

Diabetes- and angiotensin II-induced cardiac endoplasmic reticulum stress and cell death: metallothionein protection

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Received: March 30, 2009; Accepted: June 9, 2009

Abstract

We have shown cardiac protection by metallothionein (MT) in the development of diabetic cardiomyopathy (DCM) *via* suppression of cardiac cell death in cardiac-specific MT-overexpressing transgenic (MT-TG) mice. The present study was undertaken to define whether diabetes can induce cardiac endoplasmic reticulum (ER) stress and whether MT can prevent cardiac cell death *via* attenuating ER stress. Diabetes was induced by streptozotocin in both MT-TG and wild-type (WT) mice. Two weeks, and 2 and 5 months after diabetes onset, cardiac ER stress was detected by expression of ER chaperones, and apoptosis was detected by CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) and cleaved caspase-3 and caspase-12. Cardiac apoptosis in the WT diabetic mice, but not in MT-TG diabetic mice, was significantly increased 2 weeks after diabetes onset. In parallel with apoptotic effect, significant up-regulation of the ER chaperones, including glucose-regulated protein (GRP)78 and GRP94, cleaved ATF6 and phosphorylated eIF2 α , in the hearts of WT, but not MT-TG diabetic mice. Infusion of angiotensin II (Ang II) also significantly induced ER stress and apoptosis in the hearts of WT, but not in MT-TG mice. Direct administration of chemical ER stress activator tunicamycin significantly increased cardiac cell death only in WT mice. Pre-treatment with antioxidants completely prevented Ang II-induced ER stress and apoptosis in the cultured cardiac cells. These results suggest that ER stress exists in the diabetic heart, which may cause the cardiac cell death. MT prevents both diabetes- and Ang II-induced cardiac ER stress and associated cell death most likely *via* its antioxidant action, which may be responsible for MT's prevention of DCM.

Keywords: diabetic cardiomyopathy • metallothionein • ER stress • apoptosis • ATF6 • caspase-3

Introduction

The endoplasmic reticulum (ER) is recognized as an organelle that participates in the folding of secretory and membrane proteins [1, 2]. A variety of conditions, including infection, nutrient deprivation (such as the absence of glucose) as well as nutrient excess (such as the overabundance of lipids or glucose), alterations in ER lumenal Ca²⁺ or redox status, and toxic chemicals, can disrupt protein folding reactions in the ER. These conditions are collectively referred to as ER stress [1–3]. ER stress leads to accumulation of unfolded proteins in the ER, which in turn evokes the unfolded protein response (UPR). This response attempts to reduce the amount of unfolded proteins by inducing production of the ER chaperones, such as 78 kD and 94 kD glucose-regulated

protein (GRP78 and GRP94) that promote protein folding, and by reducing general protein synthesis and enhancing the degradation of misfolded proteins *via* a ubiquitin–proteasome system termed ER-associated degradation [1–4]. The persistent accumulation of these misfolded proteins beyond the capacity of ER function causes cellular dysfunction and cell death in several human disorders [1–4]. Emerging evidence indicates that a variety of diseases, such as diabetes mellitus, is related to ER stress [5–7]. Furthermore, there were reportedly tissue differences for ER stress induction by insulin resistance [8].

Several studies indicated the involvement of ER stress in heart diseases [5, 9–11], but whether ER stress is also involved in diabetic cardiomyopathy (DCM) remains unclear. DCM, a leading cause of death for diabetic patients, is caused by multiple pathogenic factors including hyperglycaemia, hyperlipidaemia, hypertension and systemic and cardiac inflammation [12–14]. However, a pivotal contributor to the pathogenic changes of DCM was considered as the oxidative stress [12, 14]. Metallothionein (MT) as a potent antioxidant was found to protect the heart from type 1 and type 2

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diabetes [15–18]. In terms of the cellular mechanisms involved in the prevention of MT against DCM, we found that MT protected the heart from diabetes mainly through inhibition of diabetic cardiac cell death [17, 19]. However, it is unclear how diabetes can induce cardiac cell death.

Studies have demonstrated the involvement of oxidative stress in ER stress [6, 20, 21]. As a potent antioxidant MT is up-regulated in response to ER stress [22], suggesting that MT may act as an adaptive mechanism to protect cells from ER stress. Therefore, we suggested that ER stress may occur in the diabetic hearts and contribute to cardiac cell death that initiates DCM as observed in our previous studies [17, 19, 23]. The aim of this study was to investigate whether ER stress involves in the development of DCM with streptozotocin (STZ)-induced diabetic models in cardiac-specific MT-overexpressing transgenic (MT-TG) and their wild-type (WT) mice. Because the renin-angiotensin system plays an important role in the development of DCM [23], we further examined whether angiotensin II (Ang II) induces cardiac ER stress along with cell death and whether MT protects the heart from Ang II-induced ER stress and cell death. We also have demonstrated a direct protection by MT against ER stress-induced apoptotic cell death and shown that MT protection against ER stress-mediated cardiac cell death caused by diabetes and Ang II is most likely mediated by its antioxidant property.

Materials and methods

Animal models

MT-TG were produced from WT (Friend virus B, FVB) mice originally obtained from Harlan Bioproducts for Science (Indianapolis, IN, USA) and characterized in previous studies [16, 17]. Both MT-TG and WT mice were housed in the University of Louisville Research Resources Center at 22°C with a 12-hr light/dark cycle and were provided free access to standard rodent chow and tap water. 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 used for the following studies.

The first study was to observe whether there was ER stress in the diabetic hearts. STZ (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in sodium citrate buffer (pH 4.5), and given intraperitoneally to both WT and MT-TG mice at 40 mg/kg body weight daily for 5 days, *i.e.* multiple low-dose STZ (MLD-STZ) model. Five days after last injection of STZ, whole blood glucose obtained from mouse tail-vein was detected using a SureStep complete blood glucose monitor (LifeScan, Milpitas, CA, USA). STZ-treated mice with whole blood glucose higher than 12 mmol/l were considered diabetic [16, 17]. Animals were killed at 2 weeks, and 2 and 5 months after the onset of diabetes.

The second study was to determine whether Ang II was also able to induce cardiac ER stress, and whether MT protects Ang II-induced cardiac ER stress and associated apoptotic cell death. Ang II (Sigma Chemical Co.) was freshly prepared in a 154 mmol NaCl vehicle immediately prior to be used. MT-TG and WT mice received a single subcutaneous injection of Ang II

at 1 mg/kg body weight. Control animals were given an equal volume of vehicle. Both control and Ang II-treated animals were killed at 7 and 24 hrs later. The selection of the Ang II dose and animal killing time was based on previous studies showing that administration of 1 mg Ang II/kg body weight to rats for 7 hrs induced a high incidence of apoptosis in the heart [23, 24].

The third study was to define the direct link of ER stress to apoptotic cell death in the heart and its prevention by MT. Both MT-TG and WT mice were intraperitoneally given a single injection of chemical ER stress activator, tunicamycin (Sigma Chemical Co.) at 1.5 mg/kg body weight. The tunicamycin was freshly prepared as a 0.05 mg/ml suspension in 150 mM dextrose. The dose selection and preparation of tunicamycin was based on a published study [25]. Twelve hours after tunicamycin treatment, animals were killed.

Cell culture

Embryonic rat heart derived cells (H9c2), purchased from (ATCC CLR-1446; Rockville, MD, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum from Atlanta Biologicals (Norcross, GA, USA) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) at 37°C in an atmosphere of 95% air and 5% CO₂.

