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Perspective

Carbenoxolone prevents chemical eye ischemia-reperfusion-induced cell death via 11β-hydroxysteroid dehydrogenase type 1 inhibition

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ABSTRACT

Glaucoma is one of the leading causes of preventable blindness diseases, affecting more than 2 million people in the United States. Recently, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) inhibitors were found to exert preventive effects against glaucoma. Therefore, we investigated whether carbenoxolone (CBX), an 11β-HSD1 inhibitor, prevents chemical ischemia-reperfusion-induced cell death in human trabecular meshwork (HTM) cells. The present study demonstrated that CBX inhibited cell death caused by iodoacetic acid (IAA)-induced ischemia-reperfusion, and its effect was associated with the inhibition of 11β-HSD1 expression and activity. Furthermore, CBX reversed the IAA-induced structural damage on filamentous actin in HTM cells. In IAA-treated cells, the levels of 11β-HSD1 and the apoptosis-related factors Bax and FASL were increased throughout the reperfusion period, and CBX was able to attenuate the expression of 11β-HSD1 and the apoptosis-related factors. CBX also effectively suppressed IAA-induced intracellular ROS formation and cytochrome c release, which are involved in the mitochondrial apoptosis pathway. In addition, IAA-induced chemical ischemia-reperfusion stimulated TNF-α expression and NF-κB p65 phosphorylation, and these effects were attenuated by CBX. 11β-HSD1 RNAi also suppressed IAA-induced cell apoptosis via reduction of oxidative stress and inhibition of the pro-inflammatory pathway. Taken together, the present study demonstrated that the inhibition of 11β-HSD1 protected the TM against chemical ischemia-reperfusion injury, suggesting that the use of 11β-HSD1 inhibitors could be a useful strategy for glaucoma therapy.

1. Introduction

Glaucoma is a chronic optic neurodegenerative disorder with common characteristics such as retinal ganglion (RG) cell loss, optic nerve degeneration, and defective visual field [1]. One of the major risk factors that cause glaucomatous development and progression is elevated intraocular pressure (IOP) [2,3]. The exact mechanism of elevated IOP is not well understood; however, IOP increase could result in diverse stresses that are transmitted to the optic nerve head of the eyes and damage the optic nerves and axons of RG cells [4]. Elevated IOP results from decreased aqueous outflow through the trabecular meshwork (TM) and Schlemm's canal, and it is associated with several morphologic changes and the loss of TM cells [2,5]. Because of its regulatory role in IOP, the TM is described as a key tissue in glaucoma pathogenesis [6]. The malfunction of the TM and cell loss can lead to damage of RG cells through increased oxidative stress, mitochondrial damage, increased nitric oxide, glial cell pathology, or defective axonal transport [7–10].

11β-Hydroxysteroid dehydrogenase (11β-HSD) regulates the intracellular level of active glucocorticoid (cortisol) and activation of glucocorticoid/mineralocorticoid receptors [11,12]. 11β-HSD is divided into two types, 11β-HSD1 and 11β-HSD2. 11β-HSD1 and 11β-HSD2 are generated from two different genes and have distinct physiological roles and tissue distributions. 11β-HSD2 is the first isoform to be identified and is a NAD^+ dependent protein that converts active cortisol to cortisone. It protects mineralocorticoid

Abbreviations: 11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; CBX, carbenoxolone; IAA, iodoacetic acid; HTM, human trabecular meshwork; TM, trabecular meshwork; RG, retinal ganglion; IOP, intraocular pressure; POAG, primary open-angled glaucoma; ROS, reactive oxygen species; LDH, lactate dehydrogenase; F-actin, filamentous actin; TNF-α, Tumor necrosis factor-alpha; NF-κB, nuclear factor-kappa B; FASL, fas ligand; HO-1, heme oxygenase-1; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; RNAi, RNA interference.

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target tissues, such as the colon and kidney, from the active form of cortisol. On the other hand, 11β-HSD1 is predominantly expressed and has an important role in key metabolic tissues, such as the liver, muscle, and adipose tissues, and preferentially converts cortisone to cortisol. At a proper dose, glucocorticoids are widely used for anti-inflammatory therapies in human clinics [13]. However, recent studies have highlighted the stimulatory effects of glucocorticoids on inflammatory responses [14,15]. The exact mechanisms of 11β-HSD1 in inflammation have been of interest because of the potential role of 11β-HSD1 in a variety of inflammatory responses. In line with this, patients with primary open-angled glaucoma (POAG) or ocular hypertension have an increased cortisol level in the aqueous humor, and the inhibition of 11β-HSD1 through carbenoxolone (CBX) treatment by oral administration was reported to result in a significant decrease in IOP [16,17]. However, the precise role of 11β-HSD1 in glaucoma has not been elucidated.

