REACTIVE OXYGEN SPECIES ARE IMPLICATED IN ALTERING MG HOMEOSTASIS IN ENDOTHELIAL CELLS EXPOSED TO HIGH GLUCOSE

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ABSTRACT

Transient Receptor Potential Melastatin (TRPM7) is important in maintaining the intracellular homeostasis of magnesium (Mg), which is instrumental for vital cellular functions. Since the upregulation of TRPM7 has been proposed as a marker of endothelial dysfunction, we evaluated the effects of high glucose, which markedly impacts on endothelial performance, on TRPM7 and intracellular Mg homeostasis in human macrovascular endothelial cells. We show that glucose-induced free radicals increase the total amounts of TRPM7 as well as total intracellular magnesium. On the contrary, the highly selective Mg transporter MagT1 is not modulated by high glucose, hydrogen peroxide and low extracellular magnesium.

We conclude that in HUVEC treated with high glucose ROS alter Mg homeostasis through the upregulation of TRPM7.

Keywords: endothelium, TRPM7, magnesium, reactive oxygen species, glucose

INTRODUCTION

Uncontrolled hyperglycaemia causes endothelial dysfunction [1,2], the first step of a cascade of events leading to vascular complications among which stroke, coronary artery and peripheral vascular diseases [3]. Several mechanisms seem to mediate hyperglycemia-dependent endothelial dysfunction such as the accumulation of reactive oxygen species (ROS), the activation of the polyol pathway and the increased amounts of non-enzymatically glycated proteins [4,5].

In vitro high concentrations of extracellular glucose affect the performance of endothelial cells of different types. Both human endothelial cells from the umbilical vein (HUVEC), widely used as a model of macrovascular endothelium, and human dermal microvascular endothelial cells exposed to high extracellular glucose are growth inhibited and show symptoms of premature senescence [6]. These results are explained, in part, by a time and dose dependent increase of ROS due to high extracellular glucose. In addition, increased levels of Ca^{2+} both in the mitochondria and in the cytoplasm have been reported and linked to the induction of apoptosis [3,7].

Rather little is known about the homeostasis of magnesium (Mg) in endothelial cells exposed to high glucose. Mg is important in numerous biochemical processes, from energy generation to the synthesis of DNA, RNA and proteins and is a natural Ca antagonist [8]. Consequently, it is involved in many physiological processes, i.e. muscle contraction/relaxation, bone formation, brain activity, and others. In the vascular tree, the maintenance of Mg homeostasis is crucial to regulate blood pressure, to prevent inflammation and thrombosis [9]. At the endothelial level, Mg is pivotal in governing barrier function [10], release of vasoactive molecules, fibrinolytic and anti-coagulation factors, chemokines and cytokines [11]. Vascular endothelial cells express both the Transient receptor potential melastatin (TRPM)7 and the specific Mg transporter MagT1, and both cooperate to ensure Mg entry in these cells [12]. TRPM7 is a ubiquitous bifunctional protein endowed with a channel for divalent cations and an α kinase domain in its C-terminal [13], while MagT1 specifically transports Mg and is also involved in N-glycosylation [14]. In HUVEC low extracellular Mg, which impairs their function [11,15,16], upregulates TRPM7 through the increase of reactive oxygen species (ROS) [17]. Accordingly, hydrogen peroxide increases TRPM7 protein level [17]. On these bases, we proposed that high levels of TRPM7 might be a marker of endothelial dysfunction [12,17] . This issue is supported by *in vivo* studies showing that elevated TRPM7 in the aortas from Mg deficient mice correlates with increased amounts of vascular cell adhesion molecule-1 and plasminogen activator inhibitor-1 [18], both implicated in developing a pro-inflammatory, pro-atherogenic environment. At the moment no data are available about MagT1 and endothelial function.

The aim of this study was to evaluate TRPM7 and MagT1 as well as intracellular Mg levels in HUVEC cultured in the presence of high extracellular glucose, known to induce endothelial dysfunction.

