

Glucose-induced regulation of novel iron transporters in vascular endothelial cell dysfunction

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Abstract

Increased iron indices have been associated with the development of diabetes and its complications. In the present study, we have investigated the glucose-induced alteration of iron transporters, divalent metal transporter-1 (DMT-1), iron regulated transporter protein-1 (IREG-1), and transferrin receptor (TfR), in endothelial cell iron accumulation and oxidative stress. Cells were exposed to high glucose levels and subjected to gene expression, protein expression, iron measurement and assessment of oxidative stress. Our results show, for the first time, expression of DMT-1 and IREG-1 in vascular endothelial cells. Our data further indicates upregulation of DMT-1 and IREG-1 mRNA and protein in response to high levels of glucose. TfR, however, exhibited a modest decrease in response to high levels of glucose. Increased expression of DMT-1 and IREG-1 was associated with iron accumulation and oxidative stress. Furthermore, our results show differential expression of iron transporters with treatment of high glucose-exposed cells with two different iron chelators. In conclusion, our study suggests that glucose-induced alteration of iron transporters may arbitrate iron accumulation and oxidative stress in endothelial cells.

Keywords: *Iron, oxidative stress, diabetes, vasculopathy*

Introduction

Sustained hyperglycemia from long standing diabetes leads to chronic complications involving various organs such as the retina, the kidney, and the heart [1,2]. The initiating and perpetuating factor in the development of such complications is vascular endothelial cell dysfunction [3–5]. In response to high ambient glucose levels, endothelial cells become activated. This activated state is characterized by the elaboration of vasoactive factors, growth factors and cytokines [3,4,6]. These factors lead to an alteration of hemodynamic parameters such as blood flow and

permeability [3,4,7]. In addition, autocrine and paracrine properties of these factors leads to increased production of extracellular matrix proteins [6,8,9] which is considered to be the structural hallmark of chronic diabetic complications.

The mechanism by which sustained hyperglycemia mediates adverse effects in endothelial cells includes increased reactive oxygen species (ROS) production and oxidative stress [10–12]. We have previously demonstrated that diabetes leads to increased oxidative stress by accumulation of iron in the heart [13]. Iron, being one of the most versatile elements and cofactor in redox reactions, has been very well documented

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to increase ROS production and subsequent oxidative stress in various *in vivo* and *in vitro* models. A possible link between altered iron homeostasis and development of diabetic complications may present a novel pathway of glucose-induced vasculopathy.

The molecular mechanism by which iron is absorbed by mammalian cells includes both receptor-mediated and transporter-mediated processes [14,15]. Biological management of iron involves rapid uptake and delivery to cells via specialized transport proteins. These transport proteins include, divalent metal transporter-1 (DMT-1) and iron regulated transporter protein-1 (IREG-1; or ferroportin) [14,15]. DMT-1 is an important iron importer. DMT-1 has been shown to be expressed on the apical surface of epithelial cells and in endosomes of erythroid precursor cells [14–16]. Functional significance of DMT-1 is evident in animals carrying a mutation in DMT-1 gene which leads to severe iron deficiency and anemia [15]. IREG-1, on the other hand, is an important exporter of iron [17–19]. Localization on the basolateral surface of epithelial cells and plasma membrane of iron utilizing and storing macrophages, suggests an efficient means of export and delivery of iron [17,18]. IREG-1 or ferroportin mutations in humans result in iron overload in the hepatic macrophages [20].

In the present study, we have examined whether vascular endothelial cells express DMT-1 and IREG-1. Furthermore, we have evaluated whether these transporters are altered leading to glucose-induced oxidative stress and vascular endothelial dysfunction.

