The role of oxidative stress and TGF-β in the alteration of retinal endothelial glycocalyx under hyperglycemic condition in primary RRMECs.

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ABSTRACT

Objective: Our purpose in the current study is to identify the role that oxidative stress has on TGF-β and glycocalyx protein changes within primary rat retinal microvascular endothelial cells (RRMECs) under hyperglycemic conditions.

Methods: The cells were cultured in normal glucose (5 mM, NG), high glucose (25 mM, HG) with or without N-acetylcysteine (NAC), an antioxidant. Alteration of oxidative stress and protein expression were evaluated by dihydroethidium (DHE) assay and immunoblot analysis, respectively.

Results: DHE assay and MDA level evaluation showed increased oxidative stress by HG. NAC treatment significantly reduced oxidative stress in HG. The protein level of TGF-β was increased by HG. Furthermore, HG induced alteration of protein levels of glypican-1 and syndecan-1 which are glycocalyx proteins. All of these were significantly reversed by NAC at 50 μM and 200 μM.

Conclusion: Oxidative stress caused by hyperglycemia may have a significant effect on TGF-β and changes in the endothelial glycocalyx.

INTRODUCTION

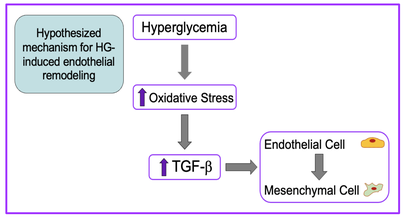
Diabetes mellitus is the origin of the most common cause of blindness among working-age adults, diabetic retinopathy (DR). Hyperglycemia, a major disease feature of diabetes, causes vascular dysfunction and inflammation leading to endothelial injury. The high glucose condition also increases membrane permeability and the formation of acellular capillaries and edema in the retinal vasculature.

A process called Endothelial-to-Mesenchymal Transition (EndoMT) is believed to contribute to the pathology seen in DR. During EndoMT, endothelial cells change their expressed genes, surface proteins, and thus, functionality to become mesenchymal tissue.

Transforming growth factor-beta (TGF-β) has been demonstrated to promote EndoMT through activin receptor-like kinase 5 (ALK5) on the plasma membrane, activating intracellular proteins to phosphorylate the Smad3 protein, promoting endothelial change.

Oxidative stress can occur due to an increased inflammatory state and due to increased lipid peroxidation, both of which are important factors in the hyperglycemic condition.

Hyperglycemia is associated with excessive oxidate stress and increased TGF-β in retinal endothelium. However, the absolute role of excessive oxidative stress and TGF–β in alteration of retinal endothelial glycocalyx has not been linked or well described.



MATERIALS & METHODS

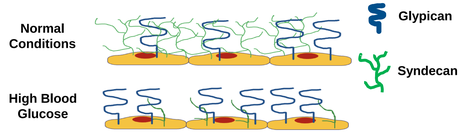
Cell Culture - Primary RRMECs were cultured in media containing 10% fetal bovine serum in a 5% CO_2 incubator under normal glucose conditions (NG; 5 mM) or high glucose conditions (HG; 25 mM).

Preparation of cell lysates – The cells washed with ice-cold PBS were lysed in RIPA buffer. After centrifugation at 14,200 rpm for 20 min at 4° C, supernatant containing the whole cell lysate was collected and protein concentration was measured with BCA protein assay kit.

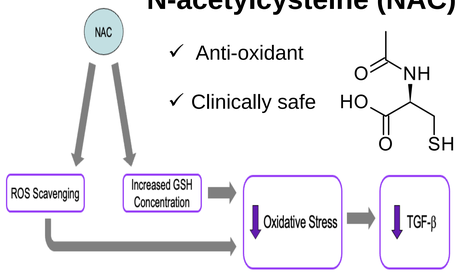
Measurement of oxidative stress – The cells incubated with desired conditions were further incubated with DHE (10 μM) for 30 min at 37° C. Florescence of oxidized DHE was measured by Agilent BioTek Gen5 microplate reader and captured by Nikon Eclipse E600 fluorescence microscope.

Immunoblot analysis - Equal volume of protein (30 ug) reduced and denatured in sample buffer were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membrane was blocked with Protein-free T20 blocking solution and incubated with primary antibody in tris-buffered saline with tween 20 (TBST) overnight. After washing membrane with TBST, horseradish peroxidase-conjugated secondary antibody in TBST was added to the membrane and incubated for an hour. After washing, protein band on membrane with enhanced chemiluminescence was captured with ChemiDoc gel imaging system and density was quantified by Image J software.

