BETA ADRENERGIC RECEPTORS STIMULATION ATTENUATES PHOSPHORYLATION OF NF-κB AND IκBα IN HYPERGLYCEMIC ENDOTHELIAL CELLS

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ABSTRACT

Background/Aims: NF-κB induces transcription of a number of genes, associated with inflammation and apoptosis. In this study, we have investigated the effect of β-adrenergic receptor stimulation on NF-κB and IκBα in HUVECs. Methods: Human umbilical vein endothelial cells (HUVECs) were cultured in high and low glucose concentrations. All HUVECs were treated with different concentrations of isoproterenol and propranolol for different time periods. The analytical procedures consisted of Western Blot, ELISA, DCFH-DA and TUNEL assays. Results: Isoproterenol (agonist of a beta-adrenergic receptor) significantly reduced phosphorylation at Ser-536 of NF-κB; and Ser-32 and Ser-36 of IκBα in hyperglycemic HUVECs. Isoproterenol also significantly reduced apoptosis and ROS generation. No effect of IκBα was observed on Tyr-42 phosphorylation. The effect of isoproterenol was reversed by the antagonist propranolol. We also checked if NF-κB inhibitor MG132 causes any change at the level of apoptosis. However, we observed an almost similar effect. Conclusion: Given data demonstrates that beta-adrenergic receptors stimulation has a protective effect on HUVECs that might be occuring via NF-κβ and IκBα pathway.

Keywords : Beta Adrenergic receptors, Hyperglycemia-induced inflammation, Apoptosis NF-κB, HUVECs

INTRODUCTION

Diabetes mellitus (DM) is considered to be a metabolic disorder characterized by hyperglycaemia, insulin resistance, pancreatic β cell dysfunction and other complications [1-10]. Worldwide, millions of people are being affected by diabetes, assigning it as one of the most common non-communicable diseases [8-10]. The molecular and biochemical mechanisms of both type I Diabetes mellitus (T1DM) and type II Diabetes mellitus (T2DM) are not necessarily be the same. However, there are numerous common similarities between both. Both types of diabetes greatly increase the risk of various other complications including inflammation and endothelial dysfunction [10-16].

Overproduction of reactive oxygen species (ROS) by mitochondria is known to be a causal link between hyperglycemia and the major biochemical pathways, including NF- kB-mediated inflammation [17, 18]. NF-κβ pathway is a well known pro-inflammatory signalling pathway [19]. NF-κβ is a transcription factor that regulates the κβ light chain expression and also regulates the genes that control cell survival and cell proliferation [19]. Studies have shown that the activation of NF-κβ pathway plays an essential role in apoptosis, inflammatory responses, cellular growth and developmental processes [20]. Upon cellular activation, phosphorylation at Ser-32/36 and Tyr-42 of IκBα releases the NF-κβ, allowing it to translocate to the nucleus where it acts as a transcription factor and up-regulates the expression of numerous pro-inflammatory genes, including cytokines and adhesion molecules [21]. Phosphorylation of NF-κβ p65 occurs on several serine residues. Upon treatment with TNFα, Ser-529 is phosphorylated by casein kinase II [22, 23], Ser-311 by protein

kinase C (PKC) [24], Ser-276 by both PKA and mitogen- and stress-activated protein kinase 1 (MSK1) [25, 26] and Ser-536 by the IκB kinase (IKK) complex [27] in a range of cell types.

Beta-adrenergic receptors are the emerging targets for seeking therapeutic interventions. Several studies have suggested that they might be the promising targets against vascular, cardiac and metabolic complications. In a study, isoproterenol treatment significantly decreased protein levels of iNOS, TNF-α, and IL-1B, in rMC-1 cells [28]. Stimulation of beta- 2 adrenergic receptor also exhibited anti-inflammatory effects in rats [29]. The aim of this study was to analyse the effect of isoproterenol (β-ARs agonist) and propranolol (β-ARs antagonist) on the hyperglycemia-induced apoptosis and phosphorylation of NF-κB at Ser- 536 and IκBα at Ser-32, Ser-36 and Tyr-42 in HUVECs.

METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in complete ECM media, supplemented with 10% FBS, 1% Penicillin-Streptomycin and animal-derived growth factors. Cells were cultured in flasks containing normal (5 mM) and high (25 mM) glucose. HUVECs were treated with agonist (isoproterenol) and antagonist (propranolol) in 5 µM, 10 µM and 20 µM concentrations for 6, 12 and 24 hours. Cells were also treated with TNF-α (10 ng/ml). Cells were grown in 5% CO2 at 37°C. Media was changed every 2 to 3 days.

Western Blot Analysis

HUVECs were lysed, centrifuged and proteins were extracted according to the manufacturer's protocol (Sigma). Total of 30 µg proteins was loaded in each well and separated on 10% SDS-PAGE (Precast gels, Bio-Rad, cat no 456-1093). Blots were incubated overnight at 4°C with primary antibodies (1:3000) against IκBα and NF-κB (Santa Cruz). Anti-β-actin (Santa Cruz, sc-7210) antibodies were used to ensure the quality of protein separation and loading contents. Membranes were incubated with HRP-conjugated IgG secondary antibodies (Santa Cruz, sc-2004) and visualized with enhanced chemiluminescence (Amersham Life Sciences, UK), using gel imaging system (Biospectrum 410, UVP).

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay DNA damage was examined and quantified, using a colorimetric apoptosis detection kit with TUNEL staining in a 96-well format (Titer TACS; R&D System). Briefly, 1 × 105 cells/well were transferred into a 96-well plate and fixed with 3.7% buffered formaldehyde for 5 minutes. After washing with PBS, cells were subjected to permeabilization with 100% methanol for 20 minutes. Labelling procedure was carried out and the reaction was stopped with 0.2 N HCl. The absorbance was measured at 450 nm with a microplate reader.

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay 2′7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) reagent was used to measure intracellular ROS (reactive oxygen species). Cells were seeded in 96-well plates for 24 hours with beta adrenergic receptors agonist and antagonist. Cells were then treated with 5 µM DCFH-DA and the readings were taken at 485 nm excitation and 530 nm emission in a fluorescence plate reader.

Statistical Analysis

Each test was performed in triplicate. The results were expressed as the mean value ± SD. One-way ANOVA test was used to determine statistical significance. A value of 0.05 was considered significant. Statistical analysis was performed using SPSS-17.0 package (IBM Corporation, Armonk, NY, USA).

RESEARCH RESULTS

Effect of beta-adrenergic receptors agonist and antagonist on apoptosis in HUVECs

We investigated the effect of isoproterenol (beta-adrenergic receptors agonist) and Propranolol (beta –adrenergic receptor antagonist) on HUVECs. TUNEL assay was employed to check the apoptosis. Cells treated with higher glucose concentration for 24 hours showed significantly high apoptosis. Isoproterenol at various concentrations significantly reduced the level of apoptosis. Isoproterenol at concentrations of 10 µM and 20 µM for 12 hours; and 5 µM, 10 µM and 20 µM for 24 hours (Fig. 1A, 1B and 1C) showed a significant effect on hyperglycemia-inducedapoptosis. No effect was observed at 6 hours' time period. Propranolol reversed the effect of isoproterenol when treated with 10 µM and 20 µM for 12 hours and 24 hours respectively.

Effect of beta-adrenergic receptors agonist and antagonist on reactive oxygen species (ROS)

Studies have reported that hyperglycemia significantly induces the level of reactive oxygen species in endothelial cells. We were keen to investigated the process by which beta- adrenergic receptors agonist and antagonists alter ROS level. As shown in Fig. 1D and 1E, high glucose-treated cells showed high ROS generation. Isoproterenol at concentrations of 5 µM, 10 µM and 20 µM for 6 hours, 12 hours and 24 hours, reduced ROS generation in hyperglycemic HUVECs. We observed a significant effect when isoproterenol was treated at a concentration of 20 µM for 24 hours. Propranolol reversed the effect of isoproterenol.

