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PRECLINICAL RESEARCH

Metallothionein Suppresses Angiotensin II–Induced Nicotinamide Adenine Dinucleotide Phosphate Oxidase Activation, Nitrosative Stress, Apoptosis, and Pathological Remodeling in the Diabetic Heart

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Objectives We evaluated metallothionein (MT)-mediated cardioprotection from angiotensin II (Ang II)–induced pathologic remodeling with and without underlying diabetes.

Background Cardiac-specific metallothionein-overexpressing transgenic (MT-TG) mice are resistant to diabetic cardiomyopathy largely because of the antiapoptotic and antioxidant effects of MT.

Methods The acute and chronic cardiac effects of Ang II were examined in MT-TG and wild-type (WT) mice, and the signaling pathways of Ang II–induced cardiac cell death were examined in neonatal mouse cardiomyocytes.

Results Acute Ang II administration to WT mice or neonatal cardiomyocytes increased cardiac apoptosis, nitrosative damage, and membrane translocation of the nicotinamide adenine dinucleotide phosphate oxidase (NOX) isoform $p47^{phox}$. These effects were abrogated in MT-TG mice, MT-TG cardiomyocytes, and WT cardiomyocytes preincubated with peroxynitrite or superoxide scavengers and NOX inhibitors, suggesting a critical role for NOX activation in Ang II–mediated apoptosis. Prolonged administration of subpressor doses of Ang II (0.5 mg/kg every other day for 2 weeks) also induced apoptosis and nitrosative damage in both diabetic and nondiabetic WT hearts, but not in diabetic and nondiabetic MT-TG hearts. Long-term follow-up (1 to 6 months) of both WT and MT-TG mice after discontinuing Ang II administration revealed progressive myocardial fibrosis, hypertrophy, and dysfunction in WT mice but not in MT-TG mice.

Conclusions Metallothionein suppresses Ang II–induced NOX-dependent nitrosative damage and cell death in both nondiabetic and diabetic hearts early in the time course of injury and prevents the late development of Ang II–induced cardiomyopathy. (J Am Coll Cardiol 2008;52:655–66) © 2008 by the American College of Cardiology Foundation

A key pathophysiological event in the development of diabetic cardiomyopathy is the excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the heart (1–3). Metallothionein (MT) is a cysteine-rich protein that scavenges a wide range of free radicals, including superoxide, nitric oxide, hydrogen peroxide, and peroxynitrite (3,4). Cardiac-specific metallothionein-overexpressing transgenic (MT-TG) mice have been shown to be protected against the development of diabetic cardiomyopathy in streptozotocin (STZ)-induced type 1 diabetes (3,5–7), spontaneous type 1 diabetes (8), and sucrose feeding-induced insulin resistant, pre-diabetic mice (9,10). We previously demonstrated that MT suppresses peroxynitrite-derived nitrosative damage in diabetic hearts (3). However, the cellular mechanisms underlying this beneficial effect remained unclear.

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Cardiac angiotensin II (Ang II) is thought to play an important role in the pathogenesis of diabetic cardiomyopathy (11–13). Angiotensin II acts via its receptors AT_1 or AT_2 to induce nicotinamide adenine dinucleotide phos-

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Abbreviations and Acronyms

Ang II = angiotensin II

ANOVA = analysis of variance

CAT-TG = catalase-overexpressing transgenic

CTGF = connective tissue growth factor

ET = endothelin

LV = left ventricle/ventricular

MT = metallothionein

MT-TG = cardiac-specific, metallothionein-overexpressing transgenic

NOX = nicotinamide adenine dinucleotide phosphate oxidase

NT = nitrotyrosine

PAI = plasminogen activator inhibitor

RNS = reactive nitrogen species

ROS = reactive oxygen species

STZ = streptozotocin

TNF = tumor necrosis factor

TUNEL = terminal transferase dUTP nick end labeling

WT = wild type

phate oxidase (NOX) activation and is involved in a wide range of pathogenic processes in the diabetic heart, including the induction of apoptosis and fibrosis (11–15). We have demonstrated that apoptosis is increased in the diabetic heart and contributes importantly to the development of cardiomyopathy (5,16). Previous studies also have indicated that Ang II–mediated ROS generation in vitro is significantly attenuated in cardiomyocytes isolated from MT-TG diabetic hearts compared with wild-type (WT) diabetic hearts (8). To what extent this occurs in vivo and the responsible underlying mechanisms are unknown. Several possible mechanisms of MT-induced Ang II modulation in diabetic cardiomyopathy may be invoked: 1) MT may down-regulate AT_1 or AT_2 receptor expression or down-regulate NOX expression; or 2) MT may directly scavenge ROS without influencing the Ang II axis. In the current study, we tested the hypothesis that the cardioprotective benefits of MT in diabetes are related in part to suppression of Ang II–induced NOX activation via the AT_1 and AT_2 receptors, resulting in attenuation of oxidative and nitrosative damage and apoptosis in the heart. Consequently, MT suppression of Ang II–mediated early cardiac cell death prevents the late development of cardiomyopathy.

Methods

Cardiac-specific MT-TG and catalase-overexpressing transgenic (CAT-TG) mice along with their WT FVB mice were used, and the animal care conditions, experimental treatments, and detailed methods are provided in the Online Appendix. In brief, 4 sets of animal studies were performed, and the hearts were harvested for protein, messenger ribonucleic acid (mRNA), and histopathological analysis. The 4 groups investigated were: study A, the effect of diabetes on cardiac Ang II receptor expression in diabetic mice induced by a single dose of STZ (150 mg/kg body weight) (16); study B, the acute cardiac effects of Ang II in WT, MT-TG, and CAT-TG mice after a single subcutaneous injection of 1 mg of Ang II/kg body weight; study C, the subchronic cardiac effects of Ang II in MT-TG and WT mice after subcutaneous injection of subpressor doses of Ang II (0.5 mg/kg body weight) every other day for 2 weeks; and study D, the subchronic effects of Ang II in diabetic hearts by similar protocols used for studies A and C.

Primary cultures of neonatal cardiomyocytes from WT and MT-TG mice were used for the in vitro study by incubating these cells with 100 nmol/l Ang II for 24 h to investigate the signaling pathways of Ang II–induced nitrosative damage and cell death.

To quantitatively determine the induction of apoptosis by Ang II, we measured deoxyribonucleic acid fragmentation by using a Cell Death Detection ELISA kit (Roche Diagnostics, Basel, Switzerland). Real-time quantitative polymerase chain reaction was used to analyze AT_1, AT_2, atrial natriuretic peptide, endothelin (ET)-1, tumor necrosis factor-alpha (TNF-α), and plasminogen activator inhibitor (PAI)-1 gene expression (11–15). Western blotting was used for analysis of NOX $p47^{phox}$ phosphorylation, the activated form of caspase-3, AT_1, AT_2, 3-nitrotyrosine (NT), and connective tissue growth factor (CTGF). For the detection of apoptosis in the heart, we performed the terminal transferase dUTP nick end labeling (TUNEL) assay by using the In Situ Apoptosis Detection Kit (Chemicon International, Temecula, California), and mouse testicular tissue was used as a positive control (16). In addition, immunofluorescent staining was used to localize activated caspase-3 by double stains for cardiomyocytes with alpha sarcomeric actin and caspase-3 (5) and also to directly detect peroxynitrite formation in cultured myocytes with its specific probe HKGreen-1 (kindly provided by Dr. Dan Yang from The University of Hong Kong) (17). Serum and cardiac Ang II were measured with the Ang II Enzyme Immunoassay Kit (SPI-BIO, Massy, France). Cardiac function was monitored as previously described, with echocardiography (18) and aortic and left ventricular (LV) pressure measurement (19) (Online Appendix).