H9c2 cells were stably transfected with a vector in which the human MT-IIA gene was placed under the control of the constitutively active β-actin promoter. Transfected cells were cultured in DMEM with 10% foetal bovine serum and G418. G418-resistant colonies were selected 10–14 days after transfection and propagated. The expression of human MT-IIA mRNA and protein was confirmed by RT-PCR and Western blotting. Among all MT-IIA expressing clones, the clone H9c2MT7 exhibited relative high MT level and maximal preservation of the WT morphology. Correspondingly, H9c2MT7 cells showed a marked reduction in reactive oxygen species production when exposed to hydrogen peroxide or to hypoxia/reoxygenation evaluated by dihydroethidium staining. In addition, this cell line was also resistant to cadmium toxicity [26]. Both H9c2 and H9c2MT7 cells were treated with Ang II at 100 nM for 24 hrs. In some experiments, H9c2 cells were pre-treated with MnTMPyP, a cell-permeable superoxide dismutase mimic, at 50 µM for 30 min. [16] or an antioxidant N-acetyl-cysteine (NAC) at 100 µM for 24 hrs [27, 28] and then exposed to Ang II at 100 nM in the presence of either MnTMPyP or NAC for 24 hrs. MnTMPyP can directly act on superoxide while NAC needs to be converted into glutathione (GSH) to act on hydrogen peroxide; therefore, their pre-treatment times are different [16, 27, 28].

Western blotting assay

As ER stress chaperons, cleaved activating transcription factor 6 (ATF6), GRP78, GRP94 and phosphorylation of eukaryotic initiation factor 2α (p-eIF2α) were examined by Western blotting assay. As one of the components of the ER stress-mediated apoptosis pathway is CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), and cleaved caspase-12 as well as cleaved caspase-3 were also examined by Western blotting assay, based on published methods [9–11, 17, 23]. Briefly, cardiac tissue homogenates were lysed and fractionated electrophoretically on sodium dodecyl (lauryl) sulphate polyacrylamide gel electrophoresis (10–15% gradient gels), and proteins were transferred to a nitrocellulose membrane. The membrane was blocked with a

5% non-fat, dried milk for 1 hr. The following antibodies were used: anti-ATF6 (1:2000, Abcam, Cambridge, MA, USA), anti-GRP78 (1:4000, Abcam), anti-GRP94 (1:1000, Abcam), anti-p-eIF2 α and anti-CHOP (1:200 and 1:500, respectively, Santa Cruz, Santa Cruz, CA, USA), anti-cleaved caspase 3 (1:2000, Calbiochem, La Jolla, CA, USA) and anti-cleaved caspase-12 (1:1000, Exalpha Biologicals, Shirley, MA, USA). Membranes were incubated with the primary antibodies overnight at 4°C. After the unbound antibodies were removed with Tris-buffered saline (pH 7.2) containing 0.05% Tween 20, membranes were incubated with the secondary antibody for 1 hr at room temperature. Antigen-antibody complexes were visualized with enhanced chemiluminescence (ECL) system.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay

Heart tissue was fixed in 10% formalin, embedded in paraffin and sectioned at 3–4 μ m. The slides were stained for TUNEL with the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, Temecula, CA, USA). Each slide was deparaffinized and rehydrated, and treated with proteinase K (20 mg/l) for 15 min. The endogenous peroxidase was inhibited with 3% hydrogen peroxide for 5 min. and then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP for 1 hr at 37°C. The TdT reaction was carried out in a humidified chamber at 37°C for 1 hr, and then 3, 3'-diaminobenzidine chromogen was applied. Methyl green was used as counterstaining. Mouse testicular tissue was used as positive control. For negative control, TdT was omitted from the reaction mixture. The apoptotic cell death was quantitatively analysed by counting the TUNEL⁺ cells randomly selected from five fields from each of the three slides for each mouse [17, 19].

MT measurement

MT expression was detected by a modified Western blotting protocol [29]. Briefly, cardiac proteins were treated with DTT at a final concentration of 20 mM at 56°C for 30 min., and then added with iodoacetamide (Sigma Chemical Co.) at 50 mM in room temperature for 1 hr in the dark. The supernatant was collected by centrifuging at 800 \times *g* for 5 min., and mixed with loading buffer and heated at 95°C for 5 min. and then subjected to electrophoresis on 18% SDS-PAGE gel at 120 V. Gel was incubated in transfer buffer for 10–20 min. The electrophoresed proteins were transferred to nitrocellulose membrane at 40 V for 2.5 hrs at 4°C in the above transfer buffer modified by addition of 2 mM CaCl₂. After transfer, the membrane was incubated in 2.5% glutaraldehyde (Fisher Scientific, Pittsburgh, PA, USA) at room temperature for 1 hr in the dark. The membrane was then blocked in blocking buffer [3%, bovine serum albumin (BSA)] at room temperature for at least 1 hr and incubated with anti-MT monoclonal antibody (Dako North America, Carpinteria, CA, USA) diluted 1:1000 in 3% BSA at 4°C for overnight. The membrane was then washed and reacted with secondary horseradish peroxidase-conjugated antibody at room temperature for 1 hr. Antigen-antibody complexes were visualized with ECL system. However, since in the transfer conditions CaCl₂ was utilized for MT protein analysis, these blots could not be stripped and re probed for GAPDH. Thus, two parallel gels were run under the same conditions. One gel (MT gel) was transferred to a membrane in transfer buffer containing CaCl₂, while another (GAPDH gel) was transferred to membrane in buffer without CaCl₂, as described in previous studies [29, 30].

Statistical analysis

Data were collected from repeated experiments and were presented as mean \pm S.D. One-way ANOVA and Student's t-test was used for statistical analysis. Origin 7.5 (OriginLab data analysis and graphing software) was used for all statistical tests. Statistical significance was considered as $P < 0.05$.

Results

Diabetes-induced cardiac ER stress and prevention by MT

Both MT-TG and WT mice were made diabetic by MLD-STZ, and showed similar patterns of persistent increase in blood glucose levels (Data not shown). Our previous studies showed cardiac cell death in the heart of WT diabetic mice, but not MT-TG diabetic mice, at 2 weeks after the onset of diabetes [17], and also significantly pathological and functional changes in WT diabetic, but not MT-TG diabetic mice, at 2 and 5 months [16, 17, 30]. Therefore, we examined whether these pathological changes are related to cardiac ER stress by analysing the expression of ER chaperones from diabetic and age-matched control mice at 2 weeks, and 2 and 5 months after the onset of diabetes (Fig. 1). Western blotting assay showed that both phosphorylated eIF2 α (p-eIF2 α) and GRP94 proteins were significantly increased in the heart of WT diabetic mice at 2 weeks, but not 2 and 5 months, after diabetes onset (Fig. 1A, B). Cleaved ATF6 protein was significantly increased both at 2 weeks and 2 months, but not at 5 months (Fig. 1C). In contrast to the above changes, GRP78 protein level was increased in the heart of diabetic mice at all three time-points with the highest expression at 2 weeks after diabetes (Fig. 1D). More importantly, none of these increased ER chaperones in the hearts of WT diabetic mice were observed in the heart of MT-TG diabetic mice (Fig. 1).

Diabetes-induced cardiac MT up-regulation

Kondoh *et al.* has shown the induction of hepatic MT in response to ER stress [22]. We next examined whether diabetic ER stress also induced MT synthesis in the heart. Western blotting assay revealed that diabetes induced a significant increase in MT contents of heart from 2 weeks to 5 months with a rapid increase at 2 weeks and then gradually decreased until 5 months after diabetes, but it remained significantly higher than control levels (Fig. 2). Interestingly, diabetes-induced cardiac MT synthesis in the heart of WT mice was not observed in the heart of MT-TG diabetic mice, suggesting that pre-existed MT prevents diabetes-induced stress and MT synthesis. The prevention of diabetes-induced MT synthesis in MT-TG heart implies that ER stress is responsible for MT

