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Increased Susceptibility to Amyloid- β Toxicity in Rat Brain Microvascular Endothelial Cells under Hyperglycemic Conditions

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Abstract

We hypothesized that hyperglycemia-induced mitochondrial dysfunction and oxidative stress are closely associated with amyloid- β peptide (A β) toxicity in endothelial cells. Brain microvascular endothelial cells from rat (RBMEC) and mice (MBMEC) were isolated from adult Sprague-Dawley rats and homozygous db/db (Lepr^{db}/Lepr^{db}) and heterozygous (Dock7^m/Lepr^{db}) mice, and cultured under normo- and hyperglycemic conditions for 7 d followed by 24 h exposure to A β ₁₋₄₀. Some experiments were also performed with two mitochondrial superoxide (O₂^{•-}) scavengers, MitoTempo and Peg-SOD. Cell viability was measured by the Alamar blue assay and mitochondrial membrane potential (Ψ m) by confocal microscopy. Mitochondrial O₂^{•-} and hydrogen peroxide (H₂O₂) production was assessed by fluorescence microscopy and H₂O₂ production was confirmed by microplate reader. Hyperglycemia or A β ₁₋₄₀ alone did not affect cell viability in RBMEC. However, the simultaneous presence of high glucose and A β ₁₋₄₀ reduced cell viability and Ψ m, and enhanced mitochondrial O₂^{•-} and H₂O₂ production. MitoTempo and PEG-SOD prevented A β ₁₋₄₀ toxicity. Interestingly, MBMEC presented a similar pattern of alterations with db/db cultures presenting higher susceptibility to A β ₁₋₄₀. Overall, our results show that high glucose levels increase the susceptibility of brain microvascular endothelial cells to A β toxicity supporting the idea that hyperglycemia is a major risk factor for vascular injury associated with AD.

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Keywords

Alzheimer's disease; amyloid- β peptide; brain endothelial cells; mitochondria; type 2 diabetes

1. INTRODUCTION

Modern improvements in health care, increased life expectancy, and the proportion of the aged population in the developed world [1] has led to an abrupt rise in the prevalence of age-associated diseases such as Alzheimer's disease (AD). Sporadic AD, which represents the majority of AD cases, is a late-onset disease primarily affecting people over 65 years old. This disease is characterized by a progressive cognitive decline with behavioral changes culminating in a complete loss of control of bodily functions and death [2]. The deposition of amyloid- β (A β) peptide as senile plaques and neurofibrillary tangles formed mainly by hyperphosphorylated tau protein are characteristic neuropathologic hallmarks of AD. Although research has focused primarily on A β peptide and the mechanisms underlying its production and toxic effects on neurons [3], evidence shows that cerebral vascular endothelium is an early target in AD [4].

Type 2 diabetes (T2D) is one of the major risk factors for AD with chronic hyperglycemia being one of its hallmarks. High levels of glucose activate several deleterious pathways culminating in several metabolic and cellular abnormalities [5]. Indeed, under a hyperglycemic state, high levels of reactive oxygen species (ROS) are produced activating several deleterious pathways, such as the activation of the transcription factor nuclear factor κ B (NF κ B) enhancing monocytes adhesion to the vessel walls, which may initiate an atherosclerotic state [6]. Furthermore, an increase in endothelin-1 and a decrease in nitric oxide levels can contribute to increased blood-brain barrier permeability, inflammation, vasoconstriction, and, consequently, neuronal damage [7].

An extensive literature shows that hyperglycemia has deleterious effects on endothelial cells. Indeed, Rogers and coworkers [8] showed that human umbilical vein endothelial cells exposed to high glucose showed the accelerated appearance of markers of senescence jointly with reduced endothelial nitric oxide synthase expression and activity. Furthermore, several studies demonstrated that hyperglycemia enhances ROS production, which plays a major role in the etiology of diabetes complications, such as endothelial dysfunction [9, 10]. Recent findings confirmed that hyperglycemia-derived ROS mediated apoptosis of vascular endothelial cells [11, 12]. It was also reported that high glucose increased the permeability of endothelial cells of umbilical veins [13] or microvessels [14] in humans.