Statistical analysis. Data were expressed as mean ± SD for normally distributed variables. For statistical analysis, 1- or 2-way analysis of variance (ANOVA) was used as appropriate. The overall F-test was performed to test the significance of the ANOVA models. The significance of the interactions and main effects were taken into consideration and then multiple comparisons were performed by the Bonferroni test. The significance level was considered at $p < 0.05$.

Results

Effect of MT on AT_1 and AT_2 expression in diabetic hearts. Diabetes was induced by STZ in MT-TG and WT mice with blood glucose levels >12 mmol/l on day 3 after STZ treatment. AT_1 and AT_2 protein levels in the hearts of diabetic mice at 2 weeks after STZ treatment were significantly increased in both WT and MT-TG diabetic hearts relative to control hearts (Online Fig. 1). Levels for AT_1 or AT_2 mRNA were also significantly increased in both WT and MT-TG diabetic hearts (data not shown). These results indicated that cardiac AT_1 and AT_2 are both up-regulated

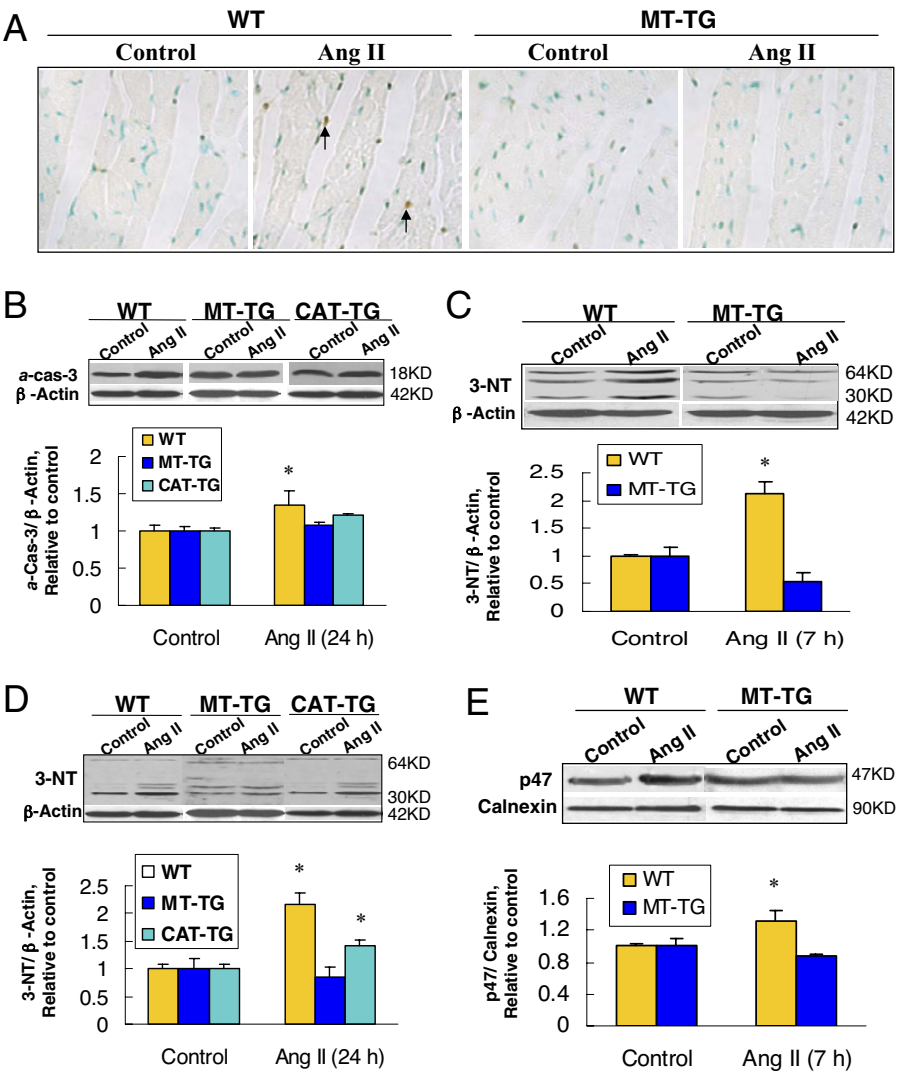

Figure 1 **MT Prevents Ang II–Induced Apoptosis, 3-NT Formation, and NOX Membrane Translocation in the Hearts of Mice (n ≥ 5) That Were Given 1 mg/kg of Ang II at 7 or 24 h Before the Experiments**

Apoptosis was detected by terminal transferase dUTP nick end labeling assay **(A)** and Western blotting activated caspase-3 **(B)**. All 3-nitrotyrosine (3-NT) formation (bands from 30 to 60 kDa) **(C and D)** and nicotinamide adenine dinucleotide phosphate oxidase (NOX) p47phox membrane translocation **(E)** were detected by Western blotting assay. *$p < 0.05$ versus corresponding control. Ang II = angiotensin II; CAT-TG = catalase-overexpressing transgenic; MT = metallothionein; MT-TG = cardiac-specific, metallothionein-overexpressing transgenic; WT = wild type.

in STZ-induced diabetes and that MT overexpression does not significantly affect this response.

Metallothionein alleviates Ang II–induced cardiac apoptosis, nitrosative stress, and NOX activation in vivo. We next examined whether Ang II induced apoptosis and nitrosative damage in the heart and whether MT protected against these effects. The WT and MT-TG mice were given a single subcutaneous injection of Ang II at 1 mg/kg body weight, and their hearts were collected 7 and 24 h later (20). The use of TUNEL staining revealed significantly increased myocardial apoptosis in Ang II–treated WT mice but not Ang II–treated MT-TG mice (Fig. 1A). Western blotting for the activated caspase-3 further confirmed the induction of apoptosis in Ang II–treated WT hearts but not in Ang II–treated MT-TG hearts (Fig. 1B). Nitrosative damage, indexed by 3-NT, was significantly increased in the hearts of Ang II–treated WT mice but not MT-TG mice (Figs. 1C and 1D).

It is known that 3-NT can be generated by multiple mechanisms, including myeloperoxidase. To ensure that Ang II–induced 3-NT formation was predominantly due to peroxynitrite-induced protein nitration, CAT-TG and WT mice were given a single injection of Ang II 1 mg/kg as described previously, and 24 h later the hearts were collected. Because catalase exerts its catalytic effects downstream of superoxide, catalase overexpression should not effectively protect against Ang II–induced superoxide gen-

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eration, peroxynitrite formation, and associated protein nitration. Western blotting data for caspase-3 activation (Fig. 1B) showed no significant interaction effects, tested by the F test for the significance of 2-way ANOVA ($p > 0.05$) but indeed showed a significant difference for the main effect ($p < 0.05$). The post-hoc multiple comparisons showed a significant difference between Ang II–treated WT and WT control groups, suggesting that Ang II–induced caspase-3 activation was attenuated by both MT and catalase. Analysis of 3-NT accumulation (Fig. 1D) showed significant interaction effects ($p < 0.05$). Under such conditions, the simple main effects of Ang II treatment were tested within each mouse model (WT, MT-TG, or CAT-TG). There was a significant difference between the Ang II–treated WT and WT control groups and also a significant difference between the Ang II–treated CAT-TG and CAT-TG control groups (Fig. 1D), suggesting that Ang II–induced 3-NT accumulation was attenuated only by MT.

