THIOREDOXIN PROTECTS HUMAN ENDOCARDIAL ENDOTHELIAL CELLS FROM HYPERGLYCEMIA INDUCED MITOCHONDRIAL HYPERFISSION

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Introduction

Introduction: Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia. It may be due to impaired insulin secretion, resistance to peripheral actions of insulin, or both. Cardiovascular complications are highly prevalent in diabetic patients, as hyperglycemia has been linked to cell injury and mitochondrial dysfunction (1). However, how hyperglycemia affects cardiac endothelial cells and their mitochondrial metabolism is not clear. Cardiomyocytes make up 70-85% of heart by volume, but endothelial cells are the most numerous and constitute up to 64% by number in the mammalian heart. Cardiac endothelial cells supply several paracrine factors that are essential for both health and function of cardiomyocytes. However, cardiac endothelial cells undergo apoptosis prior to cardiomyocytes during I/R injury, leading to complications. Mitochondrial fission has been implicated in this apoptotic process (2).

Mitochondrial fission proteins mediate the apoptosis-associated fragmentation of mitochondria. Mitochondrial fission proteins assemble around the fission site to create membrane constriction and form shorter, rounder mitochondria, as seen in Figure 1.

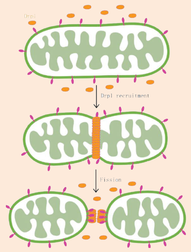

Fig. 1: Model for mitochondrial fission showing dynamin-like protein (Drp1) during mitochondrial fission. Drp1 assembles around the fission site to create membrane constriction (3).

Fig. 2: Ultrastructure of HECEC mitochondria. Mitochondria in HECECs are branched, elongated and have less cristae density compared to cardiomyocyte mitochondria.

Mitochondria in mammalian cells form reticular networks composed of filamentous tubules which constantly move and change shape through fission and fusion (4). The most studied and characterized pathway for mitochondrial fission in mammalian cells involves the dynamin-like protein DRP1 and its putative receptor Fis1. DRP1 is a dynamin-related large GTPase that binds to the mitochondrial surface for the fission reaction through the function of Fis1 (5). Fis1 resides in the mitochondrial outer membrane and recruits DRP1 during mitochondrial fission. Drp1 assembles around the fission site to create membrane constriction (3). Two dynamin-related proteins, mitofusin 1 and 2 (MFN1 & 2), have been found to mediate fusion of outer membranes in mitochondria.

Mitochondrial breakage leads to the decreased efficiency of ATP production and the increased production of superoxides, which can be harmful to the cell. The thioredoxin class of proteins, however, has been found to play a role in protecting against harmful reactive oxygen species such as superoxides. This study revolves around the efficacy of thioredoxins in protecting against glucose induced DRP1-faciliated mitochondrial fission.

Objectives & Hypothesis

This study aims to test the efficacy of human thioredoxin on protecting against high glucose induced activation of DRP1 and mitofission. Many GTPases are activated by oxidative stress. High glucose is known to cause oxidative stress in cells. We hypothesize that thioredoxin will reduce or block high glucose-induced DRP1 activation and thereby decrease hyper mitochondrial fission.

Methods

Human endocardial endothelial cells (HECECs) were cultured in EBM-2 medium with growth supplements and exposed to hyperglycemic conditions (25 mM glucose, equivalent to 450 mg/dL) for different time periods, while control group cells were treated with 5.6mM glucose, the normal blood level glucose concentration. Mannitol was used as a equimolar osmotic control. A second set of cells were treated with both hyperglycemia and recombinant human thioredoxin (rhTrx). All cells were lysed using RIPA buffer and equal amount of protein were analyzed by Western blotting.

After protein estimation through BCA reagent assay, 30-40 µL of lysate was resolved on 10-12% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to membrane using an electroblotting apparatus. DRP1 was detected by immunoblotting using a 1:1000 dilution of Anti-DRP1 mouse monoclonal antibody (BD Transduction Labs.) in TBST (10 µM Tris and 150 mM NaCl, pH 8.0 with Tween 20) with 5% dry milk overnight at 4°C. Phosphorylated DRP1 Serine 616 & Serine 637 were detected with pDRP1 rabbit polyclonal antibody (Cell Signaling Tech.). MFN2 was detected with mitofusin 2 mouse monoclonal antibody (Sigma Life). Beta Actin was detected with beta actin mouse monoclonal antibody (Sigma Life). Bound antibodies were detected using a secondary antibody, Goat Anti Rabbit (GAR) or Goat Anti Mouse (GAM) (Cell Signaling Tech.), diluted at 1:5,000 in TBST with 5% dry milk. Proteins were detected using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

Methods (cont.)

Additionally, primary HECECs were cultured on glass coverslips and exposed to high glucose (25 mM). The cells were then washed, fixed, permeabilized, and probed with rabbit anti-pDRP1 (Ser616) and mouse anti-Tom 20 antibodies, followed by Alexa Fluor 488- and Alexa Fluor 568-conjugated donkey anti-rabbit and anti-mouse secondary antibodies. Cells were counterstained with DAPI. A stack of fluorescent images was then obtained along the z-axis at 120-nm intervals via a 100x/1.40 numerical aperture objective using a Zeiss Axio Imager Z2 upright fluorescent microscope and deconvolved using AxioVision 4.9 software.

Results

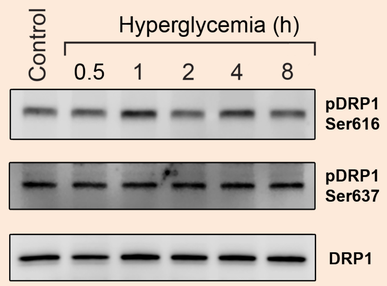

Fig. 3: DRP1 is activated by high glucose: Western blotting of HECEC cell lysates shows that DRP1 was activated via Ser616 phosphorylation while the inhibitory phosphorylation (Ser 637) level did not change.

