H₂O₂ Treatment of HUVECs Facilitates PKC Mediated Thr495 Phosphorylation on eNOS when Pre-treated with High Glucose Levels

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Abstract

Objective: Metabolic syndrome entails hypertension, hyperglycemia, obesity and hypercholesterolemia. This syndrome increases the risk of cardiovascular disease and diabetes. Hyperglycemia during coronary reperfusion is associated with a poor prognosis. Contrastingly, targeting correction of hyperglycemia in clinical trials has not improved clinical outcome or has even been detrimental. H₂O₂ is produced under hyperglycemic conditions and under reperfusion. This study aims to provide a mechanistic approach evaluating the impact of high glucose on the endothelial nitric oxide pathway in a H₂O₂-rich environment.

Methods and results: HUVECs (human umbilical vein endothelial cells) were exposed to high glucose (20 mM) for either 20 or 72 hours co-incubated with or without H₂O₂ (400 µM) for 30 minutes as models of increased oxidative stress during acute and prolonged hyperglycemia, respectively. The presence of reactive oxygen species (ROS) in both mitochondria and cytoplasm was measured by fluorescence activated cell sorting (FACS). Phosphorylation of endothelial nitric oxide synthase (eNOS) on threonine 495 (Thr495) and serine 1177 (Ser1177) was assessed by western blotting. Short-term (20 hours) high concentration of glucose alone increased ROS in mitochondria to 133.5% (p<0.05), whereas prolonged (72 hours) did not increase mitochondrial ROS. The increase in mitochondrial ROS could be attenuated by the anti-oxidant N-acetyl-L-cysteine (NAC). Incubation with H₂O₂ for 30 minutes resulted in an increase in Thr495 phosphorylation (to 425%, p<0.01) and a decrease in Ser1177 phosphorylation (to 50.6%, p<0.01). Pre-incubation for 20 hours with 10 and 20 mM glucose did not affect phosphorylation of Thr495 and Ser1177. Stimulating HUVECs that were pre-incubated with 20 mM glucose for 72 hours with H₂O₂ increased Thr495 phosphorylation to 146.6% (p<0.05). PKC inhibition attenuated the H₂O₂-induced Thr495 phosphorylation in cells incubated with high glucose levels for 72 hours.

Conclusion: Acute exposure to high glucose induces oxidative stress. H₂O₂ leads to phosphorylation of eNOS at Thr495 and dephosphorylation of Ser1177. After prolonged exposure to high glucose levels, the addition of H₂O₂ yields phosphorylation of Thr495 through the PKC pathway.

Keywords: Endothelial nitric oxide synthase; Thr495 phosphorylation; Ser1177 phosphorylation; High glucose levels; Mitochondrial radical oxygen species

Introduction

The present study aims to provide information on how chronic and acute high glucose affect the activation of endothelial nitric oxide (NO) in an oxidative-stress rich environment. An important manifestation of endothelial dysfunction is the decrease in endothelial derived nitric oxide bioavailability [1]. The production of NO by vascular endothelial cells is central in maintaining normal endothelial function and preventing the development of atherosclerosis. In clinical settings, decreased endothelium-derived NO is an independent predictor of cardiovascular events [2], which probably is related to the ability of NO to inhibit platelet aggregation, attachment of neutrophils to endothelial cells and proliferation of smooth muscle cells [3]. Diabetes is characterized by hyperglycemia and endothelial dysfunction [4,5]. Two interdependent mechanisms seem to contribute to endothelial dysfunction in diabetes: Hyperglycemia and reactive oxygen species (ROS). The experiments conducted in humans by Calver et al. and McVeigh et al. showed that forearm blood flow in diabetic patients was impaired due to decreased availability of NO [6,7]. Studies in healthy subjects during hyperglycemic clamps suggest an important role of hyperglycemia as the response of forearm vessels to methacholine, as it is attenuated during the clamps [8]. The reperfusion that follows prolonged ischemia provides oxidative stress which may contribute to an impairment of NO production [9,10]. Thus, a combination of hyperglycemia and reperfusion following myocardial ischemia has been regarded as a possible explanation for a poorer outcome in patients with diabetes who suffer from myocardial infarction [11-18]. The results of the DIGAMI-1 study seemed to support this notion, as correcting glycaemia in diabetic patients with high glucose levels (blood glucose >11 mM) at admission for acute myocardial infarction seemed to reduce mortality in these patients [19,20], even though subsequent studies do not support the use of insulin to achieve glycemic control [21]. Since phosphorylation of eNOS at Ser1177 is necessary for eNOS to synthesize NO [22], while phosphorylation at Thr495 hinders its enzymatic activity [23,24], it is expectable, that conditions where

