

## Pharmacological Inhibition of Mitochondrial Carbonic Anhydrases Protects Mouse Cerebral Pericytes from High Glucose-Induced Oxidative Stress and Apoptosis

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**Running Title:** Mitochondrial CA inhibition protects apoptosis of brain PC

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**ABBREVIATIONS:** BBB, blood-brain barrier; bFGF, basic fibroblast growth factor; CAs, carbonic anhydrases; CD13, aminopeptidase N; EC, endothelial cells; ETC, electron transport chain; ETZ, 6-Ethoxy-2-benzothiazolesulfonamide (ethoxazolamide); FACS, fluorescence Activated Cell Sorting; GFAP, glial fibrillary acidic protein; GPI, glycosylphosphatidylinositol; GSH, reduced glutathione;  $\text{HCO}_3^-$ , bicarbonate; HG, high glucose; HNE, 4-hydroxy-2-trans-nonenal;  $\text{IFN-}\gamma$ , interferon gamma; imPC, conditionally immortalized cerebral pericytes; LPS, lipopolysaccharide; mCAs, mitochondrial carbonic anhydrases; NDS, normal donkey serum; NG, normal glucose; NG2, neuron-gial 2; OxSt, oxidative stress; PC, pericytes; PDGFR- $\beta$ , platelet-derived growth factor receptor  $\beta$ ; PDS, plasma derived serum; PECAM-1, platelet

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endothelial cell adhesion molecule-1; ROS, reactive oxygen species;  $\alpha$ -SMA, smooth muscle actin  $\alpha$ -subunit; TEER, transendothelial electric resistance; TCA, trichloroacetic acid; TOP, topiramate; TUNEL, TdT-dUTP Terminal Nick-End Labeling.

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## ABSTRACT

Diabetes-associated complications in the microvasculature of the brain are caused by oxidative stress, generated by overproduction of reactive oxygen species from hyperglycemia-induced accelerated oxidative metabolism of glucose. Pericytes, essential for the viability of the microvasculature, are especially susceptible to oxidative stress. Mitochondrial carbonic anhydrases, regulators of the oxidative metabolism of glucose, determine the rate of reactive oxygen species production and inhibition of mitochondrial carbonic anhydrases rescues glucose-induced pericyte loss in the diabetic mouse brain. We hypothesized that high glucose induces intracellular oxidative stress and pericyte apoptosis and inhibition of mitochondrial carbonic anhydrases protects pericytes from oxidative stress-induced apoptosis. To validate our hypothesis, conditionally immortalized cerebral pericyte (IPC) cultures were established from immortomice to investigate the effect of high glucose on oxidative stress and pericyte apoptosis. The IPC expressed pericyte markers and induced high transendothelial electrical resistance and low permeability in brain endothelial cell monolayers comparable to pericytes in primary cultures. The IPC also secreted cytokines constitutively and in response to lipopolysacchride similar to pericytes. High glucose caused oxidative stress and apoptosis of these cells with both oxidative stress and apoptosis significantly reduced following mitochondrial carbonic anhydrase inhibition. These results provide the first evidence that pharmacological inhibition of mitochondrial carbonic anhydrases attenuates pericyte apoptosis caused by high glucose-induced oxidative stress. Carbonic anhydrase inhibitors have a long history of safe clinical use and can be immediately evaluated for this new indication in translational research. Thus, mitochondrial carbonic anhydrases may provide a new therapeutic target for oxidative stress related illnesses of the brain.

## Introduction

Diabetes mellitus is associated with chronic hyperglycemia, oxidative stress (OxSt) and diabetes-specific microvascular pathology in a variety of tissues including the brain (Giugliano, et al., 1996;Kowluru, 2001;Kowluru, et al., 2004). The microvasculature of the brain comprises the vascular blood-brain barrier (BBB), a regulatory interface that prevents the unrestricted leakage of plasma proteins into the central nervous system and has nutritive, homeostatic, and communication roles. The brain endothelial cells (EC) comprising the BBB are in immediate contact with the pericytes. The pericytes play an important role in the maintenance and homeostasis of the BBB (Dore-Duffy, et al., 2006;Dore-Duffy, 2008) and are especially susceptible to OxSt (Price TO, et al., 2011).

Oxidative stress is caused by overproduction of reactive oxygen species (ROS) primarily during mitochondrial oxidative metabolism of glucose (Du, et al., 2000;Nishikawa, et al., 2000). ROS are produced as byproducts (Chen, et al., 2003;Liu, et al., 2002) of electron transport chain (ETC) reactions during production of ATP as glucose is metabolized to H<sub>2</sub>O and CO<sub>2</sub>. In diabetes, the hyperglycemic state provides more glucose to the Krebs cycle, especially in insulin-insensitive tissues, thus increasing the rate of production of electron donors, FADH<sub>2</sub> and NADH. These electron donors generate a proton gradient across the inner mitochondrial membrane during ETC reactions. When the electrochemical potential difference generated by the proton gradient is high, the lifetime of the superoxide-generating electron-transport intermediates is prolonged (Brownlee, 2001). Beyond a threshold, superoxide production is markedly increased (Korshunov, et al., 1997), thereby causing OxSt. Therefore, a reduction in the rate of production of ROS should reduce OxSt. We recently reported reduction of OxSt in the brain of diabetic-

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mice by pharmacological inhibition of mitochondrial carbonic anhydrases (mCA) (Price TO, et al., 2011).

Carbonic anhydrases (CA) catalyze a simple yet fundamental biological reaction, CO<sub>2</sub> hydration to produce bicarbonate (HCO<sub>3</sub><sup>-</sup>) and protons (Shah, et al., 2000). This reaction forms the basis for the regulation of acid–base balance in organisms. In addition, CA participate in a number of other physiological processes such as CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> transport, bone resorption, production of body fluids, gluconeogenesis, ureagenesis and lipogenesis (Sly and Hu, 1995). During evolution, at least 13 active CA isoenzymes have emerged in both rodents and humans. These isozymes have differences in their tissue distribution, kinetic properties and subcellular localizations (Hilvo, et al., 2005). Only two of the known isozymes, designated as CA VA and CA VB, are expressed in the mitochondria (Shah, et al., 2000). These mCA play an important role in the regulation of oxidative metabolism of glucose (Price TO, et al., 2011).