Western blotting further revealed that the membrane translocation of NOX p47^{phox} was significantly increased in

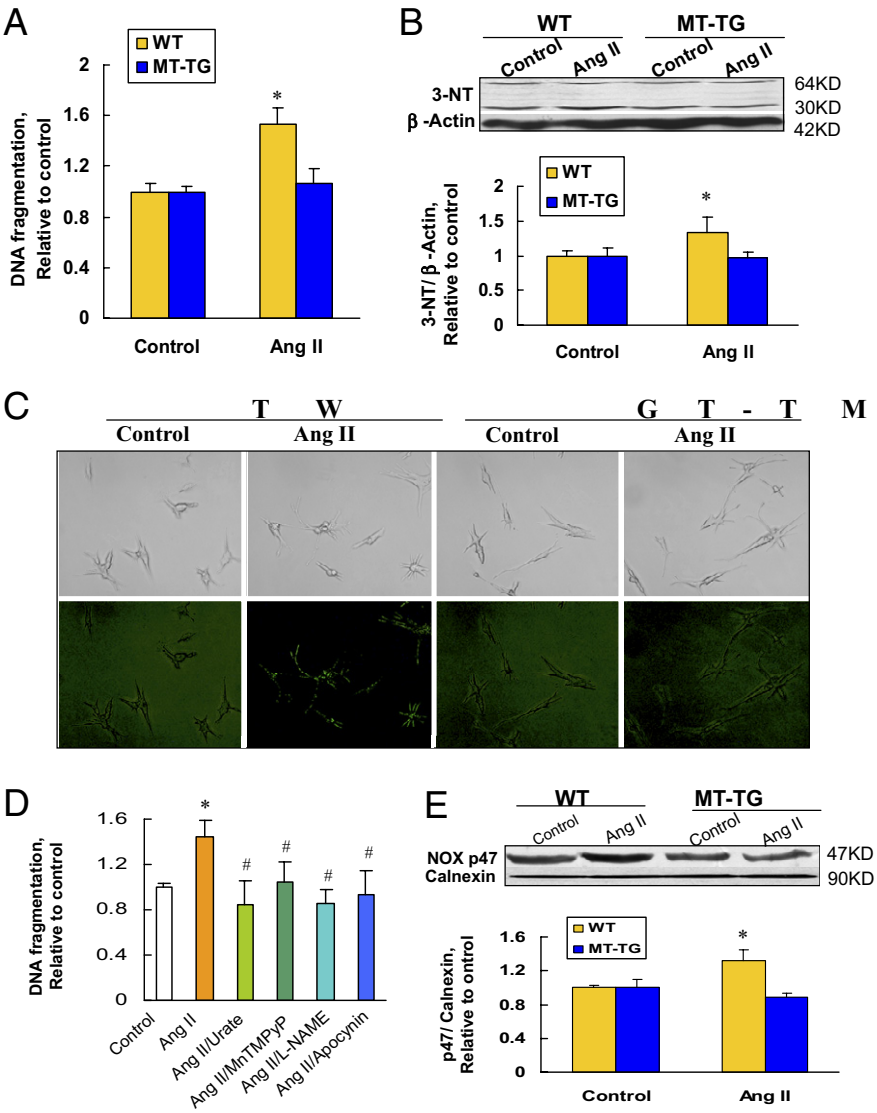

Figure 2 **MT Protects Cardiomyocyte Apoptotic Death From a 24-h Exposure to Ang II (100 nmol/l) and Role of NOX-Dependent Peroxynitrite Generation in Ang II Apoptotic Effect**

(A) Deoxyribonucleic acid (DNA) fragmentation by enzyme-linked immunosorbent assay. **(B)** 3-NT formation by Western blotting. **(C)** Fluorescent imaging of peroxynitrite in the cultured cardiomyocytes exposed to Ang II for 6 h. **Top row,** phase contrast images; **bottom row,** fluorescent images. **(D)** Cardiomyocytes exposed to Ang II for 24 h with or without 1-h pre- and coincubation with urate (peroxynitrite inhibitor), MnTMPyP (superoxide inhibitor), L-NAME (nitric oxide synthase inhibitor), and apocynin (NOX inhibitor). **(E)** Western blotting for the membrane translocation of NOX p47^{phox} induced by a 12-h Ang II exposure. Results are presented as relative to control with pooled results from 3 separate experiments with triple samples for each. *$p < 0.05$ versus control; #$p < 0.05$ versus Ang II alone. L-NAME = NG-nitro-L-arginine methyl ester; MnTMPyP = Mn(111) tetrakis 1-methyl 4-pyridylporphyrin pentachloride; other abbreviations as in Figure 1.

the hearts of Ang II–treated WT mice, an effect not observed in Ang II–treated MT-TG mice (Fig. 1E), suggesting that NOX inactivation may contribute to MT-mediated protection against Ang II–induced injury.

Metallothionein prevents Ang II–induced apoptotic signaling and 3-NT accumulation in cardiomyocytes in vitro. Neonatal cardiomyocytes were exposed to Ang II 100 nmol/l for 24 h. Apoptosis, as detected by TUNEL assay (Online Fig. 2A) and deoxyribonucleic acid fragmentation (Fig. 2A), was significantly increased in WT cells but not in MT-TG cells. Angiotensin II–induced 3-NT accumulation was also significantly increased only in WT cardiomyocytes (Fig. 2B).

Peroxynitrite levels were directly measured in Ang II–exposed (6 h) cardiomyocytes with the use of the fluorescent probe HK Green-1 (17). Confocal microscopy showed increased fluorescence in Ang II–treated WT cardiomyocytes but not in Ang II–treated MT-TG cardiomyocytes (Fig. 2C). Moreover, Ang II–induced apoptosis in WT cardiomyocytes was prevented by coincubating (1 h before and during Ang II exposure) with 100 μmol/ml urate (peroxynitrite scavenger), 50 μmol/ml Mn(111) tetrakis 1-methyl 4-pyridylporphyrin pentachloride (superoxide dismutase mimetic), and 100 μmol/ml NG-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor) (Fig. 2D), suggesting that peroxynitrite is an important mechanism of Ang II–mediated apoptosis and is generated from superoxide and nitric oxide interaction. Similarly, Ang II–mediated apoptosis was prevented by coincubation with 100 μmol/ml apocynin, a specific NOX inhibitor, supporting the primary