Since T2D is a risk factor for AD and chronic hyperglycemia is a hallmark of diabetes, we postulated that elevated blood glucose could compromise the cerebral vascular endothelium and enhance the toxicity of A β . Furthermore, we hypothesized that endothelial mitochondria might be adversely affected by hyperglycemia and initiate, via mechanisms involving enhanced production ROS, the destructive effects of A β . Therefore, we investigated whether high glucose potentiates the effects of A β ₁₋₄₀ in rat (RBMEC) and mice (MBMEC) brain microvascular endothelial cells derived from adult Sprague Dawley (SD) rats and diabetic db/db mice, respectively.

MATERIAL AND METHODS

The animal protocol was approved by the Institutional Animal Care and Use Committee of Tulane University School of Medicine. All experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For RBMEC culture, 10 week old, male SD rats ($n = 6$) were obtained from Harlan laboratory; for MBMEC culture, 11 week old db/db mice, a homozygous mouse model for the diabetes spontaneous mutation ($Lepr^{db}$), and the heterozygous mice ($Dock7^m/Lepr^{db}$) were obtained from Jackson Laboratory. Rodents were housed in the animal care facility and received standard rat or mice chow and tap water *ad libitum*.

Brain microvascular endothelial cell isolation

Briefly, animals were decapitated under deep anesthesia, and the brain cortices were freed from meninges, homogenized, and digested. The homogenate was redistributed in 20% bovine serum albumin and was centrifuged at 1,000 g for 20 min to yield cortical microvessels. The microvessels were washed in Dulbecco's modified Eagle medium (DMEM) further digested, layered on a continuous 33% Percoll gradient, and centrifuged again at 1,000 g for 10 min. The band of cerebral microvascular endothelial cells was aspirated, washed, and was then seeded onto collagen IV and fibronectin-coated glass-bottom culture dishes (MatTek, Ashland, MA, USA) and plates (BD Falcon, Bedford, MA). A 300 μ l volume of cells was added to each well and allowed to seed for 24 h. Then, the medium was changed and Puromycin (4 μ g/ml) was added for 48 h to avoid the proliferation of P-glycoprotein negative contaminating cells [15]. At day 3, the cell medium was replaced by fresh medium with different glucose concentrations and was then changed every 48 h. The cell culture medium consisted of DMEM supplemented with 20% fetal bovine plasma-derived serum, 2 mM glutamine, 1 ng/ml basic fibroblast growth factor, 50 μ g/ml endothelial cell growth supplement, 100 μ g/ml heparin, 5 μ g/ml vitamin C, and antibiotics. We have previously demonstrated the purity of our cultures consisting of >95% BMECs, verified by positive immunohistochemistry for von Willebrand factor and by negative immunohistochemistry for glial fibrillary acidic protein (GFAP) and α -smooth muscle actin [16].

Cell culture and treatments

The RBMEC were exposed to 5, 25, and 30 mM of glucose for 7 days at 37°C and MBMEC were maintained in 5 or 30 mM glucose medium for 7 days at 37°C. The glucose concentrations and incubation period were selected based upon previous studies with 5 mM being considered a normoglycemic concentration and 30 mM as hyperglycemia [17, 18]. Five or 10 μ M of $A\beta_{1-40}$ was added at day 6. To elucidate the involvement of mitochondrial ROS in $A\beta$ -induced toxicity, RBMECs were co-incubated with $A\beta_{1-40}$ and antioxidants: 100 μ M mitochondrial-targeted antioxidant (MitoTempo) or 100 units/ml of an enzyme involved in $O_2^{\bullet-}$ removal (PEG-SOD). Osmotic controls in RBMEC were done with 25 mM mannitol and $A\beta_{40-1}$ was used as peptide negative control.

Assessment of cell viability

Cell viability was determined using the Alamar Blue (a soluble, stable, and non-toxic redox indicator that is used to evaluate metabolic function and cellular health) assay. One hour

before the termination of an experiment, a 10% solution of Alamar blue was added to the culture medium. After 1 h incubation at 37°C, the supernatant was collected and the absorbance was measured at 570 nm and 600 nm using a microplate reader (SpectraMax uQuant microplate Reader, BioTek, Winooski, VT) [19]. Cell viability (% of control) was calculated according to the formula $(A570 - A600)$ of treated cells $\times 100 / (A570 - A600)$ of control cells.

