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Metallothionein rescues hypoxia-inducible factor-1 transcriptional activity in cardiomyocytes under diabetic conditions

Wenke Feng [a], Yuehui Wang [a], Lu Cai [a], Y. James Kang [a,b,*]

[a] Department of Medicine, University of Louisville School of Medicine, 511 S. Floyd Street, MDR532, Louisville, KY 40202, USA
[b] Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40202, USA

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Abstract

Metallothionein (MT) is effective in the prevention of diabetic cardiomyopathy, and hypoxia-inducible factor-1 (HIF-1) is known to control vascular endothelial growth factor (VEGF) gene expression and regulate angiogenesis in diabetic hearts. We examined whether or not MT affects HIF-1 activity in the heart of diabetic mice and in the cardiac cells cultured in high glucose (HG) media. Diabetes was induced by streptozotocin in a cardiac-specific MT overexpressing transgenic mouse model. The primary cultures of neonatal cardiomyocytes and the embryonic rat cardiac H9c2 cell line were cultured in HG media. HIF-1 and VEGF were determined by immunofluorescent staining and enzyme-linked immunosorbent assay, respectively. The H9c2 cells were transfected with a hypoxia-responsive element-dependent reporter plasmid and the HIF-1 transcriptional activity was measured by luciferase reporter assay. MT overexpression increased HIF-1α in diabetic hearts. HG suppressed $CoCl_2$-induced VEGF expression in primary cultures of neonatal cardiomyocytes and MT overexpression suppressed the inhibition. The addition of MT into the cultures of H9c2 cells relieved the HG suppression of hypoxia-induced luciferase activity. This study indicates that MT can rescue HIF-1 transcriptional activity in cardiomyocytes under diabetic conditions.

Keywords: Diabetes; Metallothionein; HIF-1; VEGF; Cardiomyocytes

It has become increasingly evident that hypoxia plays an important role in all diabetes complications [1]. Sustained damage to endothelial cells by hyperglycemia ultimately leads to cell loss, reduced blood flow, hypoxia, and tissue ischemia [2,3]. Hypoxia-inducible factor-1 (HIF-1) is a transcriptional factor that functions as a major regulator of oxygen homeostasis. HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β subunits [4]. HIF-1β is constitutively expressed, whereas HIF-1α is controlled by protein degradation mediated by prolyl hydroxylation and pVHL binding [5]. Under hypoxic conditions, HIF-1α is stabilized, translocates into nucleus, dimerizes with HIF-1β, and up-regulates genes involved in the angiogenesis, glycolytic energy metabolism, cell proliferation, and survival [6,7].

In addition to hypoxic stimulus, a variety of factors can stabilize HIF-1α, such as some transition metals, insulin, insulin-like growth factor (IGF), and advanced glycosylation end product [8–10]. Hyperglycemia is the metabolic hallmark of diabetes, which has been shown to be a major cause of diabetic cardiomyopathy. Recent studies have shown that hyperglycemia suppresses HIF-1 function [11].

We have shown that overexpression of metallothionein (MT) by zinc or through transgenics prevents diabetic cardiomyopathy [12–14]. MT is a small protein rich in cysteine content primarily for essential metal homeostasis and heavy metal detoxification. MT also plays important roles in other biological functions, such as anti-oxidation. Some

* Corresponding author. Address: Department of Medicine, University of Louisville School of Medicine, 511 S. Floyd Street, MDR532, Louisville, KY 40202, USA. Fax: +1 502 852 6904.
E-mail address: yjkang01@louisville.edu (Y.J. Kang).

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studies have shown that the absence of MT results in a reduction of the expression of several angiogenic factors in mice, such as vascular endothelial growth factor (VEGF) [15]. Down-regulation of VEGF in the heart is associated with the increased risk of cardiovascular morbidity and mortality in patients with diabetes [16].

Given that the expression of VEGF is regulated by transcriptional factor HIF-1 [17], it is interesting to investigate the role of MT in the regulation of HIF-1 activation. In the present work, we investigated the role of MT in transcriptional activity of HIF-1 in diabetic mouse hearts and in cardiac cells cultured in high glucose (HG) media. MT overexpression increased HIF-1α protein level and enhanced HIF-1 transcriptional activity in cardiomyocytes under diabetic conditions.

