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

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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

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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-

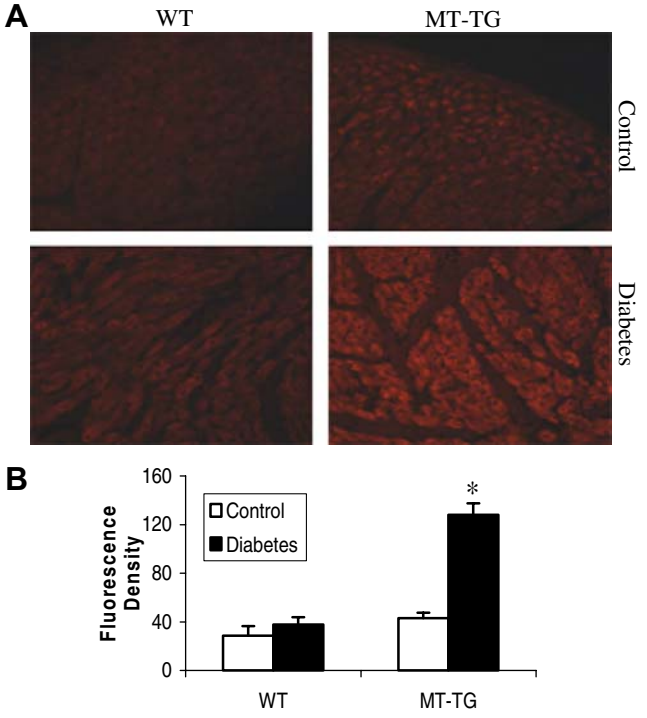


Fig. 1. MT induces HIF-1α expression in diabetic heart. (A) Immunofluorescent staining of HIF-1α was performed in the hearts of WT and MT-TG diabetic and control mice two weeks after STZ treatment. (B) Histogram of quantitative analysis of the density of fluorescence in (A). *Significant vs. WT and control.