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Activation of HIF-1 by metallothionein contributes to cardiac protection in the diabetic heart

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[1]*School of Pharmacy, Wenzhou Medical College, Wenzhou, China;* [2]*Department of Public Health, Xi'an Jiaotong University School of Medicine, Xi'an, China;* [3]*College of Chemistry and Material Sciences, Northwest University, Xi'an, China;* [4]*Department of Medicine, University of Louisville School of Medicine, Louisville, Kentucky; and* [5]*Department of Pediatrics, University of Louisville School of Medicine, Louisville, Kentucky*

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Xue W, Liu Y, Zhao J, Cai L, Li X, Feng W. Activation of HIF-1 by metallothionein contributes to cardiac protection in the diabetic heart. *Am J Physiol Heart Circ Physiol* 302: H2528–H2535, 2012. First published April 20, 2012; doi:10.1152/ajpheart.00850.2011.—Metallothionein (MT) protects against heavy metal-induced cellular damage and may participate in other fundamental physiological and pathological processes, such as antioxidation, proliferation, and cell survival. Previously, we have shown that elevation of MT by transgene or by induction with zinc protects the heart against diabetic cardiomyopathy by mechanisms such as antidiabetes-induced oxidative stress and inactivation of glycogen synthase kinase-3, which mediates glucose metabolism. We also reported that MT overexpression rescued the diabetic-induced reduction of hypoxia-inducible factor (HIF)-1α, which plays an important role in glucose utilization and angiogenesis. Here, we showed that overexpression of MT increased hexokinase (HK)-II expression under control conditions and attenuated diabetes-decreased HK-II expression. Glycolytic flux assay demonstrated that MT increased glycolysis output in high glucose-containing media-cultured H9c2 cells. The diabetes-induced reduction in cardiac capillaries was also attenuated by MT overexpression. Furthermore, MT induction significantly increased HIF-1 expression under both control and diabetic conditions. Moreover, in the present study, we demonstrated that MT-enhanced HIF-1α activity is likely through a mechanism of protein nuclear translocation. These results suggest that MT induces HIF-1α expression, leading to increased HK-II in the diabetic heart.

diabetes; hypoxia-inducible factor

DIABETES is frequently associated with hypoxia and oxidative stress and is known to impair energy metabolism (17), neovascularization (28), and other forms of adaptive cellular responses. Hyperglycemia is the driving force of such dysregulations. Previous studies (5, 27, 38, 40, 41, 44, 45) have shown that hyperglycemia induces oxidative stress in the heart and that the induction of an antioxidant, metallothionein (MT), protects the heart against diabetic cardiomyopathy. MT is a small cysteine-rich protein that primarily functions in essential metal homeostasis, heavy metal detoxification, and cellular antioxidative defense (10, 18). It has also been shown that MT is critically involved in cell survival, angiogenesis, apoptosis, and proliferation. However, the role of MT in the regulation of cellular hypoxia has not been investigated until recent studies demonstrated that MT induces the upregulation of hypoxia-inducible factor (HIF)-1 in renal (20) and brain (19) cells. In cardiomyocytes, we (13) have shown that MT could rescue HIF-1 transcriptional activity under diabetic conditions.

HIF-1 is a master transcription factor and plays an essential role in glucose metabolism, angiogenesis, and cell proliferation (29, 30). HIF-1 consists of HIF-1α and HIF-1β subunits. HIF-1β is constitutively expressed, whereas HIF-1α is the regulatory subunit and mainly senses tissue O_2 and responds to various stimuli (36, 37). Stabilized HIF-1α dimerizes with HIF-1β and translocates into the nucleus to start target gene expression. In addition to hypoxic stimuli, a variety of factors have been shown to affect HIF-1 activity even under normoxic conditions (12, 16, 35). Hyperglycemia is the metabolic hallmark of diabetes and is a major cause of diabetic cardiomyopathy. Recent studies (6, 7, 15) have shown that hyperglycemia suppresses HIF-1α protein stability, modification, and function. Studies attempting to increase HIF-1 activity have shown beneficial effects in preventing diabetic cardiomyopathy (13, 42) and in protecting the diabetic heart from ischemic insult (8).

The aim of the present study was to examine whether transgenic overexpression of MT in cardiac tissue upregulates HIF-1 along with its target genes in glycolysis and angiogenesis pathways under diabetic conditions. The results demonstrated that MT upregulates hexokinase (HK)-II, possibly via HIF-1 regulation, leading to enhanced glycolysis and angiogenesis under diabetic conditions in the heart.

MATERIALS AND METHODS

Experimental animals. Cardiac-specific MT-overexpressing transgenic (MT-TG) mice were produced from FVB mice, which have been previously characterized. Both 8- to 10-wk-old MT-TG positive mice and negative littermates [wild-type (WT) mice] were kept in the same cages with free access to rodent chow and tap water. All animal procedures were approved by the Institutional Animal Care and Use Committee, which has been certified by the American Association for Accreditation of Laboratory Animal Care. The type 1 diabetic mouse model was generated using streptozotocin (STZ) as previously described (4, 38, 40). STZ-treated mice with whole blood glucose higher than 13.9 mmol/l were considered as diabetic. Body weights and blood glucose levels were measured regularly. Mice were euthanized at the experimental end points.