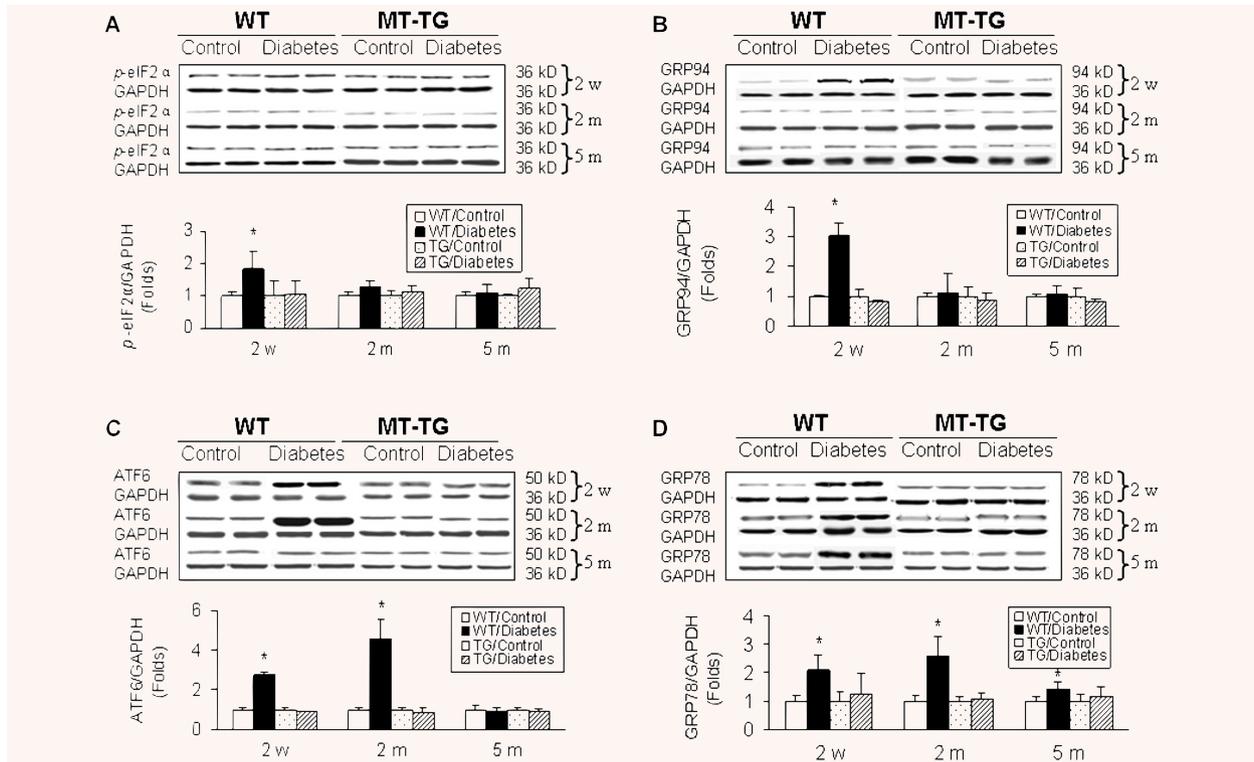


Fig. 1 Diabetes-induced cardiac up-regulation of ER chaperones. Both MT-TG and WT mice were made diabetic by MLD-STZ, and cardiac ER stress was examined by Western blotting analysing the expression of ER chaperones p -eIF2 α (A), GRP94 protein (B), cleaved ATF6 (C) and GRP78 (D) from the control and diabetic mice at indicated time-points. Experimental data were presented as means \pm S.D. ($n = 5$ at least for each group). *, $P < 0.05$ versus control.

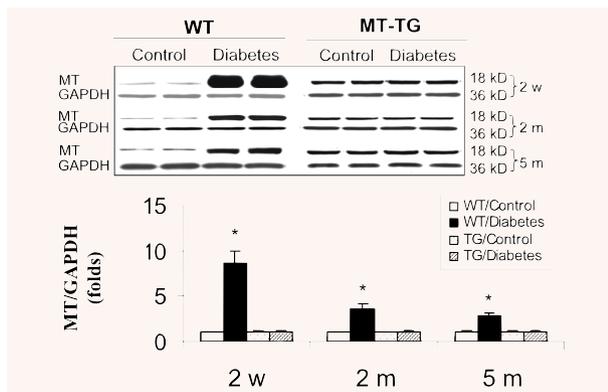


Fig. 2 Diabetes-induced cardiac up-regulation of MT protein. Animal treatment and tissue collections are same as those described in Fig. 1. Cardiac MT contents were examined by Western blotting assay. It should be noted that in order to see whether there was MT induction in the hearts of MT-TG diabetic mice, the exposure time for the films to gels was significantly shorter for samples from MT-TG mice than those from WT mice; Therefore, MT expressions in MT-TG samples looks significantly low than diabetes-induced cardiac MT levels. Experimental data were presented as means \pm S.D. ($n = 5$ at least for each group). *, $P < 0.05$ versus control.

synthesis and there is no diabetes-induced cardiac ER stress in the heart of MT-TG diabetic mice.

Diabetes-induced cardiac cell death and prevention by MT

Although we have reported the induction of cardiac cell death in the WT diabetic mice, it was only examined for the short time period (within 1 month) [17, 19]. Therefore, we extended the examination of cardiac cell death from 2 weeks to 2 and 5 months after diabetes onset with TUNEL assay for apoptotic cells (Fig. 3A) and Western blotting assay for the activated form of caspase-3 (Fig. 3B). Both assays delineated that diabetes mainly induced cardiac cell death at the early stage of diabetes (2 weeks), but not in the late stages (2 and 5 months), which is consistent with previous studies [17, 19]. However, there was no apoptotic cell death in the hearts of MT-TG diabetic mice (Fig. 3). To determine whether the cardiac cell death is ER stress-mediated, CHOP protein expression (Fig. 3C) and caspase-12 cleavage (Fig. 3D) were examined by Western blotting assay, which showed that significant increases in CHOP expression and cleaved caspase-12 were observed only in the heart of