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increased phosphorylation of eNOS-Thr495 occur, lead to a decreased eNOS enzymatic activity and, consequently, to decreased NO production. Indeed, prolonged periods (2-3 days) of exposure of high glucose levels in rat aortic endothelial cells, smooth muscle cells and bovine retina endothelial cells increase the total diacylglycerol (DAG) levels, leading to the activation of the DAG-PKC pathway, and eNOS Thr495 phosphorylation [25].

During the reperfusion that follows acute target organ ischemia, there is a substantial increase of the presence of H$_2$O$_2$ [26], which may induce specific phosphorylation of eNOS regulating the synthesis of NO. This effect may also be mediated by increases in the endothelial calcium concentrations or changes in membrane potential [27-29]. In regard to ROS, it has been shown that high glucose levels lead to increased superoxide production by inducing NADPH oxidase [30], which will decrease eNOS expression in endothelial cells [31]. Furthermore, uncoupling of eNOS results in production of superoxide, which reacts rapidly with NO producing peroxynitrite ultimately [32].

NO production though eNOS phosphorylation at Ser1177 [33,34] and later inhibition of NO production [35] while only an inhibitory effect is apparent in other [36]. Hence, it is still obscure to what extent eNOS expression, eNOS cofactor availability or oxidative stress contribute to a decreased NO activity in diabetes.

Our study aims to investigate the effects of glucose and ROS on phosphorylation of eNOS Thr495 and Ser1177 in HUVECs. We hypothesized that the presence of high glucose levels in an environment rich in H$_2$O$_2$ would lead to eNOS Thr495 phosphorylation and Ser1177 dephosphorylation. Based on previous results, in which we observed increased mitochondrial ROS after H$_2$O$_2$ incubation [36], we expected to see an increase in mitochondrial ROS generation with high glucose alone and with H$_2$O$_2$ incubation. By extension, we anticipated phosphorylation changes of eNOS after high glucose levels alone and with H$_2$O$_2$ incubation. We have previously shown that incubating HUVECs with H$_2$O$_2$ led to an ERK and ROCK mediated phosphorylation of eNOS at Thr495 [36]. We hypothesized that incubating HUVECs for longer duration (72 hours) with high glucose levels would lead to a facilitation of the PKC pathway and therefore expected to see an increase in Thr495 phosphorylation with high glucose levels alone or combined with H$_2$O$_2$ and that the phosphorylation could be hindered by inhibition of PKC.

**Materials and Methods**

**Cell culture and medium**

Pooled HUVECs (human umbilical vein endothelial cells) from 15 women were obtained from Lonza (CC-2519, Lonza, Basel, Switzerland) and were grown to confluency in EBM-2 medium (CC-3156) with growth factor and additional supplements (CC-4176, both Lonza, Basel, Switzerland) and 5% Bovine Serum (10270-106, Invitrogen, Carlsbad, CA, USA). According to vendor supplements contained ascorbic acid. Final concentration of ascorbic acid or whether it was in stable form is not stated. Cells were not serum deprived prior to experiments and only cells in passage 3 and 4 were used. The cells were grown to confluency and co-incubated with relevant chemicals in 6 well plates (92006, Techno Plastic Products AG, TGP, Trasadingen, Switzerland) coated with 10.5 µg/cm² gelatin (214340, Difco Laboratories, Beckton, USA). Cells were grown at 20% O$_2$ (ambient air). HUVECs exposed to glucose were incubated in concentrations of 5, 10 or 20 mM for either 20 hours or 72 hours. It has been shown that HUVECs grown in high glucose levels (19 or 33 mM) for at least 36 hours undergo apoptosis, which could be reverted by ascorbic acid (100 µM) [37]. Because HUVECs were grown in and treated with high glucose levels with ascorbic acid, we did not expect to induce apoptosis. In concurrent experiments, HUVECs were exposed to 400 µM H$_2$O$_2$ for 30 minutes to simulate ischemia/reperfusion. Suppressor oxidative stress was assessed by pre-incubating the relevant subset of HUVECs with 10 mM N-acetyl-L-cysteine (NAC), A9165, Sigma-Aldrich, Steinheim, Germany) for 20 hours before stimulation with H$_2$O$_2$.