MATHERIAL AND METODS

Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from the American Type Culture Collection (ATCC) and cultured in medium M199 (Euroclone, Milano, Italy) added with fetal bovine serum (FBS, 10%) (Euroclone, Milano, Italy), L-Glutamine (1 mM), Sodium Pyruvate (1 mM), Penicillin-Streptomycin (1 mM), Heparin (5 U/ml) and Endothelial Cell Growth Factor (ECGS, 150 µg/ml) on 2% gelatin-coated dishes. All culture reagents were from Thermo Fisher Scientific (Waltham, MA, USA). The cells were routinely tested for the expression of endothelial markers and used for 9-10 passages. All the experiments cells were performed on sub-confluent cells. D-glucose (Sigma Aldrich, St. Louis, Missouri, USA) was used at the concentrations of 11.1 mM and 30 mM. The same concentrations of L-glucose (Sigma Aldrich, St. Louis, Missouri, USA) were utilized as a control for osmolarity. In some experiments hydrogen peroxide (H_2O_2) (100 µM) and N-acetylcysteine (NAC) (5 mM) (Sigma Aldrich, St. Louis, Missouri, USA) were used. In some experiments HUVEC were cultured in Mg free medium (Thermo Fisher Scientific Waltham, MA, USA) supplemented with magnesium sulphate ($MgSO_4$) to reach a final concentration of 0.1 mM Mg to mimic Mg deficiency or 1.0 mM, which is the physiological concentration [19].

Western Blot Analysis

HUVEC were lysed in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1% NP40, 0.25% NaDeoxy, protease inhibitors (10 µg/ml Leupeptin, 10 µg/ml Aprotinin, PMSF 1 mM) and phosphatase inhibitors (NaF 1 mM, NaV 1 mM, NaP 5 mM). Lysates (40 µg/lane) were separated on SDS-PAGE and transferred to nitrocellulose sheets using Trans-Blot Turbo Transfer System (Biorad, Hercules, USA). Western Blot analysis was performed using antibodies against TRPM7 (Bethyl, Montgomery, USA), MagT1 (Abcam, Cambridge, UK), thioredoxin-interacting protein (TXNIP) (Invitrogen Corporation, Carlsbad, USA) superoxide dismutase (SOD)2 (BD Bioscences, Milano, Italy). After extensive washing, secondary antibodies labelled with horseradish peroxidase (GE Healthcare, Waukesha, WI, USA) were used. Immunoreactive proteins were detected by the SuperSignal Chemiluminescence Kit (Thermo Fisher Scientific Waltham, MA, USA) [20,21]. The nitrocellulose sheets are used as control loading. The densitometric anlysis was performed using Image J Lab software (Biorad, Hercules, USA). The results are the mean of three independent experiments performed in triplicate.

ROS activity

For the detection of ROS, HUVEC were cultured in 96 wells black plate (Greiner Bio-One, Kremsm¨unster, Austria) and incubate for 30 minutes with 10 mM 2’-7’-dichlorofluorescein diacetate (DCFH) solution. The DCFH dye emission was monitored at 535 nm (excitation λ= 484 nm) using VICTOR X5 multilabel plate (Perkin Elmer, Milano, Italy). ROS production was normalized on the basis of cell number as described [21]. The results are the mean of three independent experiments performed in triplicate.

Quantification of total intracellular Mg

Total intracellular magnesium was measured using the fluorescent chemosensor DCHQ5 (kindly donated by Prof. S. Iotti, University of Bologna) as described [22]. Fluorescence intensities were acquired at 510 nm. Mg concentrations were obtained by the interpolation of their fluorescence with the standard curve performed using known concentrations of $MgSO_4$. The results are the mean of three independent experiments performed in triplicate.

Statistical analysis

Statistical significance was determined using Student’s t test and set as following: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

RESULTS

1. HUVEC exposed to high extracellular D-glucose accumulate ROS and upregulate TRPM7

HUVEC were cultured for 24 h in medium containing the physiological (5.5 mM, CTR) or high concentrations of D-glucose (11.1 mM and 30 mM). 11.1 or 30 mM of L-glucose were utilized as controls of osmolarity. As shown in figure 1A, exposure to high extracellular D-glucose significantly increased ROS in a dose-dependent manner, while L-glucose exerted no effect. To understand the mechanisms involved, we evaluated the total amounts of the pro-oxidant protein thioredoxin-interacting protein (TXNIP) and the superoxide dismutase (SOD)2, the first line of defense against ROS. The cells were maintained in 5.5, 11.1 or 30 mM D-glucose for 24 h. Western blot revealed a marked upregulation of TXNIP, but no alterations of the levels of SOD2 in high glucose-treated HUVEC (Fig 1B), thereby suggesting that TXNIP has a role in the accumulation of ROS in these experimental conditions.

The total amounts of TRPM7 were then evaluated after 24h of exposure to 5.5, 11.1 or 30 mM of D-glucose. By western blot, we found an increase of TRPM7 levels in response to high extracellular D-glucose, whereas the same concentrations of L-glucose did not exert any effect (Figure 2).