Research design and methods

Animals. Cardiac MT overexpressing transgenic (MT-TG) mice were produced as described in our previous study [18]. Wide type (WT) control mice (FVB) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in the University of Louisville Research Resources Center at 22 °C with a 12-h light/dark cycle and free access to rodent chow and tap water. All animal procedures were approved by the Institutional Animals Care and Use Committee of the University of Louisville. Eight-week-old male mice were intraperitoneally given a single dose of streptozotocin (STZ; Sigma Chemical Co., St. Louis, MO) at 150 mg/kg body weight, dissolved in sodium citrate buffer (pH 4.5). On day 3 after STZ treatment, whole blood glucose obtained from mouse tail-vein was detected using a SureStep complete blood glucose monitor (LifeScan, Milpitas, CA). STZ-treated mice with whole blood glucose equal to and higher than 250 mg/dl were considered as diabetic. Mice serving as vehicle controls were given the same volume of sodium citrate [13]. Experimental measurements were performed in the control and diabetic mice 2 weeks after STZ treatment.

Cell culture. H9c2 cells were maintained in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum in a humidified atmosphere (5% CO_2) at 37 °C. Cells were grown to 40–50% confluency, the cultures were exposed to D-glucose (Sigma Chemical Co.) in a final concentration of 25 or 5.5 mM as control for 48 h and then incubated under hypoxia (1% O_2, 5% CO_2, and balanced with N_2) or with $CoCl_2$ (100 μM) for 16 h. Metallothionein II (Sigma) was added to the culture along with glucose at a concentration of 100 nM. In HIF-1 transactivity assay experiments, the cells were transfected with reporter plasmid pH3SVL containing luciferase gene [19] before treatment with glucose. After treatments, the monolayer cultures were collected using a cell policeman and then lysed. Neonatal cardiomyocytes from MT-TG and WT mice were isolated as described previously [20]. Cells were then treated as described for H9c2 cells.

Immunohistochemical assay of HIF-1α. Expression of cardiac HIF-1α in the hearts of control and diabetic WT and MT-TG mice was examined by immunohistochemical staining with polyclonal rabbit anti-HIF-1α antibody (Santa Cruz Biotech, Santa Cruz, CA) as primary antibody, followed with anti-rabbit IgG, H&L Cy3 conjugate (Abcam, Inc., Cambridge, MA) for heart tissues and anti-rabbit IgG H&L (FITC) (Abcam) for cultured cells as second antibody, respectively.

Reporter gene assay. H9c2 cells were transfected with a construct pH3SVL containing a total of six HIF-1 binding sites derived from the transferrin hypoxia-responsive element [19] by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. After recovering and treatment described above, the cells were washed with PBS, and then lyzed with the Passive Lysis Buffer provided by Promega. The protein concentration was measured by Bio-Rad protein assay reagent. The luciferase activity was determined with the luciferase assay system (Promega, Madison, WI) and normalized to total cellular protein.

VEGF expression. The VEGF levels in the media of the cell cultures were measured by commercial human enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D Systems, Minieapolis, MN).

Statistics. Data were expressed as means ± SD values and analyzed by ANOVA followed by a Duncan's multiple-range test for further determination of the significance of differences. Differences among groups were considered significant when $p < 0.05$.

Results

The effect of MT overexpression on HIF-1α levels in mouse hearts under diabetic conditions was examined in mice treated with STZ. Two weeks after STZ treatment, blood glucose levels were increased to above 250 mg/dl, occurring in about 60% of the STZ-treated mice that were considered diabetic. The STZ-treated mice that did not show high blood glucose levels were used as non-diabetic controls. There was no detectable difference in the intensity of immunohistochemical staining of HIF-1α between the non-diabetic and diabetic WT mouse hearts. Two weeks after STZ treatment, however, the intensity of HIF-1α staining in the MT-TG diabetic mice was increased about three times higher than that in the WT diabetic hearts (Fig. 1).

