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High glucose-induced mitochondrial respiration and reactive oxygen species in mouse cerebral pericytes is reversed by pharmacological inhibition of mitochondrial carbonic anhydrases: Implications for cerebral microvascular disease in diabetes

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ABSTRACT

Hyperglycemia-induced oxidative stress leads to diabetes-associated damage to the microvasculature of the brain. Pericytes in close proximity to endothelial cells in the brain microvessels are vital to the integrity of the blood–brain barrier and are especially susceptible to oxidative stress. According to our recently published results, streptozotocin-diabetic mouse brain exhibits oxidative stress and loose pericytes by twelve weeks of diabetes, and cerebral pericytes cultured in high glucose media suffer intracellular oxidative stress and apoptosis. Oxidative stress in diabetes is hypothesized to be caused by reactive oxygen species (ROS) produced during hyperglycemia-induced enhanced oxidative metabolism of glucose (respiration). To test this hypothesis, we investigated the effect of high glucose on respiration rate and ROS production in mouse cerebral pericytes. Previously, we showed that pharmacological inhibition of mitochondrial carbonic anhydrases protects the brain from oxidative stress and pericyte loss. The high glucose-induced intracellular oxidative stress and apoptosis of pericytes in culture were also reversed by inhibition of mitochondrial carbonic anhydrases. Therefore, we extended our current study to determine the effect of these inhibitors on high glucose-induced increases in pericyte respiration and ROS. We now report that both the respiration and ROS are significantly increased in pericytes challenged with high glucose. Furthermore, inhibition of mitochondrial carbonic anhydrases significantly slowed down both the rate of respiration and ROS production. These data provide new evidence that pharmacological inhibitors of mitochondrial carbonic anhydrases, already in clinical use, may prove beneficial in protecting the brain from oxidative stress caused by ROS produced as a consequence of hyperglycemia-induced enhanced respiration.

1. Introduction

Complications of diabetes in the microvasculature of the brain are caused by oxidative stress [2,3,20]. These complications include pericyte loss [20], disruption of the blood–brain barrier [1,11], and cognitive decline [4,15,22,28,30,32]. The blood–brain barrier is formed largely by endothelial cells in the microvasculature of the central nervous system. Cerebral pericytes, another type of cell in close proximity to endothelial cells, are vital for the integrity of the blood–brain barrier [1]. Pericytes are especially susceptible to oxidative stress, which leads to the cell death by apoptosis [25].

Oxidative stress in diabetes is caused by reactive oxygen species (ROS) [7,18,31], which are primarily generated during mitochondrial oxidative metabolism of glucose (respiration). These mitochondrial ROS activate other biochemical pathways such as polyol pathway [5,19], advanced glycation end products (AGE) formation [8,10,16], protein kinase C activation [13,33], and hexosamine pathway [17,24], which in turn propagate more ROS. The pathological levels of ROS produced from these pathways cause oxidative stress, leading to diabetic complications in the brain.

Abbreviations: CAIs, mitochondrial carbonic anhydrase inhibitors; ECAR, rate of acid efflux; ETZ, 6-ethoxy-2-benzothiazolesulfonamide (ethoxyzolamide); HCO^-_3, bicarbonate; HG, high glucose; IFN-γ, interferon gamma; NG, normal glucose; OCR, rate of oxygen consumption; ROS, reactive oxygen species; TOP, topiramate.

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Previously, we and others [2,3,20,25] hypothesized that a hyperglycemia-induced increase in the rate of respiration is responsible for the production of high levels of mitochondrial ROS in relatively insulin insensitive tissues such as the brain. However, there are no published reports to show an increase in the rate of respiration either in the diabetic brain in response to hyperglycemia or in cultured cerebral pericytes upon high glucose challenge.

In this study, we investigated the effect of high glucose on the rate of respiration and ROS production in cerebral pericytes in culture. In addition, we studied the effect of pharmacological inhibition of mitochondrial carbonic anhydrases on these high glucose-induced changes in cerebral pericytes.