Measurement of mitochondrial ROS production

Live staining of RBMEC for mitochondrial superoxide ($O_2^{\bullet-}$) production was performed using MitoSOX (Molecular Probes, Eugene, OR), a cell permeable probe that accumulates in mitochondria and fluoresces following oxidation by $O_2^{\bullet-}$, as described by Knorr et al. [20]. MitoSOX was dissolved in DMSO and cells were incubated with a solution of MitoSOX in DMEM phenol-free to a final concentration of 5 μ M, at 37°C, for 20 min in a light-protected coverslip chamber. Then, cells were rinsed and fresh DMEM phenol-free was added. Cells were immediately examined by confocal microscopy. Images were obtained with a Leica SP2 AOB laser confocal microscope (Heidelberg, Germany) for a period of no longer than 20 min.

Measurement of H_2O_2 levels

H_2O_2 levels were measured using the AmplexTM Red-horseradish peroxidase assay kit, as previously described [21]. This assay utilizes horseradish peroxidase to catalyze the H_2O_2 -dependent oxidation of non-fluorescent AmplexTM Red to fluorescent resorufin red. Briefly, 50 μ M AmplexTM Red reagent and 0.1 U/ml peroxidase in DMEM phenol-free were added to cells and incubated for 1 h at 37°C protected from light [22]. Fluorescence was read at 565 nm wavelength at 37°C in an automatic microplate reader (FLUOstar OPTIMA microplate reader, BMG Labtech, Offenburg, Germany) equipped with a thermally controlled compartment and results were expressed as % of control.

Measurement of mitochondrial membrane potential (Ψ_m)

Rhodamine 123 (RHD123) (Molecular Probes, Eugene, OR), a fluorescent cationic dye, was used to monitor changes in Ψ_m . After treatment, cells were washed with phosphate-buffered saline and incubated with 2.5 μ M RHD123 in DMEM phenol-free for 45 min at 37°C. After incubation, the medium was replaced with fresh DMEM phenol-free and fluorescence was read using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany) and basal fluorescence was monitored at 505 nm excitation and 525 nm emission wavelengths. Immediately after the initial reading, the mitochondrial uncoupler CCCP (5 μ M) was added to induce complete depolarization. The difference between the initial fluorescence and the final fluorescence was used to evaluate Ψ_m . Results were expressed as percentage of control fluorescence. This protocol was also performed for live imaging and cells were examined by confocal microscopy. Images were obtained with a Zeiss 7 Live laser scanning confocal microscope (Jena, Germany) for a period of no longer than 10 min.

Statistical analysis

Results are presented as means \pm SEM of the indicated number of experiments. Statistical significance was determined using the One Way ANOVA test for multiple comparisons, followed by the *post hoc* Tukey test.

RESULTS

Hyperglycemia increases the susceptibility of endothelial cells to A β peptide toxicity

Under chronic hyperglycemia, RBMEC did not show any significant alteration in cell viability (Fig. 1A). However, under hyperglycemic conditions, the viability of RBMEC decreased by approximately 40% when exposed to 10 μ M A β ₁₋₄₀ whereas cells under normoglycemic conditions (5 mM glucose) did not show any significant alterations (Fig. 1A). Interestingly, MBMEC from homozygous but not heterozygous db/db mice presented an increased susceptibility to A β ₁₋₄₀ at both 5 and 10 μ M (Fig. 1B) showing that endothelial cells isolated from mice under an *in vivo* hyperglycemic state are more vulnerable to A β ₁₋₄₀ toxicity. For RBMEC, MitoTempo and Peg-SOD were equally effective in protecting cell viability with a 1.75 fold increase in cell viability (Fig. 1C). Peg-Catalase and L-NAME were also able to modestly reverse cell death, but with less efficacy (data not shown).