The pan-PKC inhibitor -3-(N-[Dimethylamino]propyl-3-indolyl)-4-(3-indolyl)maleimide, Bisindolylmaleimide I, L-[1-[3-(Dimethylamino) propyl][1H-indol-3-yl]-4-(1Hindol-3-yl)1H-pyrrole-2,5-dione GFI09203X (GFX) in a concentration of 1 µM, (G2911, Sigma-Aldrich, Inc., Steinheim, Germany) was used to investigate the role of Protein kinase C (PKC) in Thr495-eNOS phosphorylation and was added 1 hour before harvest.

**Western blot procedure**

HUVECs were washed twice in ice-cold PBS and harvested with RIPA buffer (R0278), which contained protease (P8340) and phosphatase inhibitor cocktails I and II (P2850, P5726). Additional 1 mM sodium orthovanadate (S6508) and 1 mM phenylmethylsulfonyl fluoride (PMSF, 78830) was added to robustly preserve the phosphorylation of eNOS and inhibit serine proteases, respectively (all compounds from Sigma-Aldrich, Steinheim, Germany). The cell lysate was ultrasonicated and centrifuged for 30 minutes at 20,000 g. After discarding the pellet the solubilized protein concentration was determined with Bradford Protein Assay, according to the producer's recommendations (500-0006, Bio-Rad Laboratories, Hercules, California, USA). After obtaining the protein concentration of each lysate, the concentration was corrected with RIPA buffer to attain equivalent protein amounts. The samples were run on a 7% Novex Tris-Acetate gel (EA03585BOX, Invitrogen Corporation, Carlsbad, CA, USA). After transfer to a nitrocellulose membrane, the equal loading of the gel lanes was confirmed with protein detection Ponceau S staining. The nitrocellulose membrane was blocked by submerging it in a blocking buffer containing 5% (w/v) skim milk in Tris Buffered Saline (TBS) with 0.05% Tween-20 (TBS-T) for 1 hour at room temperature. Then the membrane was washed 3 times in TBS-T followed by overnight incubation at 4°C with relevant primary antibodies in 5% BSA in TBS-T, which consisted of 1:1000 anti-phospho-eNOS (Thr495) mouse antibody (612707, BD Transduction Laboratories, Franklin Lakes, NJ, USA), 1:1000 anti-phospho-eNOS (Ser1177) mouse antibody (612393, BD Transduction Laboratories, Franklin Lakes, NJ, USA) and rabbit polyclonal anti-eNOS (07-520, Upstate, Lake Placid, NY, USA), followed by washing of the nitrocellulose membrane three times with TBS-T. Thereafter we applied blocking buffer containing a secondary HRP-conjugated anti-rabbit antibody (1858415, Pierce Biotechnology, Rockford, IL, USA) or a HRP-conjugated anti-mouse antibody (1858413, Pierce Biotechnology, Rockford, IL, USA) for 1h at room temperature (1:5000). After washing the nitrocellulose membrane three times it was incubated in SuperSignal West Femto Maximum Sensitivity Substrate (34095, Pierce Biotechnology, Rockford, IL, USA) for 1 minute followed by densitometric quantification (LabWorks, Ultra-Violet Products Ltd, Cambridge, UK). The membranes were also analyzed for equal loading with beta-tubulin. We used the ratio between Thr495 and total eNOS for comparison of eNOS phosphorylation in all western blotting experiments.

**Fluorescent activated cell sorting (FACS)**

HUVECs were analyzed in separate wells for intracellular and intramitochondrial ROS by incubating cells in medium with 5 µM...
We wished to investigate combinations of high glucose and ROS to model the short and long term ischemic conditions and to find additive or synergistic effects between the two. We therefore analyzed Thr495 phosphorylation levels in response to increasing concentrations of glucose in combination with a high amount of H$_2$O$_2$. Figure 2 shows that H$_2$O$_2$ increased Thr495 phosphorylation (to 425% (5 mM), 370% (10 mM), 308% (20 mM)). As these increases after H$_2$O$_2$ addition in Thr495 phosphorylation were not significantly different from each other, there was no combined effect of glucose and H$_2$O$_2$ on Thr495 phosphorylation. Regarding phosphorylation of Ser1177 in response to glucose and H$_2$O$_2$, Fig. 3 shows that H$_2$O$_2$ decreased Ser1177 phosphorylation (to 50.6% (5 mM), 31.2% (10 mM), 29.4% (20 mM), p<0.01). As these decreases in Ser1177 phosphorylation were not significantly different from each other, there was no combined effect of glucose and H$_2$O$_2$ on Ser1177 dephosphorylation (Figure 3).

