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H_2O_2 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. H_2O_2 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 H_2O_2 -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 H_2O_2 (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 H_2O_2 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 H_2O_2 increased Thr495 phosphorylation to 146.6% ($p<0.05$). PKC inhibition attenuated the H_2O_2-induced Thr495 phosphorylation in cells incubated with high glucose levels for 72 hours.

Conclusion: Acute exposure to high glucose induces oxidative stress. H_2O_2 leads to phosphorylation of eNOS at Thr495 and dephosphorylation of Ser1177. After prolonged exposure to high glucose levels, the addition of H_2O_2 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_2O_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 leading to decreased NO bioavailability [32]. In some studies H_2O_2 appears to be have bidirectional effects, with an early promotion of 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_2O_2 would lead to eNOS Thr495 phosphorylation and Ser1177 dephosphorylation. Based on previous results, in which we observed increased mitochondrial ROS after H_2O_2 incubation [36], we expected to see an increase in mitochondrial ROS generation with high glucose alone and with H_2O_2 incubation. By extension, we anticipated phosphorylation changes of eNOS after high glucose levels alone and with H_2O_2 and an additive effect by combining the two. We have previously shown that incubating HUVECs with H_2O_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_2O_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, TPP, Trasdingen, Switzerland) coated with 10.5 μg/cm^2 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_2O_2 for 30 minutes to simulate ischemia/reperfusion. Suppressible 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_2O_2.

The pan-PKC-inhibitor (3-(N-[Dimethylamino]propyl-3-indolyl)-4-(3-indolyl)maleimide, Bisindolylmaleimide I,3-[1-[3-(Dimethylamino) propyl]1H-indol-3-yl]-4-(1Hindol-3-yl)1H-pyrrole-2,5dione) GF109203X (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 phenylmethanesulfonyl 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

5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (C6827, Invitrogen, Eugene, Oregon, USA) or MitoSOX Red mitochondrial superoxide indicator (M36008, Invitrogen, Eugene, Oregon, USA), respectively for 15 minutes at 37°C in the dark. HUVECs were suspended in cold PBS with 1% BSA and analyzed by flow cytometry on a FACS-Aria from BD Biosciences (New Jersey, USA), armed with a blue (488 nm), red (633 nm) and violet (405 nm) laser. CM-H2DCFDA was measured with a 525/50 bandpass filter and MitoSOX Red with a 585/42 bandpass filter. Ten thousand data points were accumulated for each round of analysis.

Statistical analysis

As the absolute signal value from each round of experiment displayed variation we normalized to the control situation in each western gel, thus reflecting the variation in the control level of phosphorylation in an increased variation in the treatment groups. Data are expressed in arbitrary units as percent changes compared to Control (unstimulated cells) and are expressed as average +SEM unless otherwise stated. Groups of data were analyzed by ANOVA followed by the Scheffé post hoc analysis (Statistica, Statsoft, Tulsa, OK, USA). To maintain variance homogeneity relevant data sets underwent logarithmic transformation. Paired comparisons were analyzed by Student's T-test for unequal variance. A p-value <0.05 was considered statistically significant. The letter "n" refers to the number of times an experiment was repeated.