Hyperglycemia-induced ROS production is enhanced by A β exposure

The RBMEC under hyperglycemic conditions presented higher levels of mitochondrial O₂^{•-} production (Fig. 2G), which were exacerbated in the presence of A β ₁₋₄₀ (Fig. 2J). As expected, MitoTempo and Peg-SOD effectively prevented the increase in O₂^{•-} levels (Fig. 2H, I, K, and L).

Hyperglycemia alone did not change H₂O₂ levels (Fig. 3). However, A β ₁₋₄₀ promoted an increase in H₂O₂ levels, in both normo- and hyperglycemic conditions, an effect that was prevented by MitoTempo and Peg-SOD (Fig. 3).

A β leads to a decrease in mitochondrial membrane potential under chronic hyperglycemia

The plate reader assay showed that RBMEC exposed to high glucose did not present significant alterations in Ψ m. However, those cells co-incubated with A β ₁₋₄₀ showed a significant loss of Ψ m (Fig. 4). The antioxidants MitoTempo and Peg-SOD prevented the loss of Ψ m; however, Peg-SOD was more effective in preserving Ψ m (Fig. 4).

DISCUSSION

The major finding of our study is that chronic hyperglycemia enhances brain microvascular endothelial cell susceptibility to A β peptide exposure through mitochondrial changes that seem to be initiated by increased mitochondrial O₂^{•-} production. Additionally, the results obtained with MitoTempo and Peg-SOD seemed to corroborate this hypothesis, since these antioxidants were proved to be effective in preventing high glucose and A β toxicity. Thus, our study provides a mechanistic basis for the linkage between T2D and the enhanced damage to cerebral endothelial cells as a cause for AD.

T2D is one of the major risk factors for the development of neurodegenerative diseases. Over 60% of T2D patients are estimated to suffer from neurological disorders [23–25]. Furthermore, studies report that people with T2D are 1.39 times more likely to develop AD [26]. In fact, previous studies from our laboratory showed that diabetic and AD mice present a similar profile of behavioral, cognitive, vascular, and mitochondrial abnormalities [27, 28]. Interestingly, diabetic mice presented a significant increase in A β levels in brain cortex and hippocampus [27, 28]. Accordingly, Kolluru and coworkers [29] reported that vascular dysfunction is a major player in the establishment of diabetes-associated complications. However, studies on the cerebral microvasculature exposed to hyperglycemia are not as advanced as those on peripheral vessels [30].

Hyperglycemia *per se* did not affect viability or other parameters in RBMEC. However, the combination of high glucose plus A β_{1-40} peptide induced a significant decrease in cell viability in a dose-dependent manner, whereas the viability of RBMEC cells was not significantly changed when exposed to A β_{1-40} alone. The absence of A β_{1-40} toxicity seems, in some extent, contradictory with most published studies describing the toxic effects of the peptide. However, when we analyzed the results, we realized that the control maintenance medium is supplemented with high levels of glucose hiding the possible non-toxic, physiological roles of A β_{1-40} [31, 32]. To prove that chronic hyperglycemia increases the susceptibility of brain endothelial cells to A β_{1-40} , we also tested the effects of this peptide in MBMEC isolated from diabetic db/db mice. The db/db mice are polyphagic, polydipsic, and polyuric and demonstrate an uncontrolled rise in blood sugar, severe depletion of the insulin-producing β -cells of the pancreatic islets, and death by 10 months of age. Interestingly, a substantial effect on db/db MBMEC viability occurred regardless of glucose level in culture when exposed to A β peptide. Additionally, the experiments on MBMEC demonstrate that these endothelial cells retain a distinct phenotype in culture consistent with chronic exposure to hyperglycemia. Previous studies also reported that A β neurotoxicity was exacerbated during hypoglycemia/hyperglycemia (≤ 2 mM/ ≥ 30 mM) [33]. Hyperglycemia alone did not interfere with Ψ m but, in the presence of A β_{1-40} , we observed a significant decrease in Ψ m (Fig. 4). These results are in agreement with a study performed by Moreira et al. [34] showing that brain mitochondria isolated from T2D Goto-Kakizaki rats did not show significant alterations in Ψ m, but a significant drop in this parameter occurred when mitochondria were exposed to A β_{1-40} peptide.