Endothelial Glycocalyx



N-acetylcysteine (NAC)



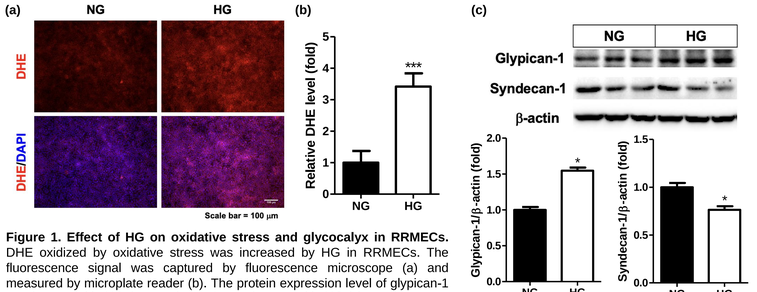


Figure 1. Effect of HG on oxidative stress and glycocalyx in RRMECs. DHE oxidized by oxidative stress was increased by HG in RRMECs. The fluorescence signal was captured by fluorescence microscope (a) and measured by microplate reader (b). The protein expression level of glypican-1 was increased in HG; but syndecan-1 was decreased in HG (c).

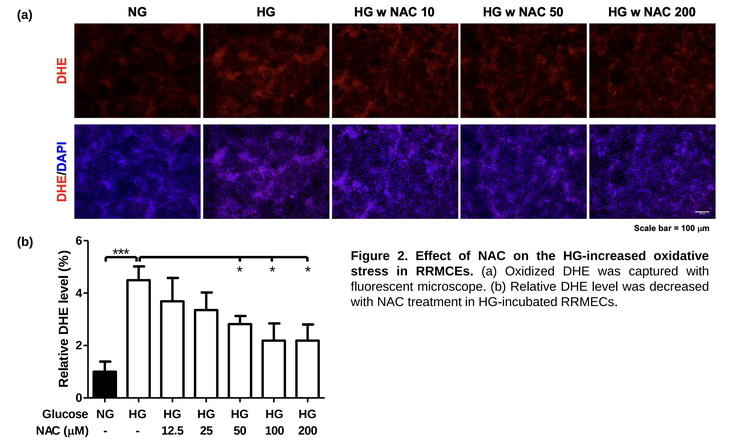


Figure 2. Effect of NAC on the HG-increased oxidative stress in RRMCEs. (a) Oxidized DHE was captured with fluorescent microscope. (b) Relative DHE level was decreased with NAC treatment in HG-incubated RRMECs.

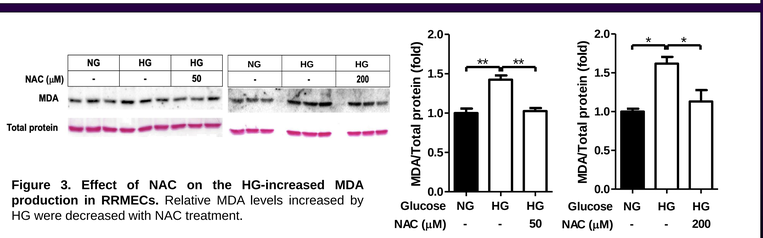


Figure 3. Effect of NAC on the HG-increased MDA production in RRMECs. Relative MDA levels increased by HG were decreased with NAC treatment.

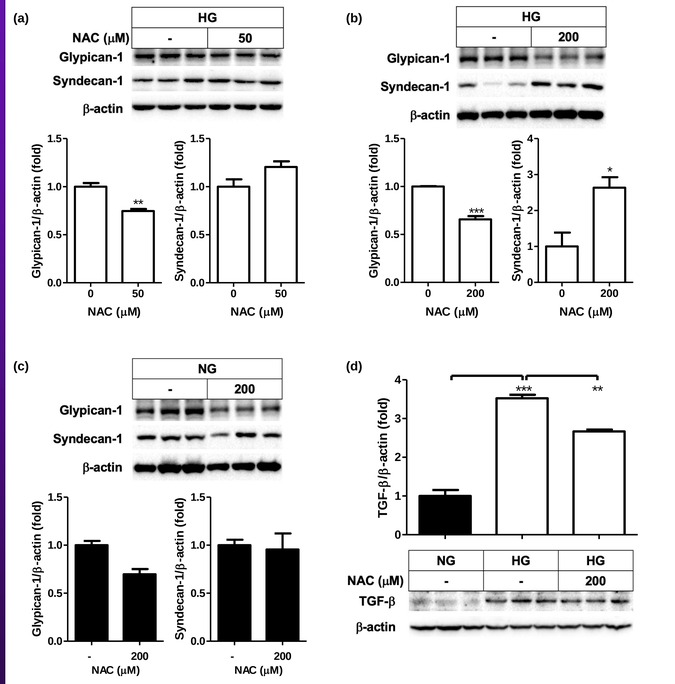


Figure 4. Effect of NAC on the protein levels in RRMECs incubated with HG or NG. (a) Low level of NAC (50 μM) decreased glypican-1 levels and might increase syndecan-1 levels in HG. (b) High level of NAC (200 μM) decreased glypican-1 levels and increased syndecan-1 levels in HG. (c) NAC might decrease the glypican-1 but does not affect on the syndecan-1 level in NG at 200 μM. (d) TGF-β was increased by HG and decreased with NAC.

CONCLUSION

Oxidative stress caused by hyperglycemia may have a significant effect on TGF-β and changes in the endothelial glycocalyx.

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