

Inhibition of Superoxide Generation and Associated Nitrosative Damage Is Involved in Metallothionein Prevention of Diabetic Cardiomyopathy

Lu Cai,^{1,2} Jianxun Wang,¹ Yan Li,¹ Xuihua Sun,¹ Lipeng Wang,¹ Zhanxiang Zhou,¹ and Y. James Kang^{1,2}

The mechanisms of metallothionein prevention of diabetic cardiomyopathy are largely unknown. The present study was performed to test whether inhibition of nitrosative damage is involved in metallothionein prevention of diabetic cardiomyopathy. Cardiac-specific metallothionein-overexpressing transgenic (MT-TG) mice and wild-type littermate controls were treated with streptozotocin (STZ) by a single intraperitoneal injection, and both developed diabetes. However, the development of diabetic cardiomyopathy, revealed by histopathological and ultrastructural examination, serum creatine phosphokinase, and cardiac hemodynamic analysis, was significantly observed only in the wild-type, but not in MT-TG, diabetic mice 2 weeks and 6 months after STZ treatment. Formations of superoxide and 3-nitrotyrosine (3-NT), a marker for peroxynitrite-induced protein damage, were detected only in the heart of wild-type diabetic mice. Furthermore, primary cultures of cardiomyocytes from wild-type and MT-TG mice were exposed to lipopolysaccharide/tumor necrosis factor- α for generating intracellular peroxynitrite. Increases in 3-NT formation and cytotoxicity were observed in wild-type, but not in MT-TG, cardiomyocytes. Either urate, a peroxynitrite-specific scavenger, or Mn(111) tetrakis 1-methyl 4-pyridyl porphyrin pentachloride (MnTMPyP), a superoxide dismutase mimic, significantly inhibited the formation of 3-NT along with a significant prevention of cytotoxicity. These results thus suggest that metallothionein prevention of diabetic cardiomyopathy is mediated, at least in part, by suppression of superoxide generation and associated nitrosative damage. *Diabetes* 54:1829–1837, 2005

From the ¹Department of Medicine, University of Louisville, School of Medicine, Louisville, Kentucky; and the ²Department of Pharmacology and Toxicology, University of Louisville, School of Medicine, Louisville, Kentucky.

Address correspondence and reprint requests to Dr. Lu Cai, Department of Medicine, University of Louisville School of Medicine, 511 South Floyd St., MDR 533, Louisville, KY 40202. E-mail: l0cai001@louisville.edu. Or Dr. Y. James Kang. E-mail: yjkang01@louisville.edu.

Received for publication 10 December 2004 and accepted in revised form 28 February 2005.

3-NT, 3-nitrotyrosine; CPK, creatine phosphokinase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; LVEDP, left ventricular end-diastolic pressure; LVMDP, left ventricular minimum diastolic pressure; MnTMPyP, Mn(111) tetrakis 1-methyl 4-pyridyl porphyrin pentachloride; MT-TG, metallothionein-overexpressing transgenic; OD, optical density; RNS, reactive nitrogen species; ROS, reactive oxygen species; SIN-1, 3-morpholinisydnonimine; SOD, superoxide dismutase; STZ, streptozotocin; TNF- α , tumor necrosis factor- α .

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Previous studies have shown that oxidative stress is critically involved in the pathogenesis of diabetic cardiomyopathy (1), a leading cause for mortality in both type 1 and type 2 diabetic patients (2–4). The pathological changes in the heart were found to be associated with increased myocardial cell death, predominately in the mode of apoptosis and accumulation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) (5–8). The roles of ROS and RNS in the diabetic complications of multiple organ systems were extensively documented (1,8–13), although the exact mechanisms of ROS/RNS-induced pathogenesis have not been fully understood.

Superoxide overproduction in the organ systems is an important feature of diabetic complications (10–12). One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxynitrite (11–15), which is a potent oxidant that causes nitrosative stress in the organ systems. A significant increase in serum and tissue 3-nitrotyrosine (3-NT), a by-product of the reaction between peroxynitrite and proteins, has been found in diabetic patients (6,15). Studies using experimental animal models and in vitro cultured cells have demonstrated that peroxynitrite is an important causative agent in diabetes-caused cardiovascular injury (16–19).

Therefore, we hypothesized that inhibition of myocardial nitrosative stress would lead to suppression of diabetic cardiomyopathy. Recent studies have shown that overexpression of metallothionein, a small molecular weight protein that has been shown to protect the heart from injury under various oxidative stress conditions (20–24), protects the heart from diabetes-induced damage (25,26), probably through suppression of oxidative stress (27,28). However, the mechanisms of metallothionein inhibition of diabetes-induced oxidative stress in the heart are largely unknown. Although we have demonstrated that metallothionein directly interacts with peroxynitrite to prevent peroxynitrite-induced lipid and DNA damage (29), whether the peroxynitrite formation is also involved in the pathogenesis of diabetic cardiomyopathy in vivo and whether metallothionein prevents peroxynitrite-induced cardiac damage in vivo remain unknown.

The present study, therefore, was undertaken to address the role of superoxide generation and associated nitrosative damage in the pathogenesis of diabetic cardiomyopa-

thy in a mouse model of type 1 diabetes induced by streptozotocin (STZ). Furthermore, an attempt was made to understand the inhibitory action of metallothionein on the nitrosative cardiac damage in vivo.

RESEARCH DESIGN AND METHODS

Diabetes model. MT-TG mice were produced from FVB mice and have been well characterized (20). Both MT-TG⁺ mice (heterozygotes) and MT-TG⁻ littermates (wild type) were kept in the same cages with free access to rodent diet and tap water and used for experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care.

Eight-week-old male mice were given a single dose of STZ (150 mg/kg body wt i.p.; Sigma, St. Louis, MO) dissolved in sodium citrate buffer (pH 4.5). Whole-blood glucose obtained from the mouse tail vein was detected using a SureStep complete blood glucose monitor (LifeScan, Milpitas, CA) 2 and 3 days after STZ treatment. STZ-treated mice with glucose levels higher than 12 mmol/l were considered diabetic, and mice serving as controls were given the same volume of sodium citrate (5). To eliminate the effects of STZ on cardiotoxicity, insulin-treated diabetic mice were used. For these mice, when hyperglycemia was diagnosed 2 or 3 days after STZ treatment, insulin was immediately given three times (8-h interval) using Humulin U (Eli Lilly, Indianapolis, IN) at a concentration of 10 units · day⁻¹ · mouse⁻¹ to maintain the blood glucose levels between 5.6 and 11.2 mmol/l with an average of 7.6 ± 1.1 mmol/l, until they were killed.