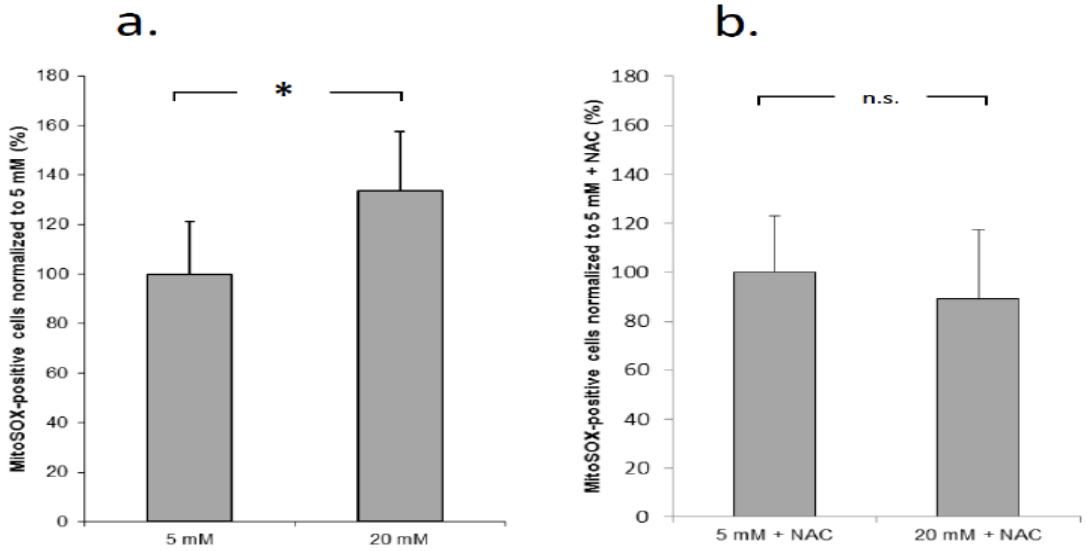
Results

To assess the capability of high glucose levels to induce ROS we exposed HUVECs to a glucose concentration of 20 mM for 20 hours and analyzed the level of ROS in mitochondria as described above. Figure 1a shows that this increased the amount of ROS in mitochondria to 133.5%, $p<0.05$. This effect could be abolished by simultaneous treatment of cells with 10 mM NAC for 20 hours (Figure 1b). We also investigated the effect of glucose on the cytoplasmic levels of ROS. FACS analysis showed that there was no difference in these levels (data not shown).

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_2O_2. Figure 2 show that H_2O_2 increased Thr495 phosphorylation (to 425% (5 mM), 370% (10 mM), 308% (20 mM)). As these increases after H_2O_2 addition in Thr495 phosphorylation were not significantly different from each other, there was no combined effect of glucose and H_2O_2 on Thr495 phosphorylation. Regarding phosphorylation of Ser1177 in response to glucose and H_2O_2, Fig. 3 shows that H_2O_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_2O_2 on Ser1177 dephosphorylation (Figure 3).

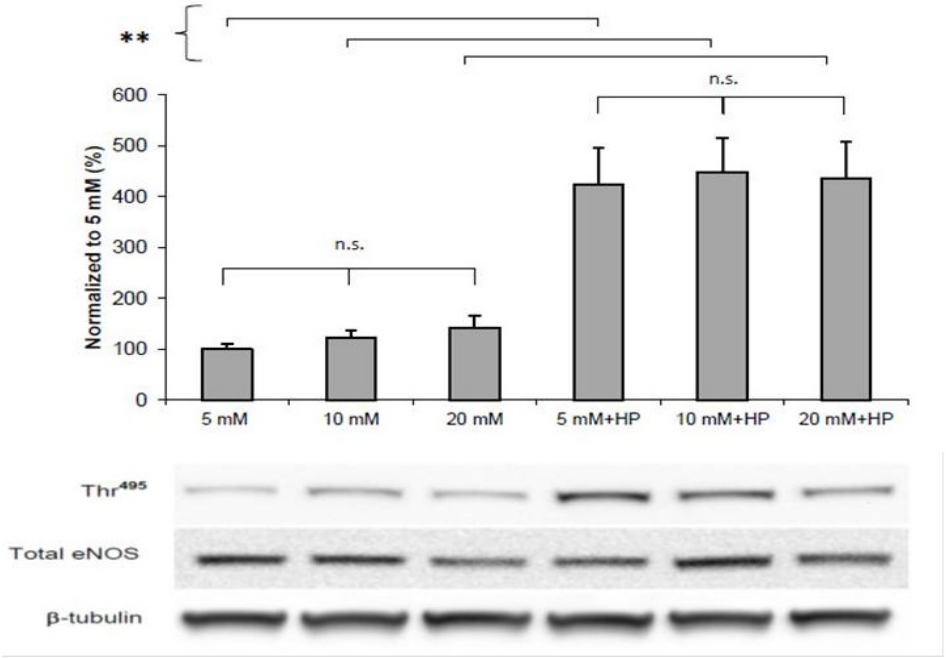
Studies have shown that the production of diacylglycerol (DAG) is significant after prolonged exposure to of high glucose concentrations (72 hours). [see Rask-Madsen and King(Rask-Madsen and King 2005) for a review]. DAG stimulates PKC which in turn is capable of phosphorylating Thr495. We therefore hypothesized that although the short term effect of H_2O_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_2O_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_2O_2.