Mitochondrial carbonic anhydrases VA and VB regulate the rate of respiration [20,25]. Mice in which these enzymes have been genetically knocked out exhibit reduced oxidative stress in the brain [26]. Pharmacological inhibition of mitochondrial carbonic anhydrases also protects the mouse brain from oxidative stress and pericyte loss [20]. The high glucose-induced intracellular oxidative stress and apoptosis of pericytes are remedied by treatment with mitochondrial carbonic anhydrase inhibitors (CAIs) [25] as well.

We now report for the first time that the rate of respiration is significantly higher in cerebral pericytes challenged with high glucose compared to those in normal glucose. The amount of ROS is also significantly higher in these cells. Furthermore, we show a reversal in high glucose-induced rapid rate of respiration as well as the amount of ROS produced upon pharmacological inhibition of mitochondrial carbonic anhydrases.

These studies provide further evidence to recognize mitochondrial carbonic anhydrases as an important pathway that can be targeted therapeutically to prevent diabetic brain injury. Pharmacological inhibitors of mitochondrial carbonic anhydrases are in clinical use for a variety of disorders, including obesity [9], and can be safely tested for this new indication in translational research.

2. Materials and methods

2.1. Cell culture

Conditionally immortalized mouse cerebral pericyte cultures were established as previously described (Shah GN, et al., 2012). The pericytes were grown in 60 mm petri dishes in growth media (DMEM, D6046, Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin/streptomycin (Sigma–Aldrich) and murine recombinant IFN-γ at 44 U/ml (R&D Systems, Minneapolis, MN) in an atmosphere of 5% CO_2 at 33 °C. The cells were fed every 2–3 days.

2.2. Measurement of the rate of oxidative metabolism of glucose (respiration)

A Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) was used to measure the rate of oxidative metabolism of glucose (respiration). This instrument measures the rate of change of dissolved O_2 and pH in medium immediately surrounding adherent cells cultured in an XF24 V7 cell culture microplate (Seahorse Bioscience). Under typical *in vitro* cell culture conditions, the rate of oxygen consumption (OCR) is an indicator of mitochondrial respiration and the rate of acid efflux (ECAR) is predominantly a measure of lactic acid produced by dehydrogenation of pyruvate.

The pericytes were seeded in XF24-well microplates at 5×10^4 cells per well (0.32 cm^2) in 100 μl of growth medium and incubated at 33°C in 5% CO_2 for 2 h. An additional 150 μl of medium was added after the cells had adhered. On the following day, assays were initiated by replacing the media with no-buffered media and incubating at 37 °C for 60 min to allow the temperature and pH to reach equilibrium. The microplate was then placed into the XF24 instrument to measure OCR and ECAR. After steady state of OCR and ECAR was obtained (20 min), normal glucose (5.7 mM), high glucose (40.7 mM), and high glucose with either topiramate (T0575, Sigma–Aldrich) or ethoxyzolamide (333328, Sigma–Aldrich) were injected through reagent delivery chambers. Mitochondrial CAIs, topiramate and ethoxyzolamide, were used at 10 and 100 μM, respectively.

2.3. Quantification of intracellular reactive oxygen species

Intracellular ROS were measured with an ROS activity assay kit (Cell Meter™ Fluorimetric Intracellular Total ROS activity assay kit, cat# 22900, AAT Bioquest, Thermo Fisher Scientific, Inc., Waltham, MA). The kit uses a cell-permeable fluorescent dye (Amplite™ ROS Green), which generates green fluorescence upon reaction with ROS. The pericytes were seeded in Costar black wall/clear bottom 96-well plate at a density of 1×10^5 cells per well in 100 μl of growth media, and were allowed to adhere overnight in a 5% CO_2, 37 °C incubator. The following morning, ethoxyzolamide or topiramate (10 μM) were added and incubations continued for 3 more hours. At the end of the treatment, 100 μl of glucose stock solution (1 M) was added to bring the final concentration of glucose to 40.7 mM. Immediately after that, 100 μl of assay loading solution was added to each well and the incubations were continued in a 5% CO_2, 37 °C incubator for 1 h. Fluorescence at excitation and emission wavelengths of 490 and 520 nm, respectively, were measured using a fluorescence plate reader (Tecan Safire II, Tecan, Männedorf, Switzerland). Hydrogen peroxide was used as a positive control and Tempo as a negative control per the supplier's instructions. The ROS produced are presented as a percent of control. Each sample was run in triplicate and experiments were repeated at least three times.