Histopathological and immunohistochemical examination by light microscopy. Heart tissues were fixed with 10% neutral and embedded in paraplast (23,30). Tissue sections of 5 μm thickness were stained by hematoxylin-eosin and examined under a light microscope. The 3-NT as a marker of peroxynitrite-induced damage was detected by immunohistochemical stain for two sections of each mouse of five mice in each group (6,30).

Ultrastructural examination by electron microscopy. The hearts 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 (20,23). The detail procedures of fixation, rinsing, postfixation, dehydration, and embedding for the tissue samples were described previously (20,23). Ultrathin sections were obtained with a LKB ultramicrotome, stained with uranyl acetate and lead citrate and observed with a Philip transmission electron microscope.

Measurements of serum creatine phosphokinase and lipid peroxidation. Whole blood was collected from the dorsal vena cava of the anesthetized animals. Serum was prepared using a serum separator apparatus (Becton Dickinson, Rutherford, NJ) to measure creatine phosphokinase (CPK) activity following the instructions provided in the kit (Sigma). Serum lipid peroxidation was measured by a thiobarbituric acid-reactive substance assay, as described previously (29).

Assessment of left ventricle performance. General measures of cardiac performance were done by in situ left ventricle (LV) hemodynamic analysis, as described previously (31). Mice were anesthetized using sodium pentobarbital (60 mg/kg i.p.). A midline incision (1–2 cm) in the neck external to the trachea and a small opening in the trachea were made for the insertion of a PE-100 catheter to ensure a patent airway. The rostra end of the artery was clamped to occlude blood flow from the heart. A small incision was then made in the artery for the insertion of a hand-stretched, fluid-filled PE-50 catheter, which was connected to a transducer and a computer recording system. The catheter was then slowly advanced through the common carotid artery, through the ascending aorta and into the left ventricle. The animal was allowed to stabilize for 20–30 min before recording of the waveform for up to 2 h. At the end of each experiment, the chest was opened to confirm the presence of catheter inside the left ventricle.

Measurement for superoxide. Superoxide generation in the heart was examined by two methods: fluorescence labeling of superoxide specific staining (32) and a cytochrome *c* reduction assay for NADPH-dependent superoxide generation (33). Dihydroethidine is oxidized to ethidine (red fluorescence) selectively by superoxide, but not by other ROS or RNS such as hydrogen peroxide, hydroxyl radicals, or peroxynitrite. Dihydroethidine at 10 mg/kg was injected via a tail vein 1 h before tissue harvest, as described previously (32), and cryostat sections of heart were cut at 5 μm and mounted on glass slides. The fluorescence was detected with a Nikon 2000S fluorescent microscope.

For cytochrome *c* reduction assay, the measurement of NADPH-dependent superoxide generation, cardiac tissues were homogenized and centrifuged at 800g for 10 min. The supernatant was incubated in the presence of 30 μmol/l succinylated ferricytochrome *c* and 1 mmol/l NADPH (both from Sigma). The

change in absorbance at 550 nm was measured. The difference in the amount of reduced succinylated ferricytochrome *c* in the presence or absence of 0.3 mg/ml superoxide dismutase (SOD) (Sigma) was used to estimate the amount of superoxide generation by using an absorbance coefficient 21.1 mmol/l⁻¹ cm⁻¹ (33).

Primary cultures of neonatal cardiomyocytes. Primary cultures of neonatal mouse cardiomyocytes were prepared by a modification of the method published previously (22,34). Briefly, hearts from 1- to 3-day-old wild-type and MT-TG mice were minced and dissociated with 0.15% trypsin. Dispersed cells were plated for 2 h in 100-mm dishes with minimum essential medium plus 10% bovine calf serum to remove noncardiomyocytes that attached to the plate within the incubation time. The myocytes that remained in the suspension were plated at a density of 500 cells/mm² with minimum essential medium plus 10% bovine calf serum, 0.1 mmol/l bromodeoxyuridine, and 20 μmol/l arabinosylcytosine. The medium was replaced 24 h later with fresh medium. On day 3, the wild-type and MT-TG cardiomyocytes were incubated with 30 μg/ml lipopolysaccharide (LPS) (Sigma) plus 100 ng/ml tumor necrosis factor (TNF-α) (PreproTech, Rocky Hill, NJ) (LPS/TNF-α) for 48 h, based on previously published studies (35–38). In some experiments, wild-type cardiomyocytes were cocultured with 100 μmol/l peroxynitrite scavenger urate (Sigma) or 50 μmol/l SOD mimic Mn(II) tetrakis 1-methyl 4-pyridyl porphyrin pentachloride (MnTMPyP) (Calbiochem, La Jolla, CA) with LPS/TNF-α.

Detection of 3-NT by Western blot. Heart tissues were homogenized in lysis buffer using homogenizer. Cardiomyocytes were sonicated in lysis buffer. The lysis buffer contains 2% SDS, 10% glycerol, and 62.5 mmol/l Tris (pH 7.0). Tissue or cell proteins were collected by centrifuging at 12,000g at 4°C in a Beckman GS-6R centrifuge for 10 min. The protein concentration was measured. The sample, diluted in loading buffer and heated at 95°C for 5 min, was then subjected to electrophoresis on 10% SDS-PAGE gel at 120 V (5). After electrophoresis of the gel and transfer of the proteins to nitrocellulose membrane, the membranes were rinsed briefly in Tris-buffered saline, blocked in blocking buffer (5% milk and 0.5% BSA) for 1 h, and washed three times with Tris-buffered saline with Tween containing 0.05% Tween 20. The membranes were incubated with rabbit anti-NT polyclonal antibody (Chemicon, Temecula, CA) at a dilution of 1:1,000 for 2 h and then washed as above and reacted with secondary horseradish peroxidase-conjugated antibody for 1 h. Antigen-antibody complexes were then visualized using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ).

