Experimental therapeutic approaches against hyperglycemia-induced mitochondrial injury in endothelial cells

PhD thesis

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1. Introduction

The global prevalence of diabetes among adults over 18 years of age has risen from 4.7% in 1980 to 8.5% in 2014. Diabetes-related healthcare expenditure accounts for 10% of the total healthcare costs but it is estimated to increase by 70% over the next 25 years leading to a serious societal and economic burden. Diabetes complications are responsible for the majority of the associated costs. Hyperglycemia-induced endothelial dysfunction is the major contributor to the development of vascular disease in diabetes mellitus. The major pathway that is responsible for endothelial damage is glucose-induced oxidative stress in diabetes.

2. Aims

The glucose-induced cell damage is mediated by oxidative stress in endothelial cells, and according to the unifying hypothesis mitochondrial reactive oxygen species (ROS) production acts as an upstream player in this process. Reactive oxygen species are produced by the respiratory chain (complexes I and III) in the mitochondria via directly transferring electrons to oxygen leaving behind extra protons in the intermembrane space.

To find potential inhibitors of hyperglycemic endothelial damage we pursued the following **Specific Aims**:

1. Establish a cell culture model of hyperglycemia-induced endothelial injury that is characterized by mitochondrial overproduction of ROS and is applicable for medium throughput cell-based drug screening
2. Screen the currently available clinical drugs and similar biologically active compounds to identify inhibitors of the glucose-induced mitochondrial ROS production in endothelial cells
3. Determine the mechanism of action of selected hit compounds against hyperglycemic mitochondrial ROS production.

3. Methods

We conducted a phenotypic screen to identify compounds that inhibit the mitochondrial ROS production induced by elevated extracellular glucose in cultured b.End3 endothelial cells. We tested a focused library consisting of 6,766 compounds, which included clinical-stage drugs, biologically active compounds with defined pharmacological activity and natural compounds.
Cellular and mitochondrial ROS production was measured by kinetic assays based on the use of mitochondrial superoxide and H_2O_2-sensitive probes MitoSOX Red and 5-(and-6)-chloromethyl-2',7'-dichlorodihydro fluorescein diacetate, acetyl ester (CM-H_2DCFDA). Mitochondrial oxidant production was also evaluated *in situ* using fluorescence microscopy following dual staining with MitoSOX Red and mitoTracker, supplemented with nuclear staining with Hoechst 33342. The mitochondrial membrane potential was measured after loading the cells with the mitochondrial membrane potential probe JC-1 dye. Oxidative damage was evaluated at the DNA, RNA and protein levels using the Comet assay, immunofluorescent labeling for 8-hydroxy-guanosine and via the Oxyblot method.
Cellular viability was determined using nuclear staining with Hoechst 33342 and the cellular metabolism was explored using thiazolyl blue tetrazolium (MTT) and lactate dehydrogenase (LDH) assays. Cellular oxygen consumption and acid production rates were also determined utilizing extracellular Flux Analysis (Seahorse, Billerica, MA).
Gene expression changes were determined by real-time PCR-based assays or macroassays at the mRNA level, and by Western blotting at the protein level. siRNA mediated gene silencing was used to suppress the expression of drug target UCP2 in separate experiments.
Isolated mitochondria assays and selected respiratory complex activity assays were used to specifically explore the positive effect of H_2S donors.
The protective effect on vascular function was also evaluated using vessel bath (myography) experiments either in diabetic samples treated *in vivo* or in vessels exposed to high glucose *ex vivo*.

4. Results

4.1. Hyperglycemia induces ROS production and oxdiative injury in microvascular endothelial cells

Extended hyperglycemic exposure induced a progressive increase in the mitochondrial ROS production in microvascular endothelial cells. The glucose-induced ROS production was associated with metabolic changes in the cells, but no alteration was detected in the expression of genes related to mitochondrial ATP production. When exposed to hyperglycemia, the cells showed a progressive increase in the mitochondrial MTT conversion indicating the stimulation of aerobic metabolism and oxidative phosphorylation (OXPHOS). On the other hand, no change was detectable in the anaerobic metabolism as measured by the cellular LDH activity. Similar to the changes in the mitochondrial metabolism, a progressive increase was detectable in the mitochondrial membrane potential coinciding with the increase in the mitochondrial ROS production. Furthermore, cytoplasmic sources of ROS generation were also stimulated in the cells.

The cells maintained a stable energy level in hyperglycemia for 7 days but a decline was detectable afterwards. Despite the elevated membrane potential, mitochondria failed to generate the necessary energy to meet the basal ATP requirement of the cells. Since no suppression was detectable in the level of assembled respiratory complexes, the metabolic failure might be explained by a functional deficit in the mitochondrial electron transfer (or in the chemiosmotic coupling).

In summary, the increased glucose load led to mitochondrial hyperpolarization that may serve as a promoter of increased ROS generation in microvascular endothelial cells and many features of this cellular injury model closely match the changes seen in diabetes.