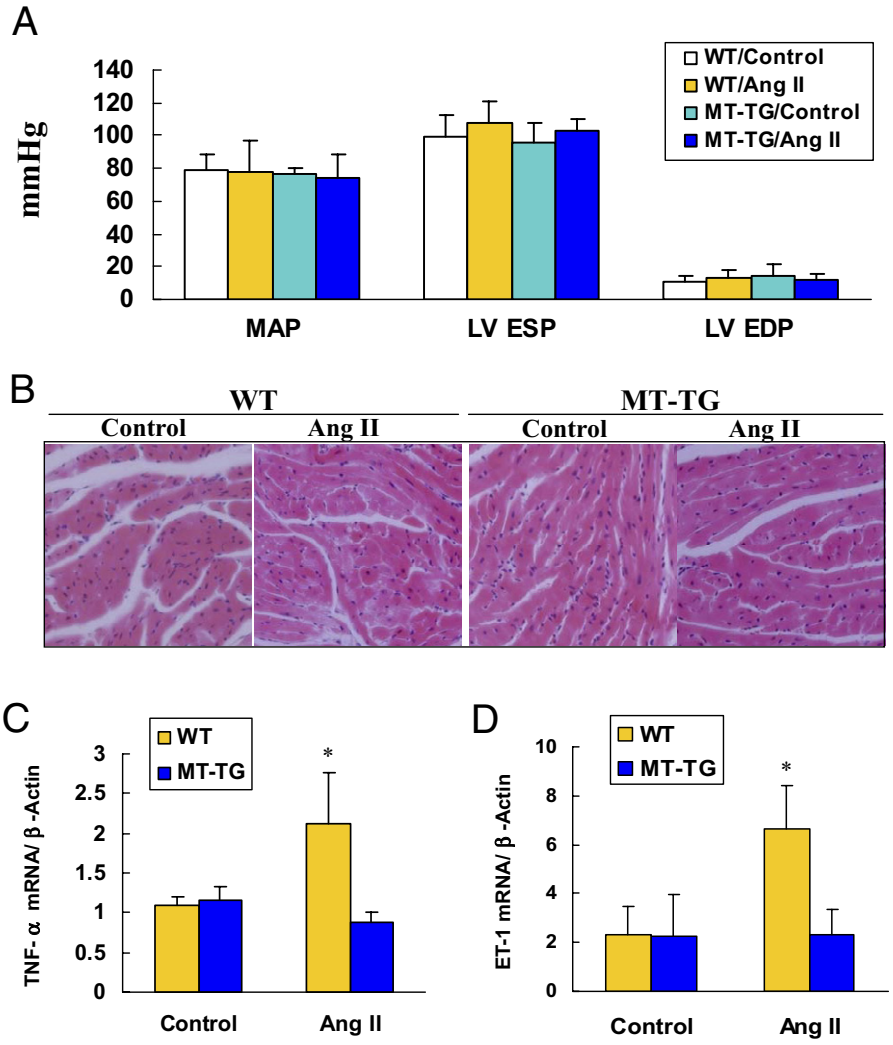

Figure 3 **Effects of Ang II on Blood Pressure, Cardiac Histopathology, and Inflammatory Factors**

Mice ($n \geq 5$) were given angiotensin (Ang) II at 0.5 mg/kg body weight for 2 weeks, and 1 month after the first dosing of Ang II, mean aortic blood pressure (MAP), left ventricular end-systolic pressure (LV ESP), and left ventricular end-diastolic pressure (LV EDP) **(A)** were measured with cardiac morphological examination by hematoxylin and eosin staining **(B)** and messenger ribonucleic acid (mRNA) expression of tumor necrosis factor (TNF)-α and endothelin (ET)-1 by real-time polymerase chain reaction **(C and D)**. *$p < 0.05$ versus corresponding control.

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involvement of NOX activation and NOX-dependent superoxide generation (Fig. 2D). In contrast, the inhibition of p38 MAPK and phosphatidylinositol 3 kinase with their inhibitors (SB203580 and LY294002, respectively) did not influence Ang II–induced apoptosis (Online Fig. 2B). Additionally, in agreement with the in vivo results (Fig. 1E), Ang II induced NOX p47^{phox} membrane translocation in WT cardiomyocytes but not in MT-TG cardiomyocytes (Fig. 2E).

Metallothionein attenuates Ang II–induced long-term cardiac remodeling and dysfunction. We next evaluated whether short-term Ang II exposure in vivo induced late manifestations of cardiac dysfunction and, if so, whether MT attenuated or prevented these events. The WT and MT-TG mice were given subpressor doses of Ang II (0.5 mg/kg) every other day for 2 weeks as in previous studies (21,22) to induce apoptosis without altering mechanical load. After the 2-week Ang II exposure, animals were followed for up to 6 months to assess for the development of cardiomyopathy.

As shown in Figure 3A, no change was found for the mean aortic blood pressure, LV end-systolic pressure, or LV end-diastolic pressure at 1 month after Ang II administration in both WT and MT-TG mice, indicating the lack of a persistent pressor effect at the dose used. Hematoxylin and eosin staining of hearts exposed to 2 weeks of Ang II revealed no significant necrotic damage in the heart aside from very rare loci of endomyocardial cardiomyocytes with

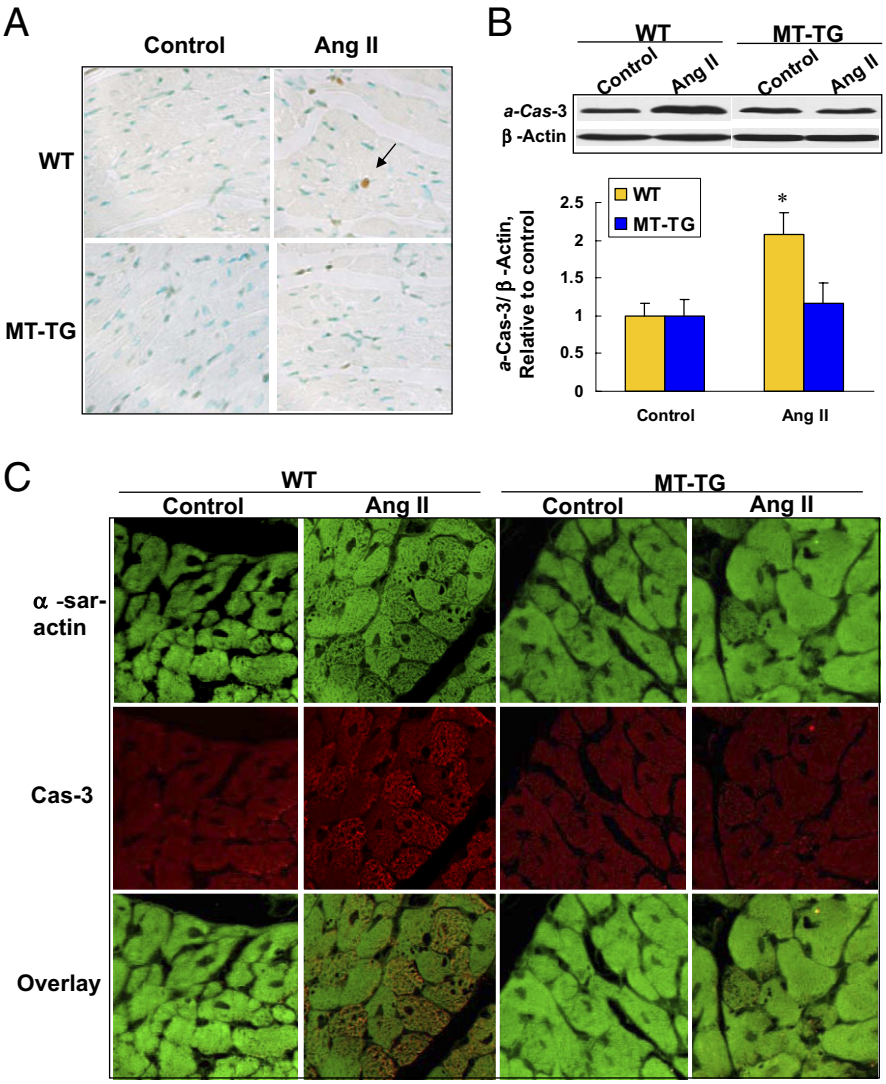

Figure 4 **Chronic Exposure to Ang II–Induced Cardiac Apoptosis**

Mice (n ≥ 5) were given Ang II at 0.5 mg/kg body weight for 2 weeks, during which the hearts were collected at 1 and 2 weeks for terminal transferase dUTP nick end labeling assay **(A)**, Western blotting of activated caspase-3 **(B)**, and colocalization of activated caspase-3 with cardiomyocytes (α-sarcomeric actin) with immunofluorescent staining **(C)**. *$p < 0.05$ versus corresponding control. Abbreviations as in Figure 1.

condensed nuclei or faint blue nuclei with eosinophilic staining (Fig. 3B). However, there were significant increases in cardiac TNF-α and ET-1 mRNA expression from these mice (Figs. 3C and 3D).