We then examined the effect of MT on VEGF, whose expression is primarily under the control of HIF-1, using primary cultures of neonatal cardiomyocytes (Fig. 2). HG and $CoCl_2$ were used to mimic diabetic and hypoxic condi-

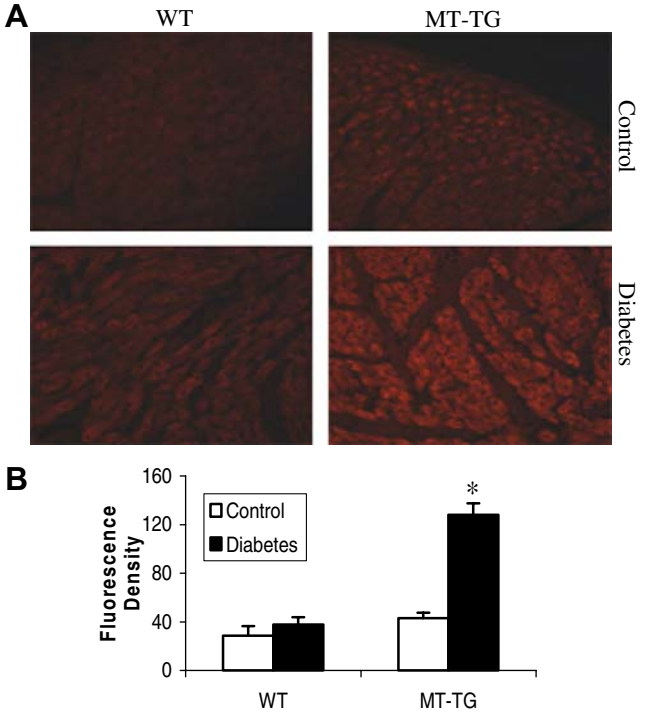

Fig. 1. MT induces HIF-1α expression in diabetic heart. (A) Immunofluorescent staining of HIF-1α was performed in the hearts of WT and MT-TG diabetic and control mice two weeks after STZ treatment. (B) Histogram of quantitative analysis of the density of fluorescence in (A). *Significant vs. WT and control.

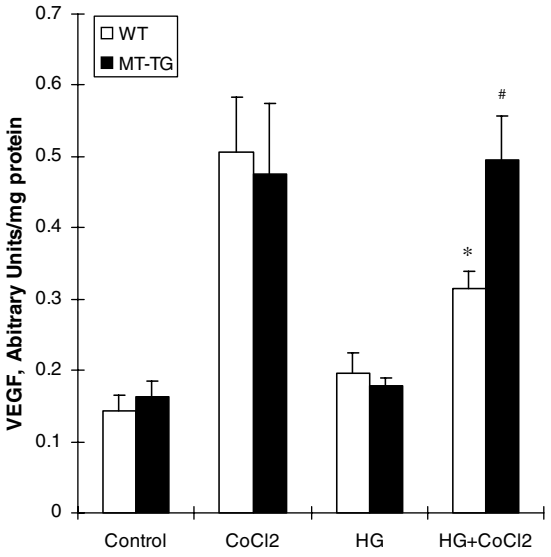

Fig. 2. Effect of high glucose and MT on VEGF expression. Neonatal mouse cardiomyocytes from WT and MT-TG mice were incubated with glucose in the concentrations indicated in the section of Research design and methods for 48 h, and then with 100 μM $CoCl_2$ for 16 h for hypoxia mimicry. VEGF levels in the culture supernatants were measured by ELISA. Data are means ± SD of at least three separate culture dishes from one representative experiment performed at least three times. *Significant vs. $CoCl_2$, #significant vs. WT.

tions, respectively. $CoCl_2$ markedly increased VEGF expression in both WT and MT-TG cardiomyocytes. However, HG suppressed the $CoCl_2$-stimulated VEGF expression in the WT, but not in the MT-TG cardiomyocytes (Fig. 2).

The effect of MT on HG inhibition of HIF-1 transcriptional activity was determined using a luciferase reporter assay. H9c2 cells were transfected with a pH3SVL reporter plasmid. As shown in Fig. 3, HG significantly decreased hypoxia-induced luciferase activities. Interestingly, when the HG-cultured cells were exposed to MT, the HG suppression of HIF-1 transcriptional activity was relieved.