2.4. Cell viability

A Cell Meter™ Cell Viability Assay Kit (cat#22784, AAT Bioquest, Thermo Fisher Scientific, Inc.) was used to determine cell viability. The kit uses a weakly fluorescent dye, CytoCalcein Violet 450, AM, which is hydrolyzed by intracellular esterase to generate a strongly fluorescent hydrophilic product that is well retained in the cell cytoplasm. The esterase activity is proportional to the number of viable cells, and thus directly related to the fluorescence intensity of the product. The pericytes were seeded in the wells of a Costar black wall/clear bottom 96-well plate and treated with normal glucose, high glucose, and high glucose with and without mitochondrial CAIs, as described earlier. Following the treatment, the CytoCalcein Violet 450, AM dye-loading solution (100 μl) was added and the cells were incubated in a 5% CO_2, 37 °C incubator for 1 h. Fluorescence intensity was measured on a fluorescence plate reader (Tecan Safire II) at excitation and emission wavelengths of 405 nm and 460 nm, respectively. The data is expressed as percentage of cell viability compared to viable cells in normal glucose. Each sample was run in triplicate and experiments were repeated at least three times.

Statistical analysis

All means are reported with their n and standard error of the mean (SEM). Two means were compared by the unpaired two-tailed Student's t test. For more than two means, ANOVA, followed by Newman-Keuls multiple comparison test was used. $p < 0.05$ was considered significant. Statistical analyses were made using

GraphPad Prism 5.0 package program (GraphPad Software Inc., San Diego, CA). For OCR and ECAR measurements over time, the area under the curve was generated by an algorithm executed by the Seahorse device.

3. Results

3.1. Effect of pharmacological inhibition of mitochondrial carbonic anhydrases on high glucose-induced rapid rate of mitochondrial oxidative metabolism of glucose (respiration)

As indicated by OCR (Fig. 1A and C), a significant increase in the respiration rate was observed in cerebral pericytes following 40 min of exposure to high glucose, which reached its peak at 2 h. Treatment with topiramate (Fig. 1A and C), a mitochondrial carbonic anhydrase inhibitor, completely blocked the effect of high glucose. High glucose also led to a significant increase in ECAR, a measure of lactic acid formed (Fig. 1B and D). However, topiramate did not alter the effect of high glucose on ECAR. Similar results were obtained with ethoxyzolamide, another inhibitor of mitochondrial carbonic anhydrases.

3.2. Effect of pharmacological inhibition of mitochondrial carbonic anhydrases on high glucose-induced reactive oxygen species

One hour exposure to high glucose significantly increased intracellular ROS (Fig. 2) in cerebral pericytes. The effect of high glucose was significantly blocked by treatment with both topiramate and ethoxyzolamide (Fig. 2).

3.3. Cell viability

The pericytes remained viable throughout the duration of the ROS determination assay in all treatment groups (Fig. 3).

4. Discussion

Excess ROS generated by hyperglycemia-induced rapid oxidative metabolism of glucose (respiration) have been implicated in oxidative stress in insulin insensitive tissues such as the brain [2,3,20]. However, there are no published reports to support these implications.

We now present data to show that the rate of respiration and ROS production in cultured cerebral pericytes is significantly increased in response to high glucose challenge. A 40-min exposure to high glucose led to a significant acceleration in oxygen consumption rate (OCR), a measure of the rate of respiration (Fig. 1A and C). A significant increase in the amount of ROS was observed following a 1 h exposure to high glucose (Fig. 2).