Cell cultures for in vitro experiments. An embryonic rat heart-derived cell line (H9c2 cells, American Type Culture Collection, Manassas, VA) and a stable human MT-IIA-overexpressing cardiac cell line derived from H9c2 cells (H9c2MT7 cells) (43) were maintained in DMEM supplemented with 10% FBS and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) at 37°C in an atmosphere of 95% air and 5% CO_2. H9c2MT7 cells showed no major morphological and physiological differences compared with WT H9c2 cells (42). Cells were subcultured at <70% confluence, and mononucleated

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myoblasts were incubated in serum-free DMEM for 24 h and then subjected to experimental procedures as indicated.

For the glucose metabolism experiments, H9c2 and H9c2MT7 cells were cultured in normal glucose (5.5 mM)-containing or high-glucose (25 mM)-containing medium for 20 passages. Cells were then plated in six-well plate in DMEM containing 1% FBS. Glycolytic flux was determined by the addition of [5-^{3}H]glucose in the presence of 5.5 μM glucose and by measuring glycolysis-generated ^3H_2O by scintillation counting as previously described (11, 39).

Real-time RT-PCR. Total RNA from hearts and treated cells were isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was reverse transcribed using reverse transcriptase (Applied Biosystems, Foster City, CA) with random hexamers. Reactions were performed in the 96-well format with the SYBR Green PCR Core Reagents kit (Applied Biosystem) using a 7500 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. The primer sequences were as follows: mouse HIF-1α, 5′-TCAAGTCAGCAACGTGGAAG-3′ (sense) and 5′-TATCGAGGCTGTGTCGACTG-3′ (antisense); rat HIF-1α, 5′-TGCTTGGTGCTGATTTGTGAA-3′ (sense) and 5′-TATCGAGGCTGTGTCGACTGAG-3′ (antisense); and mouse HK-II, 5′-CTGTCTACAAGAAACATCCCCATTT-3′ (sense) and 5′-CACCGCCGTCACCATAGC-3′ (antisense). All oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). The relative quantities of target transcripts were calculated from duplicate samples after normalization of the data against the housekeeping gene β-actin. Dissociation curve analysis was performed after PCR amplification to confirm the specificity of the primers. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method, where C_t is threshold cycle (21).

Western blot analysis. Western blot assays were used to detect HK-II and β-actin protein levels. Heart tissues were homogenized in lysis buffer using a homogenizer. Tissue or cell proteins were collected by centrifugation at 12,000 *g* at 4°C for 10 min. The protein concentration was measured by a Bradford assay. The sample (diluted in loading buffer and heated at 95°C for 5 min) was then subjected to electrophoresis on a 10% SDS-PAGE gel at 120 V. After electrophoresis and transfer of the proteins to nitrocellulose membranes, membranes were blocked in Tris-buffered saline-Tween 20 [TBST; 10 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] containing 5% nonfat dry milk and incubated with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200 at 4°C overnight with slight agitation. Membranes were then washed three times with TBST and reacted with secondary horseradish peroxidase-conjugated antibody for 1 h. Antigen-antibody complexes were then visualized using an ECL kit (GE Healthcare).

ELISA. Nuclear HIF-1α levels in heart tissues and culture cells were measured by commercial human/mouse total HIF-1α ELISA kits (Quantikine, R&D Systems, Minneapolis, MN) in accordance with the manufacturer's protocol.

Histopathology and immunofluorescence staining. Hearts were excised, washed with saline solution, and placed in 10% formalin. Heart tissues were cut transversely close to the apex to visualize the left and right ventricles. Several sections of the heart (5 μm thick) were prepared and stained with hematoxylin and eosin for histopathology and then visualized with a light microscope.

Capillaries were visualized in frozen left ventricular sections stained with rat anti-mouse CD31 (BD Biosciences, San Diego, CA) (1:50) primary antibody and Cy3-labeled secondary antibodies (Abcam, Cambridge, MA) using a Nikon 2000S fluorescence microscope. Capillary density was assessed by a computer-assisted image-analysis system (SigmaScan Pro5.0, SPSS, Chicago, IL). Ten fields at ×200 magnification/heart were analyzed, and 6–7 mice/group were examined for the calculation. Capillary density was expressed as the number of capillaries/mm^2.

For HIF-1α protein immunocytochemistry staining, H9c2 cells were seeded in chamber slides and treated as indicated. Cells were then fixed and stained with anti-HIF-1α antibody and a fluorescent secondary antibody.

Statistical analysis. In vitro experiments were performed at least three times with duplicate or triplicate samples each time. In vivo experiments included at least 6 animals/group. Data are presented as means ± SE and were analyzed by one-way ANOVA for the different groups followed by post hoc pairwise multiple comparisons by Turkey test. P values of <0.05 were considered as statistically significant.

RESULTS

MT overexpression protects against diabetes-induced cardiac injury. WT and MT-TG diabetic mice showed similar, persistent increases in whole blood glucose levels after the onset of diabetes induced by STZ over the period of 60 days (Fig. 1*A*). Histological examinations revealed significant differences in histopathological changes in the heart between WT and MT-TG diabetic mice. Disorganized array of the myocardial structure, cell necrosis, and myofibrillar discontinuation were observed in the hearts of WT diabetic mice but not in the hearts of control or MT-TG diabetic mice, as shown in Fig. 1*B*.