Stimulation with H_2O_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_2O_2 stimulation.



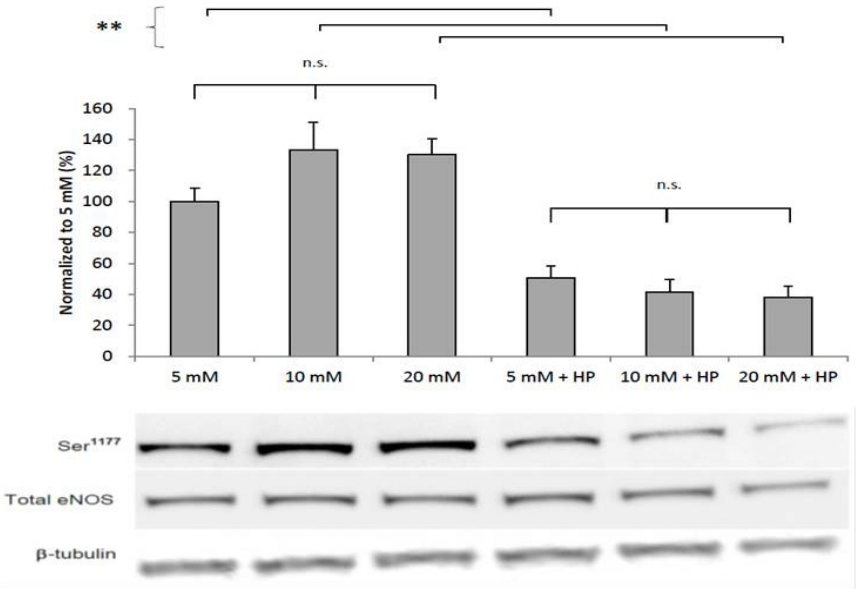
a. The results are presented as normalized means + SEM. N=5. (*p < 0.05: 20 mM glucose treatment for 20 hours vs. control (5 mM))
b. The results are presented as normalized means + SEM. N=4. (n.s. p>0.05; 5 mM + NAC: Control (5 mM glucose) co-incubated with 10 mM NAC for 20 hours. 20 mM + NAC: Cells incubated with 20 mM glucose and 10 mM NAC for 20 hours.

Figure 1: FACS analysis of the ROS formation assessed by application of MitoSOX red mitochondrial superoxide indicator.



30 minutes of H_2O_2 stimulation (400 μM) increased phosphorylation of Thr495 significantly. Incubating cells with increasing amounts of glucose (5, 10, 20 mM) did not significantly alter the Thr495 phosphorylation level. No combined effect of H_2O_2 and glucose was observed. The results are presented as densitometric means of ratios between Thr495 and Total eNOS signals (+/- SEM). Below the graph is shown Thr495, total eNOS and Beta tubulin. (HP: H_2O_2; n.s.: $p>0.05$; ** $p<0.01$).

Figure 2: Western blotting analysis of the role of H_2O_2 and glucose on phosphorylated threonine 495 residue on eNOS (p -Thr495) (n=8).

