High-glucose-induced metallothionein expression in endothelial cells: an endothelin-mediated mechanism

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Apostolova, Margarita D., Shali Chen, Subrata Chakrabarti, and M. George Cherian. High-glucose-induced metallothionein expression in endothelial cells: an endothelin-mediated mechanism. Am J Physiol Cell Physiol 281: C899–C907, 2001.—Vascular endothelial cells are constantly exposed to oxidative stress and must be protected by physiological responses. In diabetes mellitus, endothelial cell permeability is impaired and may be increased by high extracellular glucose concentrations. It has been postulated that metallothionein (MT) can protect endothelial cells from oxidative stress with its increased expression by cytokines, thrombin, and endothelin (ET)-1. In this study, we demonstrate that high glucose concentration can induce MT expression in endothelial cells through a distinct ET-dependent pathway. Exposure of human umbilical vein endothelial cells (HUVEC) to increasing concentrations of glucose resulted in a rapid dose-dependent increase in MT-2 and ET-1 mRNA expression. MT expression may be further augmented with addition of ET-1. Preincubation of the cells with the specific ET<sub>A</sub> antagonist BQ-788 blocked MT-2 mRNA expression more effectively than the ET<sub>B</sub> inhibitor TBC-11251. High glucose also increased immunoreactive MT protein expression and induced translocation of MT into the perinuclear area. Perinuclear localization of MT was related to high-glucose-induced reorganization of F-actin filaments. These results demonstrate that an increase in extracellular glucose in HUVEC can lead to a rapid dose-dependent increase in MT-2 mRNA expression and to perinuclear localization of MT protein with changes to the cytoskeleton. These effects are mediated via the ET receptor-dependent pathway.

ONE OF THE MAJOR PROBLEMS in diabetes mellitus is the disruption of glucose homeostasis, which is normally maintained by a balance of hepatic glucose production, and cellular glucose uptake and metabolism (8). Sustained hyperglycemia in diabetes may alter expression of vasoactive substances in the target organs of diabetic complications through a variety of mechanisms (16).

It has been well established that the endothelium plays a vital role in the regulation of reactivity of vascular tissues by releasing the endothelium-derived factors that act on adjacent smooth muscle cells (40). One such factor produced by vascular endothelial cells is the potent vasoconstrictive peptide endothelin (ET)-1, which is regulated by a number of inflammatory and vasoactive mediators (13, 26, 29). Regulation of ET-1 secretion by these mediators is critical in the maintenance of vascular tone in certain pathophysiological conditions. Recently, several reports have shown that insulin stimulates ET-1 secretion from endothelial cells and also enhances ET-1 binding to its receptors (35). It also has been shown that plasma ET-1 levels are elevated in patients with type II diabetes mellitus with microvascular complications, suggesting that ET-1 may be involved in diabetes-related complications such as microangiopathy (24).

The 21-amino acid ET-1 peptide is derived by proteolytic processing of prepro-ET-1, a 212-amino acid precursor protein (46). ETs are a family of three oligopeptides that includes ET-1, ET-2, and ET-3 (17). These peptides interact with a group of specific receptors such as ET<sub>A</sub>, ET<sub>B</sub>, and ET<sub>C</sub> (28, 41). The mechanism of ET expression in both constitutive and induced conditions is important for elucidating the role of ET in various diseases. Changes in ET-1 expression may affect blood flow, extracellular matrix protein synthesis, and expression of other molecules of physiological importance (32).

It has been suggested (21) that ET-1 may modulate the expression of metallothionein (MT), a zinc- and copper-binding protein (19). MT genes are expressed in most tissues and organisms, and the transcription of these genes is regulated by metals, growth factors, glucocorticoids, cytokines, and stress conditions (10, 22, 23). In experimental diabetes mellitus, increased pancreatic and hepatic MT expression has been demonstrated (6, 11, 47, 50). However, the role of MT and zinc in diabetes is not well understood. Increased expression of MT in response to cadmium, cytokines, thrombin, and ET-1 has been reported in cultured human umbilical vein endothelial cells; TBC-11251; BQ-788.

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with the use of commercially available horseradish peroxidase-conjugated secondary anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and developed with chemiluminescent substrate (Amersham Pharmacia Biotechnology, Amersham, UK).