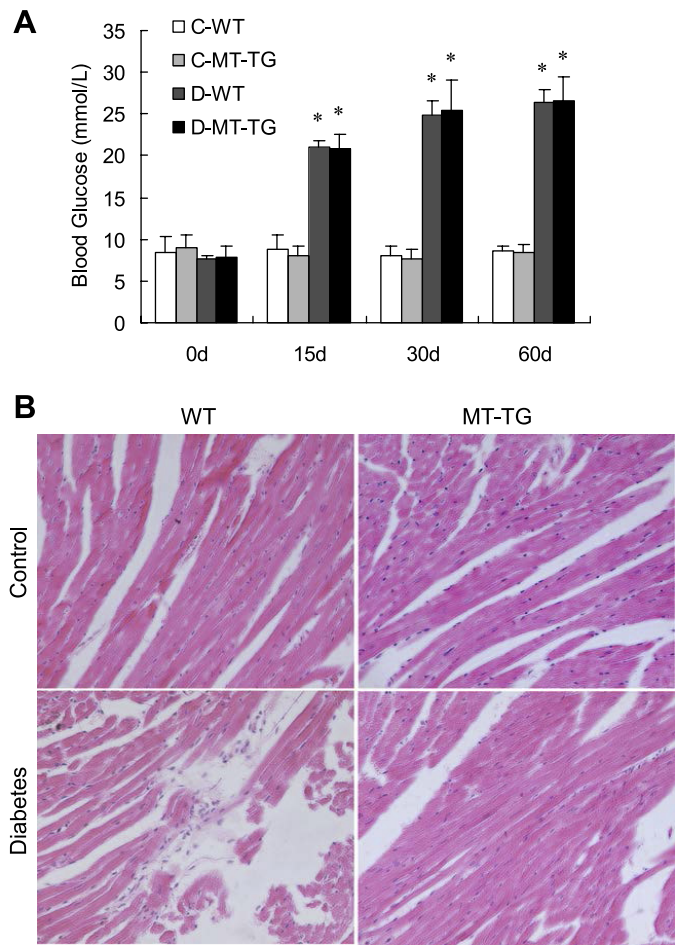

Fig. 1. Blood glucose and cardiac histological changes by metallothionein (MT) overexpression. *A*: fasting blood glucose levels of 8-wk-old wild-type (WT) mice and MT-overexpressing transgenic (MT-TG) mice treated with 150 mg/kg streptozotocin or sodium citrate buffer were measured 15, 30, and 60 days (d) after diabetes onset. C-WT and C-MT-TG, control WT and MT-TG mice; D-WT and D-MT-TG, diabetic WT and MT-TG mice. *$P < 0.05$ vs. control mice. *B*: cardiac morphological changes were observed in cross sections stained with hematoxylin and eosin. Original magnification: ×200. Bars = 50 μm.

Effects of MT on HIF-1α regulation. Next, we examined the effect of MT on HIF-1α expression in diabetic hearts and in cultured cardiac cells. Diabetes had no effect on mRNA levels of HIF-1α in both WT and MT-TG hearts. MT overexpression significantly increased HIF-1α mRNA under both control and diabetic conditions (Fig. 2*A*). However, diabetes significantly decreased HIF-1α protein levels in WT hearts but not in MT-TG hearts (Fig. 2*B*). We (38) have also previously shown that supplementation with zinc, a strong MT inducer, had a protective role in diabetic cardiomyopathy. Therefore, we also examined the effect of zinc on HIF-1α induction in WT H9c2 cells and MT stably transfected cells (H9c2MT7). Zinc treatment significantly increased HIF-1α mRNA in H9c2 cells but had no effect in H9c2MT7 cells. The likely explanation could