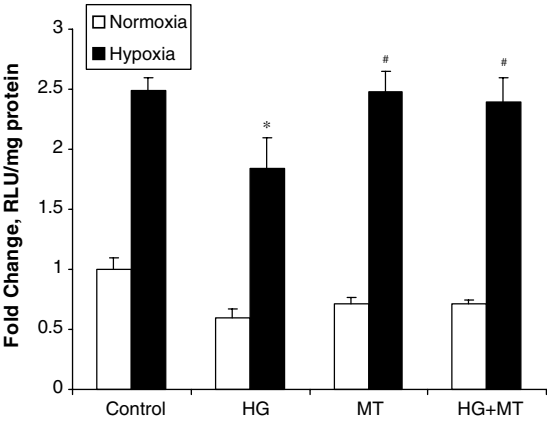

Fig. 3. Induction of HIF-1 transcriptional activity. H9c2 cells were transfected with pH3SVL luciferase reporter plasmid containing the luciferase gene under control of six HIF-1 binding sites from the transferrin 3′ enhancer, and then the cells were treated with glucose (30 μM) or MT (100 nM) for 48 h before incubation with $CoCl_2$ (100 μM) for 16 h for induction of HIF-1 transactivity. Luciferase activities were expressed as-fold induction compared with untreated normoxic cells. Data are means ± SD of at least three separate culture dishes from one representative experiment performed at least three times. *Significant vs. control, #significant vs. HG.

Discussion

Our previous studies have demonstrated that MT prevents diabetic cardiac complications. MT inhibits diabetes-induced early myocardial apoptosis through suppression of mitochondrial oxidative stress leading to a prevention of the development of diabetic cardiomyopathy [12]. Here, we report that MT overexpression increases HIF-1α in the diabetic hearts. Under hypoxia conditions, which would be accompanied with diabetic cardiomyopathy, HIF-1 activity increased, as demonstrated in primary cultures of cardiomyocytes and H9c2 embryonic rat cardiac cells. In the presence of high glucose, HIF-1 activity was suppressed. The recovery of HIF-1 activity in the presence of MT suggests that MT can rescue HIF-1 under high glucose conditions. This finding suggests a new mechanism by which MT protects the heart from diabetic complications.

The α-subunit of HIF-1 plays a pivotal role in adaptive responses of cells to hypoxia or other stress conditions. It has been shown that diabetic complications in the heart generate a pseudohypoxia condition, which would increase the stability of HIF-1α and increase HIF-1 transcriptional activity. However, hyperglycemia has been shown to inhibit hypoxia-induced HIF-1α stabilization in several cell types. In the present study, we observed that HIF-1α in diabetic WT mouse heats was not increased, indicating the dominant effect of hyperglycemia over pseudohypoxia on HIF-1α in the myocardial diabetic complication. MT overexpression appears not to affect the level of HIF-1α in the non-diabetic heart, but to overcome the inhibitory effect of hyperglycemia on HIF-1α in the diabetic mouse heart.

The *in vivo* observation was verified by *in vitro* studies. In the primary cultures of neonatal mouse cardiomyocytes, the expression of VEGF, a gene controlled directly by HIF-1, was significantly increased in response to $CoCl_2$ treatment, a condition mimicking hypoxia. Addition of high levels of glucose significantly suppressed $CoCl_2$-induced VEGF expression. MT overexpression completely rescued the VEGF expression in the presence of high levels of glucose. Further studies using the HIF-1-specific reporter gene-transfected H9c2 cells also demonstrated the rescuing effect of MT on high levels of glucose inhibition of hypoxia-induced HIF-1 activation.

It has been shown that down-regulation of myocardial VEGF expression preceded all other features of diabetic cardiomyopathy and was followed by an increased apoptosis of endothelial cells, decreased numbers of circulating endothelial progenitor cells, decreased capillary density, and impaired myocardial perfusion [21,22]. VEGF gene delivery to myocardium with diabetic complications

showed an increase in capillary density, decreased endothelial cell and cardiomyocyte apoptosis, and eventually improvement of cardiac function [22]. Our finding in the present study indicates MT plays an important role in VEGF expression in the heart under diabetic conditions through restoration of HIF-1 transcriptional activity. This finding is in an agreement with a previous observation that MT plays a major regulatory role in the angiogenesis process [15].

How can MT regulate HIF-1 function? Many studies have demonstrated that MT functions in cellular protection from oxidative stress. The accumulation of reactive oxygen species (ROS) under HG conditions has been proposed [23]. ROS has been known to destabilize HIF-1α. It is thus possible that MT, by suppressing ROS accumulation, prevents the inhibitory effect of ROS on HIF-1 under diabetic conditions. This scenario needs to be demonstrated in future studies.

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