Furthermore, we showed that the increased susceptibility of RBMEC under high glucose exposure to A β is mediated by mitochondrial O $_2^{\bullet-}$. Indeed, it was previously suggested that mitochondrial overproduction of ROS due to hyperglycemia may increase the susceptibility of endothelial cells to injury [35]. In fact, we observed a significant increase in mitochondrial O $_2^{\bullet-}$ when cells were exposed to 30 mM glucose and this increase was exacerbated by A β_{1-40} . A significant increase in H $_2$ O $_2$ levels was also observed in both normoglycemic and hyperglycemic cells exposed to A β peptide but it is unclear whether this ROS was produced directly or via dismutation of O $_2^{\bullet-}$. These results are consistent with previous studies showing that, under physiologic conditions, cells maintain the redox balance through the generation and elimination of ROS, however, when redox homeostasis is disturbed, oxidative stress may lead to aberrant cell death and contribute to disease

development including neurodegenerative diseases [36]. To confirm that mitochondrial ROS play a key role in the alterations observed in cells exposed to 30 mM glucose and A β we tested several antioxidants: MitoTempo, Peg-SOD, Peg-catalase, L-NAME, and apocynin (an inhibitor of NADPH oxidases). The MitoTempo and Peg-SOD completely prevented the loss of cell viability induced by high glucose plus A β ₁₋₄₀, contrasting with the other antioxidants which only exerted a partially protective effect (data not shown). As expected, MitoTempo and Peg-SOD normalized O₂^{•-} levels in cells exposed to high glucose or high glucose plus A β ₁₋₄₀. These observations suggest that mitochondrial O₂^{•-} has a key role in mediating high glucose and A β toxicity. Previous studies showed that MitoTempo is a SOD mimetic with a mechanism of action similar to SOD in O₂^{•-} detoxification, which is, however, specifically targeted to mitochondria [37]. Furthermore, both MitoTempo and Peg-SOD were able to prevent the drop in Ψ m and the increase in H₂O₂ levels in cells exposed to high glucose plus A β ₁₋₄₀. Interestingly, Peg-SOD was more effective in restoring Ψ m than MitoTempo. This may be due to the fact that Peg-SOD is also able to detoxify the O₂^{•-} produced at other locations besides mitochondria. Although mitochondria are major sources of ROS, we cannot exclude the potential contribution of other cytosolic sources [38], which may potentiate mitochondrial dysfunction and ROS production [39]. Concerning H₂O₂ levels, no alterations were observed in the presence of Peg-SOD in control conditions (Fig. 3). However, Peg-SOD reversed A β -induced increase in H₂O₂ levels under hyperglycemic conditions. The reduction in H₂O₂ levels seems contradictory since Peg-SOD converts O₂^{•-} to H₂O₂. Liochev and Fridovich [40] suggested that an increase in O₂^{•-} dismutation would prevent the formation of H₂O₂ by other reactions. This is supported by studies performed in cell lines overexpressing CuZnSOD, which show reduced levels of H₂O₂ [41]. It was also reported that Peg-SOD treatment increased catalase activity in cancer cells [42], an additional mechanism that could justify the decrease in H₂O₂ levels. Additionally, MitoTempo was able to decrease H₂O₂ levels (Fig. 4). Dikalova and coworkers [43] showed that in addition to O₂^{•-} scavenging, MitoTempo has the capacity to reduce mitochondrial ROS production by normalizing mitochondrial respiration. In agreement with our results, Liang et al. [44] also showed that MitoTempo was able to recover Ψ m through the inhibition of the mitochondrial permeability transition pore.

In summary, our results show that brain endothelial cells under chronic hyperglycemia are more susceptible to A β toxicity, an effect that seems to be mediated by mitochondrial ROS. Furthermore, we have shown that our *in vitro* model of chronic hyperglycemia mimics diabetic conditions. This study supports the idea that diabetes is a risk factor for AD.