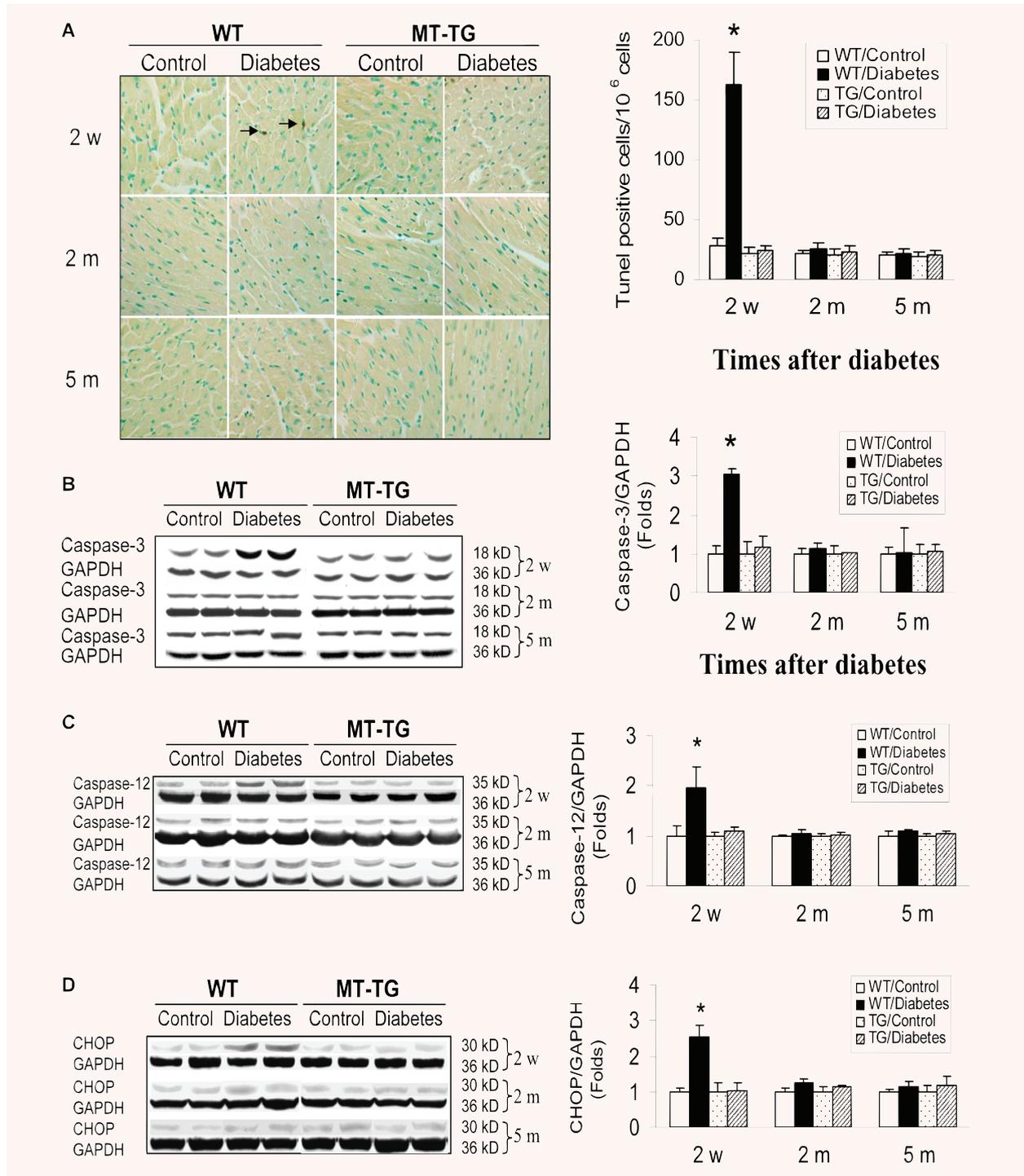


Fig. 3 Diabetes-induced cardiac cell death. Animal treatments and tissue collections are same as those described in Fig. 1. Cardiac apoptotic cell death was examined by TUNEL staining for apoptotic cells (A) and also by Western blotting analysis for cleaved caspase-3 (B) and caspase-12 (C) and CHOP expression (D). Experimental data were presented as means \pm S.D. ($n = 5$ at least for each group). *, $P < 0.05$ versus control.

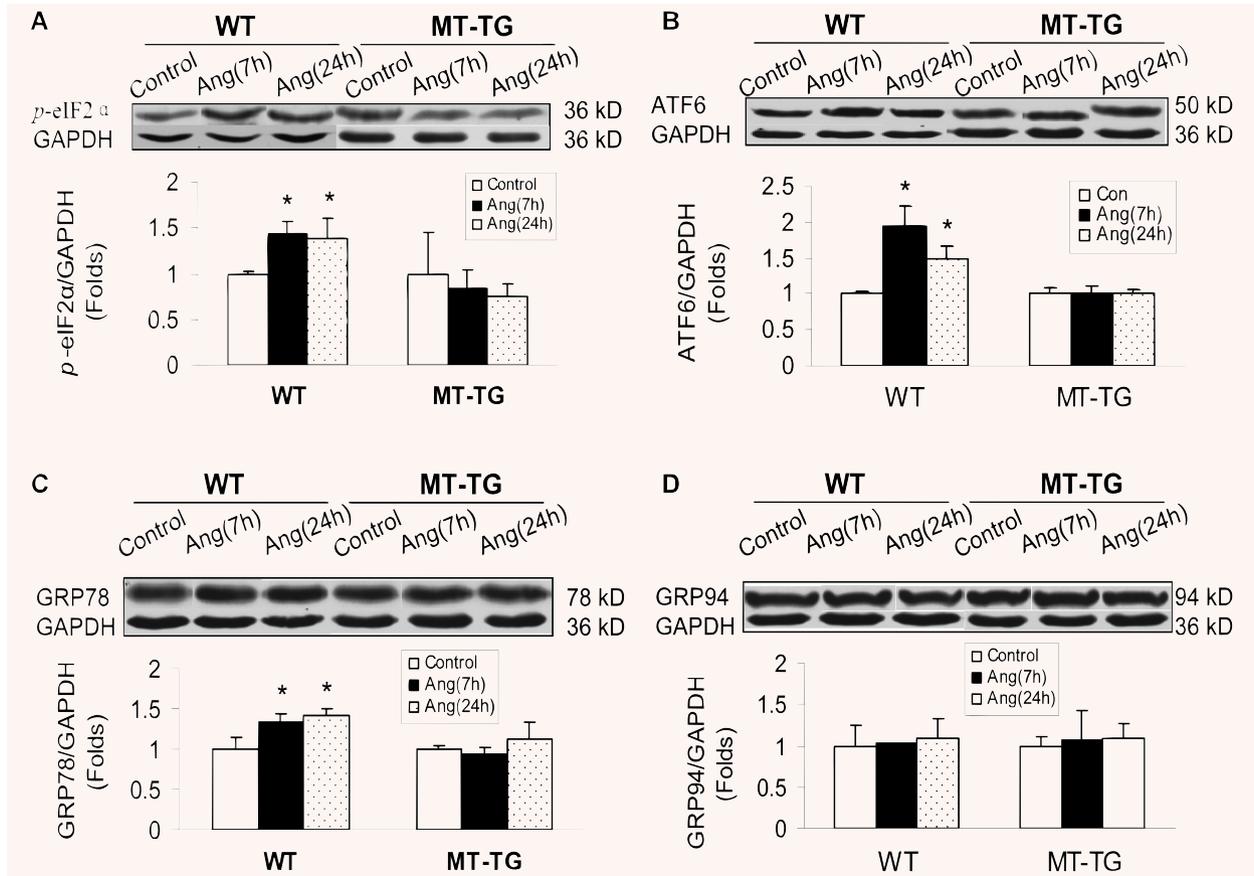


Fig. 4 Ang II-induced cardiac up-regulation of ER chaperones. Both MT-TG and WT mice were administered Ang II at 1 mg/kg and 7 and 24 hrs later cardiac ER stress were examined by Western blotting analysing the expression of ER chaperones *p*-eIF2 α (A), cleaved ATF6 (B), GRP78 (C) and GRP94 protein (D). Experimental data were presented as means \pm S.D. ($n = 5$ at least for each group). *, $P < 0.05$ versus control.

WT diabetic mice at 2 weeks, but not at 2 and 5 months after diabetes onset, suggesting the association of diabetes-induced cardiac cell death with ER stress. The ER-stress-mediated cardiac cell death was not seen in the heart of MT-TG diabetic mice (Fig. 3C, D).

MT protection against Ang II-induced cardiac ER stress, MT synthesis and cell death

To examine whether Ang II as an important pathogenic factor for DCM is also able to induce ER stress, MT induction and cardiac cell death, both MT-TG and WT mice were intraperitoneally given Ang II at 1 mg/kg. Seven and 24 hrs after Ang II infusion, cardiac ER chaperone proteins were examined (Fig. 4). Treatment of WT mice with Ang II significantly increased the expression of *p*-eIF2 α (Fig. 4A), cleaved ATF6 (Fig. 4C) and GRP78 (Fig. 4D), but not GRP94 (Fig. 4B). The up-regulated ER

chaperone proteins by Ang II were not observed in the heart of MT-TG mice (Fig. 4). Correspondingly, Ang II also significantly induced cardiac MT synthesis and cardiac cell death only in the WT mice (Fig. 5A, B), as observed in the hearts of diabetic mice (Figs. 2, 3). The association of Ang II-induced cardiac cell death with ER stress was also favoured by significant increases in CHOP protein expression and caspase-12 activation (Fig. 5C, D).

MT protection against chemical ER stressor-induced cardiac ER stress, MT synthesis and cell death

To define the direct protection by MT from ER stress-mediated apoptotic cell death, both WT and MT-TG mice were administered with tunicamycin (Tu) at 1.5 mg/kg, and 12 hrs later, cardiac ER chaperone proteins were examined. Western blotting