Previously, we reported (Price TO, et al., 2011) rescue of OxSt-induced cerebral pericyte loss in diabetic mice with topiramate, a mCA inhibitor (Nishimori I, et al., 2005). The purpose of the current study was to investigate the mechanism by which high glucose-induced OxSt causes pericyte death and to determine whether inhibition of mCA protects pericytes from OxSt-induced apoptosis. To address these questions, we established conditionally immortalized cerebral pericytes (IPC) from the Immortomouse (Scheef EA, et al., 2009). The IPC can be rapidly expanded under permissive conditions [33°C in the presence of interferon-gamma (IFN-γ)] and can be readily passaged, frozen, and thawed. We show here that similar to pericytes in primary culture (PC), these cells expressed α-smooth muscle actin (α-SMA), neuron-gial 2 (NG2) proteoglycan, platelet-derived growth factor receptor-β (PDGFR-β), and aminopeptidase N (CD13) markers in both the early and the late passages. In addition, IPC enhanced the

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transendothelial electric resistance (TEER) and reduced the permeability coefficient of brain EC monolayers in triple co-cultures (Nakagawa S, et al., 2009) similar to PC. The IPC had an expression pattern for CA's similar to PC: presence of mCA VA and VB, but lack of cytosolic CA II, two transmembrane CA's (CA XII and CA XIV), and lack of the glycosylphosphatidylinositol (GPI)-anchored CA IV.

We now report, for the first time, that high glucose causes intracellular OxSt and pericyte death by apoptosis. We also provide evidence that high glucose-induced intracellular OxSt and pericyte apoptosis are significantly reduced upon pharmacological inhibition of mCA.

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## **Materials and Methods**

### **Animals**

Immortomice expressing a temperature-sensitive simian virus (SV) 40 large T antigen and male CD-1 mice were obtained from Charles River Laboratories (Charles River, Wilmington, MA). Male C57BL/6 mice were from Jackson Laboratories (Bar Harbor, ME). All animal experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee.

### **Preparation of pericytes**

Immortalized cerebral pericytes (IPC) were isolated from immortomice back crossed to C57BL/6 mice (Scheef EA, et al., 2009) according to the method of Hayashi et al. (Hayashi, et al., 2004) with modification. Briefly, brains from 5-10 mice (both female and male), ubiquitously expressing a thermolabile strain of tsA58 of the SV40 large T antigen (tsA58 Tag), were removed and placed in DMEM (low glucose; Sigma, St. Louis, MO) on ice. The meninges were removed by gently rolling half of a brain on a sterile filter. The white matter and choroid plexus were removed and the remaining tissue was cut into small pieces and incubated in 25 ml DMEM containing collagenase Type II (1 mg/ml; Worthington, Lakewood, NJ) and DNase I (30 U/ml; Sigma) for 40 min at 37°C. Following incubation, the digest was centrifuged at 500xg for 10 min at 4°C. The supernatant was carefully removed by aspiration, the pellet was mixed with 25 ml of 20% BSA prepared in DMEM, mixed well, and centrifuged at 1 000xg for 20 min at 4°C. The lipid layer on the top was carefully removed, the pellet which contains the blood vessels was resuspended in 5 ml of collagenase/dispase (1 mg/ml in serum free DMEM containing 0.1% BSA), DNase I (30 U/ml) and incubated at 37°C for 30 min. The digestion was



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resuspended by pipetting, centrifuged, and the cell pellet was washed once with 5 ml of DMEM containing 10% FBS. The pellet was resuspended in 1 ml of growth medium consisting of DMEM with 10% FBS, 2mM L-glutamine, penicillin/streptomycin (Sigma) and murine recombinant IFN- $\gamma$  at 44 U/ml (R&D Systems, Minneapolis, MN), and plated into a well of 24 well plate. The cells were fed every 3-4 days, maintained at 33°C with 5% CO<sub>2</sub>, and when confluent expanded to larger tissue culture plates. Because microvessel fragments contain 2-3% pericytes, the pure culture of cerebral pericytes was obtained by prolonged culture of isolated brain microvessel fragments. Pericyte survival and proliferation was favored by selective culture conditions (Kis, et al., 2002). Pericytes were characterized by their large size and rhomboid morphology, positive immunostaining for  $\alpha$ -SMA and NG2 (Balabanov and Dore-Duffy, 1998) and the absence of platelet endothelial cell adhesion molecule (PECAM-1) and glial fibrillary acidic protein (GFAP) staining. By flow cytometry, 95% of the cells were positive for both NG2 and  $\alpha$ -SMA. Less than 5% of the cells were either positive for NG2 or for  $\alpha$ -SMA.

### **Characterization of IPC**

***Fluorescence activated cell sorting (FACS) analysis:*** Mouse IPC were dissociated with Trypsin-EDTA (T3924; Sigma). For immunostaining with anti-mouse PDGFR- $\beta$  (eBioscience, San Diego, CA) and rat anti-mouse PECAM-1 (Mec13.3, BD Pharmingen), the cells were blocked with 5% FBS in PBS and incubated with an appropriate antibody for 30 min. After washing twice with 5% FBS in PBS, the cells were analyzed by a FACScanCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). For anti-NG2 (MAB5384, Millipore, Temecula, CA), anti-human  $\alpha$ -SMA (MAB1420, R&D systems), and rabbit anti-CD13 (Abcam, MA) immunostaining, dissociated IPC were fixed with 2% paraformaldehyde in PBS for 30 min

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and incubated with appropriate primary antibodies in permeabilisation and blocking solution (0.1% BSA and 0.1% Triton X-100 in PBS) for 30 min. Cells were washed and incubated with the appropriate secondary antibody for 30 min, washed, and analyzed by flow cytometry, as described above. All incubations were at 4°C, all antibodies were used at the dilutions recommended by the supplier. Cells incubated with secondary antibodies only were used as controls.

There are currently no specific markers for pericytes. Staining with  $\alpha$ -SMA, PDGFR- $\beta$  and NG2 is good, but not specific. Therefore, we considered biological functions such as improvement of BBB function and IPC response to lipopolysacchride (LPS) to further characterize these cells (see below).