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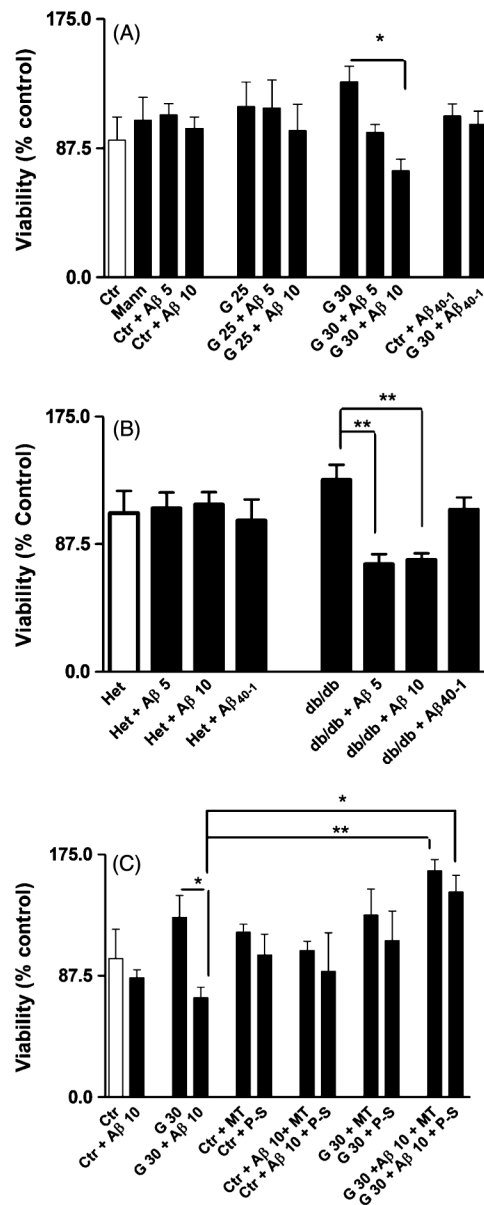
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**Fig. 1.**

Effects of glucose and A β on viability of RBMEC and MBMEC. A) High levels of glucose alone did not affect cell viability, however, high glucose increased susceptibility to A β toxicity. B) In MBMEC from db/db mice but not from lean mice, both high and low glucose increased toxicity to A β toxicity. Here we only show low glucose data since there were no differences in responses within the obese and lean groups. C) When treated with MitoTempo and PEG-SOD, cell viability was restored to values close to control treated cells. Where Ctrl is Control, Mann is Mannitol, A β 5 is A β peptide 1–40 (5 μ M), A β 10 is A β peptide 1–40 (10 μ M), G 25 is 25 mM Glucose, G 30 is 30 mM Glucose, MT is MitoTempo, P-S is PEG-SOD, A β 40-1 is A β peptide 40-1 (10 μ M), Het is heterozygote culture and db/db is diabetic mice culture. Data are combined from 5 independent cultures. Statistical significance: * p < 0.05; ** p < 0.01, and *** p < 0.001.