Measurement of lactate dehydrogenase activity. Cytotoxicity for primary cultures of cardiomyocytes exposed to LPS/TNF-α was assessed by spectrometric measurement of lactate dehydrogenase (LDH) activity in the culture media of cultured cells, and LDH activity was presented as optical density (OD) value at 490 nm, as described in the assay instruction (Promega, Madison, WI).

Statistical analysis. Data were collected from repeated experiments and are presented as means ± SE. One-way ANOVA and Student's *t* test were used for statistical analysis. Differences were considered to be significant at *P* < 0.05.

RESULTS

Diabetes-induced cardiac structural and functional abnormalities and metallothionein prevention. Diabetes was developed in both MT-TG and wild-type mice on day 3 after STZ treatment, as characterized by multiple systemic changes (Table 1). There was no significant difference between MT-TG and wild-type mice in the incidence and manifestations of the STZ-induced diabetes. Serum thiobarbituric acid-reactive substance significantly increased in both wild-type and MT-TG diabetic mice, suggesting that systemic oxidative stress exists in both kinds of diabetic mice. Insulin-treated diabetic (diabetes/insulin) mice have significantly reduced glucose levels, compared with diabetic mice, and no systemic diabetes-related changes (Table 1).

There were significant differences in the histopathological and ultrastructural changes in the heart between the wild-type and MT-TG diabetic mice. Disorganized array of the myocardial structure, foci bleeding and cell necrosis, and myofibrillar discontinuation were observed in the heart of the wild-type diabetic mice, but not in the heart of control or insulin-treated wild-type diabetic mice (Fig. 1A)

TABLE 1
General parameters of STZ-induced diabetes in wild-type and MT-TG mice

Groups	WBGL (mmol/l)	BWG (g)	HWR (%)	KWR (%)	HbA _{1c} (%)	Serum TBARS (nmol/ml)
Wild type						
Control	4.2 ± 0.3	1.28 ± 0.36	0.45 ± 0.02	0.75 ± 0.04	2.10 ± 0.04	11.0 ± 0.3
Diabetes	15.0 ± 0.3*	-0.61 ± 0.65	0.46 ± 0.05	0.92 ± 0.05*	3.07 ± 0.15*	16.6 ± 0.8*
Diabetes/insulin	7.6 ± 1.1†	1.25 ± 0.50	0.45 ± 0.10	0.77 ± 0.45	2.21 ± 0.56	12.5 ± 1.3
MT-TG						
Control	4.1 ± 0.2	1.17 ± 0.28	0.44 ± 0.01	0.76 ± 0.03	2.09 ± 0.12	12.4 ± 1.0
Diabetes	14.9 ± 0.2*	-0.02 ± 0.87	0.45 ± 0.02	0.90 ± 0.05*	2.90 ± 0.20*	16.4 ± 1.4*

Data are means ± SE (12 mice at least for each group except for diabetes/insulin group with 7 mice). BWG, body weight gain; HWR or KWR, ratio of the heart or kidney weight, respectively, to body weight; WBGL, fasting whole-blood glucose level measured from blood samples obtained from tail vein. * $P < 0.05$ vs. corresponding control; † $P < 0.05$ vs. wild-type diabetic group.

or in MT-TG diabetic mice. Under the electron microscope (Fig. 1B), the hearts of the wild-type control and insulin-treated wild-type diabetic mice did not show any abnormalities. Only the heart of wild-type diabetic mice displayed increased numbers of lipid droplets and glycogen particles around mitochondria and scattered mito-

chondrial damage (swelling and disrupted cristae). However, the mitochondrial damage was significantly prevented in the heart of the MT-TG diabetic mice, although lipid droplets remained observable. These results indicated that the histopathological and ultrastructural changes were directly related to diabetes-associated