### **TEER analysis**

**Preparation of cells:** Mouse brain EC and PC were isolated from 8-week-old male CD-1 mice according to published protocols (Coisne C, et al., 2005; Jacob A., et al., 2010) and (Nakagawa S, et al., 2009), respectively. The glial cells were obtained from neonatal CD-1 mice, by published procedures (Coisne C, et al., 2005; Nakagawa S, et al., 2009). The purity of EC was checked by positive immunostaining for factor VIII (Millipore), zona occludens-1 (ZO-1) (Invitrogen), and claudin-5 (Abcam); of pericytes for  $\alpha$ -SMA, CD13, PDGFR- $\beta$ ; and of glial cells for GFAP (Abcam).

**Construction of in vitro BBB:** The triple culture Transwell model were set up according to published methods (Nakagawa S, et al., 2009). Briefly, glial cells ( $1 \times 10^5$  cells/cm<sup>2</sup>) were seeded in the wells of Transwell plate and allowed to grow for 3 weeks. PC or IPC ( $1.5 \times 10^4$  cells/cm<sup>2</sup>) were seeded on the bottom of the collagen-coated polyester membrane (0.33 cm<sup>2</sup>, 0.4  $\mu$ m pore

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size) of the Transwell inserts (24-well type, Costar) and allowed to adhere firmly overnight. On the next day, brain EC ( $1.5 \times 10^5$  cells/cm<sup>2</sup>) were seeded on the inside of the inserts and incubated with glial cell cultures. The BBB models were maintained in an EC medium [DMEM/F12 supplemented with 10% plasma derived serum (PDS, Animal Technologies, Inc., USA), 1% GlutaMAX supplement (Gibco), basic fibroblast growth factor (bFGF, Roche Applied Sciences, 1 ng/ml), heparin (100 μg/ml), insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 ng/ml), and gentamicin (50 μg/ml)] supplemented with 500 nM hydrocortisone at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. TEER (in  $\Omega \times \text{cm}^2$ ) was measured using an EVOM resistance meter (World Precision Instruments, Sarasota, FL, USA). The TEER of cell-free Transwell<sup>®</sup>-Clear inserts were subtracted from the obtained values.

### **Transendothelial permeability**

For the transport experiments, brain EC were washed with physiological buffer (141 mM NaCl, 4.0 mM KCl, 2.8 mM CaCl<sub>2</sub>, 1.0 mM MgSO<sub>4</sub>, 1.0 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM HEPES, 10 mM D-glucose and 1% BSA, pH 7.4). The same buffer was added to the outside (abluminal chamber; 0.6 mL) of the Transwell<sup>®</sup> insert. To initiate the transport experiments, <sup>125</sup>I-albumin ( $5 \times 10^6$  cpm/mL) and <sup>14</sup>C-sucrose ( $1.5 \times 10^6$  cpm/mL) were loaded on the luminal chamber. Samples (0.5 ml) were removed from the abluminal chamber at 10, 20, 30, and 45 min and immediately replaced with an equal volume of fresh physiological buffer. <sup>125</sup>I-Albumin samples were mixed with 30% trichloroacetic acid (TCA; final concentration 15%) and centrifuged at 5 400xg for 10 min at 4°C. Radioactivity in the TCA precipitate was determined by a gamma counter, and radioactivity of <sup>14</sup>C-sucrose in the collector compartments was measured by a liquid scintillation counter (Tri Carb 1900 from Packard Instrument Company, Perkin-Elmer Life Sciences,

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Courtaboeuf, France). The permeability coefficient and clearance of TCA-precipitable  $^{125}\text{I}$ -albumin and  $^{14}\text{C}$ -sucrose were calculated as described (Dehouck MP, et al., 1992).

### **Cytokines and chemokines in IPC**

The IPC seeded on 24-well culture plate (Costar) were washed with serum-free DMEM/F12, and exposed to culture media with or without LPS from *Salmonella typhimurium* (L6511; Sigma) at 1  $\mu\text{g}/\text{ml}$  for 24 h, as described previously (Kovac, et al., 2011). Culture supernatants were collected and stored at  $-80^{\circ}\text{C}$ . The cytokines and chemokines were measured by a commercial magnetic bead Multiple ELISA kit (Bioplex, Biorad, USA) according to the manufacturer's instructions.

### **Mitochondrial CA VA and CA VB in IPC**

Immunoblotting of the whole brain and IPC lysates and RT-PCR of total RNA from the whole brain and IPC was as described previously (Price TO, et al., 2011). Briefly, proteins (50  $\mu\text{g}$ ) were separated on 4-12 % Bis-Tris reducing gels (NuPAGE Novex) and transferred to nitrocellulose membranes for immunoblotting. Polypeptides were identified by probing with mitochondrial CA VA and VB primary antibodies (Price TO, et al., 2011) and horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescent substrate (Pierce, Rockford, IL). For RT-PCR, RNA (1  $\mu\text{g}$ ) was reverse-transcribed with SuperScript III first Strand Synthesis System for RT-PCR (inVitrogen). The double stranded DNA thus produced was used as template for PCR amplification with specific primers for either CA VA or VB as described (Price TO, et al., 2011).

### **Measurement of OxSt and oxidative damage**

The IPC were seeded in 60 mm petri dishes at  $5 \times 10^4$  cells/plate. Cells were treated for 5 days with normal glucose (5.7 mM), high glucose (40.7 mM), and high glucose with mCA inhibitors ethoxzolamide or topiramate at 10 or 100  $\mu$ M. The cells were dissociated by trypsinization, resuspended in fresh media, and centrifuged. The pellets were analyzed for GSH, a measure of OxSt, and 4-hydroxy-trans-2-nonenal (HNE), a measure of oxidative damage caused by lipid peroxidation. The IPC cultured in L-glucose (D-glucose 5.7mM + L-glucose 35mM) were used for osmolarity control.

To measure GSH, the cell pellets were resuspended in a serine–borate buffer (100 mM Tris–HCl, 10 mM borate, 5 mM serine, 1 mM diethylenetriaminepentaacetic acid, pH 7.0). GSH concentrations were determined by reverse phase HPLC as described earlier (Price TO, et al., 2011).