Angiotensin II–treated WT mice, but not MT-TG mice, showed an increased number of TUNEL positive nuclei in the heart on day 7 during Ang II treatment (Fig. 4A), which was confirmed by Western blotting for activated caspase-3 (Fig. 4B). Double staining revealed that activated caspase-3 was predominantly localized in cardiomyocytes of Ang II–treated WT mice, whereas such staining was not observed in the hearts of Ang II–treated MT-TG mice (Fig. 4C). Increased expression of activated caspase-3 was also observed in the hearts of WT mice on day 14 during Ang II treatment, but not on day 30 after receiving Ang II (e.g., 2 weeks after the last injection of Ang II) (data not shown). These results suggested that cardiac apoptosis was significantly induced with Ang II exposure in vivo but was normalized upon stopping the exposure.

Figure 5A shows that, upon stopping Ang II exposure, 3-NT levels remained persistently increased in Ang II–treated WT mice, but not MT-TG mice, up to 6 months after exposure. The divergence between the apoptotic rate and nitrosative damage suggested that induction of apoptosis is multifactorial and dependent not only on nitrosative damage but also other pathophysiological factors induced by Ang II in vivo. This notion is also supported by the results in the acute model (Figs. 1B and 1D), in which 3-NT accumulation was disproportionately greater than the increase in caspase-3 activation in the Ang II–treated mice.

Because the proinflammatory cytokine PAI-1 is profibrotic as the result of its inhibition of the conversion of plasminogen to plasmin (23–25), cardiac PAI-1 mRNA

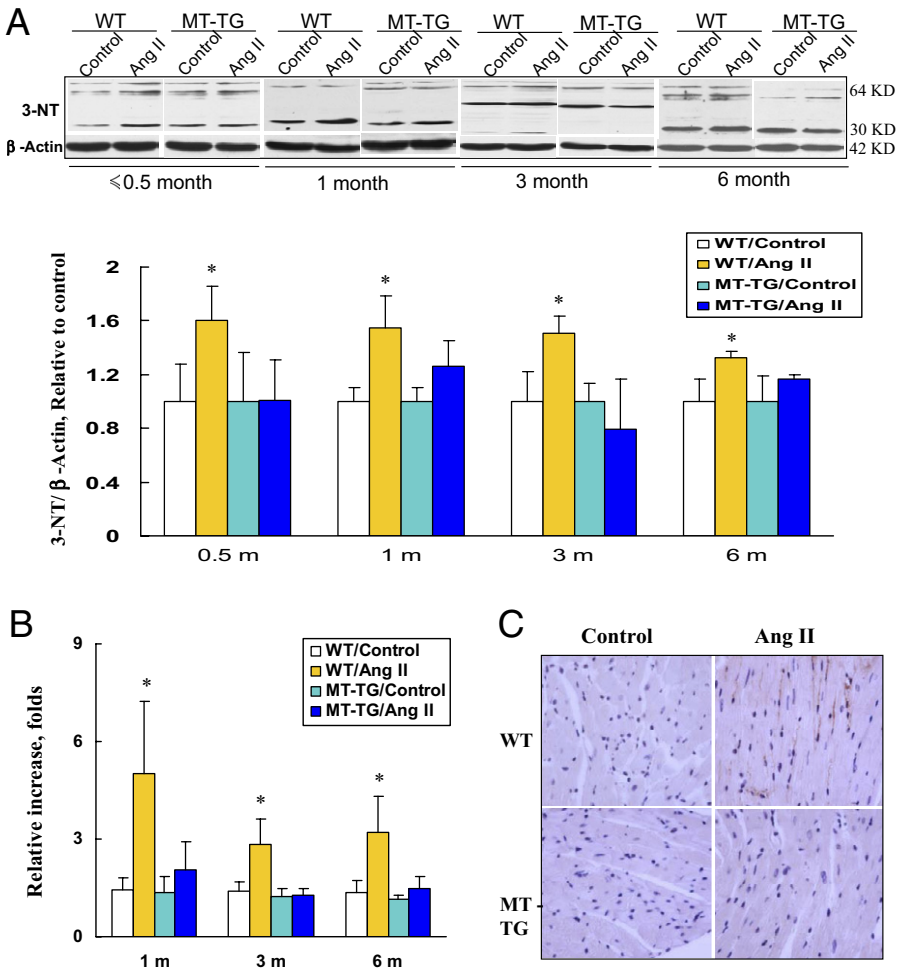

Figure 5 **MT Prevents Ang II–Induced Cardiac 3-NT Formation and PAI-1 Expression**

Mice (n ≥ 5) were given Ang II at 0.5 mg/kg for 2 weeks, and then the hearts were collected within the 2 weeks during (indicated by 0.5 m) and 1, 3, 6 months after Ang II administration for measuring 3-NT by Western blotting **(A)**, and PAI-1 mRNA **(B)** and protein expression **(C)** by real-time polymerase chain reaction and immunohistochemical staining, respectively. *$p < 0.05$ versus corresponding control. mRNA = messenger ribonucleic acid; PAI-1 = plasminogen activator inhibitor-1; other abbreviations as in Figure 1.

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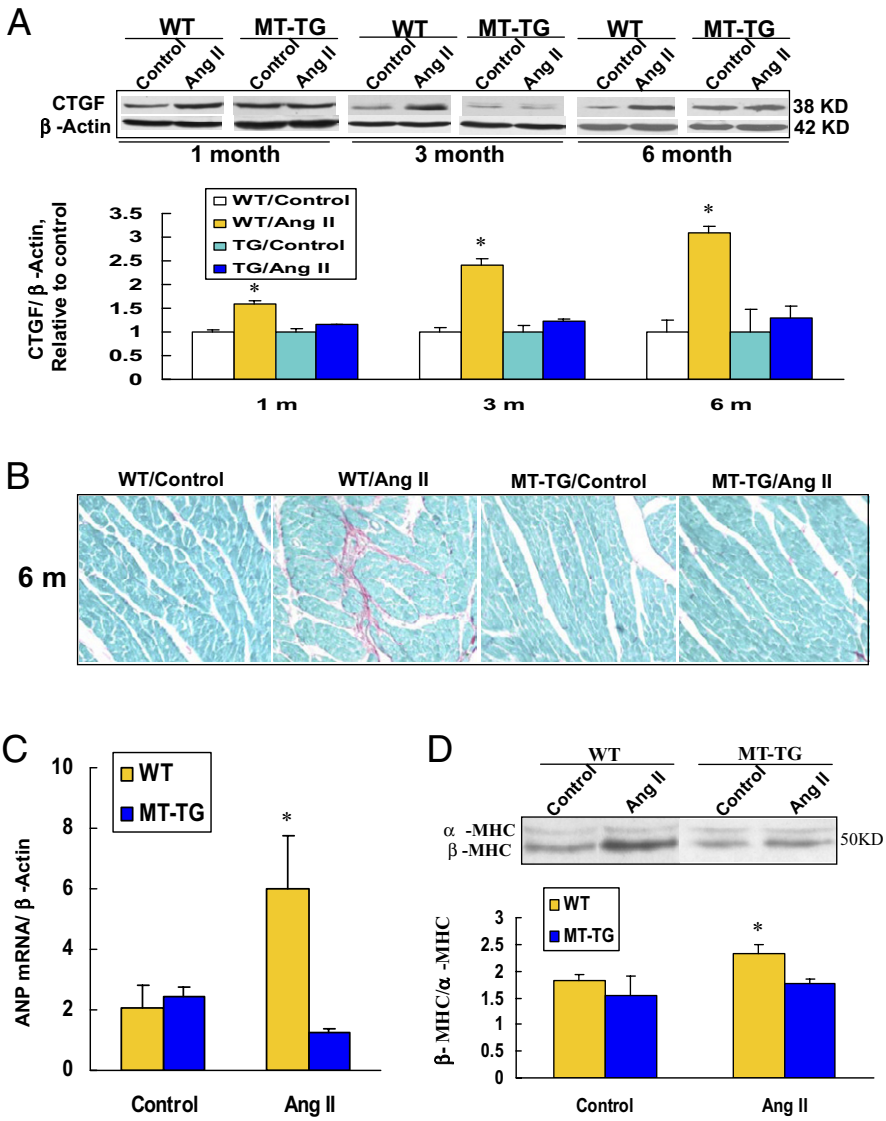