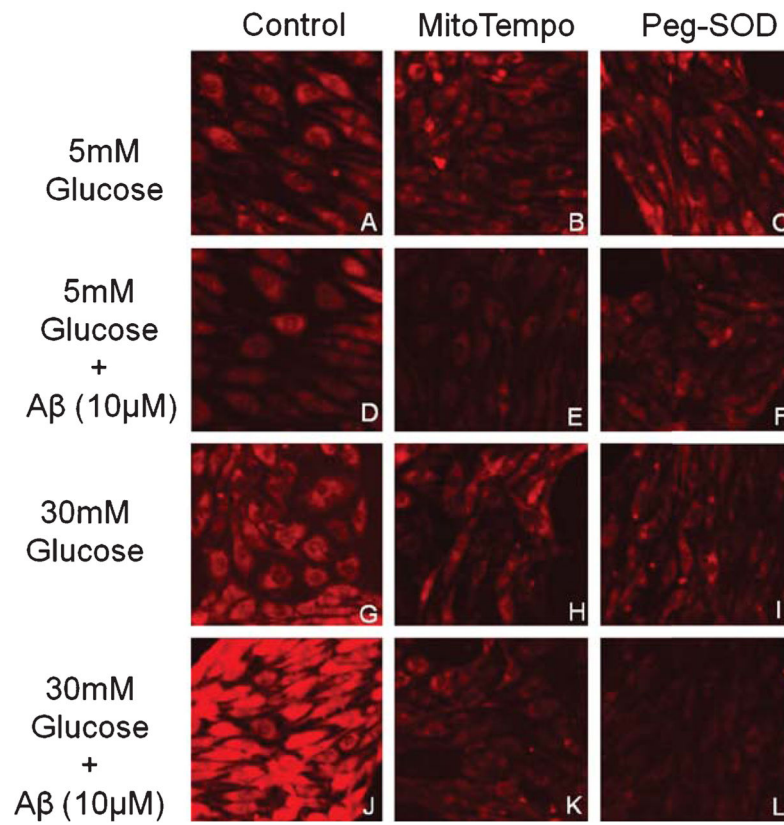


Fig. 2. Role of ROS in promoting toxicity to A β . Representative MitoSOX fluorescence images of RBMEC treated with 5 mM (A–F) or 30 mM (G–L) glucose in the presence (D–F; J–L) or absence (A–C; G–I) of A β peptide are shown. Furthermore, MitoTempo (B,E,H,K) and PEG-SOD (C,F,I,L) representative fluorescence images are also shown. Data are combined from 4 independent cultures.

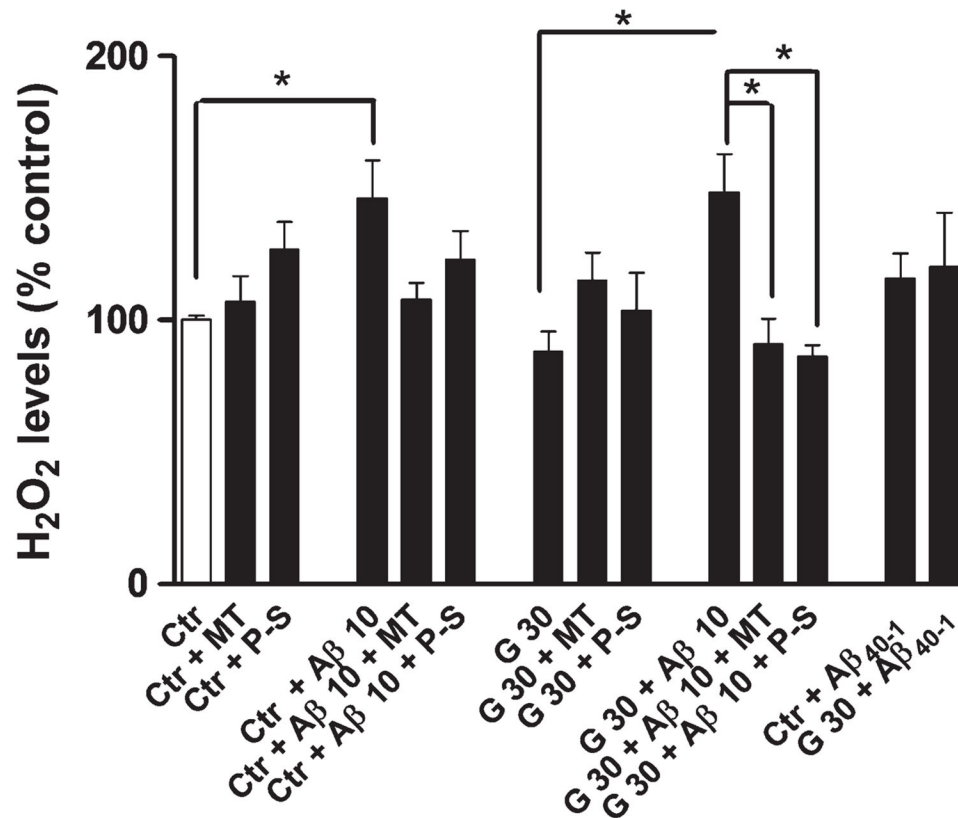


Fig. 3. Effects of hyperglycemia and A β on H₂O₂. High levels of glucose alone did not increase H₂O₂ levels however A β incubation led to a significant increase in H₂O₂ levels in both 5 mM and 30 mM glucose. When treated with MitoTempo and PEG-SOD, H₂O₂ levels reverted to values near control cells values. Data are combined from 5 independent cultures. Statistical significance: * $p < 0.05$.