Materials and methods

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs; American Type Culture Collection, Rockville MD) and human microvascular endothelial cells (HMECs; Clonetics, Walkersville, MD) were cultured as previously described [9]. Briefly, HUVECs and HMECs were plated at approximately 2500 cells/cm² in endothelial cell growth medium (EGM; Clonetics, Walkersville MD) with 10% fetal bovine serum. Endothelial growth media contains 0.278 µg/ml iron in the form of ferrousulfate (Clonetics, Walkersville MD). Fetal bovine serum, on the other hand, contains 2.49 µg/ml iron in the form of transferrin-iron (Clonetics, Walkersville MD). To determine transcriptional regulation of iron transporters by glucose, sub-confluent cells were cultured in serum-free media for 6–8 h before exposure to 5, 15, 25 and 35 mM glucose. Following 24 h of incubation, mRNA levels of DMT-1 and IREG-1 were measured. Greatest increase in transporter level was observed with 25 mM glucose, hence, subsequent experiments were carried out by exposure of cells to either 5 or 25 mM glucose.

In order to determine whether high glucose levels also lead to alteration of transferrin receptor (TfR), mRNA levels of TfR in the cells were also quantitated. To further study the role of intracellular iron in mediating glucose-induced oxidative stress, HUVECs exposed to high levels of glucose were treated with iron chelators, cell membrane permeable ferrous iron chelator dipyriddy (DP; 100 µM) or cell impermeable ferric iron chelator desferrioxamine (DFO; 100 µM). Concentrations of these chelators were in accordance with previous studies in endothelial cells [21]. Iron chelator treatments were initiated 30 min before exposure of the cells to high glucose concentrations and continued for 24 h.

Iron measurement in endothelial cells

Iron in the endothelial cells was measured by Finnigan MAT Element high resolution-inductively coupled plasma-mass spectrometry (HR-ICP-MS) (Thermo-Quest, San Jose, CA) in accordance with manufacturer's recommendations. Briefly, endothelial cells were lysed and total proteins were measured by BCA protein assay (Pierce Endogen, Rockford IL, USA) to account for differences in the number of cells. Iron content was measured essentially as described previously [22]. Iron was then expressed as µg iron/mg total protein.

RNA isolation and cDNA synthesis

Total RNA from endothelial cells was isolated using TRIZOL™ (Invitrogen Inc., Burlington, Canada) as previously described [6,9]. Following RNA isolation, DNase treatment was carried out to degrade any contaminating DNA in the samples (Invitrogen Inc.). RNA was quantified by measuring ultraviolet absorbance at 260 nm. Purity of samples was assessed by determining OD 260:280 nm. cDNA was synthesized using 3 µg of total RNA with oligo-(dT) primers and Superscript-II™ MMLV-reverse transcriptase (Invitrogen Inc.).

Real time RT-PCR

Real time RT-PCR was performed in LightCycler™ (Roche Diagnostics Canada, Quebec, Canada) as previously described [6,8]. The reaction mixture (20 µl volume) consisted of 10 µl SYBR® Green Taq ReadyMix (Sigma-Aldrich), 1.6 µl of 25 mM MgCl₂, 1 µl of each forward and reverse 10 µM primers (Table I; [23]), 4.4 µl H₂O, and 2 µl cDNA template. The data was normalized to housekeeping gene (β-actin) to account for differences in reverse transcription efficiencies and amount of template in the reaction mixtures.

Western blotting

Total proteins from endothelial cells were isolated and quantified as previously described [6]. Briefly, cells were homogenized in complete RIPA buffer (NaCl 0.877 g, deoxycholate 1 g, 1 M Tris-HCl (pH 7.5) 5 ml, Triton

Table I. Primer sequences and PCR temperature profile.

Gene	Sequence (5' → 3')	PCR Parameters*	
DMT-1	GTGGCTACGTTAAATAACAC AGTTCTAAGGTTAGGTCAGG	Amplicon	285 bp
		Denaturation	95°C in 0 s
		Annealing	53°C in 6 sec
		Extension	72°C in 10 s
		Signal	81°C in 1 s
IREG-1	CAGTTAACCAACATCTTAGC AAGCTCATGGATGTTAGAG	Amplicon	212 bp
		Denaturation	95°C in 0 s
		Annealing	54°C in 5 s
		Extension	72°C in 12 s
		Signal	81°C in 1 s
TfR	AAGGCCAATGTCACAAAACC GGTGAAGTCTGTGCTGTCCA	Amplicon	268 bp
		Denaturation	95°C in 0 s
		Annealing	58°C in 5 s
		Extension	72°C in 11 s
		Signal	83°C in 1 s
HO-1 [22]	TGATAGAAGAGGCCAAGA TTTCCAGACAGAGGGACA	Amplicon	422 bp
		Denaturation	95°C in 0 s
		Annealing	50°C in 10 s
		Extension	72°C in 17 s
		Signal	84°C in 1 s
β-actin	CCTCTATGCCAACACAGTGC CATCGTACTCCTGCTTGCTG	Amplicon	214 bp
		Denaturation	95°C in 0 s
		Annealing	58°C in 5 s
		Extension	72°C in 8 s
		Signal	83°C in 1 s