Stimulation of Beta-adrenergic receptors attenuates apoptosis and ROS via NF-κB and IκBα pathway

To elucidate the mechanisms underlying the observed reduction in apoptosis (Fig. 1A, 1B, 1C) and ROS generation (Fig. 1D) with increased cells viability, we examined NF-κB pathway. As shown in Fig. 2A and 2B, glucose stimulation induced phosphorylation of p65 at Ser-536. Cells were then subjected to various concentrations of agonist (isoproterenol) and antagonist (propranolol) for 24 hours. There was a significant reduction in the level of phosphorylation of NF-κB p65 at Ser-536 upon isoproterenol treatment at a concentration of 10 µM and 20 µM for 24 hours. Propranolol showed a reversed effect against isoproterenol- induced effect at a concentration of 10 µM and 20 µM for 24 hours.

Eukaryotic cells utilise NF-kB as a regulator of genes that control cell survival and proliferation. To test our hypothesis and to further elucidate the effect of beta-adrenergic receptor stimulation, we intended to first enhance the phosphorylation level and then use isoproterenol and propranolol to see whether high phosphorylation level could be reduced. Cells were subjected to TNF-α (10 ng/ml) for

24 hours. Enhanced phosphorylation of NF-κB p65 at Ser-536 after TNF-α treatment was observed. Isoproterenol treatment at a concentration of 5 µM, 10 µM and 20 µM for 24 hours significantly reduced the TNF-α- induced phosphorylation of NF-κB p65 at Ser-536 (Fig. 2C and 2D). Propranolol diminished the effect of isoproterenol.

It is evident that IKKb can increase phosphorylation of p65 Ser-536, as well as Ser-32 and Ser-36 of IκBα. IKKb phosphorylates at Ser-32 and Ser-36 of IκBα, which is followed by the release of NF-κB from its inhibitor and consequently its activation. Based on these reported mechanisms, we presumed that the de-phosphorylation of NF-kB at Ser-536 is actually due to the de-phosphorylation of IκBα. To confirm this hypothesis, we further studied phosphorylation at Ser-32, Ser-36 and Tyr-42 of IκBα. Interestingly, exposure to glucose increased the phosphorylation of IκBα at Ser-32 and Ser-36 with no effect on Tyr-42 (Fig. 3A and 3B). Isoproterenol at concentrations of 5 µM, 10 µM and 20 µM, significantly reduced the glucose-induced phosphorylation of Ser-32 and Ser-36. The effect of isoproterenol was validated by propranolol at the same concentrations and time periods. HUVECs were exposed to TNF-α which resulted in increased phosphorylation of Ser-32, Ser-36 and Tyr-42 (Fig. 3C, 3D). As described above, both agonist and antagonist modulated the level of TNF-α induced phosphorylation at Ser-32 and Ser-36 on the same pattern. In order to prove that beta-adrenergic receptors stimulation protects HUVECs from apoptosis, via NF-κβ and IκBα phosphorylation, we blocked NF-κB by the proteosome inhibitor MG132. After inhibition, we again conducted the apoptosis assay, and observed almost similar pattern of apoptosis in HUVECs (Fig. 1E).