Preparation of oligonucleotide primers and PCR. Oligonucleotide primers for human MT-2, ET-1, and β-actin were obtained from Life Technologies. MT-2 primer sequences were as follows: 5'-CTCTTCAGCAAGCAATTGGA-3' (sense) and 5'-CGCTTCTTACATCTGGGA-3' (antisense). The ET-1 primer sequences were as follows: 5'-GGACATCATTTGGGGAACTCC-3' (sense) and 5'-CAAGCTTGAAAGGCTTCTT-3' (antisense). The sequences of rat β-actin primers were as follows: 5'-CTCTTATGCACACAGTGC-3' (sense) and 5'-CATCAGCTACTGTGAGTGC-3' (antisense). The predicted sizes of the amplified products (cDNA) were 203 bp for MT-2, 269 bp for ET-1, and 210 bp for β-actin, respectively.

PCR for MT-2 and ET-1 were performed in accordance with methods described by Apostolova et al. (2) and Deng et
al. (9), respectively. The intensity of cDNA bands was analyzed by densitometry using Mocha (Jandel Scientific, San Rafael, CA) software. mRNA levels are expressed in arbitrary units, which are normalized with the standard probe of β-actin.

Data analysis. Statistical evaluation of data was performed by one-way analysis of variance with Origin 5.0 (Microcal Software, Northampton, MA) using Bonferroni’s multiple comparison, and results were considered statistically significant if \( P < 0.05 \). The results are presented as means ± SE.

RESULTS

Cell viability and proliferation. When the HUVEC were grown in increasing concentrations of either glucose or 2-DG (25, 50, and 100 mmol/l), there was an increase in cell growth for 64 h in the cells treated with glucose only (Fig. 1). At 72 and 96 h, there was no change in cell numbers for cells grown in glucose, but there was a decrease in cell viability for cells grown in 2-DG as shown in Fig. 1, A and B.

Effects of high concentration of glucose on MT-2 mRNA. The changes in MT-2 mRNA levels in HUVEC with increasing concentration of glucose or 2-DG (5–100 mmol/l) are shown in Fig. 2, A and B. There was a dose-dependent increase in MT-2 mRNA after exposure to glucose (25–100 mmol/l) as determined by RT-PCR. The increase was ~2-fold with 25 mmol/l glucose and >10-fold with 100 mmol/l glucose at 64 h. No such increase in MT-2 mRNA was observed after exposure to 2-DG (Fig. 2B).

The increase in MT-2 mRNA level after incubation with 25 and 50 mmol/l glucose was time dependent (Fig. 2C). The increase in MT-2 mRNA level was obtained at ~4 h after exposure to glucose and continued at 5 and 6 h. Analysis of these data shows that the increase in MT-2 mRNA was about four- to fivefold. To determine whether the induction of MT-2 mRNA with high glucose was under transcriptional control, the cells were incubated with actinomycin D and 5 or 25 mmol/l glucose. The results show a decline in MT-2 mRNA in control cells with 5 mmol/l glucose and actinomycin D (50% reduction). In addition, actinomycin D blocked the induction of MT-2 mRNA expression with 25 mmol/l glucose (Fig. 2D), suggesting a transcriptional control.

Effect of high concentrations of glucose on ET-1 mRNA level. Incubation of HUVEC with increasing concentrations of glucose (5–100 mmol/l) resulted in a
dose-dependent increase in ET-1 mRNA (Fig. 3). The mRNA transcript analyzed by RT-PCR showed a maximum increase after treatment with 25 mmol/l glucose for 64 h. This increase was about twofold compared with the ET-1 mRNA level for 5 mmol/l glucose. No such increase was observed when cells were incubated with 25 mmol/l 2-DG (data not shown).