*Initial denaturation was 1 min. Ramp rate was 20°C/s.

X-100 1 ml, and 10% sodium dodecyl sulfate 1 ml; volume adjusted to 100 ml using ddH₂O) and protease inhibitor. Total proteins were then quantified by using BCA protein assay kit (Pierce Endogen, Rockford IL, USA). Transporter protein levels were measured by DMT-1 (1:1000; Alpha Diagnostics International, San Antonio TX, USA) and IREG-1 (1:1000; Alpha Diagnostics International, San Antonio TX, USA) antibody followed by secondary antibody conjugated with horseradish peroxidase (HRP) (1:10000). ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway NJ) was used for detection.

Immunocytochemistry

Endothelial cells were cultured in 12 well plates (Fisher Scientific, Ontario) with coverslips. Sub-confluent cells were treated with high levels of glucose for 24 h as described above. Immunocytochemical analysis of oxidative stress was performed by applying monoclonal anti-mouse nitrotyrosine (1:200; Cayman Chemical, Ann Arbor MI, USA) antibody. HRP-conjugated secondary antibody was used for detection. For immunofluorescent microscopy, texas red[®] labelled secondary antibody (Vector Laboratories, Ontario Canada) was used for detection by a confocal microscope equipped with UV and FITC/TRITC filters (Zeiss LSM 410; Carl Zeiss Canada Ltd., Ontario

Canada). Slides were read by two investigators unaware of the treatment.

Statistical analysis

The data are expressed as mean ± SEM and were analyzed by ANOVA followed by HSD *post hoc t*-test. Differences were considered significant at values of $P < 0.05$.

Results

High glucose leads to iron accumulation in endothelial cells

We have previously demonstrated that diabetes leads to increased iron accumulation in the heart [13]. In order to assess whether exposure of endothelial cells to high glucose levels causes increased uptake and/or accumulation of iron, we used a highly sensitive and accurate HR-ICP-MS assay. Our results showed the greatest increase in DMT-1 and IREG-1 mRNA levels following exposure of endothelial cells to 25 mM glucose (data not shown). Therefore, we analyzed the concentration of iron in cells exposed to 5 mM glucose (control) and 25 mM glucose. Cell lysates subjected to HR-ICP-MS showed increased iron levels in both HUVECs and HMECs following 25 mM glucose treatment (Figure 1A). It is interesting to note that iron levels were significantly higher in HUVECs as compared to HMECs following glucose treatment.