The biological events involved in elevated IOP have been proposed by various studies. The most relevant findings are ocular hypoperfusion, ocular hypertension, and ischemia [18,19]. In particular, ischemia-reperfusion injury can lead to severe damages of RG and TM cells through apoptosis and necrosis. The major factors that contribute to cell death during ischemia-reperfusion include the generation of excessive reactive oxygen species (ROS) and low-grade chronic inflammation [20]. Other studies also suggested that cells are damaged by apoptosis and necrosis after ischemia-reperfusion injury, and the disruption of the blood supply causes the process of reperfusion itself, leading to metabolic confusion by generating free radicals and inflammatory cytokines [18,21].

Over the past few years, many studies have strongly suggested that chronic inflammation is closely related to glaucomatous disease [22–24]. Zhou and colleagues [22] reported that inflammatory cytokines are involved in glaucomatous development in DBA/2J mice; specifically, IL-18 expression was dramatically increased in the iris/ciliary body and aqueous humor of DBA/2J mice with age, and they suggested that the elevation of inflammatory cytokines resulted from the activation of NF-κB and phosphorylation of MAPK-related inflammatory pathways. At the same time, several studies revealed that the expression levels of inflammatory markers and NF-κB activation are markedly increased in the glaucomatous TM, suggesting a correlation between glaucoma and the inflammatory response [24,25].

In this study, we aimed to investigate the regulation, expression, and role of 11β-HSD1 in chemical ischemia-reperfusion-induced human TM (HTM) cell death. In addition, we proposed new therapeutic approaches with the 11β-HSD1 inhibitor, CBX, for the treatment of ocular hypertension-induced glaucoma. The results demonstrated the protective effect of CBX against oxidative and inflammatory stress-mediated cellular apoptosis and the role of 11β-HSD1 during chemical ischemia-reperfusion injury.

2. Materials and methods

2.1. Materials

CBX was purchased from Amfinecom Inc. (Petersburg, VA, USA). Iodoacetic acid (IAA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Tokyo, Japan). LDH assay kits were obtained from Promega Co. (Madison, WI, USA). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), calcein-AM, ethidium homodimer-1 (EthD-1), and phalloidin were obtained from Molecular Probes (Eugene, OR, USA). Stealth™ Select RNAi oligonucleotides, Stealth™ RNAi Negative Control Duplex, and Lipofectamine RNAiMAX were acquired from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

Primary HTM cells (Catalog No. 6590) and Trabecular Meshwork Cell Medium (TMCM, Catalog No. 6591) were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). HTM cells were cultured in TMCM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Gaithersburg, MD, USA). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 until confluency was achieved. The HTM cells at passage 6–8 were used for further experiments.

2.3. Chemical-induced ischemia and drug application

IAA, an irreversible inhibitor of glycolysis, was used to induce chemical ischemia as previously described [26]. Cells were seeded in culture plates at a density of 6.25×10^4 cells/cm^2 and incubated with TMCM supplemented with either 1% FBS or in the presence of IAA for 1 h to induce chemical ischemia. Then, the cells were reperfused in TMCM with 1% FBS and incubated up to 24 h. To investigate the protective effect of CBX, cells were co-treated with both ischemic and reperfusion medium.

2.4. Cell viability assays

Cell viability was determined by CCK-8, LDH leakage assay and live/dead cell staining assay. Cells were seeded in 96-well tissue culture plates, and chemical ischemia-reperfusion was induced. After reperfusion, CCK-8 reagent was added at 10 μl per well and incubated for 2–4 h. Optical density was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). For LDH leakage assay, 96-well culture plates were centrifuged at $1500 \times g$ for 5 min, and the supernatants were used to examine LDH leakage as described by the manufacturer.

Calcein-AM and EthD-1 were used for live/dead cell staining. After 1 h of ischemia and 24 h of reperfusion or ischemic induction alone, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 4% formaldehyde. Then, working solution (2 μM calcein-AM and 4 μM EthD-1) was added, and the cells were incubated for 30 min at 37 °C. Images were acquired with a fluorescence microscope (Nikon, Tokyo).

2.5. In vitro *11β-HSD1 activity assay*

For 11β-HSD1 reductase activity assay, homogeneous time-resolved fluorescence (HTRF) cortisol assay was performed (Cisbio Inc., Bedford, MA, USA). HTM cells were seeded in 96-well plates and incubated with medium containing 200 nM cortisone for 24 h in the presence or absence of CBX. After 24 h of reaction, 10 μl of the reaction mixture was removed and subjected to HTRF cortisol assay according to the manufacturer's instructions (Nihon Schering, Tokyo, Japan). The effect of CBX on *in vitro* 11β-HSD1 activity was determined from concentration-dependent inhibition curves as described previously [27]. The negative control group only consisted of cortisone medium without cells, and the positive control group consisted of cortisone-treated HTM cells. The IC_{50} values of CBX were determined from concentration-dependent inhibition curves generated from GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

2.6. Intracellular filamentous actin staining

Intracellular filamentous actin (F-actin) was stained using fluorescent phallotoxin (Invitrogen, Carlsbad, CA, USA) after ischemia-reperfusion. HTM cells were treated with 35 μM IAA for 1 h, and the medium was replaced with reperfusion medium for 24 h. After reperfusion, cells were fixed with 4% formaldehyde