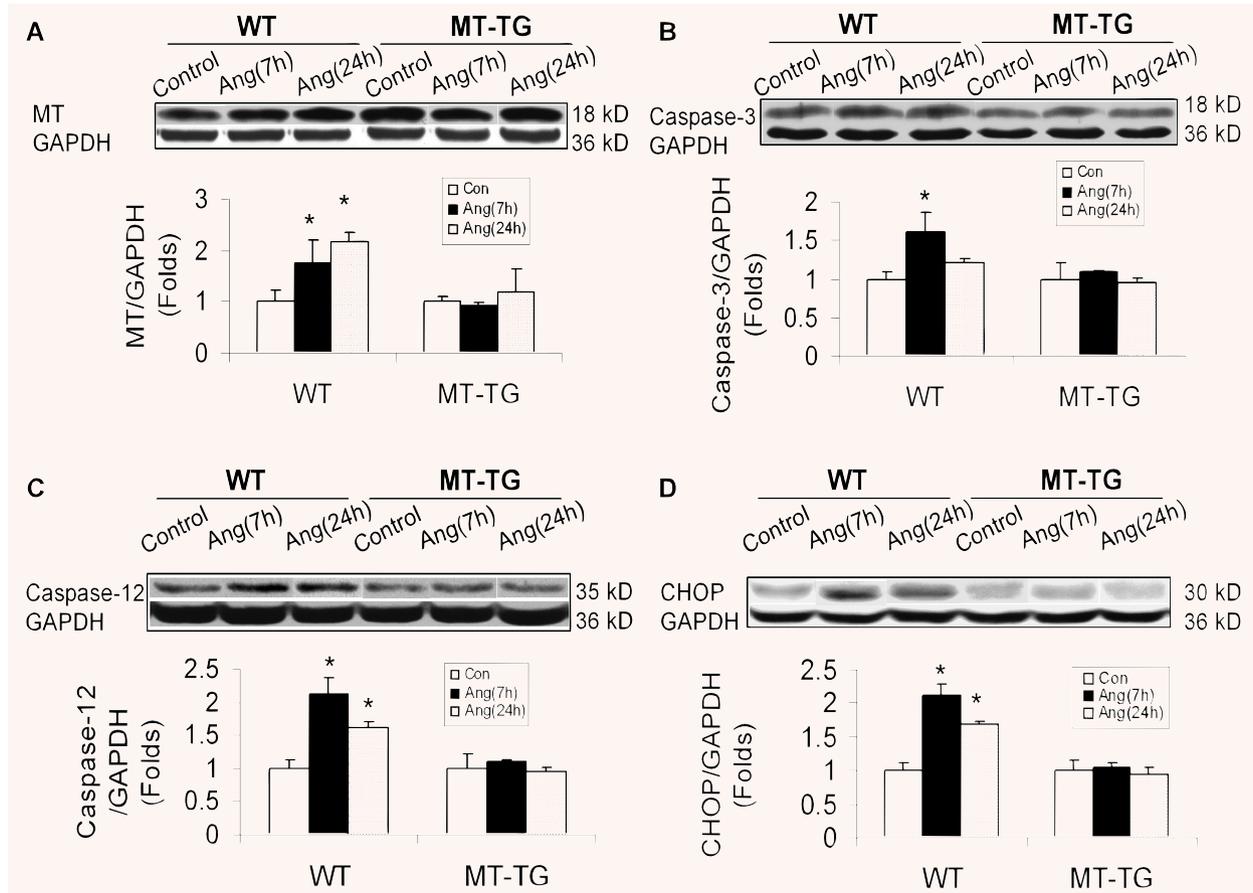


Fig. 5 Ang II-induced cardiac up-regulation of MT protein and cell death. Animal treatments and tissue collections are same as those described in Fig. 4. Cardiac MT contents were examined by Western blotting assay (A). Cardiac apoptotic cell death was examined by Western blotting analysis for the cleaved caspase-3 (B) and caspase-12 (C) and CHOP expression (D). Again in order to see whether there was MT induction in the hearts of MT-TG diabetic mice, the exposure time for the films to gels was significantly shorter for samples from MT-TG mice than those from WT mice; Therefore, MT expressions in MT-TG samples looks significantly low than diabetes-induced cardiac MT levels. Experimental data were presented as means \pm S.D. ($n = 5$ at least for each group). *, $P < 0.05$ versus control.

assay showed that proteins of p -eIF2 α (Fig. 6A), cleaved ATF6 (Fig. 6B) and GRP78 (Fig. 6C) all were significantly up-regulated, except for GRP94 protein (Fig. 6D). Similar to the above experiments in diabetic and Ang II-infusion models, MT significantly prevented chemical ER stressor-up-regulated ER chaperone proteins (Fig. 6).

It is noted that chemical ER stressor also significantly induced cardiac MT synthesis (Fig. 7A), as shown in the hearts of other ER-stress models (Figs 2 and 5A). Cardiac cell death was significantly increased in the heart of Tu-treated WT mice, but not Tu-treated MT-TG mice, examined with Western blotting for caspase-3 activation (Fig. 7B) and TUNEL assay (Fig. 7C), and confirmed by CHOP protein expression (Fig. 7D) and caspase-12 cleavage (Fig. 7E).

Ang II-induced ER stress and associated cell death was attenuated by MT and antioxidants in cultured cardiac cells

In order to understand the molecular mechanisms underlying the protective role of MT in the heart against Ang II-induced ER stress-mediated apoptotic cell death, we used stable MT-IIA over-expressing H9c2MT7 cells that exhibited similar growth kinetics and morphology, but had a remarkable increased MT protein level as compared to the parent H9c2 cells [26]. Treatment with Ang II at 100 nM for 24 hrs significantly induced ER stress, shown by increased expressions of cleaved ATF6 (Fig. 8A), p -eIF2 α (Fig. 8B), GRP78 (Fig. 8C) and GRP94 (Fig. 8D). All these ER-stress

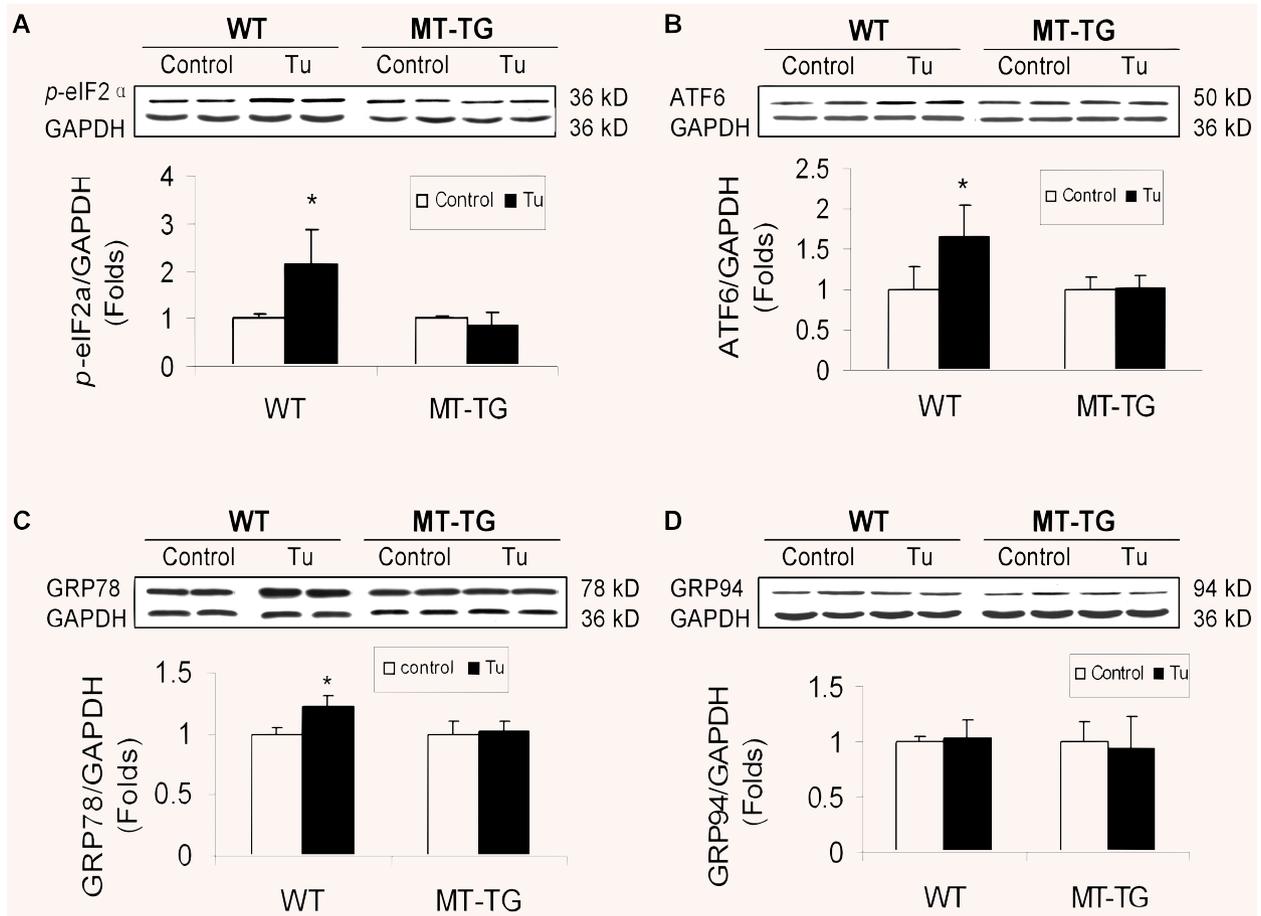


Fig. 6 Tunicamycin-induced cardiac up-regulation of ER chaperones. Both WT and MT-TG mice were directly administrated with tunicamycin at 1.5 mg/kg, and 12 hrs later cardiac ER stress were examined by Western blotting analysing the expression of ER chaperones *p*-eIF2 α (A), cleaved ATF6 (B), GRP78 (C) and GRP94 protein (D). Experimental data were presented as means \pm S.D. ($n = 6$ at least for each group). Tu: tunicamycin. *, $P < 0.05$ versus control.