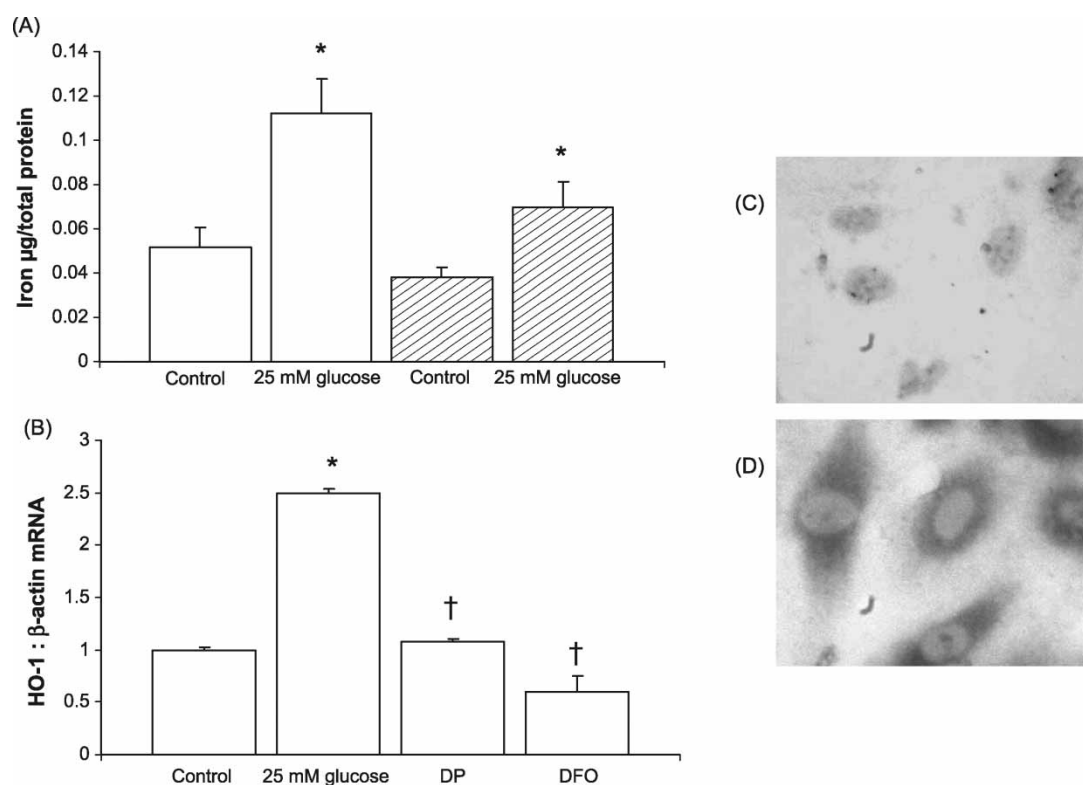


Figure 1. Glucose-mediated iron accumulation and oxidative stress in endothelial cells, as shown by (A) iron levels assessed by HR-ICP-MS analysis, (B) HO-1 mRNA levels assessed by real time RT-PCR, and nitrotyrosine staining in (C) control cells, and (D) 25 mM glucose treated cells. [Iron levels are expressed as µg iron/mg total protein; DP and DFO treatments were carried out in cells exposed to 25 mM glucose; solid bars represent HUVECs and shaded bars represent HMECs; * $p < 0.05$ compared to control, † $p < 0.05$ compared to 25 mM glucose; HMECs produced a similar nitrotyrosine staining pattern (data not shown); Original magnification $\times 400$].

Glucose-induced iron accumulation causes increased oxidative stress

To determine the functional consequence of increased iron levels in endothelial cells, we used well established markers of oxidative stress, heme oxygenase-1 (HO-1) mRNA levels and nitrotyrosine staining. HO-1 has been shown to be upregulated following oxidant toxicity and represents an early molecular marker of oxidative stress [13]. Similarly, nitrotyrosine has been extensively studied in the context of diabetes and provides another reliable means of assessing oxidative stress [13]. Our results indicate that high levels of glucose increase HO-1 mRNA in endothelial cells (Figure 1B). In parallel, endothelial cells exposed to high glucose showed nitrotyrosine positivity (Figure 1C and D). In order to determine whether such glucose-induced increase in oxidative stress is mediated by iron accumulation, we treated HUVECs in high glucose with two different iron chelators. Our results indicate that pre-treatment of cells with DP and DFO prevents high glucose-induced upregulation of HO-1 (Figure 1B) and nitrotyrosine positivity (data not shown).

Iron accumulation is associated with upregulation of iron transporters

In order to assess whether high glucose-induced iron accumulation and subsequent oxidative stress is mediated via alteration of iron transporters, we assessed expression of DMT-1 and IREG-1 by mRNA and protein analyses. Our results show glucose-induced upregulation of DMT-1 and IREG-1 mRNA in both macrovascular and microvascular endothelial cells (Figure 2A and B). Similar to our iron measurement data, quantification of mRNA revealed greater increase in iron transporter levels in HUVECs as compared to HMECs. Furthermore, expression of iron importer DMT-1 was approximately 1.5–2 fold higher as compared to IREG-1 in both HUVECs and HMECs.