Studies have shown that the production of diacylglycerol (DAG) is significant after prolonged exposure to high glucose concentrations (72 hours). [see Rask-Madsen and King(Rask-Madsen and King 2005) for a review]. DAG stimulates PKC which in turn is capable of phosphorylating Thr495. We therefore hypothesized that although the short term effect of H$_2$O$_2$ is not mediated by PKC, prolonged incubation with glucose concentrations in the range of hyperglycemia could facilitate the PKC pathway. We therefore stimulated cells with 20 mM glucose for 72 hours and added 400 µM H$_2$O$_2$ for the last 30 minutes, which increased Thr495 phosphorylation with 146.4% (from 112.9% to 165.4%) compared to 20 mM (p<0.05) (Figure 4). This effect was significantly reduced by the pan-PKC inhibitor GFX indicating an enhanced role of PKC by the combined effects of glucose and H$_2$O$_2$.

Stimulation with H$_2$O$_2$ induced phosphorylation of eNOS Thr495 after 72 hours of incubation with high levels of glucose and was paralleled by an accumulation of ROS in the mitochondria to 235% (5 mM) and 267% (20 mM) (p<0.05) (Figure 5). No significant difference of mitochondrial ROS accumulation was observed between control (5 mM) and 20 mM without H$_2$O$_2$ stimulation.
Discussion

The main findings in our study is that 72 hours after exposure of HUVECs to 20 mM glucose followed by addition of 400 µM H₂O₂ for 30 minutes induced Thr495 phosphorylation mediated by PKC.