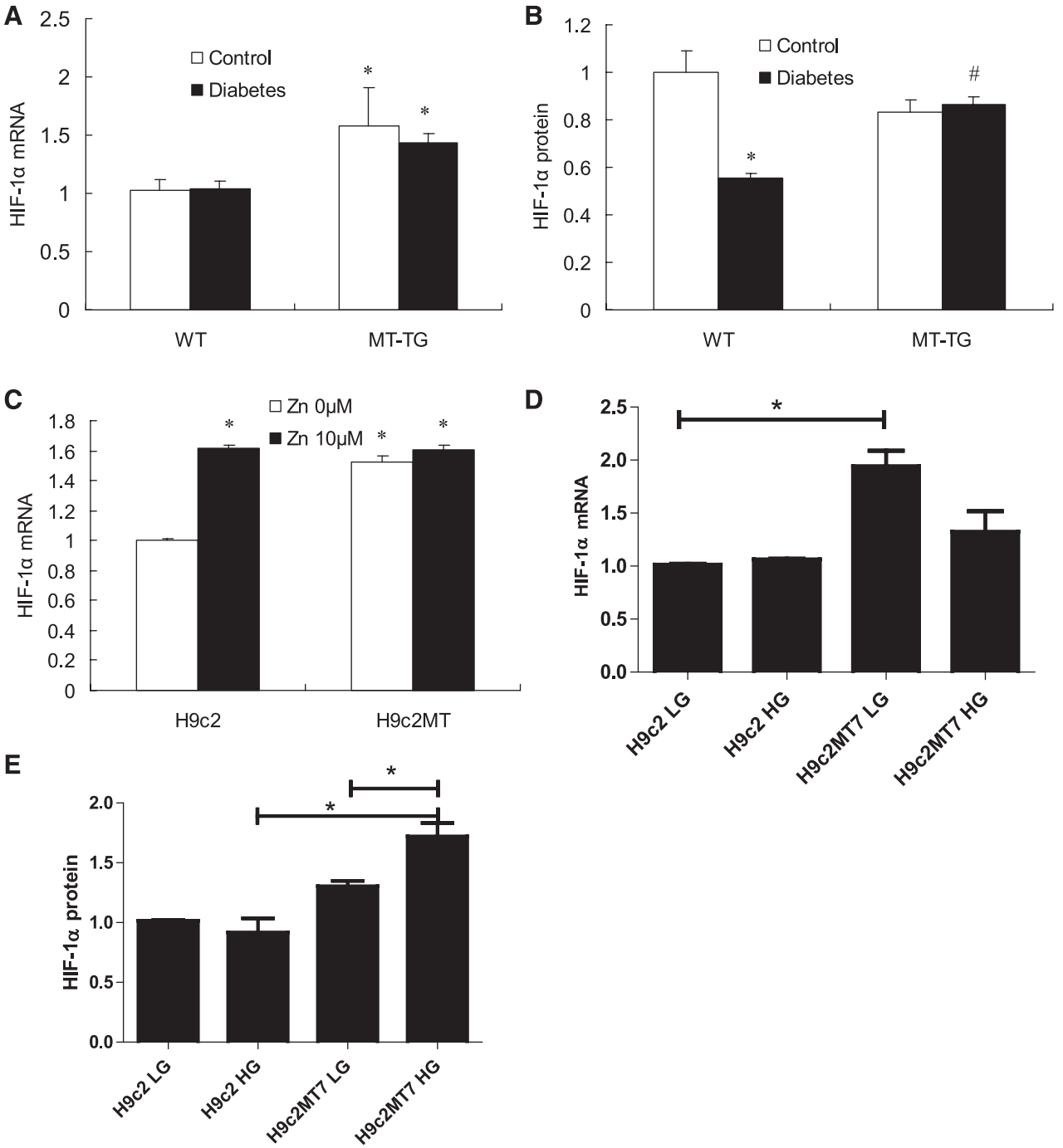

Fig. 2. Effects of MT on hypoxia-inducible factor (HIF)-1α mRNA and protein levels. *A*: HIF-1α mRNA levels in the heart of WT and MT-TG mice under control and diabetic conditions. $*P < 0.05$ vs. the respective WT value. *B*: nuclear HIF-1α protein levels in WT and MT-TG mice under control and diabetic conditions. $*P < 0.05$ vs. control WT mice; $\#P < 0.05$ vs. diabetic WT mice. *C*: HIF-1α mRNA levels in H9c2 and H9c2MT7 cells untreated or treated with 10 μM zinc for 8 h. $*P < 0.05$ vs. H9c2 with 0 μM zinc treatment. *D*: effects of glucose concentrations on HIF-1α mRNA levels in H9c2 and H9c2MT7 cells. $*P < 0.05$. *E*: effects of glucose concentrations on nuclear HIF-1α protein levels in H9c2 and H9c2MT7 cells. $*P < 0.05$. H9c2 and H9c2MT7 cells were cultured in media containing 5.5 mM glucose [low glucose (LG)] or 25 mM glucose [high glucose (HG)] for 20 passages. mRNA and nuclear protein were isolated for analysis.

be that exogenous zinc does not induce further MT expression in H9c2MT cells and therefore no further induction of HIF-1α is expected. This notion is supported in that MT overexpression itself already increased HIF-1α mRNA in H9c2MT7 cells to a comparable level as H9c2 cells by zinc treatment (Fig. 2*C*). The effects of glucose concentration on HIF-1α mRNA and protein levels were examined. Low glucose concentration induced a significant increase in mRNA levels in H9c2MT7 cells compared with H9c2 cells. However, nuclear HIF-1α protein levels were slightly, but insignificantly, increased in H9c2MT7 cells compared with WT H9c2 cells by low-glucose culture (Fig. 2*D*). In contrast, high-glucose treatment significantly increased HIF-1α nuclear protein levels in H9ce2MT7 cells, although there was no difference with high-glucose treatment in H9c2 cells (Fig. 2*E*). The discrepancy in the effect of diabetes and high glucose concentration on HIF-1α protein levels in vivo and in vitro could be the results of different treatment times under hyperglycemic conditions. Taken together, the results showed that MT transgenic overexpression or induction by zinc induces HIF-1α activation.

HIF-1α protein functions when it translocates into the nucleus, where it binds HIF-1β and starts its target gene transcription. To examine the nuclear localization of HIF-1α in response to hyperglycemia and the role of MT, H9c2 cells were cultured in high glucose-containing media for 20 passages. Hypoxia was conducted by placing the cells in a box containing 1% O_2-5% CO_2-balance N_2. As shown in Fig. 3, under normal glucose conditions, hypoxia induced a remarkable increase in nuclear HIF-1α proteins (Fig. 3, LG + H). However, when cells were treated with high concentration of glucose, hypoxia increased even more HIF-1α protein, but the majority of the proteins were localized in the cytoplasm (Fig. 3, HG + H). Importantly, when hyperglycemic cells were treated with MT, nuclear localization of HIF-1α protein was observed (Fig. 3, HG + H + MT), indicating that under hypoxic hyperglycemic conditions, MT increases HIF-1 activity, at least in part, by increasing its nuclear protein levels. Under normal O_2 concentration, HIF-1α staining was weak, but a clear nuclear translocation was observed with MT treatment in high-glucose-cultured H9c2 cells (Fig. 3, HG + N and HG + N + MT).