4.2. Cell-based screening for inhibitors of hyperglycemia-induced mitochondrial ROS production in endothelial cells

In the above cell-based assay, we tested a library of biologically active compounds against mitochondrial ROS production induced by high glucose exposure. The data sets of cellular ROS production and viability values showed Gaussian distribution and the majority of test compounds had no effect on mitochondrial ROS generation in the primary screen. Non-toxic compounds inhibiting the hyperglycemia-induced ROS production by more than 25% at 3 μM were selected as hits and were re-tested in replicates to confirm the antioxidant activity. Compounds passing the hit confirmation studies included steroids, non-steroidal anti-inflammatory agents, antioxidants, mitochondrial uncouplers and antimetabolites.
From the multiple classes of pharmacologically active compounds identified in the screen, we chose to focus our subsequent studies on paroxetine (a clinically used antidepressant compound), glucocorticoid steroids and the novel mitochondrial H_2S donor compounds AP39 and AP123. The hypothesized molecular targets of these drugs are shown on the **Figure**.

4.3. Characterization of the mode of action of hit compounds

4.3.1. Paroxetine acts as a mitochondrial superoxide scavenger in hyperglycemic endothelial cells

Paroxetine showed preference to inhibit mitochondrial ROS production, which was a unique feature of this compound among selective serotonin reuptake inhibitors. The effect of paroxetine depended on an immediate mode of action and the drug also remained effective against the hyperglycemia-induced mitochondrial ROS generation in human endothelial cells.
Paroxetine had no effect on the oxygen consumption rate in isolated mitochondria or in whole cells and it did not affect the cellular ATP content. Paroxetine was also effective against superoxide in a xanthine oxidase-based cell-free assay suggesting a direct scavenging function.

The superoxide neutralizing effect of paroxetine translated into measurable benefits in hyperglycemia. DNA fragmentation, the formation of 8-hydroxy-guanosine (an indicator of oxidative damage to the RNA) and oxidation of proteins were all attenuated by paroxetine, indicative of the ability of paroxetine to reduce the downstream consequences of mitochondrial ROS production.
The protective effect of paroxetine on hyperglycemia- and diabetes-induced endothelial dysfunction was tested in vascular rings. Paroxetine maintained the normal endothelium-dependent relaxant responsiveness of hyperglycemic vessels and similarly prevented the diabetes-induced impairment of the endothelium-dependent relaxations *ex vivo*.

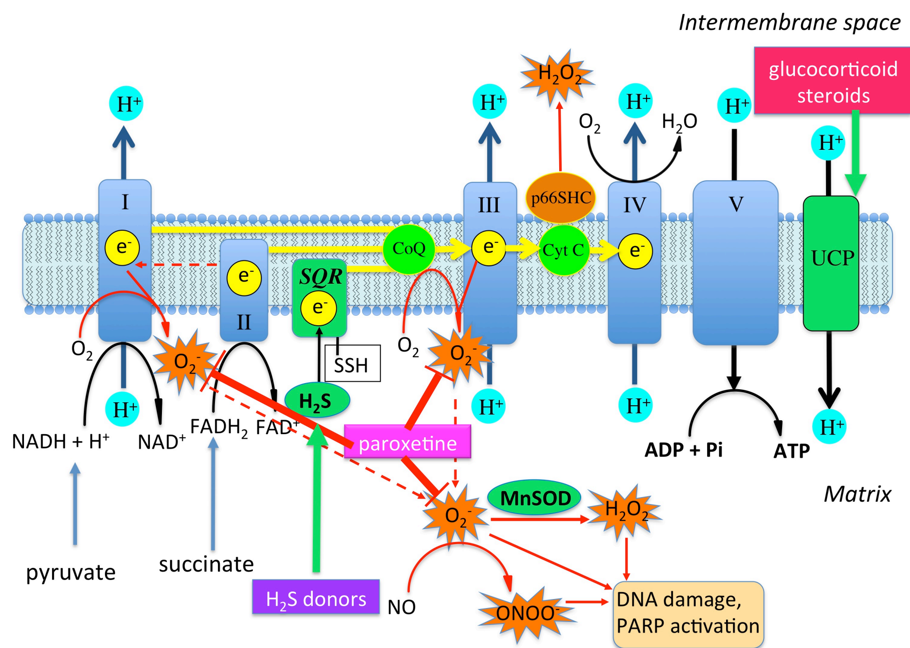

Figure. Potential targets of hit compounds against hyperglycemia-induced ROS produciton. Paroxetin acts as direct superoxide scavenger, glucocorticoids induce UCP2 expression, H_2S donor compounds act as electron donors.