Rate of respiration is regulated by mitochondrial carbonic anhydrases and their inhibition protects diabetic brain and cerebral pericytes from hyperglycemia induced oxidative stress [20,25]. The following is a brief description of the role of mitochondrial carbonic anhydrases in regulation of respiration and ROS (Fig. 4). Glucose is converted to pyruvate in the cytoplasm;

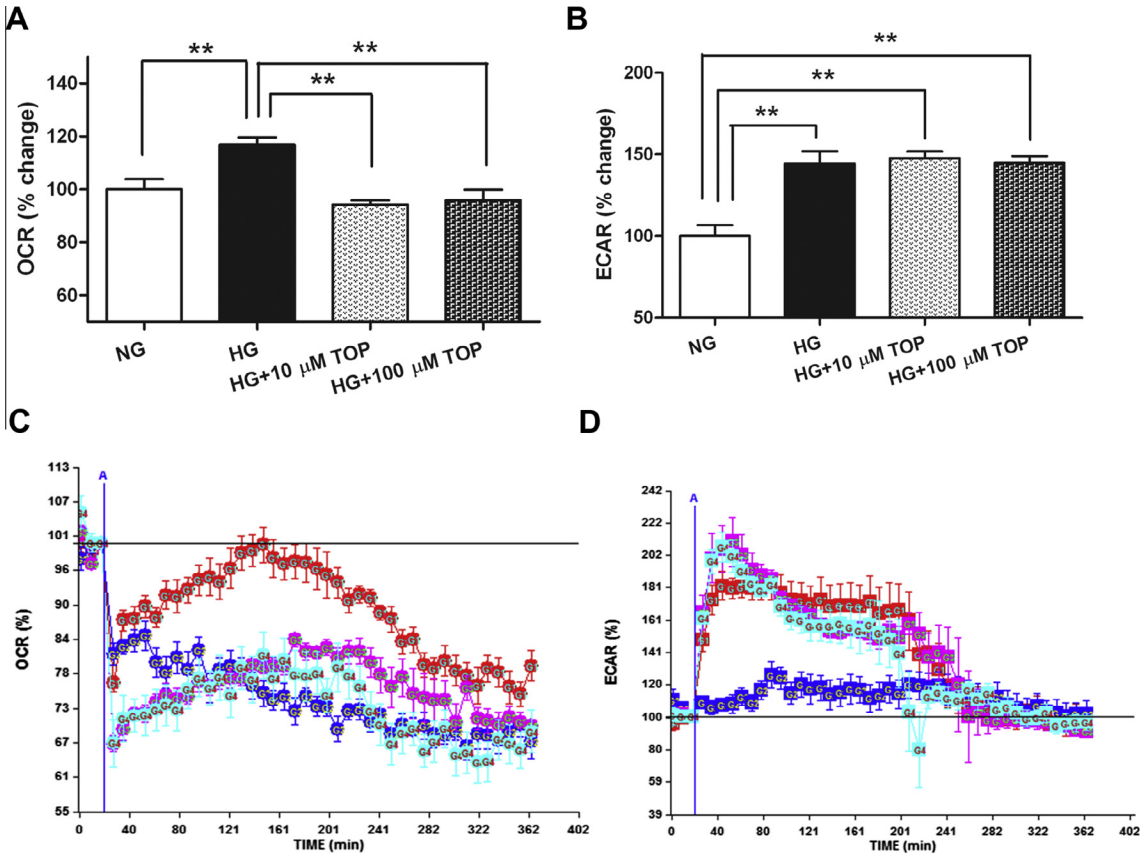

Fig. 1. Effect of mitochondrial carbonic anhydrase inhibition on rate of oxidative metabolism of glucose in cerebral pericytes. (A) shows mitochondrial respiration rate (OCR) and (B) extracellular acidification rate (ECAR). Representative graphs of OCR (C) and ECAR (D). Blue vertical line "A" indicates the timing of the injection. Lettering within data points shows the group identification number. Data represent percent change in OCR and ECAR in response to HG with or without topiramate. Results are shown as mean ± SEM ($n = 5$). The graphs are representative of three independent experiments. $^{**}p < 0.01$. HG, high glucose; NG, normal glucose; TOP, topiramate.