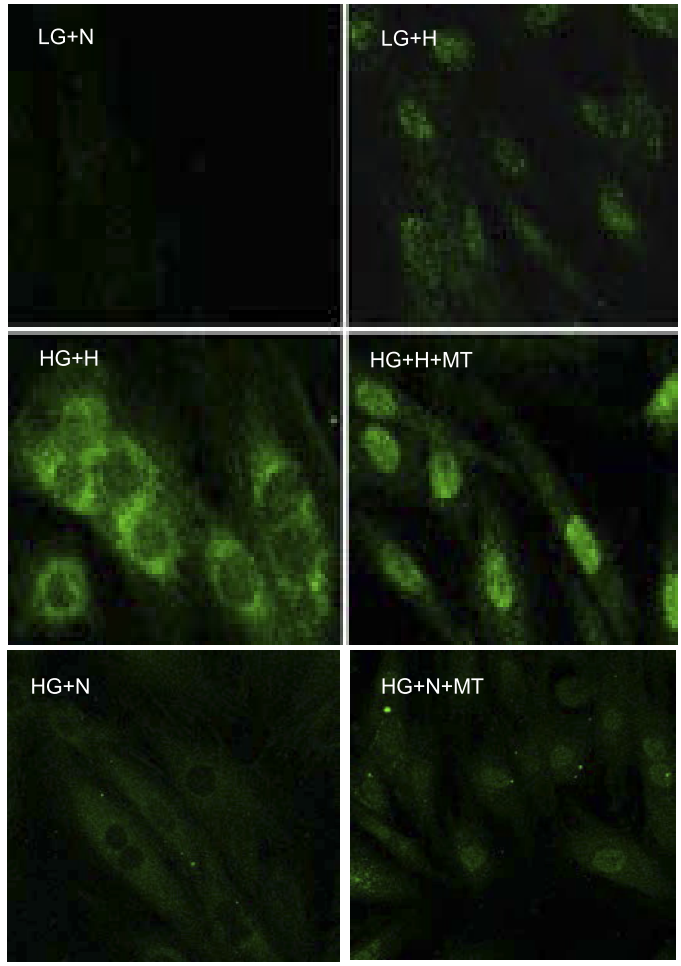

Fig. 3. Effects of MT on HIF-1α protein nuclear localization. H9c2 cells were cultured in media containing 5.5 mM glucose (LG) or 25 mM glucose (HG) for 20 passages. Cells were incubated in a tightly sealed hypoxia box, which was filled with 1% O_2-5% CO_2-balance N_2 for 8 h [hypoxic conditions (H)]. MT-II (Sigma-Aldrich, St. Louis, MO) was added to the culture media (20 ng/ml) at the beginning of hypoxia. Cells were fixed and immunofluoresence stained using an anti-HIF-1α antibody. N, normoxic conditions (control).

MT overexpression attenuates diabetic suppression of HK-II and glucose metabolism. We (42) have previously shown that cardiac-specific HIF-1α overexpression protects the heart from diabetic cardiomyopathy by increasing the glycolytic enzyme HK-II. Therefore, we explored whether MT's activation of HIF-1 is accompanied by the preservation of cardiac glucose metabolism via upregulation of HK-II. Diabetes did not change HK-II mRNA levels after 2 mo of diabetes (Fig. 4*A*). MT overexpression increased HK-II mRNA under both nondiabetic and diabetic conditions (Fig. 4*A*). However, diabetes significantly reduced HK-II protein levels by 50% after 2 wk of diabetes and 65% after 2 mo of diabetes (Fig. 4*B*). Importantly, MT overexpression attenuated this reduction (Fig. 4*B*). It should be noted that although there was a decrease in HK-II protein levels by diabetes, HK-II mRNA levels were basically not changed in WT hearts.

To further examine whether the induction of MT and HIF-1α induces HK-II expression, H9c2 cells were treated with 10 μM zinc and 100 μM $CoCl_2$, a well-known HIF-1α inducer. Western blot analysis showed a significant increase in HK-II protein levels with zinc treatment. This increase was even pronounced with $CoCl_2$ treatment. However, there was no additive or synergetic increase when zinc and $CoCl_2$ were coapplied. Importantly, stable overexpression of MT significantly increased HK-II protein levels (Fig. 4*C*). Meanwhile, HK-II mRNA levels were also assessed. Zinc and $CoCl_2$ significantly increased HK-II mRNA expression, but, again, there was no additive or synergetic effect (Fig. 4*D*).

To determine the effect of MT on glucose metabolism of cardiac cells under hyperglycemic conditions, H9c2 and H9c2MT7 cells were subcultured for 20 passages in normal glucose or high glucose-containing media (5.5 and 25 mM, respectively). Glycolysis-generated ^3H_2O was determined by the addition of [5-^{3}H]glucose in cultures by scintillation counting, as previously described. As shown in Fig. 5*A*, glycolysis was impaired in H9c2 cells cultured in high-glucose media compared with that in normal glucose media. There was a similar effect of hyperglycemia on glycolysis in H9c2MT7 cells, but MT overexpression increased basal glycolysis. Importantly, under hyperglycemic conditions, glycolysis flux in H9c2MT7 cells was