4.3.2. Glucocorticoids reduce the mitochondrial ROS production via UCP2 induction in microvascular endothelial cells

Glucocorticoid steroids inhibited the glucose-induced mitochondrial ROS production and emerged as hit compounds in our screen. Unexpectedly, the glucocorticoid antagonist mifepristone also decreased the hyperglycemia-induced ROS production in microvascular endothelial cells and at low micromolar concentrations it was more effective than dexamethasone. Both dexamethasone and mifepristone normalized the mitochondrial potential, which effect was associated with a 10-fold increase in the expression of uncoupling protein 2 (UCP2) suggesting that the induction of UCP2 expression may be responsible for the steroid-mediated anti-ROS effect in microvascular endothelial cells.

siRNA mediated UCP2 silencing partially blocked the response to steroids and also suppressed the ROS-inhibitory effect of mifepristone. It also caused an increase in the mitochondrial superoxide generation by itself. The reduced level of UCP2 led to an increase in the mitochondrial potential and partially blocked the membrane potential normalizing effect of mifepristone. Overall, these results suggest that UCP2 expression is responsible for the decrease induced by glucocorticoid steroids in the hyperglycemic endothelial cells.

Following the steroid-mediated UCP2 induction, higher proton leak was measurable in the hyperglycemic cells. UCP2 silencing diminished the increase in the proton leak. These results suggest that pharmacological induction of UCP2 expression can be achieved in select cell populations and in microvascular endothelial cells it causes distinct changes in the cellular metabolism.

4.3.3. Mitochondria-targeted H_2S-donor compounds inhibit the mitochondrial ROS production via electron donation

The mitochondrial slow-release H_2S donors AP39 and AP123 reduced the mitochondrial ROS production and also caused a slight decrease in the cellular ROS production. Both compounds significantly reduced

the hyperglycemia-induced increase in the mitochondrial membrane potential at low nanomolar concentrations. AP39 was more effective than AP123 that might be explained by the higher H_2S release of AP39.

Mitochondria-targeted H_2S donors AP39 and AP123 induced a concentration-dependent increase in the cellular ATP content in endothelial cells showing a diminished ATP pool after prolonged exposure to hyperglycemia.

The mitochondrial H_2S donors improved the coupling efficiency and significantly reduced the proton leak as measured by extracellular flux analysis that could explain the increased cellular ATP content in the cells without a measurable increase in oxygen consumption. No change was detectable in the anaerobic metabolism confirming that the compounds do not inhibit mitochondrial respiration at the tested concentrations.

To confirm that the action of mitochondrial H_2S donors directly increase the electron transfer, we performed a Complex II/III activity assay. We blocked input from Complex I by rotenone and inhibited Complex IV (cytochrome c oxidation) by potassium cyanide and measured the activity of complex III. (In the presence of substrate succinate, Complex II transfers electrons to ubiquinone that passes them to Complex III to reduce cytochrome c.) Both H_2S donors induced a concentration-dependent increase in complex III activity (cytochrome c reduction) at concentrations below 2.5 μM confirming that the compounds directly affect the respiratory complex activities. We propose that the electron donation step of mitochondrial H_2S oxidation (as electrons are fed to the quinone pool at the level of complex III) and the subsequent oxygen consumption (that occurs at a later step of H_2S oxidation) may be uncoupled and these compounds may preferentially resupply the “lost electrons” to the respiratory chain. These results are in agreement with other reports suggesting that H_2S can act as an electron donor in the electron transport chain. It is of note that mitochondrial donors were effective at 1000-fold lower concentration than previous, non-targeted H_2S donors.

5. Conclusions

In conclusion, the current studies have utilized a cell-based screening method to identify a number of drugs and drug-like molecules that beneficially affect hyperglycemic ROS production in endothelial cells.

One of these compounds, the antidepressant paroxetine, has been tested in a variety of *in vitro* and *in vivo/ex vivo* models of hyperglycemic endothelial injury and diabetic vascular complications. We found that paroxetine shows effect at submicromolar concentrations and superoxide scavenging is involved in its mode of action against mitochondrial ROS. It is interesting to note that paroxetine has previously been shown to afford certain cardiovascular benefits in terms of protection from myocardial infarction in humans.

The antioxidant effect of mifepristone, a glucocorticoid receptor antagonist, is associated with mild mitochondrial uncoupling, which is achieved by induction of UCP2 expression. This compound was also found to be effective in a clinically relevant concentration range.

Mitochondrial slow release H_2S donors also provided protection against the prolonged low level oxidative stress induced by hyperglycemia in endothelial cells. They increase the electron transfer rate at respiratory complex III and have beneficial effect on cellular bioenergetics. These compounds showed positive effect in the nanomolar concentration range, which is more than two orders of magnitude lower than their maximum tolerated concentration, suggesting a safer alternative compared to non-targeted H_2S donors and natural sources.

The current results may lay the conceptual foundation for future exploratory clinical trials in patients with diabetes, with the potential ultimate goal of re-purposing for the experimental therapy of diabetic complications. However, such studies must be preceded by careful investigation of the safety profile of this compound in diabetic patients.

6. Publications

Most closely related to the thesis:

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