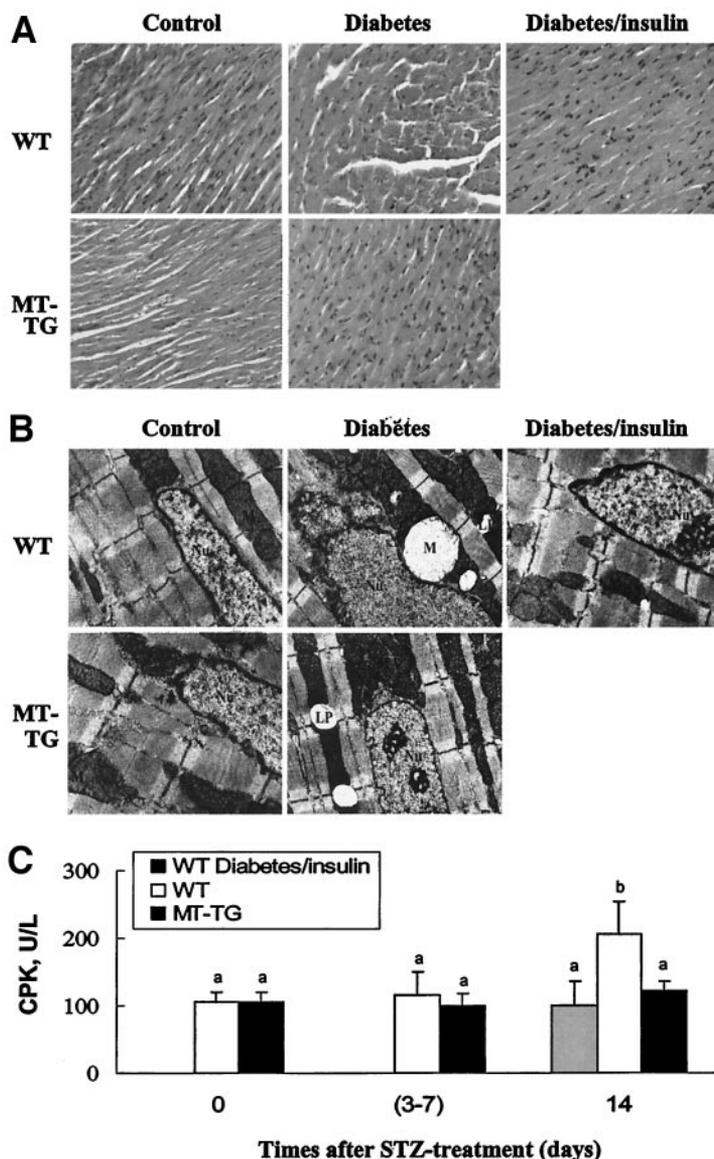


FIG. 1. Metallothionein prevention of diabetes-induced cardiac structural changes and serum CPK increase. Heart tissues from control and diabetic mice 2 weeks after STZ treatment were examined under the light microscope after hematoxylin-eosin staining (A, $\times 60$) and under electron microscope (B, $\times 11,000$). Morphological changes were described in the text. C: Serum was collected from wild-type and MT-TG control or diabetic mice at the indicated times to measure serum CPK using a CPK kit as described in RESEARCH DESIGN AND METHODS. Treatment groups that do not share the same letter were significantly different ($P < 0.05$). WT, wild type; LP, lipid droplets; Nu, nuclei; M, mitochondria.

TABLE 2
Blood pressure and cardiac function in the mice 2 weeks after STZ treatment

Cardiac function parameters	Wild type			MT-TG	
	Control	Diabetes	Diabetes/ insulin	Control	Diabetes
<i>n</i>	11	9	3	10	10
Blood pressure (mmHg)					
Systolic pressure	94.5 ± 5.6	102.4 ± 6.7	97.5 ± 5.6	94.1 ± 4.7	95.6 ± 1.0
Diastolic pressure	70.7 ± 8.1	76.1 ± 5.5	72.4 ± 6.1	73.7 ± 3.1	70.0 ± 8.4
Cardiac function					
Heart rate (bpm)	402.3 ± 10.0	451.2 ± 18.7*	406.0 ± 3.6	421.8 ± 7.8	434.4 ± 13.1
LVEDP (mmHg)	5.8 ± 1.0	17.1 ± 3.0*	5.7 ± 0.5	7.7 ± 2.2	7.8 ± 1.2
LVMDP (mmHg)	-0.6 ± 1.2	1.5 ± 1.4	-0.9 ± 1.0	-0.6 ± 2.2	-0.7 ± 1.8
LVPSP (mmHg)	103.9 ± 1.9	98.9 ± 1.6	107.6 ± 4.1	103.4 ± 4.9	103.1 ± 5.6
Maximum dP/dt (mmHg/s)	7,155.7 ± 318.8	5,054.9 ± 532.8*	7,487.1 ± 273.5	7,311.2 ± 503.8	7,380.7 ± 702.0
Maximum dP/dt DCON (ms)	37.6 ± 3.4	33.3 ± 4.9	40.4 ± 0.6	36.1 ± 3.8	37.5 ± 4.1
τ (ms)	20.4 ± 1.8	29.1 ± 3.9*	22.2 ± 0.7	20.1 ± 1.9	21.4 ± 3.1
½ R (ms)	44.3 ± 2.6	43.0 ± 3.6	44.8 ± 2.8	40.9 ± 2.8	40.3 ± 3.0

Data are means ± SE. LVPSP, left ventricular peak systolic pressure; DCON, duration of contraction; τ, time constant of relaxation; ½ R, time constant duration of half-relaxation. **P* < 0.05 vs. wild-type control.

pathogenesis rather than STZ toxicity, as documented in other studies (5,30,39), and metallothionein significantly prevented diabetes-induced cardiac toxicities.

To further evaluate the myocardial injury caused by diabetes, serum CPK was measured (Fig. 1C). Serum CPK levels were not changed in both wild-type and MT-TG diabetic mice on days 3–7 after STZ treatment, but significantly increased in the wild-type diabetic mice on day 14 after STZ treatment. This phenomenon implies that the increase in serum CPK levels was not directly related to STZ toxicity, rather than related to diabetes. More importantly, the diabetes-induced increase in serum CPK levels did not occur in the MT-TG diabetic mice. It should be mentioned that CPK is not a measurement specifically for cardiac muscle injury because skeletal muscle injury also releases it to serum; however, it may indicate the cardiac injury in the present case because metallothionein in the MT-TG mice was overexpressed only in the cardiac myocytes. If the measured CPK was derived from skeletal muscle, it would not be protected in the MT-TG diabetic mice. The fact that there was no change in serum CPK levels in the MT-TG diabetic mice may indicate that the

cardiac injury was the predominant source of serum CPK in the current experimental model.

The prevention of early structural damage (2 weeks after STZ treatment) in the MT-TG diabetic mice would result in a significant prevention of cardiomyopathy. Therefore, left ventricular functional changes were assessed by left ventricular hemodynamic analysis in diabetic mice 2 weeks (early stage) (Table 2) and 6 months (late stage) (Table 3) after STZ treatment. The heart rate was significantly increased in wild-type diabetic mice both 2 weeks and 6 months after STZ treatment and was not changed in insulin-treated diabetic or MT-TG diabetic mice. The assessment of left ventricular function changes induced by diabetes further revealed that significant defects occurred in the wild-type diabetic mice: decreased maximum dP/dt, a measurement of the mechanical ability of the heart to generate force for ejection of blood from the left ventricle; increased left ventricular end diastolic pressure (LVEDP), an index of the ventricular wall compliance; and increased τ, an index of the stiffness of the left ventricle (Tables 2 and 3). These changes were not observed in the MT-TG diabetic mice. Furthermore, the left

TABLE 3
Blood pressure and cardiac function in the mice 6 months after STZ treatment