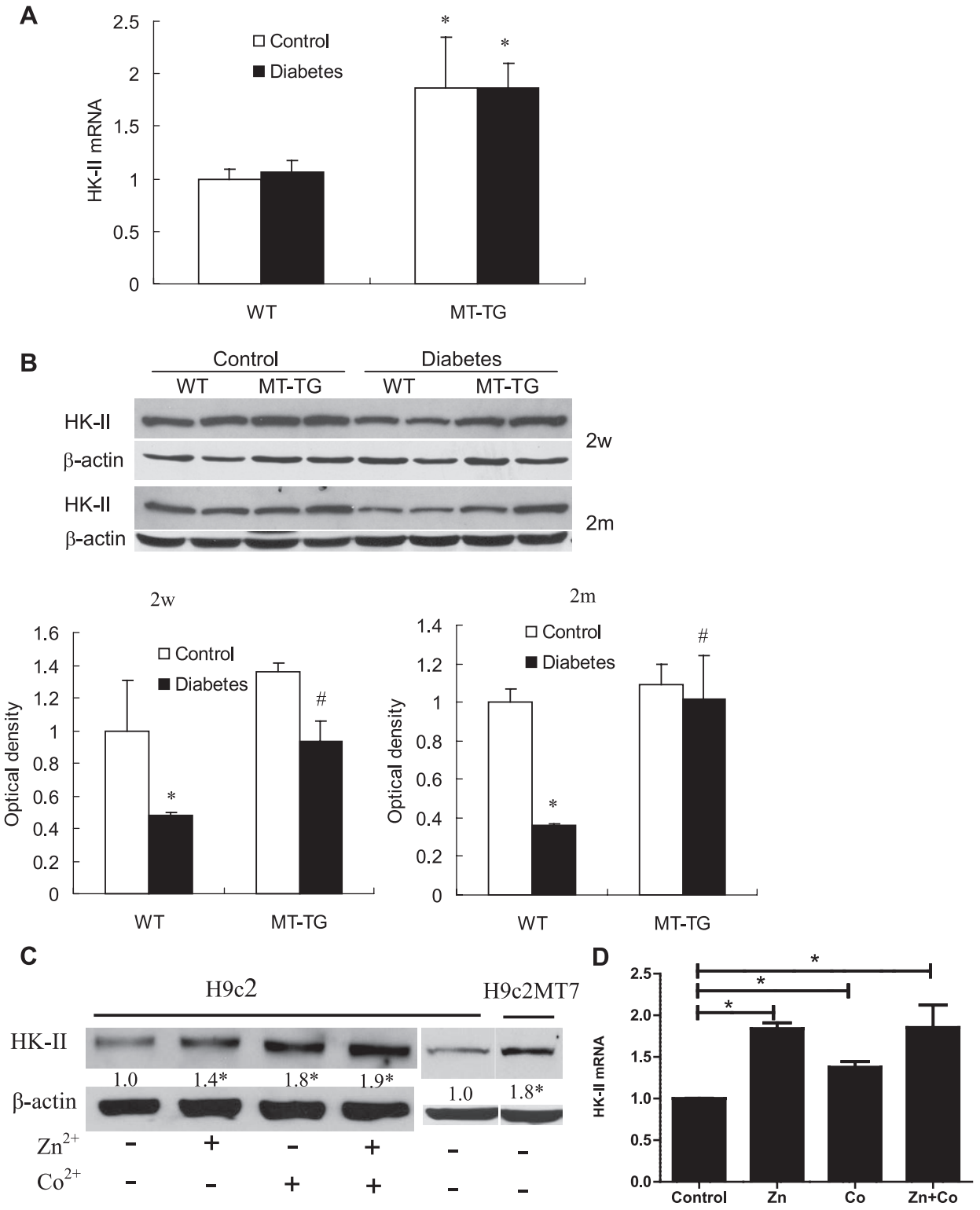

Fig. 4. Effects of MT on hexokinase (HK)-II expression. *A*: mRNA levels of HK-II in WT and MT-TG mouse hearts under control and diabetic conditions. *$P <$ 0.05 vs. the respective WT value. *B*, *top*: HK-II protein levels of WT and MT-TG mouse hearts under control conditions of 2-wk or 2-mo diabetes by Western blot analysis. β-Actin was used as a loading control. *Bottom*, quantitative analysis of the blots. *$P < 0.05$ vs. control WT mice; #$P < 0.05$ vs. diabetic WT mice. *C*: HK-II protein levels in cultured H9c2 and H9c2MT7 cells. Cells were treated with zinc (10 μM) for 24 h and then with $CoCl_2$ for 8 h as indicated. Total cell lysates were used for HK-II Western blot analysis. β-Actin was used as a loading control. Numbers under HK-II bands are results from densitometry analysis. *$P < 0.05$ vs. control H9c2 cells. *D*: effects of treatments on HK-II mRNA levels. *$P < 0.05$.