effects were not observed in H9c2MT7 cells, suggesting the protection of cardiac cells by MT from Ang II-induced ER stress. Treatment with Ang II at 100 nM for 24 hrs also significantly induced apoptotic cell death in H9c2 cells, measured by caspase-12 activation (Fig. 9A), and CHOP protein expression (Fig. 9B), but not in H9c2MT7 cells. This *in vitro* finding is consistent with those of *in vivo* findings (Fig. 5C, D).

Considering that Ang II apoptotic effect was related to oxidative stress since exposure of H9c2 cells to Ang II directly induced peroxynitrite formation due to over-generation of superoxide [23], whether MT protection against Ang II-induced ER stress and associated apoptosis was also attributed to its antioxidant action was evaluated. Pre-exposure of H9c2 cells to MnTMPyP at 50 μ M for 30 min. or NAC at 50 μ M for 24 hrs significantly prevented Ang II-induced ER stress and associated apoptosis (Fig. 9A, B), while same pretreatments did not have any effect on normal H9c2 cells

and H9c2MT7 cells (Fig. 9A, B). These results suggest that both ER stress and apoptotic effects caused by Ang II are mediated by oxidative stress, and also that MT protection against Ang II-induced ER stress and associated apoptotic effects is most likely mediated by its antioxidant action.

Discussion

The present study provided the following innovative findings: (1) Diabetes induces cardiac ER stress along with induction of cardiac cell death; (2) As one of the major pathogenic factors for DCM, Ang II also induces cardiac ER stress and cell death; (3) Cardiac overexpression of MT rescues diabetes-, Ang II- and even chemical

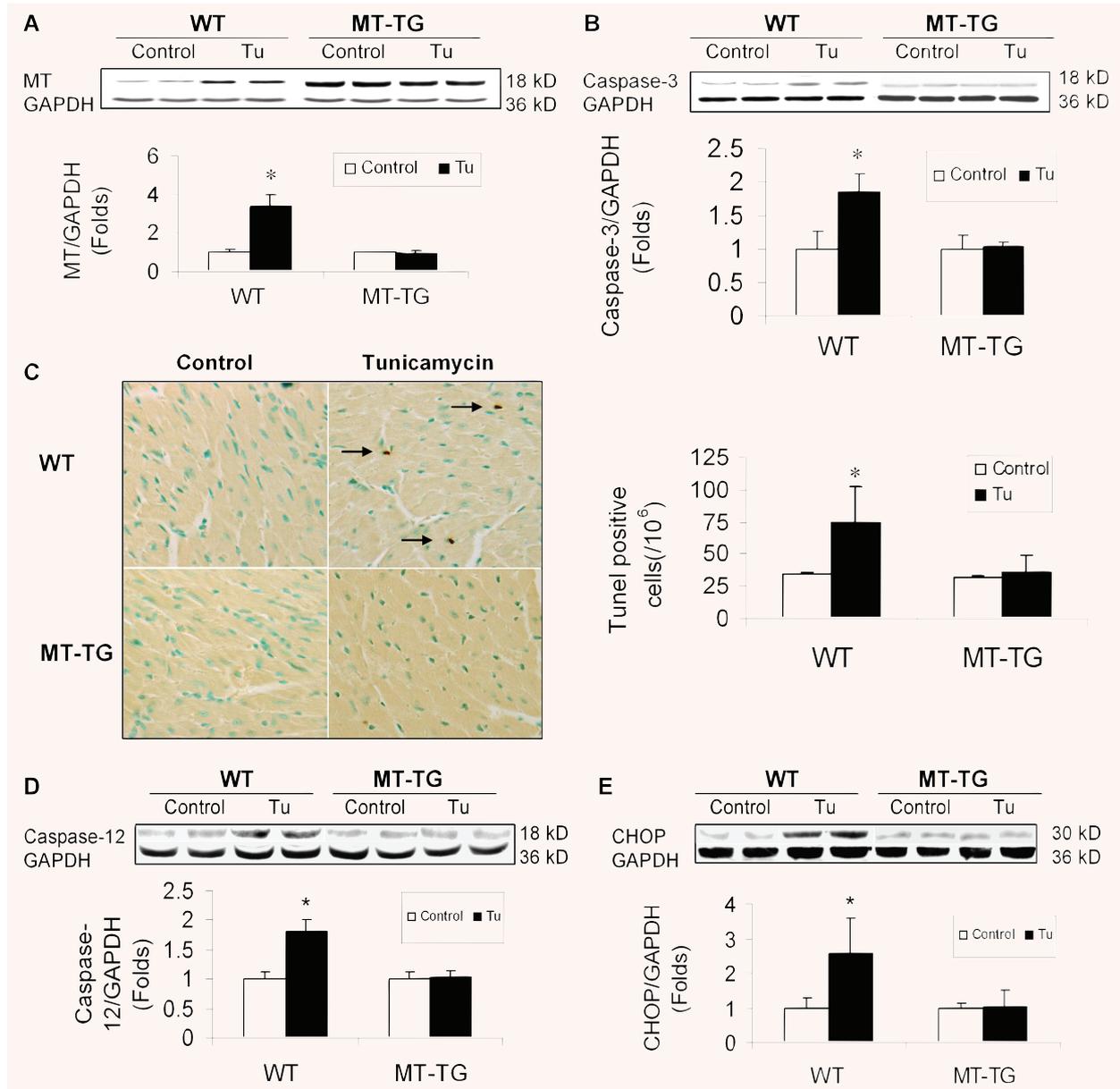


Fig. 7 Tunicamycin-induced cardiac up-regulation of MT protein and cell death. Animal treatments and tissue collections are same as those described in Fig. 6. Cardiac MT contents were examined by Western blotting assay (A). Cardiac apoptotic cell death was examined for the cleaved caspase-3 by Western blotting analysis (B) and apoptotic cells with TUNEL staining (C). Cleaved caspase-12 (D) and CHOP protein expression (E) were also examined by Western blotting assay. Experimental data were presented as means \pm S.D. ($n = 6$ at least for each group). Tu: tunicamycin. *, $P < 0.05$ versus control.

ER stressor-induced cardiac cell death *via* suppression of cardiac ER stress; (4) Oxidative stress plays a pivotal role in Ang II-induced both ER stress and associated apoptotic effects and (5) MT protection against Ang II-induced ER stress and associated apoptotic effects are most likely mediated by its antioxidant action.

DCM is a chronic and complex pathogenesis, caused by abnormal cellular metabolism and defects in organelles such as myofibrils, mitochondria and sarcolemma [12–14]. Apoptotic cell death as an early cellular event in response to diabetes has been reported to play a critical role in the development of DCM [13, 17, 19].