Cardiac function parameters	Wild type		MT-TG	
	Diabetes	Control	Diabetes	Control
Blood pressure				
Systolic pressure (mmHg)	94.9 ± 5.8	95.6 ± 7.0	92.1 ± 2.7	94.8 ± 8.4
Diastolic pressure (mmHg)	69.7 ± 3.7	76.1 ± 5.5*	68.9 ± 3.3	66.6 ± 8.5
Cardiac function				
Heart rate (bpm)	382.3 ± 12.9	436.5 ± 19.1*	386.2 ± 10.4	399.2 ± 26.7
LVEDP (mmHg)	8.3 ± 1.8	18.4 ± 4.8*	9.5 ± 2.1	8.4 ± 1.5
LVMDP (mmHg)	-0.9 ± 2.2	12.3 ± 5.5*	-1.4 ± 3.6	-4.9 ± 1.8
LVPSP (mmHg)	98.8 ± 4.0	93.8 ± 5.2	103.2 ± 5.4	108.0 ± 4.9
Maximum dP/dt (mmHg/s)	8,262.4 ± 524.7	4,183.1 ± 1,287.1*	8,132.0 ± 675.6	9,113.6 ± 692.3
DCON (ms)	30.1 ± 4.2	29.0 ± 6.3	28.0 ± 2.1	29.6 ± 5.8
τ (ms)	23.7 ± 2.5	33.1 ± 4.2*	22.0 ± 1.0	24.2 ± 1.1
½ R (ms)	45.2 ± 3.6	40.8 ± 3.7	45.7 ± 3.7	47.2 ± 2.2

Data are means ± SE (*n* = 6 or 7). LVPSP, left ventricular peak systolic pressure; DCON, duration of contraction; τ, time constant of relaxation; ½ R, time constant duration of half-relaxation. **P* < 0.05 vs. wild-type control.

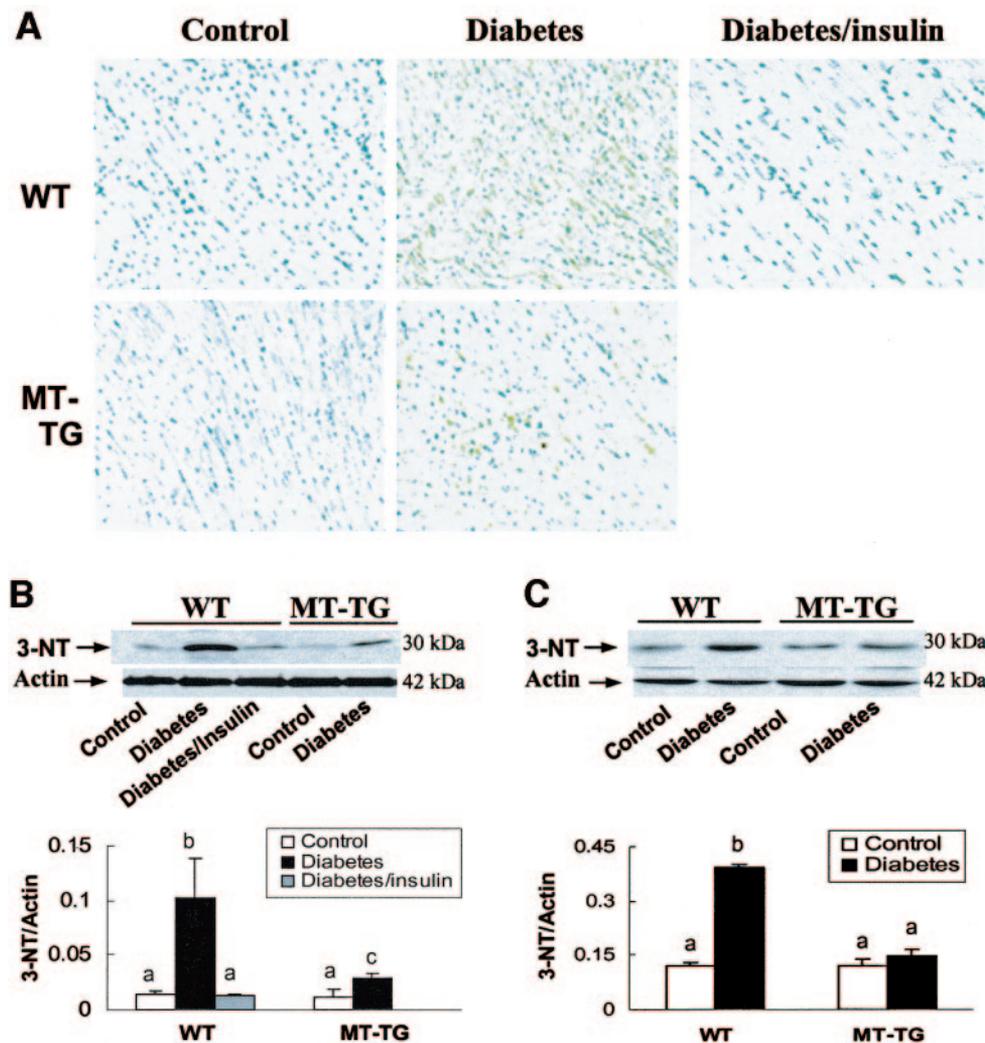


FIG. 2. Metallothionein inhibition of diabetes-induced 3-NT formation in the heart. Immunohistochemical staining for 3-NT (A) was performed in the hearts of wild-type and MT-TG diabetic mice 2 weeks after STZ treatment. Western blotting for 3-NT followed by quantitative analysis was also performed in the heart of wild-type and MT-TG diabetic mice 2 weeks (B) and 4 weeks (C) after STZ treatment. Treatment groups that do not share the same letter were significantly different ($P < 0.05$).

ventricular minimum diastolic pressure (LVMDP), although not significantly changed in wild-type diabetic mice 2 weeks after STZ treatment, was significantly increased in the wild-type diabetic mice 6 months after STZ treatment (Table 3), suggesting the development of significant diastolic dysfunction in the wild-type diabetic mice. The increased LVMDP was not observed in the MT-TG diabetic mice (Table 3).