Figure 6 **MT Prevents Ang II–Induced Fibrosis and Cardiac Hypertrophy**

Animal treatment and tissue sampling were the same as in Figure 5. Cardiac fibrosis was examined via CTGF expression with western blotting **(A)** and Sirius red staining for collagen **(B)**. **(C)** Atrial natriuretic peptide (ANP) messenger ribonucleic acid (mRNA); **(D)** α- and β-MHC protein. *$p < 0.05$ versus corresponding control. CTGF = connective tissue growth factor; other abbreviations as in Figure 1.

levels at 1, 3, and 6 months after Ang II infusion were examined with the use of real-time polymerase chain reaction (Fig. 5B), and PAI-1 protein was assessed with the use of immunohistochemistry (Fig. 5C). We found that PAI-1 expression was significantly increased in the hearts of the WT mice, but not MT-TG mice, from 1 to 6 months after 2 weeks of Ang II exposure, exactly paralleling the pattern of 3-NT accumulation (Fig. 5A).

The aforementioned results suggest that although Ang II–derived oxidative and/or nitrosative damage (Fig. 5A) does not cause significant cardiac necrosis (Fig. 3B), it can still induce apoptosis (Fig. 4) and inflammatory responses (Figs. 3C, 3D, 5B, and 5C), as reported in a previous study (23). We next defined whether Ang II–induced nitrosative damage, cell death, and inflammation heralded the development of late cardiac remodeling and dysfunction. Western blotting revealed a time-dependent increase in CTGF expression in the hearts of WT mice, but not MT-TG mice, at 1, 3, and 6 months after Ang II administration (Fig. 6A). Moreover, Sirius red staining for collagen revealed a significant increase in cardiac fibrosis in Ang II–treated WT mice but not Ang II–treated MT-TG mice (Online Fig. 3 for samples from 1 and 3 months and Fig. 6B for 6 months). Cardiac hypertrophy was also significantly induced in WT mice 6 months after Ang II exposure, as indexed by increases in atrial natriuretic

Table 1 Effects of 2 Weeks of Ang II Administration on Cardiac Function in WT and MT-TG Mice

		1 Month		3 Months		6 Months	
	Baseline	Control	Ang II	Control	Ang II	Control	Ang II
WT mice							
HR (beats/min)	492 ± 17	546 ± 32	556 ± 29	572 ± 19	592 ± 41	525 ± 41	515 ± 36
LVEDD (mm)	4.2 ± 0.2	3.8 ± 0.6	3.7 ± 0.2	3.6 ± 0.1	3.7 ± 0.2	4.2 ± 0.3	3.8 ± 0.4
LVESD (mm)	2.1 ± 0.3	2.0 ± 0.3	1.8 ± 0.3	1.4 ± 0.1	1.7 ± 0.2	2.1 ± 0.2	1.7 ± 0.3
FS (%)	49 ± 5	47 ± 2	49 ± 8	61 ± 3	56 ± 3*	49 ± 1	56 ± 4
AWT (mm)	0.70 ± 0.03	0.76 ± 0.09	0.81 ± 0.06	0.83 ± 0.06	0.84 ± 0.04	0.73 ± 0.04	0.92 ± 0.05*
PWT (mm)	0.71 ± 0.03	0.77 ± 0.08	0.80 ± 0.06	0.83 ± 0.06	0.85 ± 0.05	0.73 ± 0.05	0.91 ± 0.05*
RWT	0.34 ± 0.02	0.42 ± 0.11	0.44 ± 0.05	0.45 ± 0.03	0.45 ± 0.02	0.35 ± 0.03	0.49 ± 0.07*
V_{cf} (circ/s)	9.4 ± 1.0	9.5 ± 0.4	10.0 ± 1.9	11.4 ± 0.7	11.3 ± 1.2	9.2 ± 1.7	11.3 ± 1.3
MT-TG mice							
HR (beats/min)	556 ± 53†	548 ± 47	545 ± 47	578 ± 5	559 ± 28	539 ± 35	551 ± 32
LVEDD (mm)	3.9 ± 0.3	3.7 ± 0.6	3.7 ± 0.4	3.6 ± 0.2	3.6 ± 0.1	3.9 ± 0.2	3.8 ± 0.2
LVESD (mm)	1.8 ± 0.2†	1.8 ± 0.4	1.8 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.7 ± 0.2	1.7 ± 0.2
FS (%)	55 ± 4†	52 ± 5†	51 ± 5	58 ± 2	59 ± 2†	56 ± 3	56 ± 3
AWT (mm)	0.69 ± 0.03	0.79 ± 0.08	0.79 ± 0.06	0.79 ± 0.03	0.80 ± 0.05	0.89 ± 0.08†	0.82 ± 0.08
PWT (mm)	0.71 ± 0.02	0.79 ± 0.07	0.79 ± 0.06	0.79 ± 0.03	0.79 ± 0.06	0.88 ± 0.08	0.82 ± 0.08
RWT	0.36 ± 0.02	0.44 ± 0.10	0.43 ± 0.06	0.43 ± 0.04	0.44 ± 0.03	0.45 ± 0.06	0.43 ± 0.05
V_{cf} (circ/s)	11.7 ± 1.3†	10.3 ± 1.3	10.0 ± 1.3	11.4 ± 0.7	11.7 ± 0.7	11.4 ± 0.6	11.2 ± 0.3

*$p < 0.05$ versus age-matched control; †$p < 0.05$ versus age-matched and treatment-matched WT.

Ang II = angiotensin II; AWT = anterior wall thickness; FS = fractional shortening; HR = heart rate; LVEDD = left ventricular end-diastolic diameter; LVESD = left ventricular end-systolic diameter; MT-TG = cardiac-specific, metallothionein-overexpressing transgenic; PWT = posterior wall thickness; RWT = relative wall thickness; WT = wild-type.

peptide mRNA expression (Fig. 6C) and the ratio of β-MHC to α-MHC protein expression (Fig. 6D). Analogous to cardiac fibrosis, these hypertrophic responses were also abrogated in the Ang II–treated MT-TG mice.