For lipid peroxidation determination, the cell pellets were resuspended in lysis buffer [0.5% Triton X-100, 20 mM Tris (pH 7.4), 0.15 M NaCl, 2 mM EDTA, 1 mM EGTA, and a protease inhibitor cocktail (#78429, Thermo Scientific)], sonicated, and centrifuged at 14 000xg for 15 min: final supernatants were used for detection of HNE by immunoblotting as described earlier (Price TO, et al., 2011).

### **Measurement of mitochondrial ROS**

Mitochondrial ROS in the IPC were measured using MitoSOX<sup>TM</sup> Red (Invitrogen, USA), a fluorogenic live-cell permeant dye which is chemically and selectively targeted to mitochondria. Once in the mitochondria, MitoSOX is oxidized by superoxide and exhibits red fluorescence (excitation 510 nm/emission 580 nm). The IPC were plated on coverslips at a

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density of 2 000 cells/cover slip and allowed to adhere overnight. The cells were then treated with normal glucose (5.7 mM) or normal glucose with or without ethoxzolamide or topiramate at 100  $\mu$ M, overnight. After incubation the cells were washed with Hank's balanced salt solution (HBSS, with Ca/Mg) and exposed to normal glucose or high glucose (40.7 mM) for 30 min. At the end of the incubation, cells were washed as before and incubated for 5 min with 2.5  $\mu$ M MitoSOX, prepared in DMSO immediately prior to the experiment. The cells were thereafter washed with HBSS and counterstained with Hoechst (1:5 000). The digital images were taken by a microscope Olympus BX60 (Tokyo, Japan) with fluorescence equipment. Images were captured using 40x oil immersion objective lens.

### **Programmed cell death analysis**

Apoptotic cell death was determined by TdT-dUTP terminal nick-end labeling (TUNEL) staining. The IPC were grown for 5 days on the fibronectin-coated (2  $\mu$ g/ml) 4-well Nunc chamber slides (Fisher) under normal glucose, high glucose or high glucose containing 10 or 100  $\mu$ M of either ethoxzolamide or topiramate. Following incubation, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with PBS twice. TUNEL staining was performed using Click-iT® TUNEL Alexa Fluor® 594, as recommended by the supplier (Invitrogen). Cells were counterstained with Hoechst for the nuclei and photographed using a Zeiss fluorescence microscope (Axiophot, Zeiss, Germany) equipped with a digital camera. TUNEL-positive cells were counted and calculated as a percentage of the total cell number. The results are from three independent experiments.

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## Statistical analysis

All means are reported with their  $n$  and 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).

## Results

### Characterization of IPC

**Morphological characterization of IPC:** The IPC exhibited the same irregular and stellate morphology as the PC (Fig. 1A). To further assess similarities between IPC and PC, we analyzed IPC for the expression of pericyte markers by FACS analysis. The pericyte markers PDGFR- $\beta$  (Fig. 1B),  $\alpha$ -SMA (Fig. 1C), NG2 (Fig. 1D), and CD13 (Fig. 1E) were all expressed by IPC in both early and late passages. PECAM-1, an EC marker, was not detected in IPC (Fig. 1F).

**Physiological relevance of IPC:** To ascertain the physiological relevance of IPC, triple culture Transwell BBB models were set up with brain EC, glial cells, and either PC or IPC. Double co-culture BBB models, without pericytes (w/o PC, Fig. 2A) were used as controls. Both PC and IPC (Fig. 2A) caused statistically higher TEER compared to the double co-culture model ( $p < 0.001$ ).  $^{14}\text{C}$ -Sucrose permeability of brain EC was significantly decreased in the models constructed with either PC or IPC compared to the BBB model w/o PC ( $p < 0.01$ , Fig 2B). A slight decrease in permeability to  $^{125}\text{I}$ -albumin (Fig. 2C) observed in the presence of PC or IPC, did not reach statistical significance (Fig. 2C).

### LPS stimulated release of cytokines and chemokines by IPC

To further ascertain the functional similarity between PC and the IPC, cytokine and chemokine release in response to LPS treatment was assessed. Ten of twenty-two cytokines and chemokines were secreted constitutively by IPC and sixteen of twenty-two were secreted with stimulation by LPS (Table 1).



Together, these results showed that IPC have morphological and physiological characteristics similar to those of cerebral PC.

### **Mitochondrial CA in IPC**

Previously, we showed by RT-PCR and immunoblotting that cerebral pericytes express mCA (Price TO, et al., 2011). Using the same techniques, we now confirm the expression of both mitochondrial CA VA and VB (Fig. 3) in IPC. Also similar to PC, CA II (a cytosolic), CA XII and CA XIV (two transmembrane), and CA IV (a GPI-anchored) CA were not detected in IPC.

### **Effect of inhibition of mCA on OxSt in IPC**

The cells were incubated under normal glucose (5.7 mM) or high glucose (40.7 mM) conditions for 5 days. Mitochondrial CA inhibitors, ethoxzolamide or topiramate (10  $\mu$ M and 100  $\mu$ M) were added to the high glucose media at the start of the experiment. Compared to normal glucose, high glucose caused significant OxSt in IPC, as is indicated by a decrease in intracellular GSH (Fig. 4A and B). Treatment with ethoxzolamide significantly increased GSH at 10  $\mu$ M ( $p < 0.001$ ), and at 100  $\mu$ M ( $p < 0.0001$ ), compared to high glucose alone (Fig. 4A). Similarly, topiramate treatment reduced high glucose-induced OxSt (Fig. 4B) by significantly increasing GSH at both 10  $\mu$ M ( $p < 0.01$ ) and 100  $\mu$ M ( $p < 0.001$ ).

Lipid peroxidation, an indicator of oxidative damage, was assessed by the levels of protein-bound HNE, a stable byproduct of lipid peroxidation. The protein-bound HNE levels were significantly increased in IPC exposed to high glucose (Fig 5A and B). The treatment with ethoxzolamide significantly reduced high glucose-induced protein-bound HNE (Fig. 5A) at

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both concentrations used ( $p < 0.01$  and  $p < 0.0001$  at 10  $\mu\text{M}$  and 100  $\mu\text{M}$ , respectively). Topiramate treatment significantly reduced high glucose induced protein-bound HNE at 100  $\mu\text{M}$  ( $p < 0.0001$ , Fig. 5C).