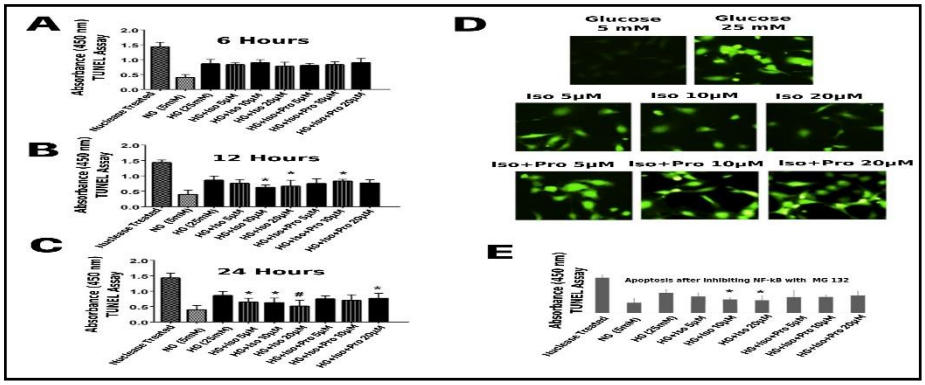

Fig. 1. Effect of isoproterenol on apoptosis and ROS generation. This Fig. shows increases apoptosis in human umbilical vein endothelial cells, treated with high glucose. Apoptosis was reduced by isoproterenol(showed iso in labelling) at different concentrations (5 uM, 10 uM and 20 uM) and time periods 6 hours (1A), 12 hours (1B) and 24 hours (1C). ROS generation was also reduced after isoproterenol treatment (1D). This effect was diminished by the antagonist of beta-adrenergic receptor propranolol (showed pro in labelling). Fig. 1E shows the level of apoptosis after inhibiting NF-κB with MG 132.

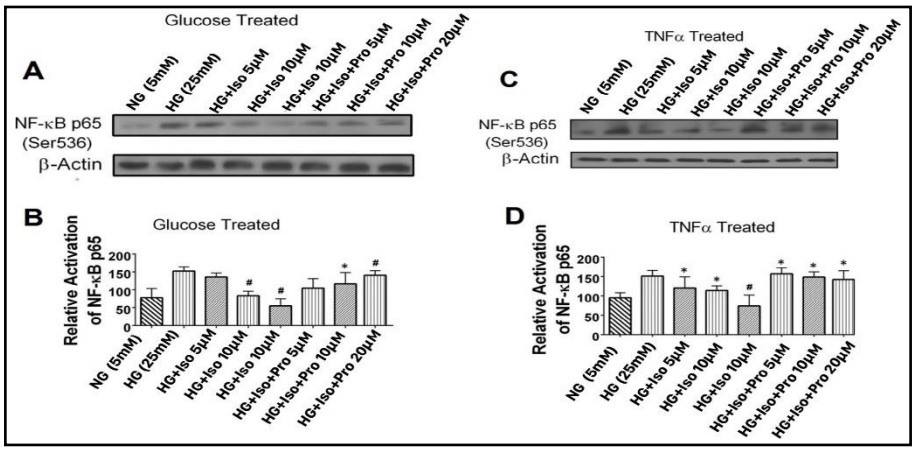

Fig. 2. Phosphorylation of NF-κB. This Fig. demonstrates the effect of isoproterenol and propranolol on glucose (2A, 2B) and TNF-α induced (2C, 2D) phosphorylation of NF-κB p65 at Ser-536 in HUVECs. High glucose and TNF-α treated cells showed increased phosphorylation of NF-κB p65 at Ser-536 which was significantly reduced by isoproterenol. The effect of isoproterenol was reversed by the agonist propranolol.