MT inhibition of diabetes-induced 3-NT and superoxide generation in the heart. To determine the possible involvement of nitrosative damage in the pathogenesis of diabetic cardiomyopathy and the effect of metallothionein, 3-NT formation was assessed both qualitatively and quantitatively in the heart of wild-type and MT-TG diabetic mice 2 weeks after STZ treatment. As shown in Fig. 2A, immunohistochemical staining for 3-NT formation showed a strongly positive signal in the heart of wild-type diabetic mice, but a very weak signal in the heart of MT-TG diabetic mice or insulin-treated wild-type diabetic mice. To validate the antibody specificity, two sections were stained using the 3-NT antibody and preincubated overnight with nitrotyrosine (nitrated-BSA; Alpha Diagnostic International, San Antonio, TX). These two sections did not show 3-NT-positive signals (data not shown). Quantitative analysis by Western blot method further confirmed a significant increase in 3-NT formation in the heart of wild-type

diabetic mice, which was significantly inhibited both in MT-TG or insulin-treated wild-type diabetic mice (Fig. 2B). A control membrane for 3-NT antibody specificity was also performed by blocking 3-NT antibody with nitrotyrosine and did not show the positive signals (data not shown). In addition, the significant increase in 3-NT formation in the wild-type diabetic heart and its prevention in the MT-TG diabetic heart was also evident in diabetic mice 4 weeks after STZ treatment (Fig. 2C).

To explore whether the inhibitory effect of metallothionein on 3-NT formation results from direct inhibition of the interaction between peroxynitrite and proteins and/or from the inhibition of the reaction between superoxide and nitric oxide, immunofluorescent staining for superoxide was performed using fluorescent superoxide probe (dihydroethidine) and showed a significant increase in superoxide generation in the heart of wild-type diabetic mice 2 weeks after STZ treatment, but not in the heart of wild-type control or insulin-treated wild-type diabetic mice (Fig. 3A). The diabetes-induced superoxide formation in the heart of the wild-type diabetic mice and its inhibition in the heart of MT-TG diabetic mice or insulin-treated wild-type diabetic mice were confirmed by quantitative measurement using cytochrome *c* reduction assay, a measurement of NADPH-dependent superoxide generation (Fig. 3B).

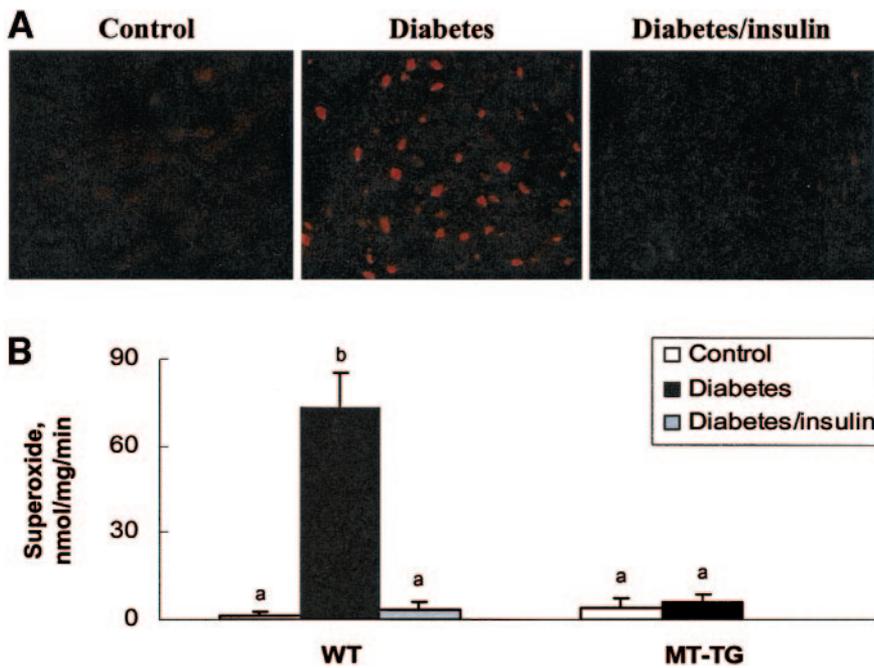


FIG. 3. Metallothionein prevention of diabetes-induced superoxide generation in the heart. **A:** Superoxide generation was probed by immunofluorescent staining using dihydroethidine, which specifically reacts with superoxide, in the heart of wild-type control, diabetic, and insulin-treated diabetic mice. **B:** In addition, the whole heart was also collected from the mice 2 weeks after STZ treatment and freshly processed for measuring NADPH-dependent superoxide generation using a cytochrome *c* reduction method, as described in the RESEARCH DESIGN AND METHODS. Treatment groups that do not share the same letter were significantly different ($P < 0.05$).

Urate and MnTMPyP inhibition of 3-NT formation in the LPS/TNF- α -treated cardiomyocytes. The *in vivo* results suggested that the suppression of superoxide and 3-NT formations in the MT-TG diabetic heart was accompanied by significant prevention of diabetes-induced cardiomyopathy. However, there is no direct link of the prevention of peroxynitrite formation and associated nitrosative damage to the inhibition of cardiomyopathy. In addition, although we have demonstrated the preventive effect of metallothionein on peroxynitrite-induced DNA and protein damage in a cell-free system (29), whether metallothionein exerts the same effect on intracellular peroxynitrite-induced nitrosative damage remains unclear. To this end, the primary cultures of neonatal cardiomyocytes from MT-TG and wild-type mice were exposed to LPS/TNF- α for intracellularly generating superoxide and nitric oxide (35–38). LPS/TNF- α was selected not only because it is a well-defined intracellular peroxynitrite generation system, but also because we have found the increase in systemic and cardiac TNF- α levels in the diabetic mice (30) and TNF- α also play a critical role in various cardiomyopathy including diabetic cardiomyopathy (7,37,38). The results in Fig. 4 show that MT-overexpressing cardiomyocytes were resistant to the LPS/TNF- α -induced cytotoxicity, detected by cell morphology (Fig. 4A) and medium LDH activity (Fig. 4B). The protective action of metallothionein was associated with the inhibition of LPS/TNF- α -induced 3-NT formation in the cells (Fig. 4C), as observed in the *in vivo* studies (Fig. 2). Importantly, when the wild-type cardiomyocytes were treated with LPS/TNF- α for 24 h in the presence of a peroxynitrite-specific scavenger urate or a cell-permeable SOD mimic MnTMPyP, the LPS/TNF- α -induced 3-NT formation and cytotoxicity both were abolished (Fig. 5A and B), as observed in the MT-overexpressing cardiomyocytes (Fig. 4).