Echocardiography (Table 1) revealed that MT-TG mice were mildly hypercontractile at baseline, as indicated by the increased fractional shortening and velocity of circumferential fiber shortening (V_{cf}), but were without significant hypertrophy compared with WT mice. One and 3 months after Ang II exposure, no significant differences in LV structure or function were observed in either the WT or the MT-TG Ang II–treated mice compared with the control mice. In contrast, at 6 months after exposure, Ang II–treated WT mice exhibited significantly increased wall thickness and a trend toward reduced chamber size, which is consistent with concentric hypertrophy. These changes were absent in Ang II-treated MT-TG mice. Representative M-mode echocardiograms at 6 months after Ang II exposure are shown in Online Figure 4, illustrating marked hypertrophy in Ang II–treated WT mice that is not observed in Ang II–treated MT-TG mice. Taken together, these results indicate that cardiac MT overexpression protects against late, load-independent, Ang II–mediated fibrosis and hypertrophy.

Metallothionein attenuates Ang II–induced pathologic effects in the diabetic heart. We next performed similar experiments in diabetic mice to define whether MT-mediated protection against Ang II–induced cardiac injury extends to diabetes. One month after STZ treatment, WT and MT-TG diabetic mice were administered Ang II at 0.5 mg/kg for 2 weeks and sacrificed 12 h after the 2-week Ang II exposure. Diabetes slightly increased serum Ang II and significantly increased cardiac Ang II in both WT and MT-TG mice compared with control mice (Figs. 7A and 7B). After 2 weeks of Ang II exposure, there was no further increase in serum Ang II levels, but there was additional augmentation of cardiac Ang II levels in diabetic WT and MT-TG mice. However, there was no significant difference for the augmentation of cardiac Ang II levels between the WT and MT-TG mice, suggesting that cardiac MT overexpression did not impact the tissue clearance of Ang II. Moreover, when comparing Ang II–induced effects in diabetic versus nondiabetic hearts, there was no additional augmentation of 3-NT accumulation (Fig. 7C), caspase-3 activation (Fig. 7D), inflammatory response (Fig. 7E), and CTGF up-regulation (Fig. 7F) in WT diabetic mice. In addition, cardioprotection against these detrimental pathophysiological responses was maintained in MT-TG hearts, even in the presence of diabetes (Figs. 7C to 7F), suggesting that diabetes does not diminish MT-mediated protection against Ang II–induced cardiac injury.

Discussion

We report for the first time that MT is cardioprotective against Ang II–induced cardiac cell death and nitrosative damage, which play a critical role in the development of cardiac remodeling under both diabetic and nondiabetic conditions. Although we previously reported that the prevention of peroxynitrite-mediated nitrosative damage and early cardiac cell death in MT-TG diabetic mice abrogated the development of cardiomyopathy (3,5,26), how apoptotic cell death led to the development of cardiomyopathy and whether cardiac cell death was directly related to nitrosative

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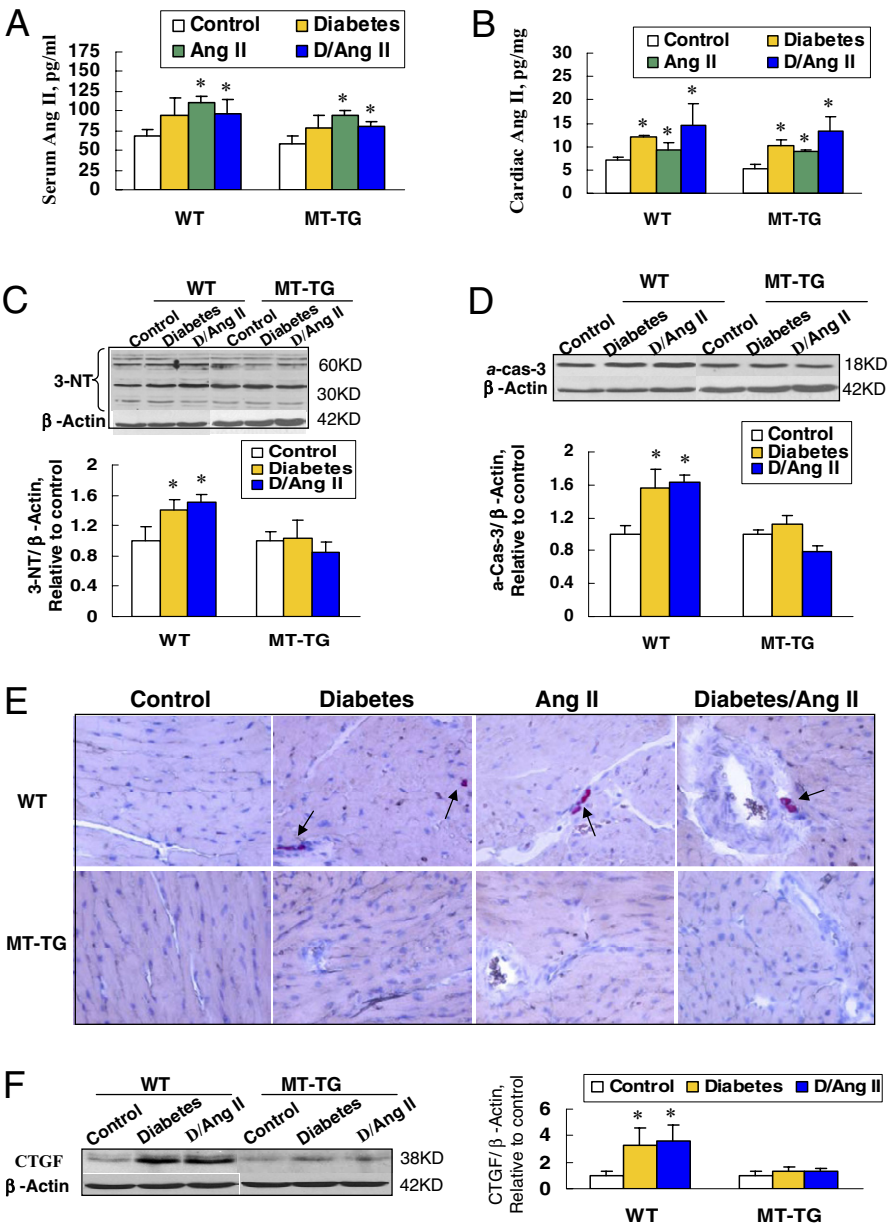

Figure 7 **MT Prevents Ang II–Induced Nitrosative Damage, Apoptosis, Granulocyte Infiltration, and Fibrotic Responses in Diabetic Hearts**

One month after streptozotocin treatment, diabetic mice were given Ang II at 0.5 mg/kg for 2 weeks. Mice ($n \geq 5$) were then sacrificed 12 h after the 2-week Ang II exposure to measure serum and cardiac Ang II levels by enzyme-linked immunosorbent assay **(A and B)**, cardiac 3-NT accumulation **(C)**, caspase-3 activation **(D)**, CTGF up-regulation **(F)**, and granulocyte infiltration by Naphthol AS-D chloracetate esterase staining **(E)**. *$p < 0.05$ versus corresponding control. Abbreviations as in Figures 1 and 6.

damage remained unclear. The finding that up-regulation of Ang II expression in the heart of diabetic patients and animals was accompanied with 3-NT accumulation and the loss of cardiac cells (14,15) suggests that Ang II might induce cardiac cell death via induction of oxidative and/or nitrosative damage in diabetic cardiomyopathy. The present study establishes that Ang II–induced cardiac apoptosis is directly mediated by NOX-activation dependent peroxynitrite formation and associated nitrosative damage, as indicated by the schematic outline in Figure 8.