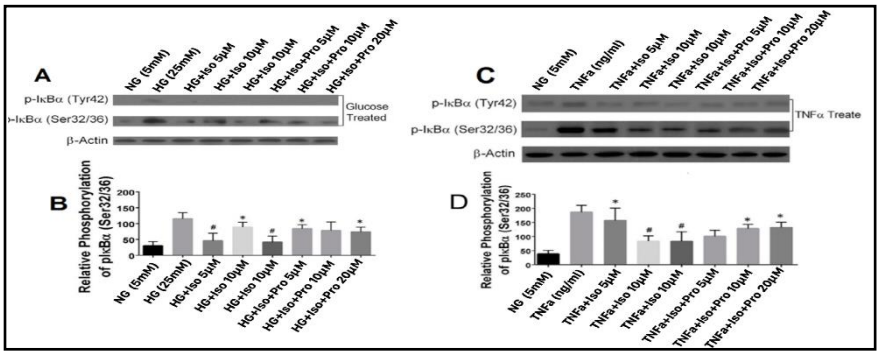

Fig. 3. phosphorylation of IκBα at Ser-32, Ser-36 and Tyr-42. This Fig. shows the effect of isoproterenol and propranolol on glucose (3A, 3B) and TNF-α induced (3C, 3D) phosphorylation of IκBα at Ser-32, Ser-36 and Tyr-42. Glucose and TNF-α treated cells showed increased phosphorylation of IκBα at Ser-32, Ser-36 and Tyr-42 which was significantly reduced by isoproterenol. The effect of isoproterenol was reversed by the agonist propranolol.

DISCUSSION

In this study, we investigated the effect of beta-adrenergic receptors stimulation on apoptosis and ROS generation, followed by the role of NF-κB and IκBα phosphorylation. Our data demonstrates that hyperglycaemia could be an apoptotic stimulus which triggers NF-κβ release and activation. These mechanisms get halted due to beta-adrenergic receptor stimulation through its agonist isoproterenol. Our data showed a disruption of NF-κβ pathway through de-phosphorylation which may ultimately lead to reduced apoptosis and low level of ROS generation.

Studies [30] implicate a general dysregulation of the endothelium with apoptosis, mitochondrial dysfunction and increased ROS generation. So we hypothesized that endothelial dysfunction might also be detected in endothelial cells under hyperglycemic conditions which may reflect the degree of apoptosis and increased level of reactive oxygen species [31]. Our results showed reduced ROS generation and decreased apoptosis after beta-adrenergic receptor stimulation. These results are in line with the previous studies [28, 32, 33] done elsewhere. Studies have also reported that blockade of TNF-α by sh- RNA and induction of the beta 2 adrenergic receptor by its agonist salmeterol,

significantly reduced the apoptosis of retinal Muller cells [34, 35]. These studies support our findings that demonstrate that beta-adrenergic receptor stimulation increases the cell viability. A recent study, that also support our findings, has reported that activation of the beta-2 adrenergic receptor could stimulate anti-inflammatory properties of dendritic cells; [36].

To elucidate the observed results, we hypothesized that beta-adrenergic receptors activation shall exert a suppressive effect on NF-κβ and its inhibitor IκBα as it is well documented that NF-κB plays a key role in modulating the gene expression [37, 38]. Phosphorylation of NF-κB p65 at Ser-536 was markedly reduced after beta-adrenergic receptor stimulation. Stimulation of TNF-α further induced the phosphorylation of NF- κB p65 at Ser-536, which was reversed by isoproterenol. Our results suggest that beta- adrenergic receptors stimulation inhibits the activation of NF-κB in HUVECs, leading to reduced apoptosis and low level of reactive oxygen species. Increased phosphorylation of IκBα at Ser-32 and Ser-36 by IKKβ causes dissociation and activation of NF-κβ [39]. We were expecting the same dephosphorylating effect on IκBα. Our results displayed the same effect by significantly reducing the phosphorylation of Ser-32 and Ser-36 in glucose and TNF-α induced HUVECs. Furthermore, we observed a reduction in the glucose-induced phosphorylation of Ser-32 and Ser-36 after beta-adrenergic receptor stimulation through isoproterenol. This demonstrates that the anti-apoptotic effect of isoproterenol is possibly due to the suppression of IκBα phosphorylation at Ser-32 and Ser-36 in HUVECs.

CONCLUSION

Results of the given study reveal that beta-adrenergic receptors stimulation exert a positive effects by reducing apoptosis and by lowering the level ROS generation. The effect of beta-adrenergic receptors agonist appears to be involved in the dephosphorylation of NF- κβ and IκBα in hyperglycaemic HUVECs.

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