2. MagT1 is not modulated by high D-glucose, hydrogen peroxide or different concentrations of extracellular Mg

We then evaluated the total amounts of MagT1 in HUVEC exposed to 5.5, 11.1 or 30 mM glucose for 24 h and found no significant modulation (Figure 3A). Since very little is known about MagT1 in endothelial cells, we asked whether MagT1 is modulated in response to low extracellular Mg (0.1 mM), which induces oxidative stress and increases cardiovascular risk [11], and hydrogen peroxide (100 μM), from which hydroxyl radicals are produced by the Fenton reaction. Figure 3B shows that culture in low Mg or in the presence of hydrogen peroxide (H_2O_2) for 24 h did not exert any effect on MagT1 levels (Figure 3B).

3. The anti-oxidant NAC prevents TRPM7 upregulation by high glucose

We then focused on TRPM7. To investigate if its upregulation by high glucose is due to the increase of ROS, HUVEC were treated with the anti-oxidant NAC (5 mM) in medium containing high or physiological concentrations of D-glucose for 24 h. NAC prevented ROS accumulation (Figure 4A) and, in parallel, TRPM7 upregulation (Figure 4B), thereby suggesting that the increased amounts of TRPM7 are due to D-glucose-induced ROS.

4. Evaluation of total intracellular magnesium in HUVEC exposed to high glucose condition

The concentration of intracellular total Mg was measured using the fluorescent chemosensor DCHQ5 in HUVEC cultured in medium containing 5.5, 11.1 or 30 mM D-glucose for 24 h. We found that intracellular Mg increased in response to high glucose, an effect that is prevented by the addition of NAC (Figure 5). L-glucose did not modulate intracellular magnesium.

DISCUSSION

High extracellular glucose alters Mg homeostasis in human macrovascular endothelial cells. In our experimental model, we found a direct correlation between high glucose-induced ROS and TXNIP. TXNIP inhibits thioredoxin, a ubiquitous redox protein that reduces thiol and controls levels of ROS to limit damage from oxidative stress. In agreement with our data, the overexpression of TXNIP has been described in a rat model of type 1 diabetes as well as in cultured human aortic endothelial cells [23]. We propose that increased TXNIP has a role in generating oxidative stress in response to high glucose, but more experiments are necessary to validate this hypothesis. It is also of interest that no modulation of the anti-oxidant enzyme SOD2, which has a critical role in protecting the cardiovascular system [24], occured. Therefore, we propose that in HUVEC high glucose favours the acquisition of a pro-oxidant phenotype, which is responsible for the upregulation of the Mg channel TRPM7. Our data confirm and broaden a previous study describing the upregulation of TRPM7 in HUVEC exposed for 72 h to high concentrations of extracellular D-glucose [25]. We also show that an antioxidant, i.e., NAC, prevents high-glucose-induced ROS and TRPM7 accumulation. We have previously shown that TRPM7 is modulated by ROS [17]. Indeed, TRPM7 increased in HUVEC cultured in low Mg [17], which promotes oxidative stress and is a risk factor for coronary artery disease [26], or exposed to hydrogen peroxide, to simulate a condition of oxidative stress. On these bases we proposed that high levels of TRPM7 might be considered as a marker of endothelial dysfunction. The results reported in this paper reinforce this view.

On the contrary, MagT1 is not modulated by high glucose, low Mg or hydrogen peroxide. Therefore, we propose that the increase of total intracellular Mg in HUVEC exposed to high concentrations of D-glucose is mainly due to the upregulation of TRPM7. This result is puzzling, since, most mammalian cells retain their basal Mg content virtually unchanged [27]. Moreover, no alterations of intracellular Mg concentration were detected in HUVEC stably silencing TRPM7 [28]. We hypothesize that other transporters or channels vicariate TRPM7 knock down, and work is in progress to test this issue. The consequences of high intracellular Mg on cell function are not clear. Since it is reported that high D-glucose results in increased intracellular Ca (Hou 2014, [3]), it is feasible to propose that the balance Ca/Mg, essential in regulating several cell functions, is maintained through a higher transport of Mg via TRPM7.