### **Effect of inhibition of mCA on high glucose-induced mitochondrial ROS in IPC**

The IPC were grown overnight in normal glucose (5.7 mM) with or without ethoxzolamide or topiramate and then treated with high glucose (40.7 mM) for 30 min. The cell treated with high glucose showed an increase in mitochondrial ROS as indicated by the intensity of the signal as well as by the number of cells exhibiting the signal (Supplemental Figure 1). Treatment with both ethoxzolamide and topiramate attenuated high glucose-induced mitochondrial ROS in these cells (Supplemental Figure 1).

### **Effect of pharmacological inhibition of mCA on high glucose-induced IPC apoptosis**

For TUNEL assays, cells were incubated under normal glucose, high glucose, and high glucose with or without mCA inhibitors conditions, as described above. The percent of TUNEL positive IPC grown in normal glucose, high glucose, and high glucose with ethoxzolamide or with topiramate are shown in Fig. 6A and 6B, respectively. Exposure (5 days) to high glucose caused a significant increase in the percentage of apoptotic cells (Fig. 6A and B) compared to normal glucose. Ethoxzolamide treatment significantly reduced the number of high glucose-induced apoptotic cells (Fig. 6A) at both concentrations ( $p < 0.01$ ). Similarly, topiramate treatment resulted in a significant decrease in the percent of apoptotic IPC ( $p < 0.0001$ ) as compared with high glucose (Fig. 6B). Representative TUNEL images of IPC in normal glucose, high glucose, and high glucose with 10 and 100  $\mu\text{M}$  ethoxzolamide or topiramate are presented in

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Supplemental Figure 2. The cells cultured in L-glucose (osmolarity control D-glucose 5.7mM + L-glucose 35mM) for 5 days did not show OxSt or apoptosis.

## Discussion

The main findings of this study showed that the pericyte exhibit OxSt and apoptosis responses when exposed to high glucose. Both high glucose-induced OxSt and apoptosis of pericyte were rescued by pharmacological inhibition of mCA.

The pericytes, in immediate contact with the specialized EC of cerebral microvasculature comprising the BBB, are vital for integrity of the latter and are especially susceptible to OxSt. Loss of pericytes results in a focal increase in BBB permeability (Bonkowski, et al., 2011). A close correlation between pericyte density and BBB permeability has been reported in pericyte deficient adult mice (Armulik, et al., 2010). Recently, we reported hyperglycemia-induced OxSt in the brain and cerebral pericyte loss in diabetic mice (Price TO, et al., 2011). Clinical evidence suggests that diabetes-induced changes in the BBB may be a predisposing factor for Alzheimer's disease (Ristow, 2004) and people suffering from both types of diabetes have an increased risk of cognitive impairment (Bruce, et al., 2008;Tiehuis, et al., 2008;Whitmer, 2007). Thus, determining the pathophysiological role of hyperglycemia on pericytes is an important step toward understanding the altered neuronal function and increased susceptibility to cerebrovascular diseases.

Hyperglycemia causes OxSt by generating pathological levels of ROS during accelerated oxidative metabolism of glucose (Du, et al., 2000;Nishikawa, et al., 2000). ROS are produced as a byproduct of oxidative metabolism of glucose and play important physiological functions (Droge, 2002). Following is a brief description of the mechanism of production of ROS in the mitochondria during oxidative metabolism: Glucose is metabolized to pyruvate in the cytosol by glycolysis. Pyruvate enters mitochondria and is carboxylated to oxaloacetate, a key intermediate in the Krebs cycle/ETC pathways. The carboxylation of pyruvate to oxaloacetate requires  $\text{HCO}_3^-$ .

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Since mitochondria are impermeant to  $\text{HCO}_3^-$  (Dodgson, et al., 1980; Hazen, et al., 1997; Parkkila, et al., 1998) the latter must be produced inside the mitochondria. Mitochondrial CA provide  $\text{HCO}_3^-$  inside the mitochondria by catalyzing reversible hydration of  $\text{CO}_2$  ( $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$ ). Pharmacological inhibition of mCA slows down the production of  $\text{HCO}_3^-$ , limits Krebs cycle/ETC, and thus slows down the production of ROS and subsequent OxSt. Recently we showed that pharmacological inhibition of mCA rescues OxSt-induced pericyte loss in diabetic mouse brain (Price TO, et al., 2011). These findings provide evidence that mCA inhibition may protect BBB from the damage caused by OxSt, thus making mCA a new therapeutic target for OxSt related illnesses of the brain. Pyruvate that builds up in the process is shuttled through anaerobic metabolism (aerobic glycolysis) to produce ATP without producing superoxide. Aerobic glycolysis is not harmful and indeed is preferred by fast growing cells (Vander Heiden, et al., 2009).

To facilitate the determination of the effect of high glucose on intracellular OxSt in pericytes and to investigate a mechanism for pericyte death, we prepared conditionally immortalized pericytes (IPC) cultures from immortomice (Fig. 1A). Unlike PC, the IPC can be readily expanded and manipulated in culture for numerous passages without loss of their characteristics. The immortomouse expresses a thermolabile strain (tsA58) of the SV40 large T antigen (tsA58 Tag) driven by an inducible major histocompatibility complex H-2K promoter, thus eliminating many intrinsic problems with immortalized lines (Lidington, et al., 2002). T antigen expression is functionally evident at the reduced temperature of 33°C and was enhanced in the presence of IFN- $\gamma$ . Generally, incubation at 37°C in the absence of IFN- $\gamma$  results in a loss of large T antigen within 48 hours (Lidington, et al., 2002). Recently, Dore-Duffy et al., (Dore-Duffy P, et al., 2011) reported a conditionally immortalized cerebral pericyte cell line established

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from immortomice. However, unlike IPC these cells are  $\alpha$ -SMA negative and proliferate slowly under permissive conditions (33°C, in the presence of IFN- $\gamma$ ) and are largely quiescent.