We next determined whether pre-treatment of endothelial cells with DP and DFO may also alter glucose-induced expression of DMT-1 and IREG-1. Interestingly, cell permeable DP decreased IREG-1 mRNA expression but not DMT-1 (Figure 2C and D). However, DFO treatment decreased both DMT-1 and IREG-1 mRNA levels.

In order to elucidate whether mRNA levels coincide with protein alteration, we carried out western blot

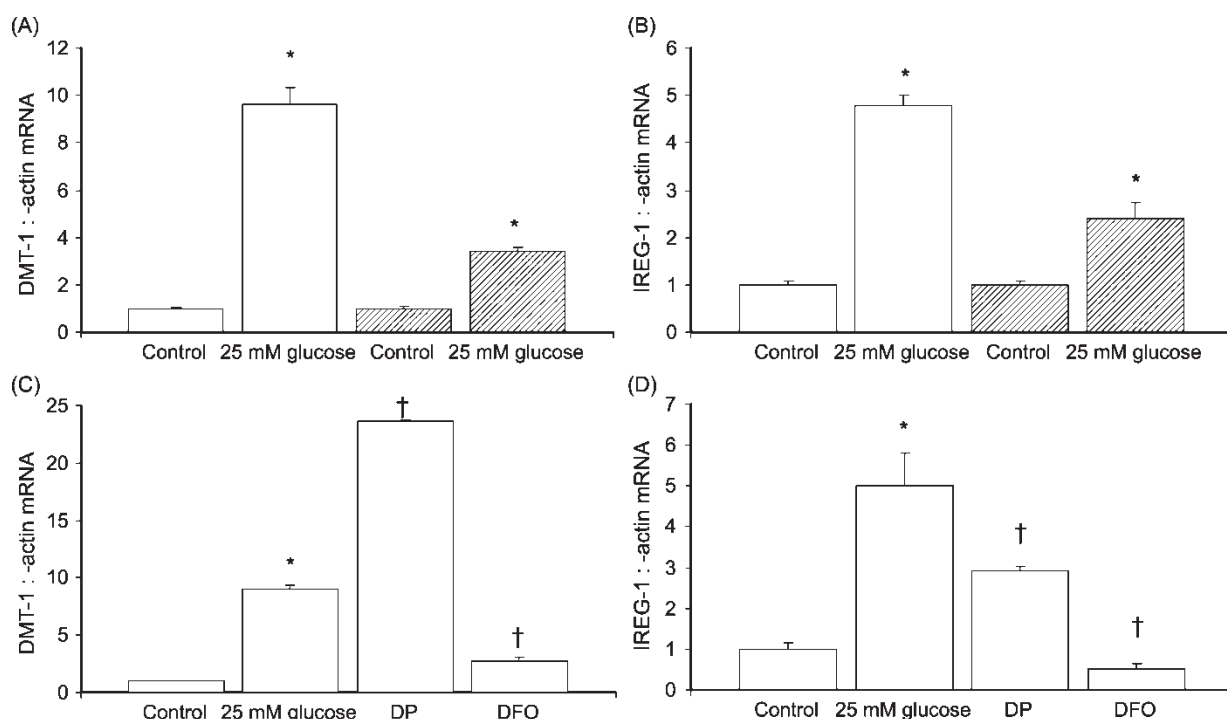


Figure 2. Quantification of DMT-1 and IREG-1 transcript levels by real time RT-PCR showing, (A,C) DMT-1 mRNA levels, and (B,D) IREG-1 mRNA levels. Transporter mRNA levels are expressed as ratio of target to β -actin [solid bars represent HUVECs and shaded bars represent HMECs; * $p < 0.05$ compared to control; † $p < 0.05$ compared to 25 mM glucose].

analyses. Our results show that upregulation of transporter mRNA levels was concurrent with protein expression (Figure 3A–C). In parallel to these western blot data, immunofluorescence also revealed increased expression of IREG-1 and DMT-1 protein in endothelial cells (Figure 3D–G).