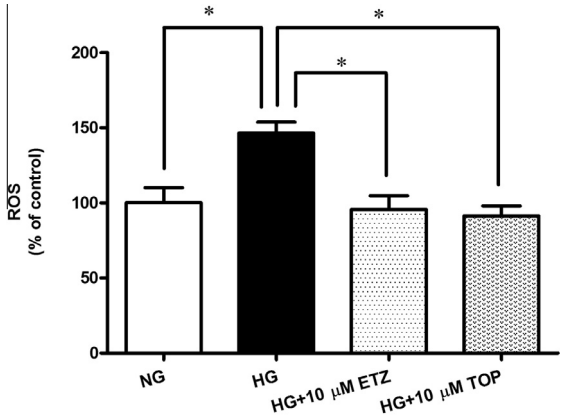

Fig. 2. Effect of mitochondrial carbonic anhydrase inhibition on high glucose-induced reactive oxygen species in cerebral pericytes. Results are presented as percentage of cells treated with NG. Data are shown as mean ± SEM ($n = 3$). The graphs are representative of three independent experiments. $^{*}p < 0.05$. ETZ, ethoxyzolamide; HG, high glucose; NG, normal glucose; TOP, topiramate.

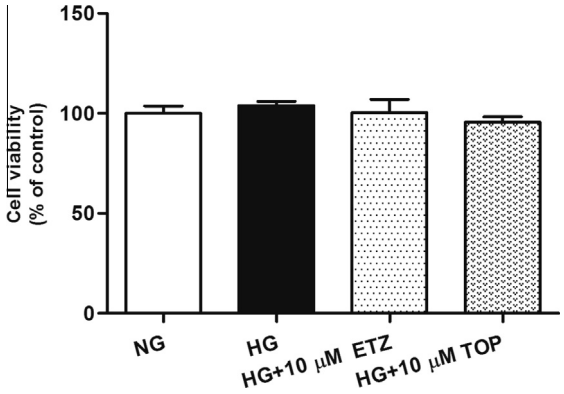

Fig. 3. Cerebral pericyte viability in NG, HG (40.7 mM), and HG with ethoxyzolamide or topiramate. Cell viability is shown as percentage of viable cells in NG. Data are shown as mean ± SEM ($n = 3$). The graphs are representative of three independent experiments. ETZ, ethoxyzolamide; HG, high glucose; NG, normal glucose; TOP, topiramate.

pyruvate enters the mitochondria and combines with HCO_3^- to form oxaloacetate, a key enzyme in the oxidative metabolism of glucose. Mitochondrial membranes are impermeant to HCO_3^-; therefore, the latter must be produced inside the mitochondria. Mitochondrial carbonic anhydrases, the carbonic anhydrases inside the mitochondria, generate HCO_3^- by reversible hydration of CO_2 ($CO_2 + H_2O \Longleftrightarrow HCO_3^- + H^+$). Oxaloacetate enters the Krebs cycle and generates electron donors, $FADH_2$ and NADH, which are carried to electron transport chain reactions to generate ATP and superoxide, the precursor of all ROS [29]. Though small fluctuations in the steady-state concentration of these oxidants may actually play a role in intracellular signaling [6], uncontrolled increases in the steady-state concentrations of these oxidants lead to free radical mediated chain reactions, which indiscriminately target proteins [27], lipids [23], polysaccharides [12], and DNA [14,21].