It is noteworthy that TRPM7 upregulation in response to elevated concentrations of high D-glucose is a common denominator is all the principal cell types implicated in atherogenesis. In rabbit smooth muscle cells, high D-glucose enhances TRPM7 by increasing oxidative stress and this is associated with the development of a proliferative phenotype [29]. In human monocytes, high glucose-induced

oxidative stress leads to the overexpression of several transient receptor potential channels, including TRPM7 [30]. These two studies do not offer any insight into intracellular Mg concentrations. While increased amounts of TRPM7 seem to be involved in endothelial dysfunction and atherogenesis, a potential contribution of MagT1 is hard to envisage since MagT1 is not modulated by high D-glucose, low Mg or hydrogen peroxide. A recent paper has reported the contribution of this transporters in altering endothelial barrier function [12], a crucial early step in endothelial dysfunction, but more efforts are needed to define MagT1 role in the vasculature. We only considered the total amounts of the protein. However, it is possible that high glucose alters its function as a component of the N-oligosaccharyl transferase (OST) complex.

In conclusion, we propose that 24 h exposure to high D-glucose suffice to edit Mg homeostasis in HUVEC.

DISCLOSURE

The authors declare no conflict of interest.

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LEGENDS

Figure 1. High D-glucose stimulates ROS accumulation

HUVEC were cultured under glucose physiological concentration (5.5 mM) (CTR) or two different high D-glucose concentrations (11.1 mM and 30 mM).

A) ROS were measured by DCFH. Data are shown as percentages of ROS levels in HUVEC cultured in physiological or high concentrations of D-glucose vs CTR. L-glucose was used as a control.

B) Western blot was performed on cell lysates using specific antibody against TXNIP and SOD2. The relative control loading is shown in the lower panel. Densitometric analysis was performed using Image J Lab software.

Figure 2. High D-glucose upregulates TRPM7.

Western blot was performed on cell lysates of HUVEC exposed to different concentrations of D-glucose using antibody against TRPM7. The relative control loading is shown in the lower panel. Densitometric analysis was performed using Image J Lab software.

Figure 3. High D-glucose does not modulate MagT1.

HUVEC were exposed to physiological (5 mM, CTR) and high concentrations of D-glucose (11.1 and 20 mM) for 24 h. L-glucose was used as a control.

HUVEC were cultured for 24 h in medium containing physiological (1mM) and low (0.1 mM) concentrations of Mg (left panel) or in control medium (CTR) in the presence or not of 100 µM of hydrogen peroxide (H_2O_2) (right panel).

In A and B western blot was performed on cell lysates using anti MagT1 antibody. The relative control loading is shown in the lower panel. Densitometric analysis was performed using Image J Lab software.

Figure 4. NAC prevents TRPM7 upregulation by high D-glucose.

HUVEC were pre-treated for 1h with 5mM NAC. The cells were then cultured for 24 h in the presence of 5.5, 11.1 and 30 mM with or without of NAC.

A) ROS production was evaluated by DCFH. Data are shown as percentages of ROS levels in HUVEC cultured in different concentration of glucose in the presence or not of NAC vs CTR.

B) Western blot was performed on cell lysates using anti TRPM7 antibody. The relative control loading is shown in the lower panel. Densitometric analysis was performed using Image J Lab software.

Figure 5. High D-glucose impacts on total intracellular Mg concentration.

HUVEC were exposed to high concentrations of extracellular D- and L-glucose in the presence or not of NAC for 24 h. Total intracellular Mg was measured using the fluorescent chemosensor DCHQ5.