Glucose-induced alteration of TfR receptor expression

Our next objective was to determine whether high glucose-induced DMT-1 and IREG-1 expression was a specific effect in mediating iron accumulation or whether it represents a general phenomenon in iron transporter expression. Therefore, we carried out mRNA analysis of TfR in endothelial cells exposed to high levels of glucose with or without iron chelators. Our results show a modest decrease in mRNA levels of TfR in cells exposed to high glucose. Interestingly, pre-treatment of endothelial cells with DP increased the level of TfR mRNA whereas DFO decreased the level (Figure 4).

Discussion

In the present study, we have for the first time demonstrated expression of iron transporters, DMT-1 and IREG-1 in both macrovascular and microvascular endothelial cells. We have shown that high levels of

glucose lead to increased expression of DMT-1 and IREG-1 at the mRNA and protein levels. Furthermore, such altered expression of iron transporters was found to be associated with increased iron accumulation and oxidative stress in endothelial cells.

A potential cross-talk between diabetes and iron dysmetabolism has also been suggested by evidence indicating that diabetes is a common manifestation of hemochromatosis [24–27]. A recent large scale study has shown that elevated ferritin concentration is associated with increased risk of type II diabetes in healthy individuals [28]. It is not clear how increased body iron stores may lead to the development of insulin resistance and diabetes. It is plausible that increased iron levels may cause augmented production of hydroxyl radicals which may contribute to cellular dysfunction and insulin resistance [29–31]. Furthermore, a number of studies have demonstrated that treatment of animals with iron chelators prevents alloxan-induced diabetes [32].

In addition to increased risk of developing diabetes, iron may also potentiate ROS production and development of chronic diabetic complications. Studies in animal models of vascular injury have demonstrated improvement of vascular structure and function with iron depletion [33,34]. These findings prompt the investigation of whether important elements of iron metabolism, namely the transporters, DMT-1 and