The IPC share the morphological (Fig. 1) and physiological (Fig. 2) characteristics of PC. The IPC were also capable of secreting cytokines and chemokines both constitutively and in response to LPS (Table 1), a characteristic similar to that recently reported by us in PC (Kovac, et al., 2011). When grown in high glucose media, IPC showed significant OxSt (Fig. 4 and 5) and apoptosis (Fig. 6). Both high glucose-induced OxSt (Fig. 4 and 5) and apoptosis (Fig. 6) were rescued by the pharmacological inhibition of mCA with either ethoxzolamide or topiramate.

Previously we used topiramate (Nishimori I, et al., 2005) to reduce OxSt and pericyte loss in vivo in the mouse brain (Price TO, et al., 2011). However, topiramate in addition to its CA inhibitory activity has effects on Na<sup>+</sup>-independent Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> channels, sodium channels, and calcium channels (Leniger, et al., 2004). Therefore, in this study we included ethoxzolamide (Hazen, et al., 1997) which is viewed as a relatively pure CA inhibitor. Use of both of these clinically available drugs increases the assurance that effects found are attributable to CA inhibition. The significant reduction of intracellular OxSt (Fig. 4 and 5), mitochondrial ROS (Supplemental Figure 1) and apoptosis (Fig. 6 and Supplemental Figure 2) of IPC is most likely from the ability of ethoxzolamide and topiramate to inhibit mCA rather than their discordant mechanisms.

Both ethoxzolamide and topiramate also inhibit isozymes of carbonic anhydrases other than mCA. Previously, we published that a cytosolic (CA II), two transmembrane (CA XII and CA XIV) and a GPI-anchored (CA IV) carbonic anhydrases are not expressed in cerebral PC (Price TO, et al., 2011). We now report the absence of these same CA isoforms in IPC. Lack of

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CA II, CA XII, CA XIV, and CA IV expression in IPC gives credence to our hypothesis that the effects of ethoxzolamide and topiramate on the reduction of OxSt and apoptosis are due to the inhibition of mCA, amply expressed in IPC (Fig. 3). Studies are under way in our labs to investigate the effect of high glucose on OxSt and apoptosis in cerebral IPC that are null for both mitochondrial CA VA and CA VB.

Topiramate in combination with phentermine has recently been approved for the treatment of obesity. We suggest that an additional benefit of such treatment will be protection from the glucotoxicity of diabetes, such as diabetic retinopathy and other central nervous system manifestation, beyond that accountable from changes in blood glucose levels.

In addition to providing important mechanistic information about diabetic neuropathy, our studies define mCA as a novel molecular pathway that could be targeted in OxSt-related illnesses of the central nervous system. Carbonic anhydrase inhibitors are commercially available, are in clinical use for other conditions, and are relatively well tolerated (Gadde, et al., 2011). The therapeutic use of carbonic anhydrase inhibitors in conditions where glucose-induced OxSt contributes to pathology such as the damage to microvasculature of the brain seen in diabetes mellitus deserves further study.

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### **Authorship Contributions**

*Participated in research design:* Shah, Banks, and Sheibani

*Conducted experiments:* Price, Morofuji, Kovac, and Shin

*Contributed new reagents or analytic tools:* Sorenson and Sheibani

*Performed data analysis:* Shah, Price, Morofuji, Kovac, and Shin

*Wrote or contributed to the writing of the manuscript:* Shah, Price, Banks, Kovac, Ercal, and Sheibani.

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## Footnotes

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## Figure Legends

**Figure 1.** Characterization of conditionally immortalized cerebral pericytes (IPC). Morphology of IPC cultured on uncoated plates (magnification x10) (**A**). IPC were stained for PDGFR- $\beta$  (**B**),  $\alpha$ -SMA (**C**), NG2 (**D**), and CD13 (**E**), all of which were expressed in IPC. PECAM-1 (**F**) an EC marker is not detected in IPC. Histogram data are presented as cell numbers versus log fluorescence intensity of individual cell markers. The controls, shaded histograms, were either cells without antibody (**B** and **F**) or treated with a secondary antibody (**C**, **D**, **E**) only. These experiments were performed three times with similar results.

**Figure 2.** Comparison of TEER and transendothelial permeability of BBB models. The triple models constructed with IPC imposed the same TEER (**A**) and transendothelial permeability (**B** and **c**) as the ones constructed with PC compared to the double models without pericyte (w/o PC). The TEER (expressed as  $\Omega \times \text{cm}^2$ ) was higher (**A**) and transendothelial permeability coefficient ( $P_e$ ) [expressed as  $(\text{cm}/\text{minute}) \times 10^{-3}$ ] was lower for paracellular markers,  $^{14}\text{C}$ -sucrose (**B**) and  $^{125}\text{I}$ -albumin (**C**), in the presence of either PC or IPC. All data are presented as mean  $\pm$  SEM ( $n=4$ ). \*\* $p<0.01$ , \*\*\* $p<0.001$ .

**Figure 3.** Mitochondrial CA VA and VB in IPC. Transcripts of CA VA and CA VB in IPC and the brain (**A**). + and – indicate with and without reverse transcriptase, respectively. Polypeptides of CA VA and CAVB in IPC and the brain (**B**).

**Figure 4.** Effect of pharmacological inhibition of mCA on high glucose-induced GSH levels in IPC. The IPC were exposed to normal glucose (NG, 5.7 mM), high glucose (HG, 40.7 mM), or

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high glucose with mCA inhibitors ethoxzolamide (ETZ) or topiramate (TOP) for 5 days. The GSH levels were measured by HPLC. High glucose-induced significant reduction in GSH levels (**A** and **B**) was restored upon treatment with ETZ (**A**) and TOP (**B**) at both (10 and 100  $\mu$ M) concentrations of the inhibitors used. The values are expressed as mean  $\pm$  SEM ( $n=3$ ). \* $p<0.01$ , \*\* $p<0.001$ , \*\*\* $p<0.0001$ . The graph is representative of three independent experiments.

**Figure 5.** Effect of pharmacological inhibition of mCA on high glucose-induced lipid peroxidation in IPC. After exposure to normal glucose (NG, 5.7 mM), high glucose (HG, 40.7 mM), or high glucose with mCA inhibitors ethoxzolamide (ETZ) or topiramate (TOP) for 5 days. The HNE levels in IPC were measured by immunoblotting. High glucose-induced significant increase in HNE (**A** and **C**) was almost completely reversed upon treatment with ETZ (**A**) and TOP (**C**) at both concentrations (10 and 100  $\mu$ M) of the inhibitors used. **B** and **D** are the representative immunoblots of protein-bound HNE. The values are expressed as mean  $\pm$  SEM ( $n=3$ ). \* $p<0.01$ , \*\* $p<0.001$ , \*\*\* $p<0.0001$ . The graph is representative of three independent experiments.