DISCUSSION

Studies aiming for mechanistic insights into the metallothionein inhibition of diabetic cardiomyopathy are impor-

tant undertakings. This study focused on the effect of metallothionein on peroxynitrite-induced damage in the mouse model of type 1 diabetes induced by STZ. The results obtained clearly showed that peroxynitrite-induced protein damage, as measured by 3-NT, was involved in the diabetic cardiomyopathy. The formation of 3-NT most likely resulted from the overproduction of superoxide in the diabetic heart, which in turn reacts with nitric oxide to produce peroxynitrite, the causative agent for the formation of 3-NT. That the increased superoxide was responsible for the formation of 3-NT was revealed by the fact that suppression of superoxide formation both *in vivo* and *in vitro* resulted in reduced formation of 3-NT.

The involvement of oxidative and/or nitrosative stress in the pathogenesis of diabetic cardiomyopathy has been implicated in experimental animal studies and in patients (5–9,13–16,40,41). The overproduction of superoxide has been previously recognized as an important contributor to diabetic vascular complications; however, superoxide alone cannot be considered as a strong oxidant toward most types of biological molecules (18). Recent studies indicate that peroxynitrite, which results from reaction of superoxide and nitric oxide, may play a central role in the pathogenesis of diabetic vascular complications (15–19, 40), because tyrosine nitration changes the structure and function of the proteins (18,40–43). The direct evidence for the causative effect of peroxynitrite-caused protein nitration on diabetic nephropathy has been documented by two recent animal studies (44,45). In the present study, we are unable to identify the proteins that are nitrated in the heart of wild-type diabetic mice; however, based on the molecular size (~ 30 kDa; Fig. 2), the nitrated proteins may belong mainly to mitochondrial proteins (42,43). Studies showed that high susceptibility of mitochondrial proteins, including energy production-related proteins (succinyl-CoA:3-oxoacid CoA-transferase and creatine kinase) and apoptosis-related protein, voltage-dependent anion channel-1 (molecule weight, 32 kDa), to tyrosine nitration in the heart from STZ- and alloxan-induced

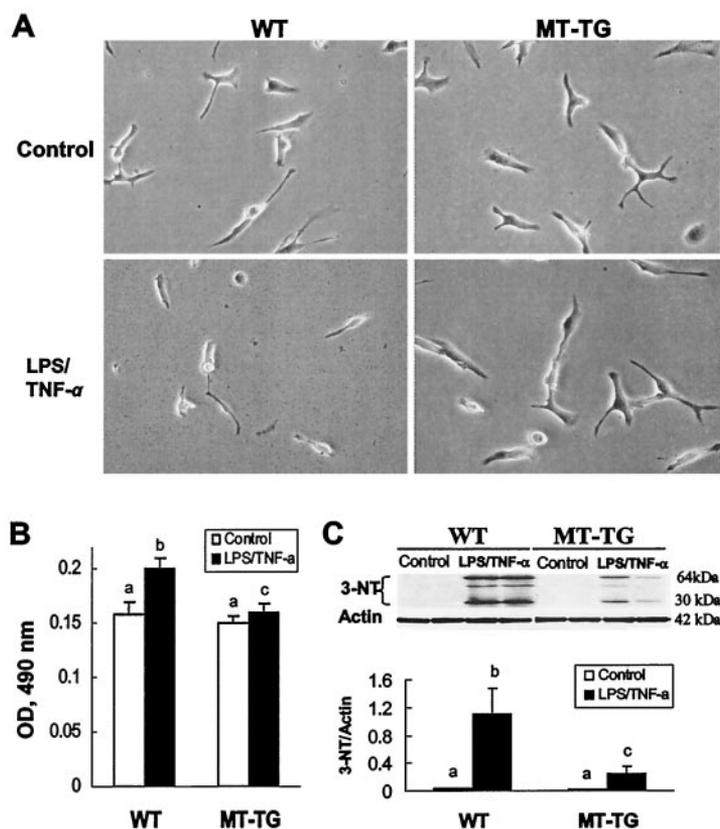


FIG. 4. Metallothionein inhibition of LPS/TNF- α -induced cytotoxicity in primary cultures of neonatal mouse cardiomyocytes. **A:** The cultures were exposed to a final concentration in the cultures of 30 μ g/ml LPS plus 100 ng/ml TNF- α for 48 h and then photographed under a microscope. **B:** The media were collected and spun at 10,000g for 5 min, and the supernatant was used for the measurement of LDH activity, shown by the OD value at 490 nm. **C:** The attached cells were removed by policeman for the measurement of 3-NT by Western blot method. Quantitative analysis of 3-NT contents (band density) from three samples of each treatment is presented relative to actin content. Treatment groups that do not share the same letter were significantly different ($P < 0.05$).

diabetic rats may be predominantly responsible for the mitochondrial and eventually myocyte dysfunction, leading to cardiomyopathy (41–43).

In a recent study (6), by immunohistochemical staining, 3-NT as an index of peroxynitrite-induced protein nitration was significantly increased along with endothelial and myocyte cell death in the heart of diabetic patients, suggesting the possible association of peroxynitrite-induced protein nitration with cardiomyopathy. Metallothionein as a potent antioxidant significantly protects the heart from diabetes-induced damage in a spontaneously developed (26,28) and STZ-induced (25; present study) type 1 diabetic mouse model. In the STZ-induced diabetic mouse model, we further demonstrated that 3-NT was significantly increased in the heart of wild-type diabetic mice, but not MT-TG diabetic mice, 2 and 4 weeks after STZ treatment by Western blot assay. The innovative finding of the present study is that inhibition of superoxide and 3-NT formation is accompanied by a significant protection from diabetes-induced cardiac diastolic dysfunction observed 6 months after STZ treatment, indicating the involvement of nitrosative damage in the pathogenesis of diabetic cardiomyopathy. To confirm this *in vivo* observation, we used primary cultures of cardiomyocytes treated with LPS/TNF- α in the presence of the cell-permeable SOD mimic MnTMPyP or urate, a peroxynitrite specific scavenger, to dissect the direct effect of superoxide or peroxynitrite on LPS/TNF- α -induced cytotoxicity. The direct link between peroxynitrite-induced nitration and cardiomyocyte cytotoxicity was then defined because both urate and MnTMPyP significantly prevented 3-NT and cytotoxicity.