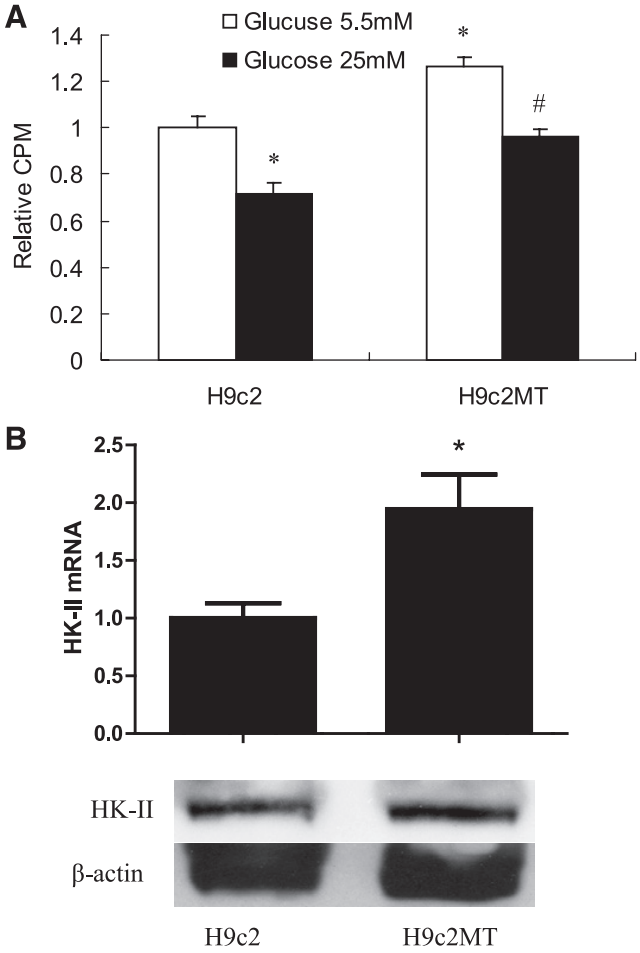

Fig. 5. Effects of MT on glucose metabolism and HK-II gene expression. *A*: H9c2 and H9c2MT7 cells were cultured in 5.5 and 25 mM glucose-containing media, respectively, for 20 passages. Cells were incubated with [5-^{3}H]glucose for 3 h. Glycolysis-produced ^3H_2O was measured by scintillation counting and normalized to cell number. *$P < 0.05$ vs. H9c2 cells with 5.5 mM glucose; #$P < 0.05$ vs. H9c2 cells with 25 mM glucose. *B*: HK-II mRNA and protein expression in H9c2 and H9c2MT7 cells under HG treatment. *$P < 0.05$ vs. H9c2 cells.

comparable with that in H9c2 cells under normal glucose conditions, indicating that MT overexpression attenuates the decrease of glycolysis under hyperglycemic conditions by increasing basal glycolytic effects.

Due to the important role of HK-II in cellular glucose uptake, we also measured HK-II mRNA and protein levels in H9c2 and H9c2MT7 cells treated with high glucose concentration. There was about a twofold increase in HK-II mRNA levels in H9c2MT7 cells compared with WT H9c2 cells. Western blot analysis showed a significant increase in HK-II protein levels (Fig. 5*B*).

Effect of MT overexpression on angiogenesis. Our previous work (13) demonstrated that MT affects VEGF expression, which is a well-known HIF-1 target and critical proangiogenic factor (31, 32). Activation of the VEGF pathway leads to enhanced angiogenesis. Here, we examined capillary densities in the hearts of WT and MT-TG mice. Immunofluorescence staining with anti-mouse CD31 antibody showed that MT overexpression did not change the capillary density under nondiabetic conditions; however, it significantly prevented the diabetes-induced reduction in capillary density (Fig. 6).

DISCUSSION

Our earlier work demonstrated that MT is protective in diabetic cardiomyopathy in mouse models and documented that antioxidative stress plays an important role in this protection. Cardiac overexpression of MT significantly reduced diabetes-induced oxidative and nitrosative stress by acting as an effective antioxidant. Zinc supplementation induced MT expression and prevented the development of diabetic cardiomyopathy (37). As shown in our recent work, in addition to antioxidative function, MT upregulates HIF-1α protein in the diabetic mouse heart. HIF-1α is a master transcription factor and plays a central role in glucose metabolism and cardiac tissue angiogenesis. Defective glycolysis and angiogenesis have been observed in cardiac tissues of diabetic patients. Glycolytic enzymes, such as HK-II, are decreased in the diabetic myocardium and in cultured cardiomyocytes. In this study, we demonstrated that MT overexpression in the diabetic heart increases HIF-1α activity and HK-II expression. The enhancement of the HIF-1α-HK-II pathway by MT induction in the diabetic heart might be a new mechanism for MT-mediated protective effects in diabetic cardiomyopathy.

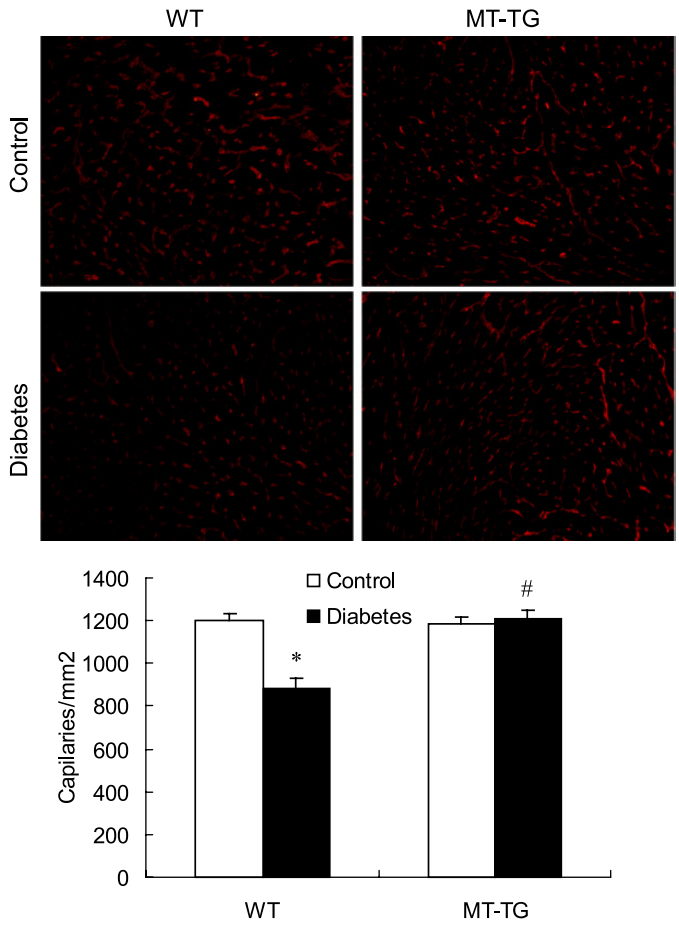

Fig. 6. Effects of MT on cardiac capillary density. *Top*: capillary density of WT and MT-TG heart sections was assessed by CD31 staining. Original magnification: ×200. Bars = 50 μm. *Bottom*: quantification of capillary numbers/mm^2. *$P < 0.05$ vs. control WT mice; #$P < 0.05$ vs. diabetic WT mice.