Apoptotic cell death is a pivotal trigger for the development of Ang II–induced cardiomyopathy. It was reported that Ang II infusion for 2 and 6 weeks stimulated the cardiac expression of apoptosis signal-regulating kinase-1 and cardiac apoptosis along with cardiac hypertrophy and remodeling in the WT mice but not in the apoptosis

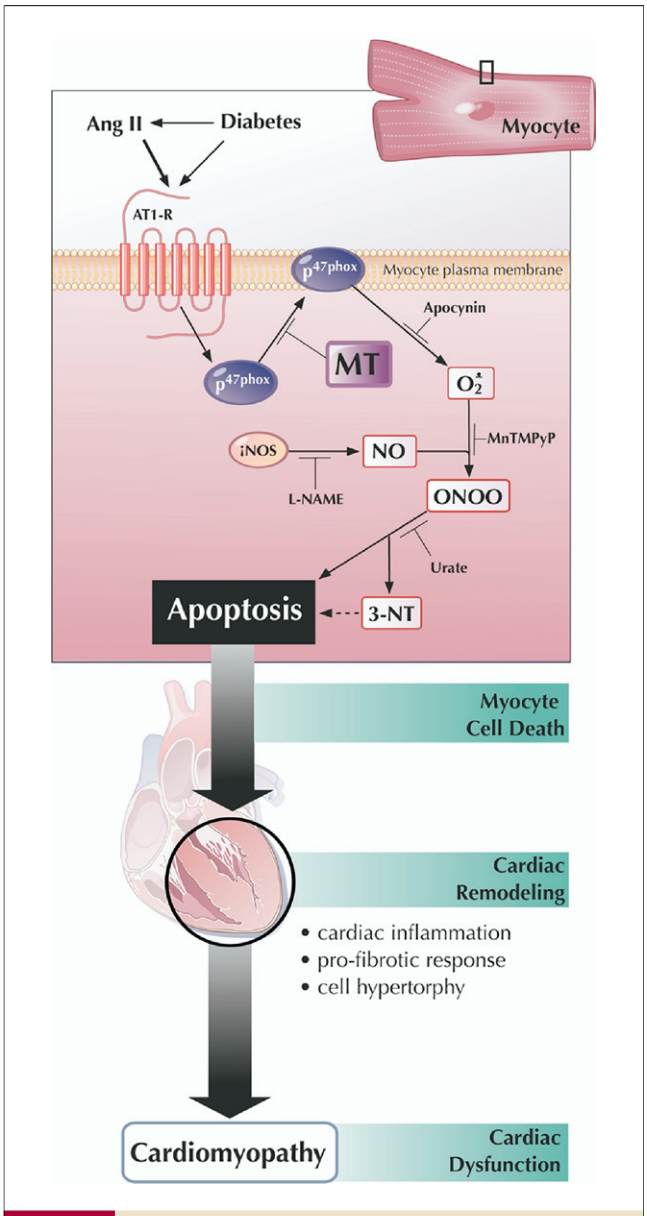

Figure 8 **Schematic Illustration for the Protection of MT Against Ang II Pathogenic Effects on the Heart**

iNOS = inducible nitric oxide synthase; NO = nitric oxide; ONOO = peroxynitrite; other abbreviations as in Figures 1 and 2. Figure illustration by Rob Flewell.

signal-regulating kinase-1–deficient mice (27). Mice deficient in poly (ADP-ribose) polymerase-1, a chromatin-bound enzyme activated by oxidative stress that mediates apoptosis (28), are protected from Ang II–induced cardiac hypertrophy (21). However, these 2 studies only observed cardiac remodeling within the Ang II infusion period of 2 weeks or 6 weeks. A novel finding of the present study is that transient induction of cardiac apoptosis and nitrosative damage induced by a limited, 2-week exposure to Ang II induced significant late remodeling with cardiac fibrosis and hypertrophy 6 months after exposure, further supporting the critical role of apoptosis in the development of cardiomyopathy (Fig. 8).

The renin-angiotensin aldosterone system plays a central role in the development of diabetic cardiomyopathy (11–13). Diabetes stimulates systemic and cardiac Ang II elaboration, which stimulates aldosterone release from the adrenal cortex and ET-1 release from vascular endothelial cells. Both aldosterone and ET-1 play a critical role in the pathogenesis of hypertension, endothelial dysfunction, and direct tissue damage (11,25). Pharmacological renin-angiotensin aldosterone system blockade with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers attenuated diabetes-related cardiac dysfunction without significant affecting blood pressure (11–13) and decreased glucose-induced NOX activation (8). Moreover, cardiac-specific overexpression of endogenous Ang II, a mouse model without hypertension, developed cardiac hypertrophy and dysfunction with aging (29).

The principal effects of Ang II and aldosterone in the heart include the excessive generation of ROS and/or RNS, which leads to oxidative and/or nitrosative stress, cell death of endothelial and fibroblast cells and myocytes (mainly as necrotic death in perivascular regions), and stimulation of genes that promote vasoconstriction (such as ET-1), inflammation (TNF-α, intercellular adhesion molecule-1, and PAI-1), endothelial dysfunction, cell hypertrophy, excess extracellular matrix deposition, and fibrosis (PAI-1, transforming growth factor-β, and connective tissue growth factor) (23–25). Although we identified an important role for NOX-dependent ROS/RNS generation in Ang II–mediated injury in vitro (Fig. 2), we cannot exclude the contribution of intermediary pathways linking Ang II and NOX activation in vivo. Potential intermediaries can include ET-1, aldosterone, and alterations in intracellular calcium in response to Ang II (13,24,30).

As shown in Figure 8, cardiac dysfunction may be predominantly attributed to the cardiac fibrosis resulting in abnormal cardiac stiffness, impairment of cell–cell communication, and cardiac arrhythmia (12,31). Several studies have demonstrated that Ang II–induced microinfarction (predominantly necrotic death of myocytes) triggers cardiac inflammatory and fibrotic responses and cardiac remodeling (23–25). In the present study, we demonstrated predominantly apoptotic cell death (Fig. 4) rather than necrosis (Fig. 3B). This does not exclude the presence of microscopic necrotic injury which escaped detection by the relatively insensitive hematoxylin and eosin staining. Indeed, the up-regulation of the inflammatory cytokines TNF-α (Fig. 3C) and PAI-1 (Figs. 5B and 5C) suggested a predilection for microinfarction and necrotic injury (23). Therefore, we assumed that MT prevents Ang II–induced oxidative and/or nitrosative damage that leads to cardiac cell death, inflammatory and fibrotic responses, and cardiac remodeling and dysfunction (Fig. 8).

We also provide evidence that MT suppresses Ang II–induced NOX 47^{phox} activation and attendant oxidative

and nitrosative damage in nondiabetic hearts. Moreover, cardiac MT expression does not influence the augmentation of systemic and cardiac Ang II levels (Fig. 7A) and the Ang II receptor expression that occurs in diabetes (Online Fig. 1), suggesting that MT-induced cardioprotective signaling occurred distal to the Ang II receptors. Indeed, subpressor doses of Ang II augmented serum and cardiac Ang II levels comparable to those seen in diabetes alone (Fig. 7A). Furthermore, although both diabetes and subpressor doses of Ang II induced similar cardiac pathology, including nitrosative damage, apoptosis, inflammation, and fibrotic responses, exogenous Ang II administration did not result in additive damage to that seen in diabetic hearts (Fig. 7). The lack of additive effects suggests that cardiac injury in diabetes shares common mechanisms with Ang II (8), which are counteracted by MT (Fig. 8).

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Key Words: metallothionein ■ diabetic cardiomyopathy ■ angiotensin II ■ cardiomyocyte apoptosis ■ NADPH oxidase ■ nitrosative damage.

APPENDIX

For an online Materials and Methods section and supplementary figures, please see the online version of this article.