These findings support earlier reports from other groups. Inoguchi et al. observed increased DAG content and PKC activation in aortas of streptozotocin induced diabetic rats and BAECs grown in 22.2 mM glucose for four days also displayed the same characteristics [38]. The
source of the increased DAG has in bovine or rat aortic endothelial and smooth muscle cells grown under the same circumstances for three days been shown to derive from de novo synthesis [39]. Xia et al. also demonstrated that endothelial and smooth muscle cells grown in 22 mM glucose for two to three days increased DAG levels which originated from de novo synthesis [25]. This increase in DAG levels and activity has the pivotal effect of activating PKC [25,38]. Similarly, Craven et al. detected an increased PKC activity which correlated with increased DAG content in non-diabetic rat glomeruli incubated in 30 mM glucose [40]. Consequentially an increased PKC activity leads to phosphorylation of eNOS Thr495 [41]. Our study failed to show that elevated glucose per se would lead to increased phosphorylation of eNOS Thr495. We have previously shown [36] that incubation of HUVECs with H_{2}O_{2} increased phosphorylation of Thr495 not through PKC activation but ROCK and MEKK/ERK activation. In the current study, however, stimulation with H_{2}O_{2} in cells incubated 72 hours after exposure to high glucose levels demonstrated that this phosphorylation can be inhibited by application of the pan-PKC inhibitor GFX. As DAG production is increased after high glucose exposure for 72 hours [3] we therefore expected phosphorylation of Thr495 after glucose exposure alone. Although we failed to observe this, we obtained results that suggest that elevated glucose concentrations for 72 hours facilitate signaling via the DAG-PKC pathway when cells are exposed to H_{2}O_{2}, something we failed to see previously [36]. The applied concentration of the pan-PKC inhibitor GFX of 1 µM exceeds the IC_{50} value of around 20 nM [42], which should completely inhibit PKC with the applied concentration. In pilot experiments we observed that pre-incubation with 1 µM GFX inhibited Thr495 phosphorylation in both HUVECs and BAECs stimulated with PMA. We do recognize that GF109203X may have off-target effects with the applied concentration of 1 µM as it has been shown that GF109203X is not a selective inhibitor of PKC isoforms α, β and γ. Both MAPKAP-K1β and p70 S6 kinase are inhibited by similar potency of GF109203X with MAPKAP-K1β having IC_{50}=50 nM and p70 S6 kinase IC_{50}=100 nM [43]. This is crucial...
because these kinases are involved in signaling pathways activating PKC. In conducting research applying GFX one should ensure that the effects obtained are not due to MAPKAP-K1β and p70 S6 kinase inhibition conducting control experiments with specific inhibitors PD 98059 and rapamycin, respectively. The medium was not changed during the 72 hours of incubation. Studies have shown that HUVECs consume approximately 0.1 mM glucose per hour regardless of outset glucose concentration, yielding a concentration of about 18 mM after 20 hours and 13 mM after 72 hours, without affecting the morphology of the cells [44,45]. This relationship is well established and robust among other cell types also. Altamirano et al. measured consumption rates of glucose in CHO cells. If grown initially at 20 mM the glucose concentration after 20 hours was approximately 17-19 mM, whereas after 72 hours the concentration dropped to around 13 mM [46]. Rheinwald et al. observed in V79 cells grown with complex carbohydrates, when these complex carbohydrates were depleted the concentration of glucose would decline from around 20 mM to approximately 11 mM after 3 days [47]. In all cases the 72 hour time point was still hyperglycemic. Cells incubated with normoglycemic medium would on the other hand experience a slight hypoglycemic environment after 72 hours [45]. It is possible that the used glucose concentration was insufficient to elicit a phosphorylation response without concomitant H2O2 stimulation. To convey this, cells could be incubated with higher glucose concentrations, and assess whether it is possible to obtain a dose dependent relationship in both mitochondrial ROS generation and Thr495 phosphorylation. Higher mortality has been shown in patients with myocardial infarction with an admission glucose levels above 8.44 [48], 9-10 [49,50], 11 [51-53] and 11.7 [54]. mM. Based on these values we incubated HUVECs with high glucose levels with an outset concentration of 20 mM reaching calculated concentrations of 18 mM and 13 mM after 20 and 72 hours, respectively. We thus calculate that the cells remained within the concentration range that is associated with higher mortality in clinical trials. Williams et al. showed that the minimum concentration of glucose to activate PKC is 15 mM [55]. Based on the calculations expressed above, such a concentration is reached after 50 hours. This may explain why we observe mitochondrial increase in ROS after 20 hours, but not after 72 hours. Furthermore, we found in preliminary studies, that HUVECs that have been either serum deprived or have not had changed medium (5 mM glucose) for several days could not elicit a phosphorylation response upon stimulation with H2O2 (data not shown). The rationale behind starvation is poorly defined and it induces an artificial condition that inhibition of PKC could prevent endothelial dysfunction which is seen in conjunction to reperfusion damage and high glucose levels. Oxidative stress leads to mitochondrial dysfunction [9,71,72] and is seen in conjunction to reperfusion damage and high glucose levels. Because a decrease in eNOS derived NO bioavailability is an important manifestation of endothelial dysfunction, it could be argued that we should have attempted to measure the available NO after HUVECs had been exposed to high glucose levels or H2O2. However, we have previously described [36] a decrease in NO production (DAF-2DA chemiluminescence) in acetylcholine stimulated HUVECs.
pre-incubated with H₂O₂ and thus showed a correlation between an increase in Thr495 phosphorylation and a decrease in NO production.

**Conclusion**

Incubation of HUVECs with 20 mM glucose for 20 hours increased mitochondrial ROS but did not induce phosphorylation of Thr495 or dephosphorylation of Ser1177, and did not act synergistically with H₂O₂. Exposure to 400 µM of H₂O₂ for 30 minutes in a physiologic glucose concentration phosphorylated Thr495 and dephosphorylates Ser1177 along with increased ROS in mitochondria. High glucose levels were neither able to increase mitochondrial ROS after 72 hours nor did it induce phosphorylation of Thr495. Addition of H₂O₂ elicited phosphorylation of this residue, which could be prevented by PKC inhibition. This suggests that 72 hours exposure to high glucose levels facilitates the PKC pathway in opposition to our previous studies where HUVECs naïve to high glucose levels phosphorylated Thr495 through MEK/ERK and ROCK in response to H₂O₂. Thus, this model points toward two different pathways being involved in ENOS phosphorylation in response to acute ROS as a model of ischemia and reperfusion in the normoglycemic and hyperglycemic state. Our findings support that high glucose levels induce changes in ENOS phosphorylation which leads to a decrease in enzymatic activity and thus NO production. These findings have clinical implications with respect to metabolic syndrome underscoring the importance of optimizing glycemic control in these patients thus minimizing development of endothelial dysfunction and by extension the incidence of cardiovascular events.

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