The major finding of this study was that cardiac MT overexpression-attenuated diabetes decreased HK-II mRNA and protein levels. Impaired regulation of glucose utilization is a main feature in the heart of both type 1 and type 2 diabetes (3, 22). We have shown that one of the regulators of glycogen synthesis, glycogen synthase kinase-3, is inactivated by MT in the diabetic heart (40). Since HK-II is the initial enzyme in the glycolysis pathway and plays a critical role in glucose metabolism, defective HK-II activity represents reduced glucose utilization in the diabetic heart. It is well known that HIF-1 is a major transcription factor of HK-II; HIF-1α dysfunction may lead to reduced HK-II expression. Indeed, studies have shown that sustained hyperglycemia induced by diabetes has a profound impact on HIF-1 activation in several cell types, such as endothelial cells (6) and muscle cells (15), and that several pharmacological interventions can increase HIF-1 activity in diabetic subjects. The present study shows that MT is one of the inducers of HIF-1 activity and that this induction leads to an increase of HK-II and glucose utilization.

Although diabetes does not alter mRNA levels of HIF-1α and HK-II, protein levels of HK-II and nuclear HIF-1α are significantly decreased in diabetic hearts. HIF-1α is a protein whose activity is mainly controlled at the protein level. After being synthesized, HIF-1α will not translocate into the nucleus and will be quickly degraded under normoxic conditions or when other inducers are absent. It seems that diabetes damages HIF-1 activity not by reducing HIF-1α transcription but by HIF-1α protein nuclear translocation. The diabetes-decreased HIF-1α nuclear protein level did not lead to lowered HK-II mRNA expression but to decreased HK-II protein levels. This differential regulation of HK-II mRNA and protein is likely due to defected HK-II protein synthesis in the STZ-induced diabetic heart. A previous study (26) has demonstrated that insulin mediates HK-II protein synthesis in skeletal muscle cells. STZ-induced diabetes is caused by defective β-cell function and consequently reduced insulin production. Therefore, it is likely that the low insulin concentration in WT mice decreased HK-II protein synthesis, although the mRNA level was not affected.

Numerous studies have demonstrated reduced expression of VEGF and its receptors and defected angiogenesis in the diabetic heart in humans and experimental animals (9, 34). Since HIF-1α is the major transcription factor of VEGF and receptors, stimulation of HIF improves angiogenesis in the diabetic heart. Our previous study (13) showed that MT increases VEGF expression in cardiac cells. This increase is likely to be mediated by MT induction of HIF-1 activity. The present study further demonstrated that MT induction of HIF-1 activity results in enhanced angiogenesis under diabetic conditions.

Several studies have shown the interaction between MT and HIF-1. Hypoxia induces MT expression in a HIF-1α-dependent manner in cancer cells, which is mediated by metal transcription factor-1, a major transcription factor of MT genes (24, 25). On the other hand, MT-induction of HIF-1α expression has been demonstrated in brain and kidney cells. In brain endothelial cells, MT induces HIF-1α activation, leading to an upregulation of VEGF via the phosphatidylinositol 3-kinase/Akt signaling pathway (19). In tubular endothelial cells, the addition of MT to culture media increased HIF-1-dependent transcriptional activity without changes in mRNA levels, suggesting HIF-1α protein stabilization (20). In this study, we demonstrated that diabetes did not affect HIF-1α mRNA expression, but it significantly reduced HIF-1α nuclear protein levels. The changes in mRNA levels of HIF-1α in the MT-TG heart, however, were moderate. In contrast, zinc treatment induces a significant increase in HIF-1α mRNA expression in high-glucose media-cultured H9c2 cells. This discrepancy may be attributed to the involvement of other zinc-dependent regulators, such as zinc-dependent transcription factor-1 and metal transcription factor-1, in addition to MT induction by zinc.

Under normoxic conditions, HIF-1α protein undergoes rapid proteasomal degradation. Hypoxic conditions inhibit this process and lead to HIF-1α protein accumulation in the nucleus. Our data showed that high-glucose treatment led to a decrease of hypoxia-induced HIF-1α nuclear protein levels and that the addition of MT resulted in an increase of nuclear HIF-1α protein, suggesting a role of MT in hyperglycemia-regulated HIF-1α nuclear localization. It is still unclear how high glucose inhibits and MT promotes HIF-1α nuclear translocation, but studies have demonstrated that hyperglycemia causes p53 activation (14) , which may inhibit HIF-1α activation (2), and MT overexpression captures free zinc and may inactivate p53, a zinc-dependent protein (23). In addition, HIF-1α protein methylgloxalation by high glucose concentration likely inhibits HIF-1α nuclear translocation and activation as well as proteasomal protein degradation pathways (1, 33). Further studies are needed to elucidate the potential mechanisms of HIF-1α regulation by high glucose and MT in diabetic subjects.

In summary, the data presented in this study suggest that the increase of HK-II by MT is likely through the activation of HIF-1α via enhanced nuclear localization under diabetic conditions. Increased glycolysis and angiogenesis may contribute to the protection of MT against diabetic cardiomyopathy via a HIF-1-mediated mechanism.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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