**Figure 6.** Effect of pharmacological inhibition of mCA on high glucose-induced apoptosis of IPC. The cells plated on chamber slides were treated with normal glucose (NG, 5.7 mM), high glucose (HG, 40.7 mM), or high glucose with 10 and 100  $\mu$ M of either ethoxzolamide (ETZ) or topiramate (TOP) for 5 days and stained for TUNEL. A significant increase in TUNEL-positive IPC (**A** and **B**) was reversed by ETZ (**A**) and TOP (**B**) treatment at both concentrations. The nuclei were counterstained with Hoechst. The TUNEL positive cells are presented as a

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percentage of total cell numbers. The values are expressed as mean  $\pm$  SEM ( $n=3$ ). \* $p<0.01$ , \*\* $p<0.001$ , \*\*\* $p<0.0001$ .



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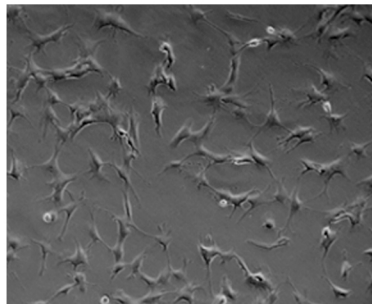
**Table 1.** Effect of lipopolysaccharide (LPS) on the release of cytokines and chemokines by IPC.

<b>Cytokine/Chemokine</b>	<b>Control (pg/ml)</b>	<b>Treated (pg/ml)</b>
IL-1a	0.26 ± 0.05	0.42 ± 0.03*
IL-1b	N.D.	5.54 ± 0.56
IL-4	0.55 ± 0.06	1.42 ± 0.02**
IL-5	1.66 ± 0.09	2.58 ± 0.11**
IL-6	2.34 ± 0.49	522.55 ± 50.06**
IL-12 (p40)	N.D.	0.73 ± 0.29
IL-12 (p70)	N.D.	5.24 ± 0.70
IL-13	N.D.	20.08 ± 2.30
IL-17	1.47 ± 0.07	2.44 ± 0.14**
GM-CSF	1.12 ± 0.06	22.65 ± 1.64**
KC	306.56 ± 22.91	37631.75 ± 16842.57
MCP-1	3544.39 ± 209.10	11930.87 ± 386.03**
MIP-1a	1.38 ± 0.14	15.50 ± 1.32**
MIP-1b	N.D.	3.04 ± 0.25
RANTES	147.46 ± 13.88	844.01 ± 122.29**
TNF-α	N.D.	5.85 ± 0.09

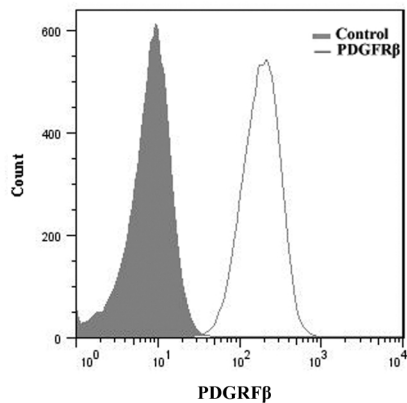
IPC were exposed to LPS (1 µg/ml) for 24 h. IL; interleukin, GM-CSF; granulocyte-macrophage colony-stimulating factor, KC; keratinocyte-derived chemokine, MCP-1; monocyte-chemo-attractant protein-1, MIP; macrophage inflammatory protein, RANTES; regulated upon activation, normal T-cell expressed, and secreted, TNF-α; tumor necrose factor. Values are mean ± SEM, n = 5. N.D.; not detected. \*p<0.05, \*\*p<0.01 for difference from controls.

**Figure 1**

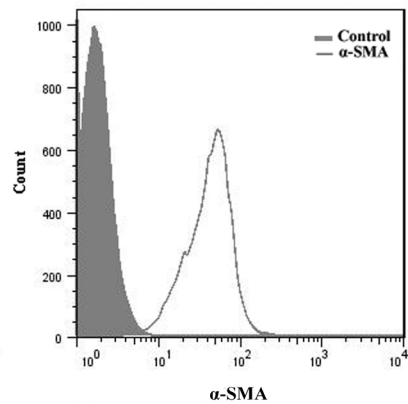
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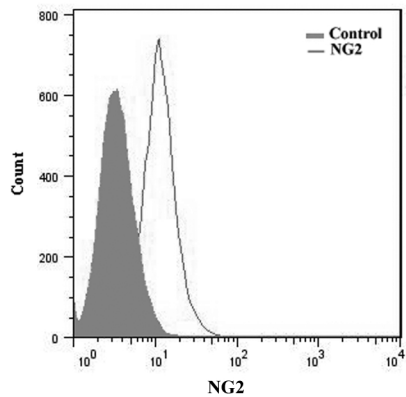
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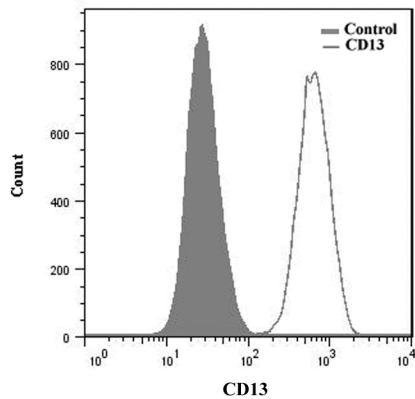
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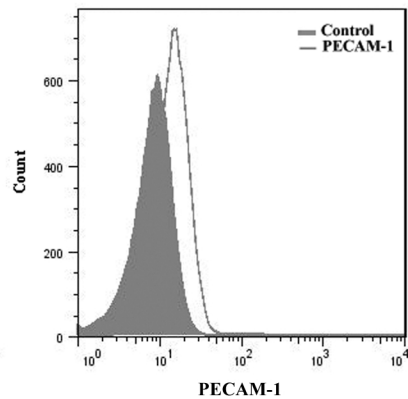
**D**