Role of ET-1 in MT expression. To investigate the direct role of ET-1 in MT expression, we examined the expression of MT-2 mRNA after exposure to 25 mmol/l glucose in the presence or absence of ET-1. The results showed that addition of ET-1 to cells grown in 5 mmol/l glucose can induce the MT-2 mRNA expression in a concentration-dependent manner (Fig. 4A). An additive effect of MT-2 mRNA expression was observed when the HUVEC were treated with both ET-1 (0.25–0.75 μmol/l) and 25 mmol/l glucose (Fig. 4B). When HUVEC treated with 25 mmol/l glucose were coincubated with TBC-11251, a specific antagonist of the ETA receptor, at three different concentrations (0.5, 1, and 10 μmol/l), a partial block in glucose-induced MT-2 mRNA expression was observed (Fig. 4C). At concentrations >1 μmol/l, this antagonist can block both ETA and ETB receptors (44). To distinguish the subtype of ET receptors involved in MT-2 mRNA expression with high glucose, we treated the HUVEC grown in 25 mmol/l glucose with 250 nmol/l BQ-788, a specific inhibitor of ETB receptor. This treatment also decreased the MT mRNA expression (Fig. 4D) similarly to that observed with 10 μmol/l TBC-11251.

Effect on MT protein induction. The results in MT mRNA expression are supported by changes in MT protein levels as shown by Western blot analysis with a monoclonal MT-2 antibody (Fig. 5). The basal level of MT in HUVEC grown in 5 mmol/l glucose was increased more than threefold after addition of ET-1 (0.5 μmol/l) or by growing the cells in 25 mmol/l glucose. This increase in MT protein was blocked after addition of both inhibitors (10 μmol/l TBC-11251 and 250 nmol/l BQ-788) of ET receptors. These results demonstrate that both MT mRNA and protein expressions are increased with high glucose and ET but can be blocked by inhibition of ET receptors.

Effects of high glucose and ET-1 on subcellular localization of MT. Immunofluorescence confocal microscopy showed that HUVEC cultured in normal medium with 5 mmol/l glucose or 2-DG had immunoreactive MT randomly distributed in the cytoplasm (Fig. 6A). The increase in MT immunoreactivity with high glucose was concentration dependent. The cells treated with 25 and 50 mmol/l glucose exhibited a higher intensity of immunofluorescence for MT (green) in the perinuclear area (Fig. 6, B and C), indicating a translocation of MT to the perinuclear area.

ET-1 alone (Fig. 7, B and C) or in combination with glucose (Fig. 7, D and E) increased MT fluorescence in the nuclear and perinuclear area. Sections obtained by laser scan microscopy in a vertical direction clearly demonstrated the presence of MT in the nucleus and perinuclear area (Fig. 7, F and G). The increased MT immunofluorescence after incubation with 25 mmol/l glucose (Fig. 8B) was blocked by addition of ET receptor antagonists TBC-11251 (10 μmol/l; Fig. 8C) and BQ-788 (250 nmol/l; Fig. 8D).

Fig. 4. MT-2 mRNA levels in HUVEC. A: effect of variable concentration of ET-1 (0.25, 0.5, and 0.75 μmol/l) in 5 mmol/l glucose on MT-2 mRNA expression for 64 h. B: effect of cotreatment of HUVEC with different concentrations of ET-1 (0.25, 0.5, and 0.75 μmol/l) and 25 mmol/l glucose on MT-2 mRNA expression for 64 h. C: HUVEC treated with 25 mmol/l glucose with or without TBC-11251 (top) or BQ-788 (bottom) for 64 h.
Effect of high glucose on actin cytoskeleton organization. At glucose concentrations of 5 mmol/l, HUVEC showed the typical normal distribution of actin filaments with most of the actin filaments arranged along the periphery of the cells and only a few filaments in the middle (Figs. 6 and 9A). High glucose caused major changes in the microfilament arrangement that were characterized by reorganization of F-actin from cortical microfilaments into transcytoplasmic stress fibers (Fig. 9, B and C). The stress fiber formation upon exposure to high glucose concentration was associated with the relocalization of MT in perinuclear area (Fig. 6, B and C). Treatment of the cells with 2-DG had no effect on either loss of membrane

Fig. 6. Subcellular localization of MT in HUVEC. The cells were treated with glucose and 2-DG for 64 h. A: 5 mmol/l. B: 25 mmol/l. C: 50 mmol/l. HUVEC were triple labeled with polyclonal antibody against MT (green), Alexa Fluor 568 phalloidin for F-actin (red), and 4,6-diamidino-2-phenylindole (DAPI; blue) for DNA. Arrows indicate MT localization in cytoplasm (solid) and in the perinuclear area (open). Bars, 50 μm.
protrusions or distribution of F-actin and localization of MT (Figs. 6 and 9, D–F).