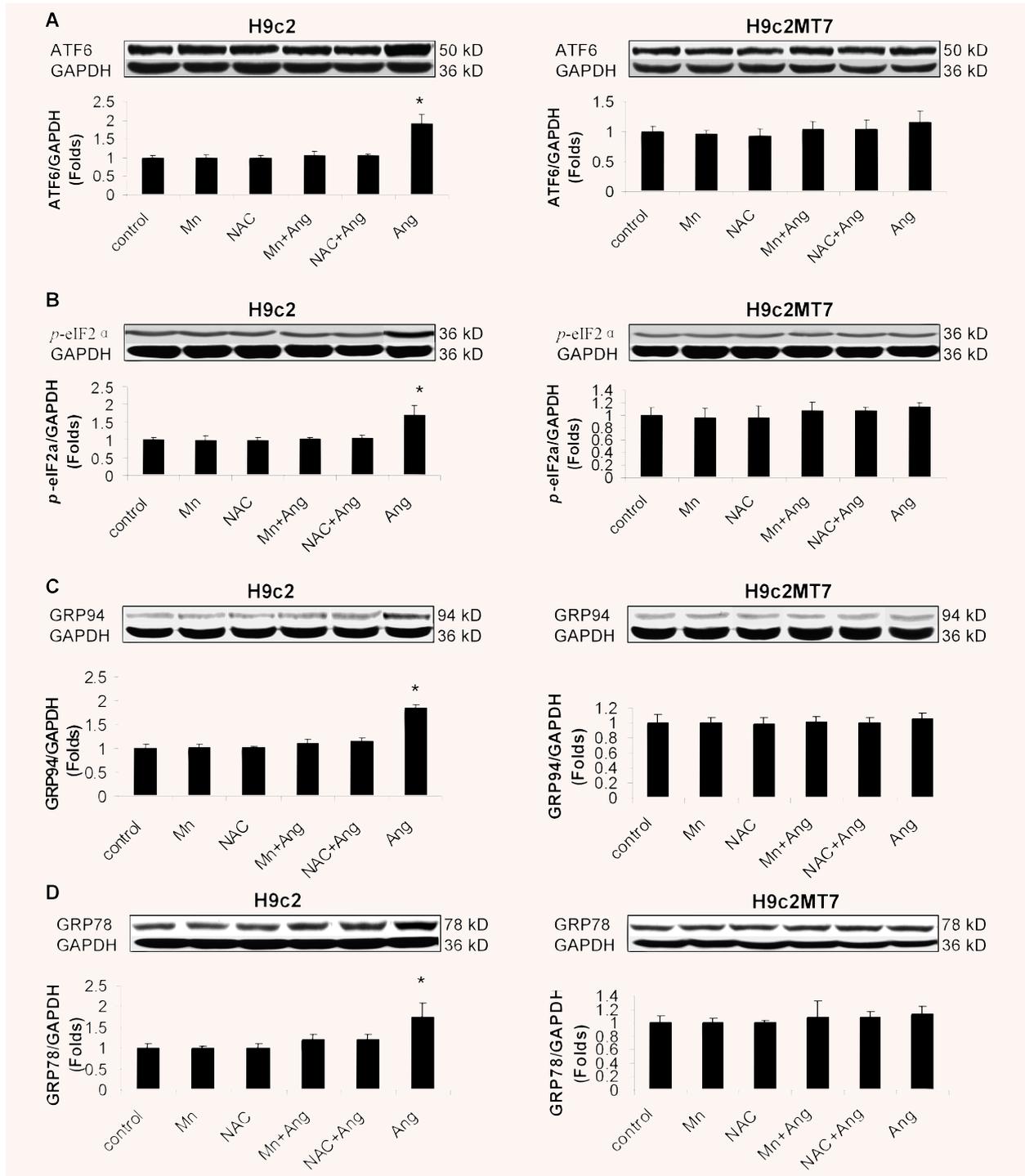


Fig. 8 Protective effect of antioxidants and MT against Ang II-induced ER stress. H9c2 and H9c2MT7 were exposed to Ang II at 100 nM for 24 hrs with or without pre-existence of MnTMPyP at 50 μ M for 30 min. or NAC at 50 μ M for 24 hrs. ER stress was examined by measuring the cleaved ATF6 (A), p-eIF2 α (8B), GRP78 (C) and GRP94 (D) with Western blotting assay. Experimental data was presented as means \pm S.D. from three separate experiments with triple samples for each experiment. *, $P < 0.05$ versus control.

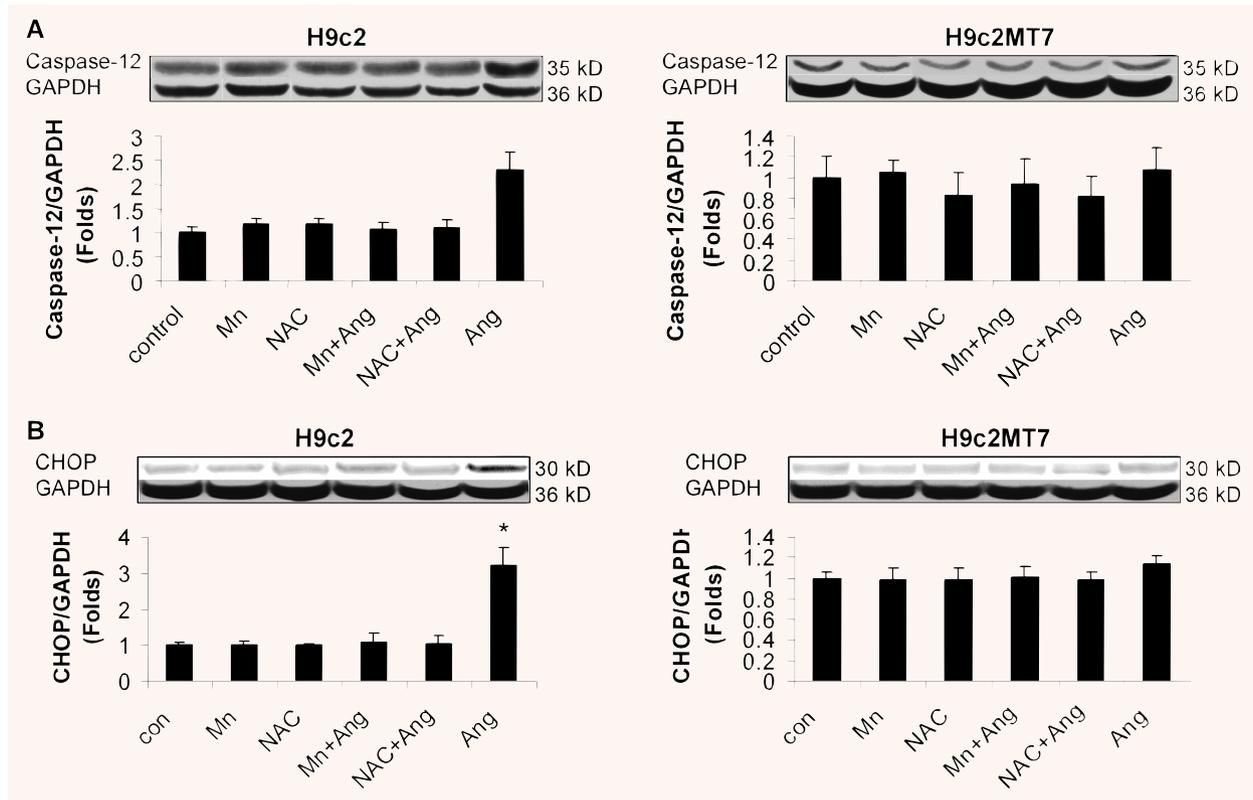


Fig. 9 Protective effect of antioxidants and MT against Ang II-induced ER stress-mediated cell death. H9c2 and H9c2MT7 were treated with the same condition as described in Fig. 8. ER stress-mediated cell death was detected by measuring the cleaved caspase-12 (**A**) and CHOP expression (**B**) with Western blotting assay. Experimental data was presented as means \pm S.D. from three separate experiments with triple samples for each experiment. *, $P < 0.05$ versus control.