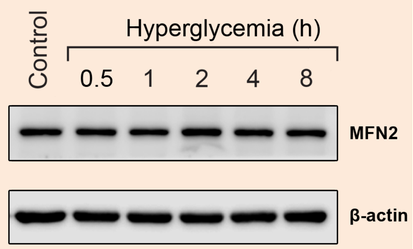

Fig. 4: High glucose did not affect mitofusin: To test the effect of high glucose on mitofusion machinery, we tested the level of MFN2 in HECECs. Its expression levels did not change due to high glucose treatment.

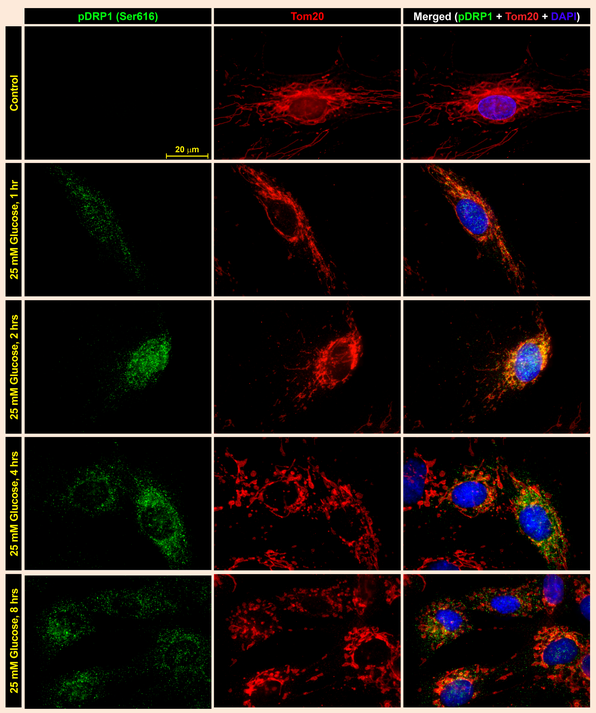

Fig. 5: High glucose activates DRP1 and induces hyper mitofission in HECECs. HECECs were exposed to high glucose (25 mM) for up to 8 hours. At the end of exposure, cells were fixed, permeabilized, and immunofluorescence staining was performed using anti-Tom20 and anti-pDRP1 (Ser616) antibodies.

DRP1 was activated in HECECs within 1 hour of high glucose exposure. But, a significant level of mitochondrial fission was observed from 2 hours. Beyond 4 hours of exposure to high glucose, HECECs appeared to lose mitochondrial level due to hyperglycemia.

Results (cont.)

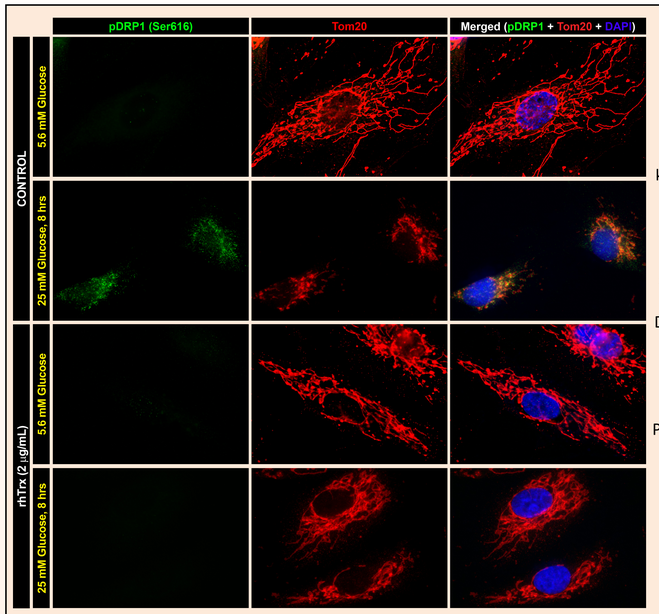

Fig. 6: rhTrx reduces DRP1 activation and attenuates hyper mitofission in HECECs: HECECs were treated with or without 2 µg/mL rhTrx for 6 hours and then exposed to high glucose for 8 hours. At the end of exposure, cells were subjected to IF staining as described in Fig. 5. DRP1 was activated and mitochondria were highly fragmented in HECECs exposed to 25 mM Glucose alone. Pretreatment of HECECs with 2 ug/mL rhTrx completely blocked DRP1 activation, but it resulted in only partial blockage of mitochondrial fragmentation.

Conclusions

- Mitochondria were shorter and more fragmented in cells after 4 hours of exposure to hyperglycemic conditions.
- Cells not treated with thioredoxin were more fragmented compared to cells treated with thioredoxin, as seen by immunofluorescence staining with Tom20.
- Western blotting also revealed activation of DRP1 (in the form of rising bands in pDRP1 Ser616) as the cells were incubated in high glucose for more time.
- These findings indicate that fission-mediated fragmentation of mitochondria is tied to hyperglycemic conditions and can lead to cardiovascular complications in diabetic patients through apoptosis.
- Furthermore, it also points to possible protective effects of human thioredoxin against damage caused by mitochondrial fission under hyperglycemic conditions in diabetic patients, as less fission was observed when cells were treated with thioredoxin.

Future Directions

- Future experiments can be conducted with thioredoxin overexpressing transgenic mice treated with hyperglycemic food/high sucrose diet, measuring mitochondrial breakage in HECECs *in vivo*.

Literature Cited

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(5) https://www.ncbi.nlm.nih.gov/pmc/articles/PMC25766/

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