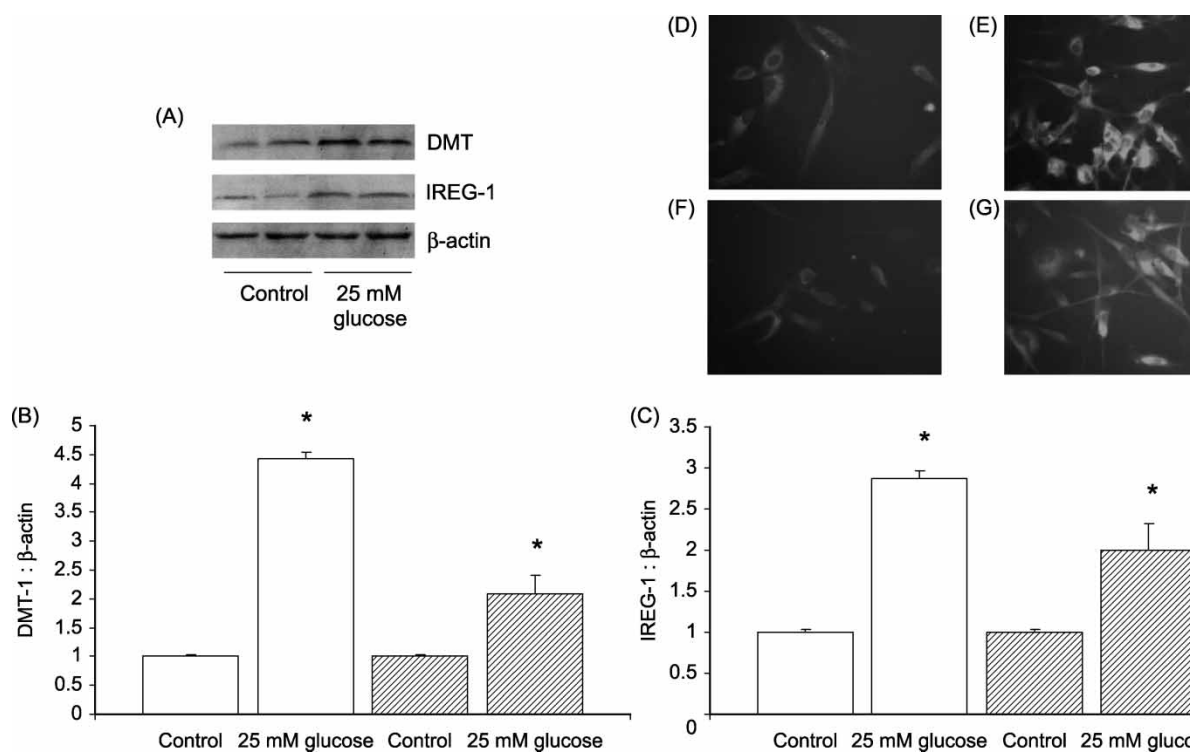


Figure 3. Iron transporter protein levels as assessed by western blot analysis, showing (A) representative immunoblots, (B) quantification of DMT-1 protein expression, and (C) quantification of IREG-1 protein expression [Immunoblot band intensities were normalized by β -actin to account for differences in protein loading; solid bars represent HUVECs and shaded bars represent HMECs; * $p < 0.05$ compared to control]. Immunofluorescent analysis of DMT-1 and IREG-1 showing, (D) DMT-1 in control cells, (E) DMT-1 in cells exposed to 25 mM glucose, (F) IREG-1 in control cells, and (G) IREG-1 in cells exposed to 25 mM glucose. [HMECs showed a similar pattern of expression (data not shown); Original magnification $\times 400$].

IREG-1, are altered in diabetes. We have demonstrated that vascular endothelial cells do express both DMT-1 and IREG-1. Furthermore, our data shows that glucose increases the expression of these transporters at both the mRNA and protein levels. The mechanistic basis of DMT-1 and IREG-1 induction is still obscure. However, under serum-free conditions, we have demonstrated that high levels of glucose increase the expression of both DMT-1 and IREG-1. It is plausible that glucose-mediated alteration of growth factors and intracellular kinases, which are very well established and

include protein kinase C and mitogen activated protein kinase pathway, may regulate the expression of these iron transporters [2,3]. The effect of the glucose-induced biochemical changes may be direct or via the iron regulatory proteins which are intimately involved in the expression of the iron transporters. In addition, transcription factors including nuclear factor- κ B and activating protein-1 may also be involved in the regulation of DMT-1 and IREG-1 expression [35]. However, such notions require further investigation.

Altered expression of these transporters may result in aberrant utilization of iron and subsequent cellular dysfunction. Our studies do support such a notion. We have shown that endothelial cells of both the macrovascular and microvascular origin increase intracellular iron levels and oxidative protein damage. Such changes were in complete agreement with increased expression of IREG-1 and DMT-1. It is interesting to note that higher upregulation of iron transporters in macrovascular endothelial cells compared to microvascular cells suggests that iron dysmetabolism may be of greater significance to diabetic macrovasculopathy. Studies have indicated an intricate relationship between iron levels and lipid peroxidation, an initial step in atherosclerosis which is a highly pronounced complication of chronic diabetes [35]. Furthermore, atherosclerosis has been suggested to be a manifestation

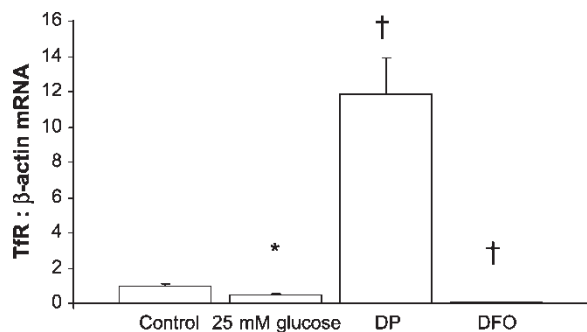


Figure 4. Tfr transcript levels in endothelial cells exposed to high levels of glucose as assessed by real time RT-PCR. [HUVECs; * $p < 0.05$ compared to control; † $p < 0.05$ compared to 25 mM glucose].

