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Regulation of high glucose-induced apoptosis of brain pericytes by mitochondrial CA VA: A specific target for prevention of diabetic cerebrovascular pathology

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ABSTRACT

Events responsible for cerebrovascular disease in diabetes are not fully understood. Pericyte loss is an early event that leads to endothelial cell death, microaneurysms, and cognitive impairment. A biochemical mechanism underlying pericyte loss is rapid respiration (oxidative metabolism of glucose). This escalation in respiration results from free influx of glucose into insulin-insensitive tissues in the face of high glucose levels in the blood. Rapid respiration generates superoxide, the precursor to all reactive oxygen species (ROS), and results in pericyte death. Respiration is regulated by carbonic anhydrases (CAs) VA and VB, the two isozymes expressed in mitochondria, and their pharmacologic inhibition with topiramate reduces respiration, ROS, and pericyte death.
Topiramate inhibits both isozymes; therefore, in the earlier studies, their individual roles were not discerned. In a recent genetic study, we showed that mitochondrial CA VA plays a significant role in regulation of reactive oxygen species and pericyte death. The role of CA VB was not addressed.
In this report, genetic knockdown and overexpression studies confirm that mitochondrial CA VA regulates respiration in pericytes, whereas mitochondrial CA VB does not contribute significantly. Identification of mitochondrial CA VA as a sole regulator of respiration provides a specific target to develop new drugs with fewer side effects that may be better tolerated and can protect the brain from diabetic injury. Since similar events occur in the capillary beds of other insulin-insensitive tissues such as the eye and kidney, these drugs may also slow the onset and progression of diabetic disease in these tissues.

1. Introduction

Hyperglycemia-induced diabetic complications of the brain include pericyte loss [1,2], microaneurysms [3,4], and cognitive impairment [5–7]. Pericytes in close proximity to endothelial cells are vital to the integrity and function of cerebral microvasculature. High blood glucose levels lead to intracellular hyperglycemia [8], rapid respiration (oxidative metabolism of glucose) [9], and pathologic levels of superoxide in pericytes from insulin-insensitive tissues such as brain [8]. Superoxide is the precursor to all reactive oxygen species (ROS) [10] and causes pericyte apoptosis [11,12]. Pericyte death leads to endothelial cell death, microaneurysms, and cognitive decline [5–7].

Carbonic anhydrases (CAs) IX and XII were recently implicated in ischemia-induced cerebrovascular pathology [13]. We previously showed that genetic knockout [14] and pharmacological inhibition [1,2] of mitochondrial carbonic anhydrases (CA), VA and VB, the two isozymes expressed in mitochondria [14], reduce respiration [9], ROS [9,12], and pericyte apoptosis [11,12]. Topiramate, the pharmacologic inhibitor of mitochondrial CAs [15,16], used in earlier studies, is in clinical use for other diseases [17–19] and can be used for this new indication in translational research. Since topiramate is not well tolerated by many patients, it is imperative to develop newer drugs with fewer side effects. One way of accomplishing this goal is to identify specific targets for drug development. Topiramate inhibits both mitochondrial CAs; therefore, the individual roles of the two isozymes were not discerned. More recently, we published data collected using genetic techniques that identified mitochondrial CA VA [11] as a potential target to treat diabetic brain injury.

Abbreviations: BPC, brain pericytes; CAs, carbonic anhydrases; CA VA-BPC, mitochondrial CA VA overexpressing brain pericytes; CA VB-BPC, mitochondrial CA VB overexpressing brain pericytes; CA VA KD-BPC, mitochondrial CA VA knockdown brain pericyte cell line; CA VB KD-BPC, mitochondrial CA VB knockdown brain pericyte cell line; HG, high glucose; NG, normal glucose; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; siRNA, small interfering RNA; shRNA, small hairpin RNA; TUNEL, terminal deoxynucleotidyl transferase-dUTP nick end labeling.

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In this report, we present novel data confirming a major role of mitochondrial CA VA in the regulation of ROS and pericyte apoptosis. The genetic knockdown of mitochondrial CA VA significantly reduced high glucose-induced ROS and apoptosis in pericytes, the two parameters of diabetic injury. The other isozyme, mitochondrial CA VB, seems inconsequential since its overexpression and knockdown had no effect on either parameter of diabetic injury.

The identification of mitochondrial CA VA as a major contributor to brain diabetic disease may lead to novel strategies to prevent diabetic complications in the brain and possibly in other insulin-insensitive tissues such as eye and kidney.

2. Materials and methods

2.1. Cell culture

Conditionally immortalized mouse brain pericyte (BPC) cultures were established as previously described [12]. The pericytes were grown in 60 mm petri dishes in growth media (DMEM, D6046, Sigma–Aldrich, Saint Louis, MO) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin/streptomycin (Sigma–Aldrich), and murine recombinant interferon-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. Knockdown of mitochondrial CA VA and CA VB in the brain pericytes