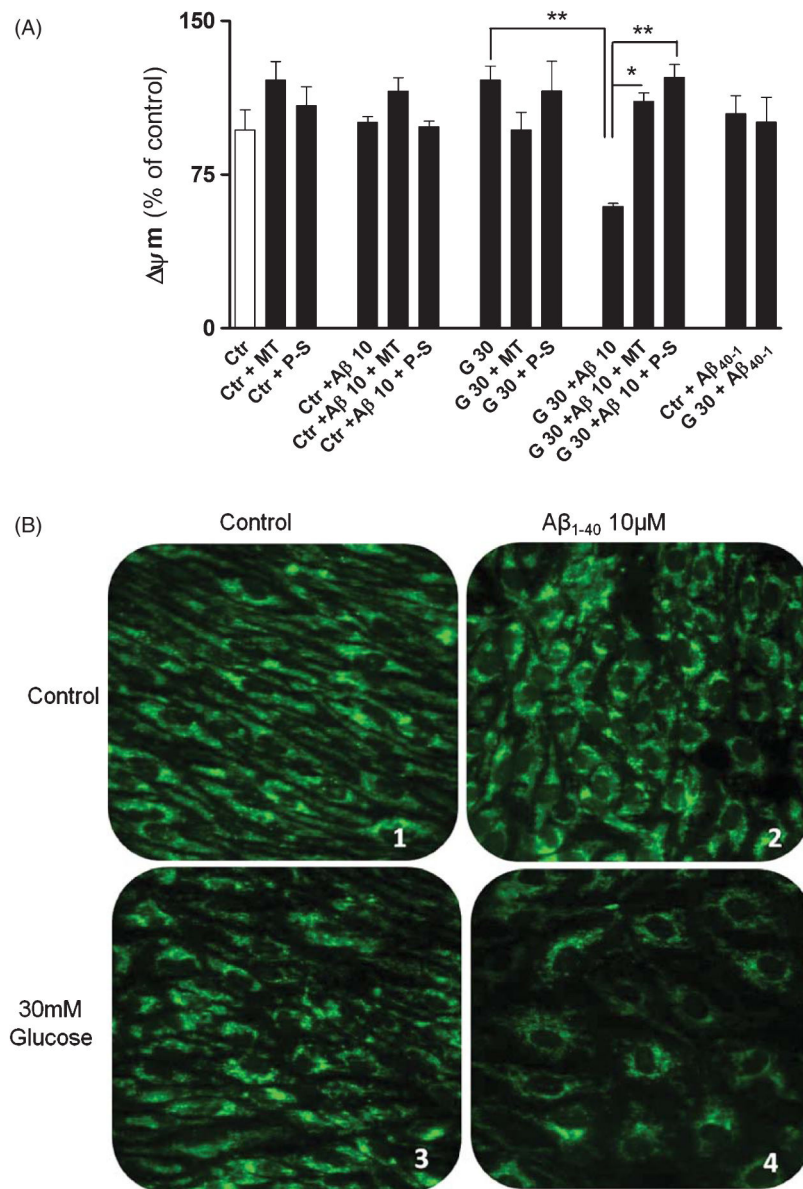


Fig. 4. Effects of hyperglycemia and A β on Ψ m. A) High levels of glucose did not affect mitochondrial membrane potential (Ψ m). However, in the presence of A β peptide a significant drop in Ψ m was observed. MitoTempo and PEG-SOD restored Ψ m in 30 mM glucose. Data are combined from 5 independent cultures. Statistical significance: ** p < 0.01. Also representative images of RHD123 fluorescence images of RBMEC treated with 5 mM (B1, B2) or 30 mM (B3, B4) glucose in the presence (B2, B4) or absence (B1, B3) of A β peptide (10 μ M) are shown.