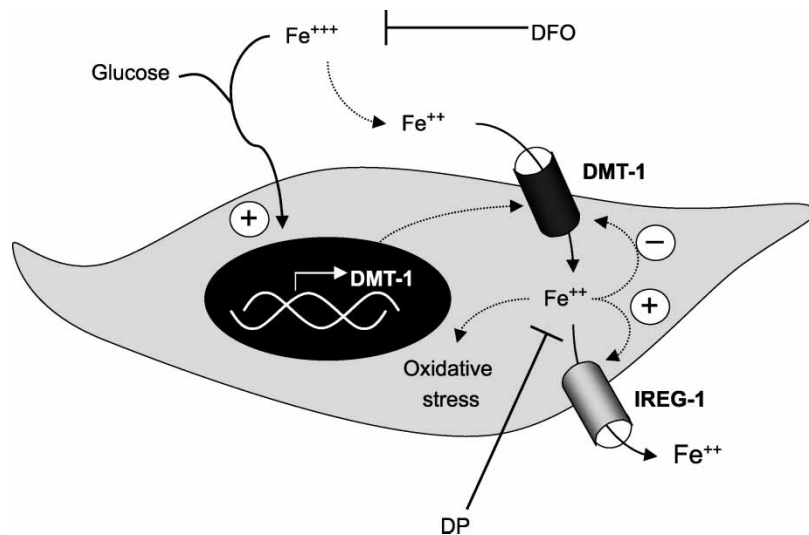


Figure 5. Schematic illustrating the proposed mechanism of glucose-induced oxidative stress via alteration of iron transporters. Glucose increases the level of DMT-1 mRNA and protein leading to increased influx of iron. Intracellular iron accumulation causes an adaptive increase in the expression of IREG-1 via the iron response element in IREG-1 mRNA. However, the level of increase in DMT-1 is more significant than the increase in IREG-1 leading to iron accumulation and oxidative stress. The schematic also illustrates the sites of iron chelation by cell-permeable Fe^{++} chelator DP and impermeable Fe^{+++} chelator DFO.

of chronic iron toxicity [35]. Interestingly, large population studies have also shown that humans heterozygous for hemochromatosis gene and iron overload have increased risk of developing carotid artery disease [36,37].

DMT-1 has been shown to be an important protein involved in iron uptake [14–17]. Upregulation of DMT-1 mRNA and protein by glucose suggests an important role of this iron importer in diabetic angiopathy. Contrary to DMT-1, IREG-1 studies in epithelial cells have revealed an extrusion property of this transporter [18–20]. Whether IREG-1 exhibits a similar iron export mechanism in endothelial cells requires further investigation. IREG-1 mRNA contains an iron response element which is important in the rapid translation of the IREG gene product in the presence of free intracellular iron [18,20]. Such a finding indicates that IREG-1 upregulation via glucose may represent an adaptive mechanism secondary to DMT-1 alteration and iron accumulation. In support of such a notion, we have demonstrated that DP, an intracellular iron chelator, prevents IREG-1 upregulation. Although TfR exhibited reduction as compared to DMT-1 and IREG-1, pre-treatment of cells with DP increased the levels of TfR mRNA, a phenomenon similar to DMT-1. DFO, however, reduced the expression levels of TfR. These findings may provide important clues as to the nature of TfR, DMT-1, and IREG-1 proteins in endothelial iron regulation. Our studies do show more pronounced upregulation of DMT-1 mRNA and protein in endothelial cells as compared to IREG-1. It is possible that iron accumulated in these endothelial cells may largely be due to DMT-1 alteration. In addition, increased intracellular levels of iron may

increase IREG-1 as an adaptive mechanism. However, the level of upregulation is significantly lower than the importer DMT-1, thereby leading to iron accumulation (diagrammatically represented in Figure 5). Such a mechanistic possibility requires investigation upon the development of DMT-1 and IREG-1 inhibitors. It is interesting to note that physiologically transferrin-iron is of importance in order to draw conclusions regarding the functional significance of DMT-1 and IREG-1 *in vivo*. As our serum-free model system mainly derives iron from ferrousulphate, it may show a novel mechanism which could underlie adverse effects of glucose in *in vitro* models of endothelial dysfunction.

In conclusion, we have demonstrated that cultured endothelial cells upregulate iron transporters in response to high ambient glucose levels. Such upregulation is associated with increased intracellular iron levels and oxidative stress. Furthermore, these findings provide novel insight into the glucose-induced endothelial cell dysfunction.

Acknowledgements

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