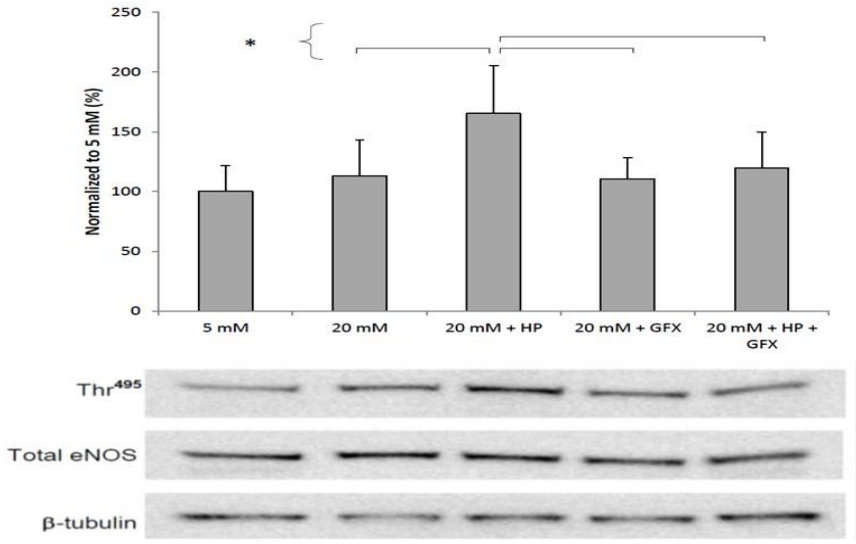


30 minutes of H_2O_2 stimulation (400 μM) decreased Ser1177 phosphorylation 30 minutes of H_2O_2 stimulation (400 μM) decreased phosphorylation of Ser1177 significantly. Incubating cells with increasing amounts of glucose (5, 10, 20 mM) did not significantly alter the Ser1177 phosphorylation level. No combined effects of H_2O_2 and glucose was observed. The results are presented as densitometric means of ratios between Thr495 and Total eNOS signals (+/- SEM). Below the graph is shown Ser1177 as a representative blot as well as the same blot reprobed with total eNOS. Beta tubulin is shown below. (HP: H_2O_2; n.s.: $p>0.05$; ** $p<0.01$).

Figure 3: Western blotting analysis of Ser1177 phosphorylation in HUVECs stimulated with H_2O_2 and glucose (n=8).

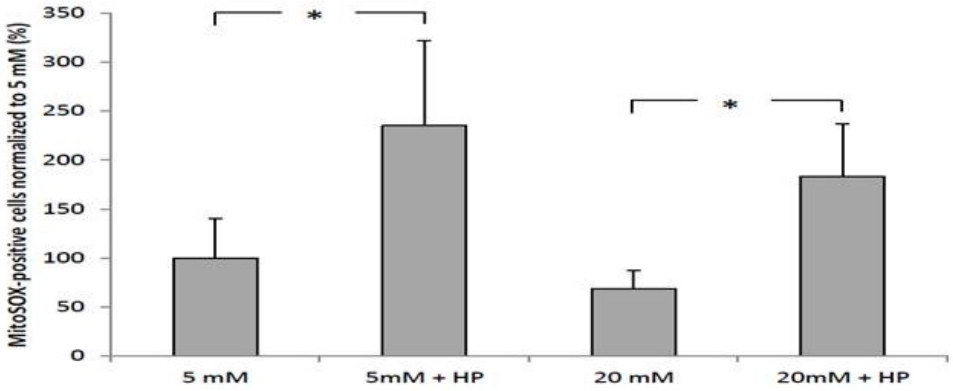
Discussion

The main findings in our study is that 72 hours after exposure of HUVECs to 20 mM glucose followed by addition of 400 μM H_2O_2 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



In order to assess the role of PKC some cells were incubated with 1 μM of the pan-PKC inhibitor GFX for the last 60 minutes (n=3). H_2O_2 induced phosphorylation of Thr495, which could be inhibited by GFX. (HP: H_2O_2; GFX: GF 109203X ; *p<0.05)

Figure 4: Western blotting analysis of Thr495 phosphorylation in HUVECs incubated in 20 mM glucose for 72 hours and stimulated with H_2O_2 for the last 30 minutes.



The results are presented as normalized means +/- SEM (n=4). HUVECs were stimulated with 20 mM glucose for 72 hrs and relevant cells were stimulated with 400 μM H_2O_2 for the last 30 minutes. H_2O_2 increased the concentration of ROS in the mitochondria whereas 72 hrs of 20 mM glucose failed to do so. (HP: H_2O_2; *p<0.05)

Figure 5: FACS analysis of the ROS formation assessed by application of MitoSOX red (mitochondrial superoxide indicator).

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_2O_2 increased phosphorylation of Thr495 not through PKC activation but ROCK and MEKK/ERK activation. In the current study, however, stimulation with H_2O_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_2O_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 1μM 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 H_2O_2 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 exposed 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 H_2O_2 (data not shown). The rationale behind starvation is poorly defined and it induces an artificial condition where most of the cells are in an arrested state. The effects of serum starvation seem grave as it induces phosphorylation of many signaling molecules, such as a more than tenfold change in ERK phosphorylation in different cell lines [56]. Therefore, we chose not to starve our cells prior to the studies.

In the micromolar concentration range, H_2O_2 induces changes in membrane potential [57] and intra-cellular calcium in human endothelial cells [57,58], which is not the case at higher concentrations [58]. Thus, intracellular calcium changes are probably not part of the changes that we have observed in our studies.