Although emerging evidence has indicated that ER stress-mediated apoptosis is involved in the pathogenesis of diabetic eye and kidney as well as non-diabetic heart failure [31–36], there was no direct evidence for the involvement of ER stress in diabetes-induced cardiac cell death and DCM. The present study shows an association of diabetes- and Ang II-induced cardiac cell death with cardiac ER stress, and also MT protection against cardiac ER stress and associated cell death caused by diabetes, Ang II and chemical ER stressor in mouse model.

Cardiac cells contain abundant ER, which is a central organelle of each eukaryotic cell as the place of protein folding, maturation and secretion [4]. Properly folded proteins are trafficked from the ER to the Golgi, lysosomes or to the plasma membrane. Conditions interfering with the function of ER are collectively called ER stress. ER stress is induced by UPR accumulation. The UPR deals with the adverse effects of ER stress and enhances cell survival through three stress-sensing proteins found on the ER membrane: PKR-like eIF2 α kinase (PERK), inositol-requiring kinase-1 α (IRE-1 α) and ATF-6 [4, 37]. Chaperone GRP78 binds the N-termini of IRE-1 α , PERK and ATF6, preventing their activa-

tion. Unfolded proteins in the ER cause GRP78 to release IRE-1 α , PERK and ATF6, leading to their oligomerization and activation in ER membranes. Among these events, the activated IRE-1 α activates its downstream kinases that activate TRAF2, ASK1 and JNK, leading to activation of caspase-9 as mitochondrial cell death pathway. Failure of GRP78 binding to PERK causes activation of caspase-12 and CHOP-dependent ER stress-mediated apoptosis as a mitochondrial independent cell death pathway [4, 37]. Therefore, these chaperons are up-regulated to activate both mitochondria-dependent and independent cell death pathways in response to different stresses [38–40].

Consistent with the above concept, we have shown the induction of apoptotic cell death via mitochondria-cytochrome c release pathway in the heart of diabetic mice [19, 41]. In the present study, we further showed the up-regulated two of the three arms of ER stress signalling, PERK- and ATF6-mediated pathways. We found that one of PERK signalling members, eIF2 α phosphorylation, was significantly increased in the heart of WT diabetic mice at 2 weeks after diabetes onset (Fig. 1A), and cleaved ATF6 expression was also significantly increased in the heart of WT diabetic

mice at both 2 weeks and 2 months after diabetes onset (Fig. 1C). The increased ER stress was accompanied with a significant increase in cardiac cell death, measured by TUNEL assay and Western blotting for the activated caspase-3 and caspase-12, and CHOP as the markers of ER stress-mediated apoptotic cell death (Fig. 3). We also demonstrated the significant increases in GRP78 and GRP94 (Fig. 1). Several studies have indicated the involvement of either eIF2 α or ATF6, along with up-regulation of GRP78 and/or GRP94 in the induction of apoptotic cell death caused by several pathogenic conditions [42–44]. However, our finding is the first time to systemically examine the cardiac ER stress and ER-stress-mediated cardiac cell death under diabetic and Ang II-treated conditions. Our studies also indicate that diabetes induces cardiac cell death *via* both ER-stress-dependent and mitochondria-dependent pathways ([19, 41] and present study).

Although both GRP78 and GRP94 protein up-regulation can be considered as an index of ER stress, these proteins can also act as an adaptive mechanism to protect the cells against stress-induced death [45–47]. We found that cardiac cell death mainly occur at 2 weeks after diabetes (Fig. 3) along with significant increases in eIF2 α phosphorylation only at 2 weeks (Fig. 1). We found that cleaved ATF6 was increased in the hearts of diabetic mice (Fig. 1). Although increase in cleaved ATF6 was also observed in the heart of WT diabetic mice at 2 months after diabetes, there was no significant increase in apoptotic cell death, suggesting that ATF6 cleavage may not be the direct cause for cardiac cell death although it is an ER stress response.

We also demonstrated that a significant and persistent up-regulation of GRP78 as an adaptive and protective regulator was also significantly increase in the hearts of diabetic mice from 2 weeks until 5 months (Fig. 1C). Cardiac adaptive response in the hearts of diabetic mice, particularly in the early stage, was mirrored by the up-regulation of cardiac expression of MT as an antioxidant and ER stress protein (Fig. 2). The response pattern of cardiac MT is exactly parallel with that of GRP78 (Fig. 1). Consistent with this finding, MT and GRP78 were up-regulated in the heart of WT mice with Ang II infusion (Fig. 5A) or with chemical ER stressor (Fig. 7A) although there was induction of apoptosis (Figs. 5B and 7B) that may be mainly due to the induction of eIF2 α phosphorylation (Figs. 4A, B and 6A, B). Interestingly, there was no increase in GRP94 protein in the heart of WT mice treated with either Ang II (Fig. 4D) or chemical stressor (Fig. 6D). We do not have any explanation for this discrepancy in these three models for the up-regulation of GRP94, and complicated functions of GRP94 have been documented although it also acts as protective factor in certain cells [48–51].

It could be questioned that if MT or GRP78 expression is up-regulated in response to diabetes as an adaptive response to protect the heart from ER stress-mediated cell death, why it does not prevent the occurrence of diabetic or Ang II-induced ER stress and cell death. We assume that the up-regulated MT or GRP78 expression observed in the WT mice is secondary to diabetes- or Ang II-induced ER stress. This stress induces MT or GRP78 expression but also simultaneously initiates the signalling of ER stress-

mediated cardiac cell death. Therefore, the ER stress-induced MT or GRP78 expression in the heart does not prevent ER stress-initiated apoptotic cell death, but prevents ER stress further-induced cell death; therefore, there was no significant increase in apoptotic cell death at the late stages of 2 and 5 months. To prevent the initial occurrence of ER stress and associated apoptosis, pre-enhanced cardiac MT content is requested. To support this notion, MT-TG mice were significantly resistant to diabetes-, Ang II- and Tu-induced cardiac ER stress and associated cell death (Figs 3, 5 and 7). H9c2MT7 cells are highly resistant to Ang II-induced ER stress-associated cell death (Fig. 8). Fu *et al.* also showed that proteasome inhibition induced ATF6 activation and cardiomyocyte death along increases in CHOP expression and caspase-12 activation. Supplement and/or pharmacological induction of GRP78 could attenuate cardiac cell death by proteasome inhibition [52].

Regarding the mechanisms by which MT prevents cardiac cell death under different ER stresses, we assume that anti-oxidative action of MT plays a critical role in preventing diabetes- and Ang II-induced cardiac ER stress and associated cell death. Our results showed that pre-treatment with antioxidant significantly attenuated Ang II-induced ER stress (Fig. 8) and ER stress-mediated cell death, measured by CHOP expression and caspase-12 activation (Fig. 9). Both oxidative stress and ER stress were implicated in the diabetic complications and a few studies have reported that accumulation of reactive oxygen species as the cause of ER stress [6, 20, 21]. For instance, Xue *et al.* demonstrated the induction of UPR by tumour necrosis factor- α in L929 cells *via* inducing reactive oxygen species accumulation [53], and Yan *et al.* also found that ER stress and UPR were attributable to oxidative stress [54]. To support these previous studies, a recent study showed that WT and MT-TG mice were received the GSH synthase inhibitor buthionine sulfoximine in drinking water for 2 weeks. Buthionine sulfoximine led to a robust decrease in the GSH and increased reactive oxygen species production, consolidating oxidative stress, along with cardiac ER stress and dysfunction in the WT mice, but not in the MT-TG mice [55]. Taken together, all these data suggested that MT is a potent antioxidant to protect cells and tissues from diabetes, Ang II and Buthionine sulfoximine (BOS) *via* suppression of reactive oxygen species accumulation [16, 17, 23, 55]. Therefore, MT protection against Ang II-induced ER stress and associated apoptotic effects is most likely mediated by its antioxidant property.

Acknowledgements

The work was supported, in part, by research grants from American Diabetes Association (05–07-CD-02, to L.C.). J.X. and Q.L. are recipients of Scholarship under State Scholarship Fund from China Scholarship Council. The authors thank Dr. Michelle Barati, Department of Medicine, University of Louisville, for her generosity in providing certain reagents. The authors are also grateful to Dr. M. George Cherian, Professor Emeritus, University of Western Ontario, Canada, for his insightful reading and editing.

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