The metallothionein inhibition of peroxynitrite-induced protein nitration, shown by an increase in 3-NT formation,

could result from its direct interaction with peroxynitrite (29), from its inhibition of superoxide generation (46,47), or from both. The results obtained from the *in vivo* study showed that both the formation of 3-NT and the NADPH-dependent generation of superoxide were inhibited to the same extent, suggesting that metallothionein inhibition of superoxide generation may be responsible for the decreased formation of 3-NT; in particular, metallothionein almost completely abolished the NADPH-dependent generation of superoxide in the heart. Based on the present study, we still do not know how metallothionein prevents the NADPH-dependent superoxide generation.

The use of LPS/TNF- α to treat the cultured cells to produce peroxynitrite-induced damage, rather than to use 3-morpholininosydnonimine (SIN-1) for the same purpose is important. Several studies (48,49) have used SIN-1 to treat cultured cells to produce peroxynitrite-induced damage. These studies have observed that although SIN-1 caused cell injury, the formation of 3-NT in the cell was not observed. Addition of SIN-1 to the medium causes an immediate formation of peroxynitrite extracellularly (29). This leads to a direct damage to the cell membrane and cell death, without the intracellular process. In the present study, an obstacle is to develop an *in vitro* model to define the role of peroxynitrite in hyperglycemia-induced cardiomyopathy *in vivo*. Although the occurrence of peroxynitrite and its "footprint" of 3-NT were observed in the tissue of diabetic animals or patients (6,15,44,45) and in the high-glucose perfused hearts (16), there is no evidence that shows the peroxynitrite generation in cultured cardiomyocytes by exposure to high levels of glucose (17). We also failed to induce 3-NT formation in the neonatal cardiomyocytes exposed to 22.5 mmol/l for 48 or 72 h

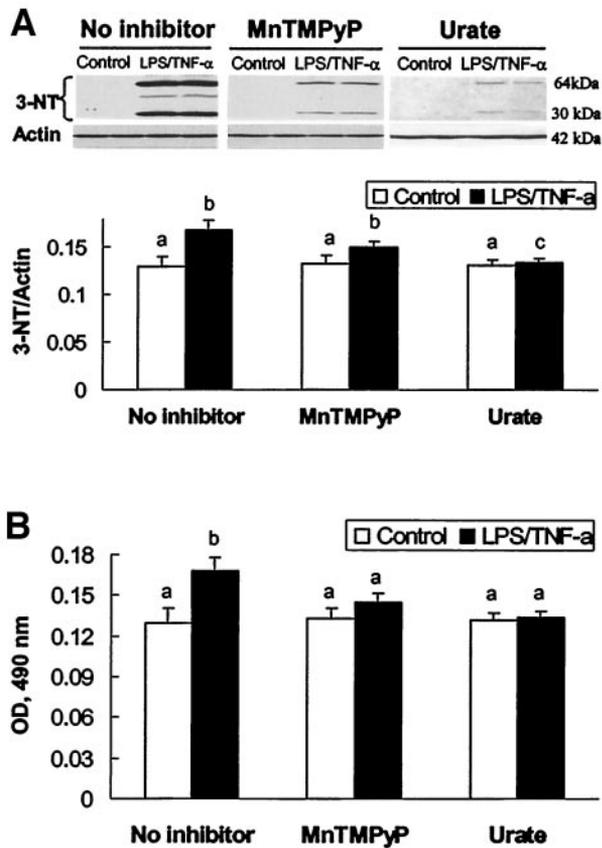


FIG. 5. Urate and MnTMPyP inhibition of 3-NT formation in the LPS/TNF- α -treated cardiomyocytes. Primary cultures of neonatal cardiomyocytes from wild-type mice were exposed to LPS/TNF- α for 48 h with or without cell-permeable urate or MnTMPyP. Coincubation of the cells with urate or MnTMPyP significantly inhibited LPS/TNF- α -induced 3-NT formation (A) and also significantly abolished LPS/TNF- α -induced cytotoxicity LDH, shown by the OD value at 490 nm (B). Quantitative analysis of 3-NT contents (band density) from three samples of each treatment is presented relative to actin content. Treatment groups that do not share the same letter were significantly different ($P < 0.05$).

(data not shown). Under in vivo conditions, endothelial cells in the heart play a critical role in the formation of peroxynitrite due to hyperglycemia (6,15,44,45). It has been shown that if endothelial cells (mostly cell lines) were exposed to high levels of glucose for several days, a significant induction of peroxynitrite and 3-NT was observed (19,40,50). Therefore, the lack of peroxynitrite formation in the cultured cardiomyocytes may result from the omission of endothelial cells from the cultures. Thus, to mimic the role of peroxynitrite in vivo, we adapted the established intracellular peroxynitrite formation model by exposing the cardiomyocytes to LPS/TNF- α (35–38).

The use of the mouse model of type 1 diabetes induced by STZ has been criticized for the possible nonspecific effect of STZ. However, this should not be a major concern in the present study. We have used insulin-treated STZ-induced diabetic mice with a range of blood glucose levels (<8 mmol/l) as controls, as in our previous study (5). Our results demonstrated that only the diabetic mice with persistent high levels of blood glucose developed significant cardiomyopathy (Fig. 1; Table 2). In addition, we also found that serum CPK was not significantly increased in the wild-type diabetic mice until 2 weeks after STZ treatment (Fig. 1), at which time hyperglycemia has been well

established. Therefore, these results indicated that cardiac toxicity is related directly to diabetes, rather than STZ toxicity. Several studies (6,16,42,44,45) and the results obtained here have shown that superoxide generation and associated nitrosative damage indeed play a critical role in the pathogenesis of diabetic cardiomyopathy. Metallothionein prevents diabetic cardiomyopathy at least in part through suppression of diabetes-caused superoxide generation and the associated nitrosative cardiac damage.

ACKNOWLEDGMENTS

This work was supported in part by Jewish Hospital Foundation Grant JHF 010808, Philip Morris USA Grant PM020187, American Diabetes Association Grant ADA020667 (to L.C.), and National Institutes of Health Grants HL63760 and HL59225 (to Y.J.K.). Y.J.K. is a Distinguished University Scholar of the University of Louisville.

We greatly appreciate the technological assistance of Yibo Du and Don Mosley.

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