**E**

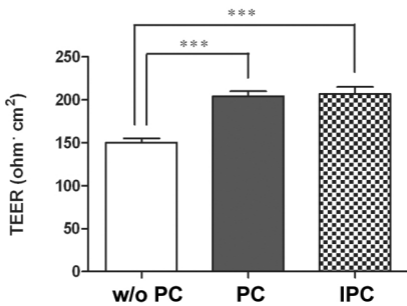


**F**

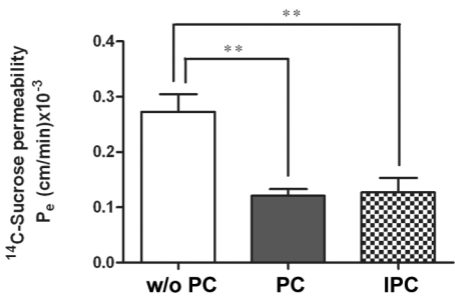


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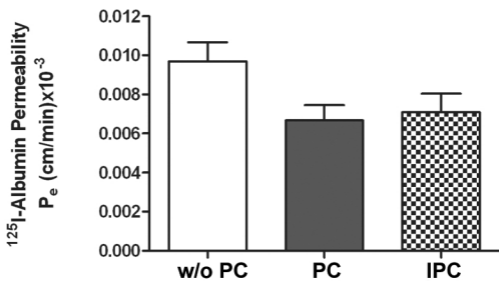
**A**



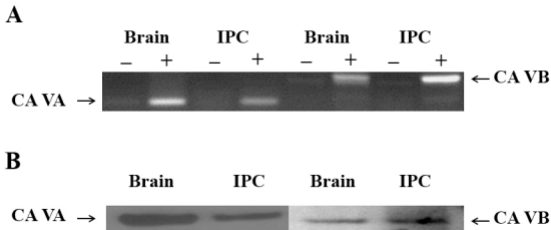
**B**



**C**

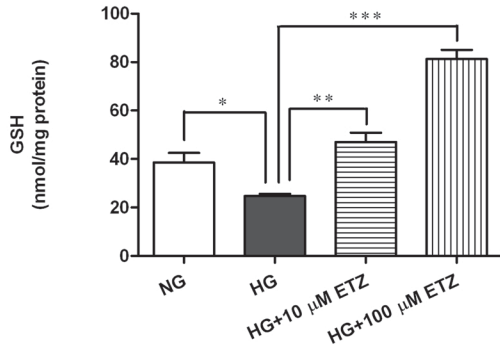


# Figure 3

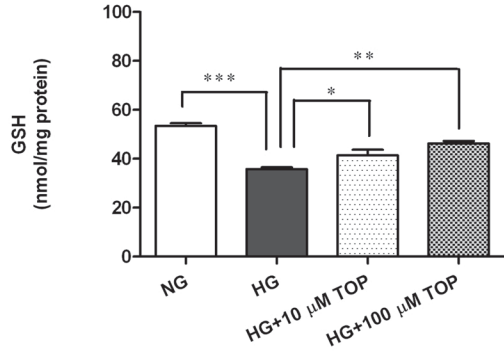


**Figure 4**

**A**



**B**



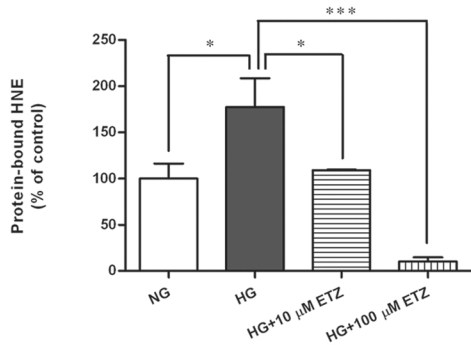
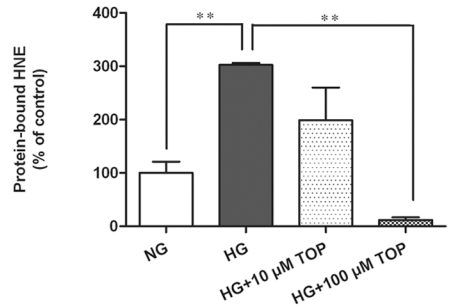
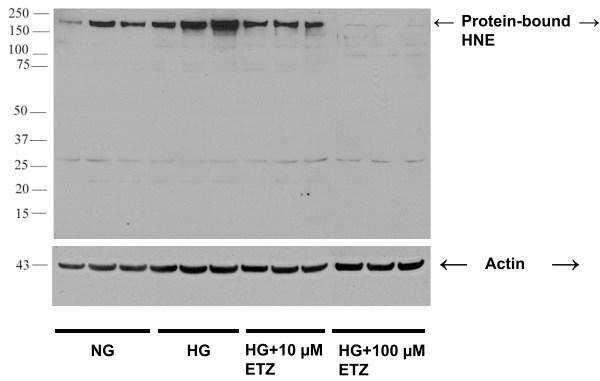
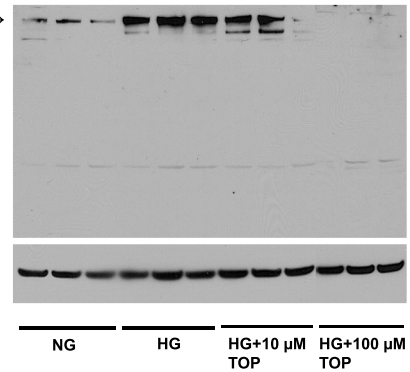
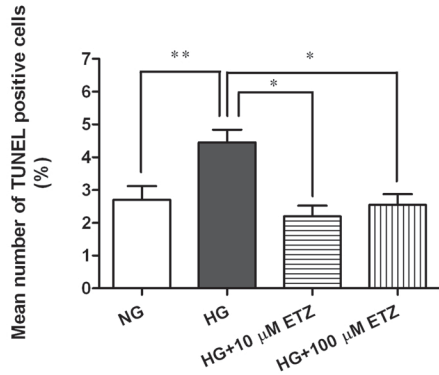
**Figure 5****A****C****B****D**

Figure 6

A



B