The applied concentration (400 μM) of H_2O_2 is enough to cause apoptosis in HUVECs [59] and PKC inhibition could inhibit PKC-dependent cell apoptosis [60]. It is in our model not elucidated whether apoptotic pathways play a role in eNOS phosphorylation on Thr495.

Activation of eNOS by phosphorylation at Ser1177 is accompanied by a decrease in the dependence of eNOS for Ca^{2+}/calmodulin [61]. The role of high glucose levels on NO production and the effect on Ser1177 phosphorylation are somewhat unclear. Carneiro et al. showed that diabetic rats had decreased eNOS phosphorylation levels at Ser1177 in corpora cavernosa [62] and Schnyder et al. showed that high glucose levels in HUVECs (15-30 min; 25 mM) inhibited NO production [63]. Salt et al., however, showed in Human Aortic Endothelial Cells (HAECs) that 25 mM glucose for 48 hours inhibited insulin stimulated NO production although phosphorylation at Ser1177 was not reduced [64]. Furthermore even shorter periods (5 hours) of high glucose levels inhibits Thr495 dephosphorylation and phosphorylation of Ser1177 in bradykinin-stimulated PAECs (porcine aortic endothelial cells) [65]. A recent study on adult cardiac myocytes showed that 10 μM hydrogen peroxide stimulation increased phosphorylation of eNOS at Ser1177 [66]. Likewise, Thomas et al. incubated PAECs with 130-300 μM H_2O_2 for 30 minutes yielding the same result [34]. Also Urao et al. showed that endogenous H_2O_2 increases Ser1177 phosphorylation in a mouse hind limb ischemia model [67]. Our experiments did not show an effect of increased ROS generated by high glucose concentrations on the phosphorylation of Ser1177. In fact, high concentrations of hydrogen peroxide had the opposite effect with a decrease in Ser1177 phosphorylation regardless of the concomitant glucose concentration. The explanation for these apparently contradictory results is suggested by a study by Hu et al. who described a dose-dependent biphasic response to H_2O_2 in which 500 μM initially increased Ser1179 phosphorylation in eNOS transfected HEK 293 cells and BAECs grown in serum-containing medium followed by a drastic decline in phosphorylation after 30 minutes [35]. It is possible that the same mechanism has taken place in our cells, even though this remains speculative as we only have assessed the phosphorylation response after 30 minutes of H_2O_2 stimulation. A clinical trial showed that infusion of a peptide inhibitor of PKCδ (delcasertib) given at reperfusion reduced infarct size when given to STEMI patients [68]. IC_{50} of GFX on PKCδ inhibition is 0.21 μM. Thus a substantial suppression of PKCδ to about 10% activity is achieved with 1 μM used in our study [69]. The findings, however, could not be replicated in the PROTECTION-MI trial, which was a multicenter, double-blind trial was performed in patients presenting within 6 hours undergoing primary PCI for STEMI [70]. We confirm here in an *in vitro* model of reperfusion that PKC is involved in Thr495 phosphorylation giving an indication 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 H_2O_2 has been suggested as a retrograde signaling molecule deriving from the cytoplasm, as it seems to be supported by the finding that neuronal mitochondria release H_2O_2 in response to incubation with H_2O_2 [73]. Nevertheless, our previous studies failed to confirm this hypothesis [36]. Here we also found no clear indication of a possible retrograde signaling from the mitochondrion to eNOS, since 20 mM of glucose elicited ROS accumulation in the mitochondrion that was not paralleled by phosphorylation or dephosphorylation of eNOS, while externally applied H_2O_2, both accumulated eNOS and induced phosphorylation changes of eNOS. We cannot conclude whether 72 hours of high glucose levels have an effect on mitochondrial ROS accumulation since the final glucose concentrations probably were insufficient to provoke this response. To amend this problem, future studies with higher glucose levels are warranted.

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 H_2O_2. However, we have previously described [36] a decrease in NO production (DAF-2DA chemiluminescence) in acetylcholine stimulated HUVECs

pre-incubated with H_2O_2 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 H_2O_2. Exposure to 400 μM of H_2O_2 for 30 minutes in a physiologic glucose concentration phosphorylates 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 H_2O_2 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 H_2O_2. 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 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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