyopathy. Diabetes 2002;51:174–81.
25. Der Sarkissian S, Marchand EL, Duguay D, Hamet P, deBlois D. Reversal of interstitial fibroblast hyperplasia via apoptosis in hypertensive rat heart with valsartan or enalapril. Cardiovasc Res 2003; 57:775–83.
26. Chen TS, Richie JP Jr., Lang CA. Life span profiles of glutathione and acetaminophen detoxification. Drug Metab Dispos 1990;18: 882–7.
27. Cai L, Kang YJ. Oxidative stress and diabetic cardiomyopathy: a brief review. Cardiovasc Toxicol 2001;1:181–93.
28. Wu HY, Kang YJ. Inhibition of buthionine sulfoximine-enhanced doxorubicin toxicity in metallothionein overexpressing transgenic mouse heart. J Pharmacol Exp Ther 1998;287:515–20.
29. Maret W. Cellular zinc and redox states converge in the metallothionein/thionein pair. J Nutr 2003;133 Suppl 1:1460–2S.
30. Feng W, Benz FW, Cai J, Pierce WM, Kang YJ. Metallothionein disulfides are present in metallothionein-overexpressing transgenic mouse heart and increase under conditions of oxidative stress. J Biol Chem 2006;281:681–7.
31. Wang J, Song Y, Elsherif L, et al. Cardiac metallothionein induction plays the major role in the prevention of diabetic cardiomyopathy by zinc supplementation. Circulation 2006;113:544–54.