DISCUSSION

The results reported in this study demonstrate a novel mechanism of induction of MT, an intracellular zinc-binding protein, that can regulate the bioavailability of zinc and may influence the redox status of the cell (1). The synthesis of MT can be induced by various stress conditions, and these proteins are also considered acute phase proteins (18). In this study, we have examined the expression of MT-2 mRNA, MT-2 protein, and the subcellular localization of MT in HUVEC after incubation with high glucose (>5 mmol/l). The results demonstrate an increased expression of MT and its translocation to the perinuclear area when HUVEC are treated with high glucose. Furthermore, these changes are probably mediated by an ET receptor-dependent pathway, since they can be prevented by ET receptor blockade. The study also shows that the induced synthesis of MT with high glucose concentrations is under transcriptional control.

In cells, several biochemical changes can occur secondarily to hyperglycemia, including augmented polyol-myoinositol-related metabolic defects, nonenzymatic glycation, augmented protein kinase C (PKC) activity, and altered redox potential (25, 27). The PKC activation may affect the expression of several vasoactive factors such as ET-1 and vascular endothelial growth factor (9, 25). Endothelial damage and altered endothelium-derived factors can induce vascular complications in diabetes (27). The changes in ET-1 expression have been demonstrated in both kidneys and heart (32). Increased ET-1 expression can have several effects, including increased extracellular matrix protein gene expression. The induction of MT in HUVEC may be one of these effects.

In this study, we also found an additive effect on MT-2 mRNA and protein expression when the HUVEC were incubated with increasing concentrations of ET-1 in 25 mmol/l glucose. Because ET-1 can stimulate glucose uptake in different cell lines (38, 45), the additive effect on MT expression could be related to an increase in glucose uptake by HUVEC.

The increased MT-2 mRNA expression that results from incubation with high glucose concentrations appears to be mediated by the ET_A and ET_B receptor-dependent pathway. Blocking of either ET_A or ET_B...
receptors can inhibit the increase in MT-2 mRNA and protein levels (Figs. 4 and 5). This effect is probably mediated through the ET<sub>B</sub> receptor. The results show that BQ-788 is 40 times more effective than TBC-11251 in reducing glucose-induced MT-2 expression. Incubation of cells with 2-DG did not cause any changes in MT-2 expression. These results demonstrate conclusively that the induction of MT in HUVEC by high glucose is directly related to ET and its receptors.

The exact role of increased synthesis of MT or its nuclear and perinuclear localization after exposure to high glucose is not yet understood. However, these changes may be related to the observed cytoskeleton activation and reorganization (Figs. 5 and 8). Alterations in F-actin filament assembly have been observed in cultured 3T3-L1 mesangial cells and HUVEC in response to increased glucose levels (7, 43, 49). Thus, the changes in MT localization in the perinuclear area can be related to decreased resistance to shear force in diabetic endothelial cells. The alterations in cell shape and permeability also occur with changes in intracellular actin pattern (43a, 49). All these metabolic changes may result in increased endothelial cell permeability, which is characteristic of diabetes mellitus (7).

However, it is unclear whether the changes in MT synthesis and localization can alter either intracellular or membrane zinc levels, which can affect membrane properties. It has already been shown that zinc can protect free radical-induced apoptosis in endothelial cells (15, 30). The induction of MT synthesis, observed in our present study, can be considered as a stress response to protect the endothelial cells from high-glucose exposure, and it is mediated by ET-1.

There are reports on endothelial dysfunction and the impairment of nitric oxide (NO) release by endothelial cells in diabetes mellitus (12). A reduction in NO-dependent vasodilation of mesenteric arteries has been demonstrated in diabetic rats (39). Studies have shown that NO production can modulate MT expression in mouse liver and brain under both normal and stress conditions (31). MT can directly react with NO, resulting in release of zinc, and may regulate NO signaling in endothelial cells (3, 34). Hence, there may be a feedback regulatory mechanism among ET-1, NO, and MT.

In summary, we demonstrate a novel mechanism on induction of MT synthesis involving ET receptors in endothelial cells when exposed to high glucose concentrations. However, further studies are required to un-
understand the physiological significance of these metabolic changes in these cells.

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