Association between Insulin and Nitric Oxide in Human Retinal Microvascular Endothelial Cells in vitro

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Obesity and type 2 diabetes is characterized by insulin resistance which has been reported as the major risk factors associated with the development of the endothelial dysfunction and vascular complications such as atherosclerosis. Induction of the vascular dysfunction is obviously a proved metabolic consequence of insulin resistance. Diabetes leads to altered retinal microvascular function and ultimately diabetic retinopathy. Insulin signaling may play a role in this process, and animal studies indicated a role of the insulin in the pathogenesis of retinal neovascularization through its effect on endothelial cells. Endothelial dysfunction impairs ocular hemodynamics by reducing the bioavailability of NO and increasing the production of reactive oxygen species (ROS) and may be responsible for the pathogenesis of vascular dysfunction in retinopathy. Diabetic retinopathy (DR) a major consequence of diabetes is considered the leading cause of vision loss and blindness worldwide among working adults. Endothelial dysfunction expediting imbalance in vascular homeostasis, is one of the primary manifestation leading to the pathogenesis of DR. NO a major vasodilator involved in the regulation of vascular homeostasis is reported to be released by insulin dependent PI3K/Akt signaling pathway. Endothelial dysfunction impairs ocular hemodynamics by reducing the bioavailability of NO and increasing the production of reactive oxygen species (ROS) and may be responsible for the pathogenesis of vascular dysfunction in retinopathy. Insulin stimulated NO productions are reported to be well established in cardiovascular and macrovascular endothelium and its association with PI3K/Akt pathway. Contrary to this insulin signaling in retinal endothelium have minimal reports with some studies suggesting it to be an important physiology player in hyperglycemia or insulin resistance induced DR. This prompted us to investigate the association between hyperglycemia and insulin mediated PI3K/Akt pathway eNOS directed NO production and associated multivariate effect on HRMECs survival, proliferation, angiogenesis, adhesion, apoptotic and inflammatory markers. The aim of this study is to examine the examine the effect of insulin on NO production in human retinal microvascular endothelial cells cultured in hyperglycemic conditions. In the current study in order to examine the
effect of insulin on NO production, HRECs cells were cultured and grown in high glucose (30 mM) and normal (5 mM) glucose for 24 hours. Subsequently, the cells were treated with 100 nM insulin for 10 minutes, 1, 2, and 4 hours. The various parameters of PI3K/Akt signaling pathway were analyzed. IRS-1, IRS-2, PI3K, Akt, eNOS, VEGFA, NFkB were analyzed for gene expression. Adhesion molecules such as P-selectin and ICAM-1 were assessed by flow cytometry. ROS and NO production were measured by immunofluorescence and fluorometry respectively. The cell viability, cell cycle, apoptosis and total oxidative stress were evaluated by imaging flow cytometry. This study demonstrated that hyperglycemia causes an increase in ROS/oxidative stress and apoptosis, while insulin promotes a significant decrease in ROS and apoptosis. eNOS mediated NO production increases with hyperglycemia but remarkably decreases with insulin treatment after 1 hour, 2 hours and 4 hours. This dissimilarity of the results to previously reported studies could be due to different endothelial cell types used or varied duration of experimental hyperglycemia. The most significant reason for the variation may be due to different method of NO measurement where, most previous studies measured NO production by isolated NO meters. However, in the current study fluorometry a more sensitive method to measure oxidized product of NO, nitrite an indicator direct NO production was utilized and confirmed by the immunofluorescence technique using the dye DAF-FM Diacetate. The assessment of PI3K/Akt pathway gene expression revealed that this study demonstrates a slight increase but insignificant elevation of IRS-1 and IRS-2 genes expression in hyperglycemic condition compared to the basal control condition, while gene expression of PI3K, Akt and eNOS were significantly upregulated in the presence of high glucose. Insulin treatment caused an up regulation of IRS-1 and IRS-2 genes after 1 hour however, PI3K, Akt and eNOS were significantly reduced. The analysis of angiogenic and proapoptotic markers VEGFA and NFkB by RT-PCR showed no significant change in the expression of NFkB in hyperglycemic alone, hyperglycemic and normoglycemic cells with insulin treatment at all-time points. However, hyperglycemia significantly increased the expression of VEGF. Though compared to basal control group insulin treatment significantly increased the expression of VEGFA in both hyperglycemic and normoglycemic cells yet the expression was low compared to HG state. Consistent with the VEGFA gene expression HG significantly increased the increase the cell migration and angiogenesis while insulin treatment significantly improves barrier function. Hyperglycemia significantly increased adhesion protein P-selectin with significant reduction at 4hrs insulin treatment in the current study. This study demonstrated a significant reduction in P-selectin after insulin suggesting that insulin could participate in preventing leukocyte adhesion thereby attenuating the progression of DR. This study could not demonstrate significance change in the ICAM-1 protein. This could be due to difference in the endothelial cells used, the duration and the type of insulin treatment used. This study reported that short acting insulin commonly used in the treatment of DR could control the metabolic fluxes thereby leading to improvement in oxidative stress and apoptosis. This could prevent early changes in vasodilator, adhesion and angiogenic markers such as NO, VEGFA, P-selectin involved in the angiogenesis, inflammation and neovascularization involved in retinal vascular functioning. The study shows the potent effect of short-acting insulin treatment to counteract these biomarkers and factors involved in the pathogenesis of DR and conserving microvascular function in HRMECs exposed to hyperglycemia (30 mM) and were reported to be improved. Thus, the diabetic interventions using insulin as a key therapy with others may have the potential to be utilized as a readily available, safe and inexpensive medicine to protect against microvascular complications of DR and delay its onset. In conclusion, this study demonstrated that Hyperglycemia causes an increase in ROS/oxidative stress and apoptosis, while insulin promotes a significant decrease in ROS and apoptosis, eNOS mediated NO production increases with hyperglycemia but remarkably decreases with insulin treatment after 1 hour, 2 hours and 4 hours, insulin could counteract the hyperglycemic effect on AKT/pi3 kinase which mediates NO production and VEGF-A, decreased adhesion molecules p-selectin involved in barrier disorder of retinal endothelial cells. Thus it could be proposed that insulin could be considered as regulators of angiogenesis.