Figure 1

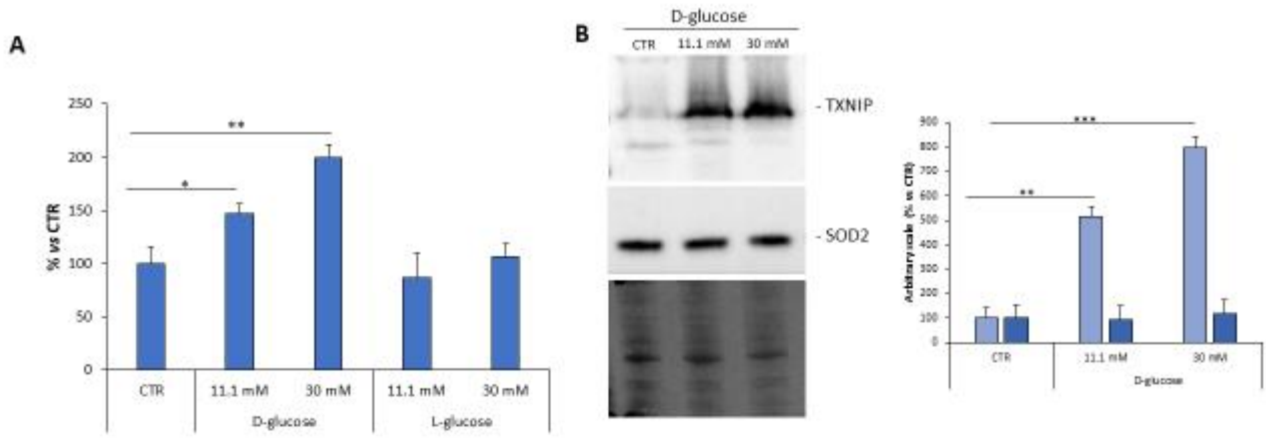


Figure 2

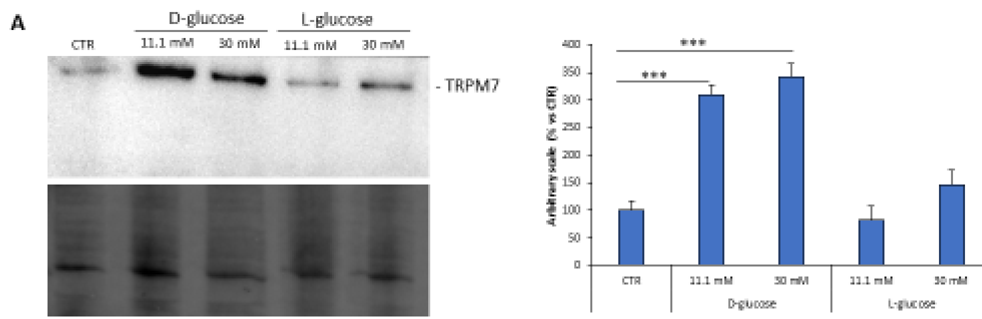


Figure 3

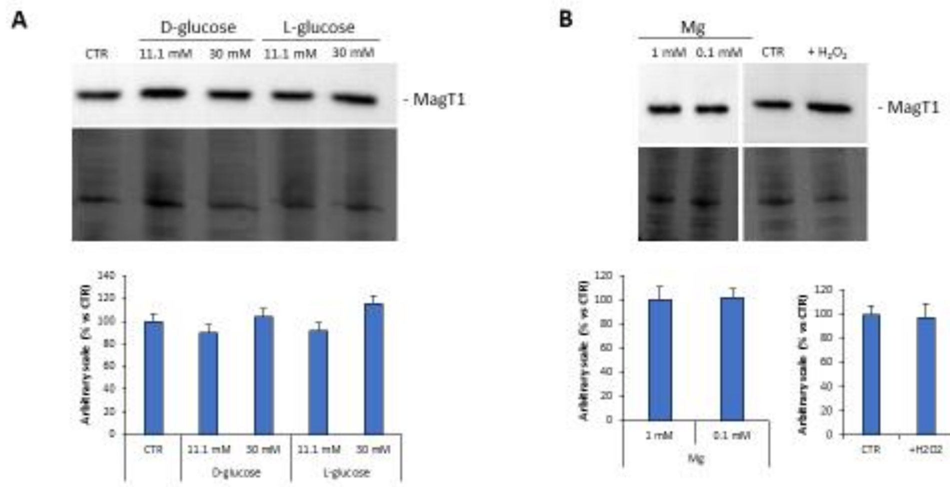
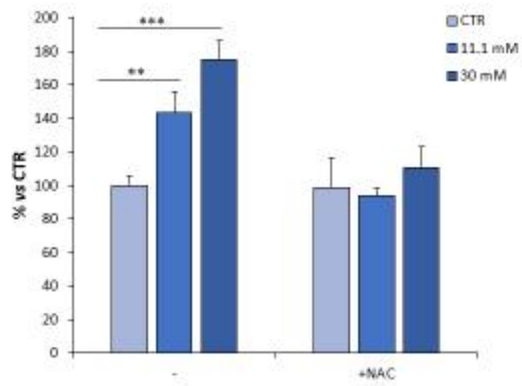


Figure 4



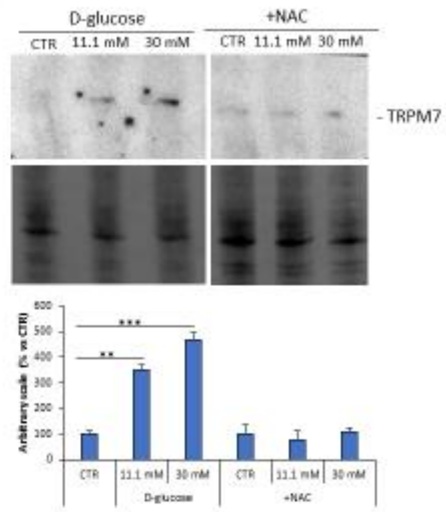


Figure 5