In diabetes, constant influx of glucose in relatively insulin insensitive tissues such as brain causes an overproduction of ROS as follows: The excess electron donors produced during the Krebs cycle generate high mitochondrial membrane potential by pumping protons across the inner mitochondrial membrane (Fig. 4). The high mitochondrial membrane potential inhibits electron transport at complex III, increases the half-life of the free radical intermediate of coenzyme Q, which reduces O_2 to superoxide, leading to production of ROS above physiological levels. Inhibition of mitochondrial carbonic anhydrases slows down the production of HCO_3^-, limits oxaloacetate, electron donors, superoxide, ROS, and consequent oxidative stress.

Our current findings clearly demonstrate that treatment of pericytes with mitochondrial CAIs [20,25] blocks high glucose-induced increases in the respiration rate (Fig. 1A and C). This inhibition is accomplished by slowing down the production of the HCO_3^- that is essential for the conversion of pyruvate to oxaloacetate, the key intermediate in energy production and generation of ROS (Fig. 4). To maintain "pyruvate lactate" equilibrium, in the absence of sufficient HCO_3^-, excess pyruvate is metabolized to lactate. As expected, the extracellular acidification rate (ECAR), predominantly a measure of lactic acid, increased significantly in pericytes in high glucose (Fig. 1B and D). The ECAR remained unaffected by mitochondrial carbonic anhydrase inhibition. This inability of mitochondrial CAIs to alter ECAR is not surprising; the conversion of pyruvate to lactate is a dehydrogenation reaction, does not require HCO_3^- and, therefore, remains unaffected by inhibition of mitochondrial carbonic anhydrase activity.

As anticipated, the high glucose-induced increase in the amount of total ROS was also blocked by inhibition of mitochondrial carbonic anhydrases (Fig. 2). The viability of the cells throughout the duration of the ROS assays remained unchanged in all treatment groups (Fig. 3). Previously, we reported ROS in live mitochondria [25]. The number of mitochondria exhibiting ROS was

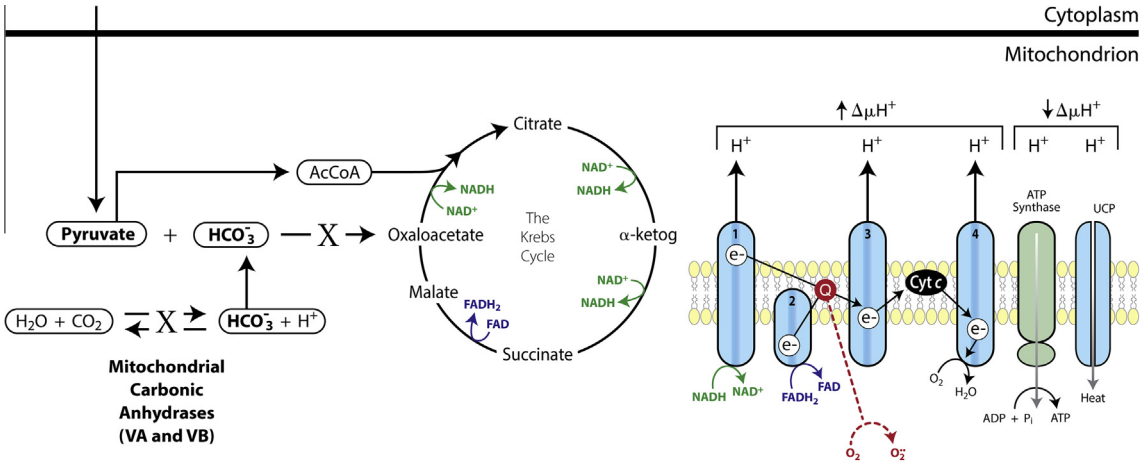

Fig. 4. Role of mitochondrial carbonic anhydrases in oxidative metabolism of glucose and superoxide (O_2^-.) production. $\Delta\mu H^+$, mitochondrial membrane potential; HCO_3^-, bicarbonate; UCP, uncoupling protein.

significantly higher in pericytes exposed to high glucose. The high glucose-induced changes were blocked upon treatment with mitochondrial CAIs.

In the light of the previous and current findings, pharmacological manipulation of mitochondrial carbonic anhydrases to protect the brain from diabetic injury seems increasingly promising.

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