Small interfering RNAs (siRNA) for the mitochondrial CA VA and CA VB genes (OriGene Technologies, Rockville, MD) were used to transiently transfect pericytes, according to the manufacturer's instructions. The siRNAs were used at 10 nM with siRNA transfection reagent (siTran, OriGene Technologies Rockville, MD). The siRNA that resulted in more than 50% decline in expression of the respective genes were given to Blue Heron Biotech (Bothell, WA) for subcloning into pSicoRpuro vector [20] to generate shRNA plasmids. The pSicoRpuro vector lacks a simian virus (SV40) origin. The commercially available shRNAs are subcloned into vectors containing an SV40 origin and are not recommended for transfection of immortalized cells containing large T antigen, as described in our earlier publication [11] and later in the Materials and methods section(see Section 2.3.1 Plasmid preparation). These shRNA plasmids were used to produce stable pericyte cell lines as follows: the day before transfection, the cells were plated on 6-well plates at 50% confluence in growth medium. The transfections were as described earlier for siRNA. Cells were harvested 28 h after the transfection. Individual clones were selected by serial dilutions with 4 μg/ml puromycin (Sigma-Aldrich, Saint Louis, MO). Media was changed every 2–3 days. Expression of mitochondrial CA VA and CA VB mRNAs were determined by quantitative real-time PCR (qRT-PCR) as described previously [11]. Briefly, total RNA was isolated from cultured pericytes using the RNeasy kit (QIAGEN, Valencia, CA). For mRNA quantification, complementary DNAs (cDNAs) were synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative RT-PCR analysis was performed in triplicate dishes of cells using Power SybrGreen reagent (Applied Biosystems, Carlsbad, CA) in a LightCycler-480 (Roche, Basel, Switzerland). The levels of mRNA were normalized to 36B4 as a housekeeping gene and calculated using the comparative $\Delta\Delta C_t$ method.

The sequences of the primer used were: mitochondrial CA VA forward primer 5′-GTC TCC CAT CAA CAT CCA-3′ and reverse primer 5′-GGA AGA AGT AGC CAG TGT-3′;

mitochondrial CA VB forward primer 5′-AAT GGC TTG GCT GTG ATA G-3′ and reverse primer 5′-GTG TCC TTG TGC TTA ATT GAT G-3′; and 36B4 forward primer 5′-CAC TGG TCT AGG ACC CGA GAA G-3′ and reverse primer 5′-GGT GCC TCT GGA GAT TTT CG-3′.

The levels of mitochondrial CA VA and CA VB proteins were assessed by immunoblot by standard procedures as previously described [1]. Briefly, cultured cells were homogenized in lysis buffer [25 mM Tris (pH 7.5), 0.15 M NaCl, 1 mM PMSF], sonicated, and cleared by centrifugation. Protein concentration in the final supernatants was determined by BCA Protein Assay (Pierce, Rockford, IL). The proteins (25 μg) were separated on NuPAGE Novex 4–12% Bis-Tris reducing gels (ThermoFisher Scientific, Waltham, MA) and then transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies overnight at 4 °C followed by blocking with 5% non-fat milk. Rabbit anti mouse CA VA carboxyl terminal (C-tail) antibodies were the same as described [21]. Affinity-purification was accomplished with a C-tail CA VA peptide-Affigel 10 column [14]. Rabbit anti mouse CA VB C-tail antibodies and affinity-pure anti-CA VB C-tail–specific IgG were as described [22]. The titer and specificity of these affinity purified antibodies was ascertained by western blotting [14]. Anti-gamma-tubulin antibodies were from Cell Signaling Technology (MA1-850, Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies, goat anti-rabbit and goat anti-mouse were for mitochondrial CAs and gamma-tubulin, respectively. Band visualization was by chemiluminescent substrate (Pierce, Rockford, IL). The quantitative estimation was obtained using ImageJ software.

Once established, mitochondrial CA VA knockdown brain pericytes (CA VA KD-BPC) and CA VB knockdown brain pericytes (CA VB KD-BPC) were maintained in puromycin containing media.

2.3. Overexpression of mitochondrial CA VB in the brain pericytes

As we previously published for mitochondrial CA VA [11], we developed a mitochondrial CA VB overexpressing cell line (CA VB-BPC) to show the effect of overexpression of mitochondrial CA VB on pericyte ROS and apoptosis.

2.3.1. Plasmid preparation

The construct for mitochondrial CA VB was designed as we described previously for mitochondrial CA VA [11]. Briefly, a 1201 base pair coding sequence of mitochondrial CA VB cDNA was directionally cloned into the pDream2.1 mammalian expression vector (GenScript, Piscataway, NJ) at Bam HI/Hind III sites. The pDream vector was selected because it lacks an SV40 origin. Immortalized pericytes express large T antigen and a plasmid with an SV40 origin is not suitable for stable transfection of such cells. DNA rearrangements, such as deletions and duplications found within and near the integrated SV40 DNA in cells overexpressing large T antigen, change the cells morphologically and physiologically [23].

2.3.2. Transfection

The BPC were transfected with jetPRIME transfection reagent (Polyplus-transfection, Illkirch, France) according to supplier's protocol. At 55 h post transfection, cells were incubated in growth media containing 4 μg/ml puromycin (Sigma–Aldrich) to select for mitochondrial CA VB overexpressing cells. Media was changed every 2–3 days. Expression of mitochondrial CA VB mRNA was determined by qRT-PCR and the level of mitochondrial CA VB protein was assessed by immunoblot as described previously [1] and in Section 2.2. Once established, mitochondrial CA VB overexpressing cells (CA VB-BPC) were maintained in puromycin containing media.

2.4. Reactive oxygen species (ROS) analysis

Intracellular ROS were measured with a ROS activity assay kit (Cell Meter™ Fluorimetric Intracellular Total ROS activity assay kit, cat# 22900, AAT Bioquest, Thermo Fisher Scientific, Inc., Waltham, MA) as described previously [9]. Briefly, the cells were seeded in Costar black wall/clear bottom 96-well plates at a density of 1×10^5 cells per well in 100 μl of growth media containing normal glucose (5.7 mM), and were allowed to adhere overnight in a 5% CO_2, 37 °C incubator. The following morning, 100 μl of glucose stock solution (1 M) was added to