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TSPO activation modulates the effects of high pressure in a rat *ex vivo* glaucoma model

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ABSTRACT

We previously reported that elevated pressure induces axonal swelling and facilitates the synthesis of the neurosteroid, allopregnanolone (AlloP), in the *ex vivo* rat retina. Exogenously applied AlloP attenuates the axonal swelling, suggesting that the neurosteroid plays a neuroprotective role against glaucomatous pressure-induced injuries, although mechanisms underlying neurosteroidogenesis have not been clarified. The aim of this study was to determine whether AlloP synthesis involves activation of translocator protein 18 kD (TSPO) and whether TSPO modulates pressure-induced retinal injury. *Ex vivo* rat retinas were exposed to various pressures (10, 35, or 75 mmHg) for 24 h. Expression of TSPO, 5α-reductase (5aRD), and AlloP was examined by quantitative real-time RT-PCR, ELISA, immunohistochemistry, and LC-MS/MS. We also examined the effects of TSPO ligands on AlloP synthesis and retinal damage. In this acute model, quantitative real-time RT-PCR and ELISA analyses revealed that elevated pressure facilitated TSPO expression. Similarly, these methods also detected enhanced 5aRD (mostly type II), which was observed in retinal ganglion cells (RGC) and the inner nuclear layer (INL). Atriol, a TSPO antagonist, suppressed pressure mediated AlloP synthesis and induced more severe histological changes in the inner retina when combined with elevated pressure. PK11195, a TSPO ligand that facilitates AlloP synthesis by itself, remarkably diminished pressure-mediated retinal degeneration. These results suggest that AlloP synthesis is induced by sequential activation of TSPO and 5aRD in an *ex vivo* glaucoma model, and that TSPO agonists may serve as potential therapeutic agents for the prevention of pressure-induced retinal damage.

1. Introduction

Glaucoma is a leading cause of irreversible blindness (Pascolini and Mariotti, 2012; Tham et al., 2014). Among types of glaucoma, acute angle closure attacks (AAC) are an ophthalmic emergency and can lead to blindness (Aung et al., 2001). AAC involves a sudden rise in intraocular pressure (IOP) that can reach 80 mmHg (Ritch, 2000). If treatment is delayed, sustained IOP elevation damages retinal ganglion cells (RGC), and leads to severe visual disturbances (Lowe, 1973). However, the pathogenesis underlying RGC damage in AAC remains unclear, although glutamate excitotoxicity likely contributes to RGC death (Diekmann and Fischer, 2013; Louzada-Junior et al., 1992; Neal et al., 1994; Harada et al., 2007; Seki and Lipton, 2008). Glutamate is the most prevalent neurotransmitter in the retina (Thoreson and Witkovsky, 1999; Massey and Miller, 1987, 1990). When glutamate is in excess, it can become toxic to retinal neurons by overstimulation of glutamate receptors (Lucas and Newhouse, 1957; Olney, 1982).

Recently, we developed a new experimental model of AAC using an *ex vivo* rat retinal preparation to examine the effects of elevated hydrostatic pressure on retinal morphology and glutamate metabolism (Ishikawa et al., 2010, 2011). In this model, high pressure (75 mm Hg) induces axonal swelling in the nerve fiber layer (Ishikawa et al., 2010), accompanied by impaired glial glutamate

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transporters and metabolism (Ishikawa et al., 2011).

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter used by horizontal and amacrine cells in the lateral retinal pathway to modulate outer and inner synaptic layers (Kalloniatis and Tomisich, 1999; Yang, 2004). Although data indicate a role for glutamate in glaucoma (Naskar et al., 2000; Martin et al., 2002; Moreno et al., 2005; Nucci et al., 2005), the role of GABA has been less thoroughly investigated (Bailey et al., 2014), and prior work indicates that the balance between glutamate and GABA is important for maintaining retinal function (Kishida and Naka, 1968). However, there are only a few reports describing significant dysfunction of the GABAergic system in glaucomatous retinas (Bailey et al., 2014; Kishida and Naka, 1968; Moreno et al., 2008).

Allopregnanolone (AlloP) is a neurosteroid that can be locally synthesized in the central nervous system in response to stressful events. In the retina, AlloP is synthesized in response to pressure elevation (Ishikawa et al., 2014), and exerts neuroprotective effects via $GABA_A$ receptors in the *ex vivo* glaucoma model (Ishikawa et al., 2014). How acute stress promotes neurosteroid production is not certain.

In the brain, neurosteroids are generated from cholesterol by a series of steps that include shuttling of cholesterol to the inner mitochondrial membrane by translocator protein 18 kDa (TSPO) and conversion to pregnenolone by CYP11A1, a P450 side-chain cleavage enzyme (Weir et al., 2004; Belelli and Lambert, 2005; Rone et al., 2009; Gunn et al., 2011). Pregnenolone exits the mitochondria and is converted to neurosteroids including AlloP. A major pathway involves the conversion of pregnenolone to AlloP by the sequential actions of 3β-hydroxysteroid dehydrogenase, 5α-reductase (5aRD) and 3α-hydroxysteroid dehydrogenase (Fig. 1a) (Belelli and Lambert, 2005; Rone et al., 2009). The translocation of cholesterol to the inner mitochondrial membrane by TSPO (Rupprecht et al., 2010) and the catalytic reaction by 5aRD (Dong et al., 2001; Agis-Balboa et al., 2007) in the endoplasmic reticulum membrane are considered rate limiting steps. Because elevated pressure enhances AlloP synthesis, we hypothesized that TSPO induction followed by 5aRD is required for AlloP synthesis when retinas are exposed to high pressure. In the present study, we tested this hypothesis by determining effects of pressure elevation on the expression of TSPO and 5aRD in an *ex vivo* glaucoma model. Moreover, we demonstrate that activation of TSPO is a key step in regulating retinal toxicity and that a TSPO antagonist promotes retinal excitotoxicity, while TSPO activation exerts neuroprotective effects via AlloP production.

2. Material and methods

Protocols for animal use were approved by the Akita University Animal Studies Committee in accordance with the guidelines of the Policies on the Use of Animals and Humans in Neuroscience Research.

2.1. Rat ex vivo eyecup preparation

Rat *ex vivo* eyecups were prepared from 28 to 32 day old male Sprague-Dawley rats (Charles River Laboratories International Inc., Wilmington, MA) as previously published (Ishikawa et al., 2010, 2011). The anterior half of the enucleated eyes was carefully removed to make eyecup preparations. Eyecups were placed at the bottom of a 100 ml glass beaker filled with aCSF (artificial cerebrospinal fluid) containing (in mM): 124 NaCl, 5 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 22 $NaHCO_3$, and 10 glucose, and incubated at 30 °C for 24 h using a closed pressure-loading system (Fig. 1b). pH was maintained at 7.35 to 7.40. In the closed-pressure system, a glass beaker with the eyecup was placed at the bottom of an acrylic pressure chamber (2000 ml volume). A 95% O_2–5% CO_2 gas mixture was delivered through disposable plastic tubing with an infusion valve and a control dial on the lid of the pressure chamber and an air filter (Cat#SLGP033RS, Merk Millipore, Billerica, MA). The plastic tubing delivering the gas terminated 1 cm above the bottom of the beaker.

Acutely prepared eyecups were incubated in gassed aCSF for at least 1 h at 30 °C before pressure loading. In some experiments, (3β,17β)-androst-5ene-3,17,19-triol (atriol) (1 μM), dizocilpine (MK801) (1 μM), AlloP (1 μM), isoquinoline carboxamide (PK11195) (50 μM) and dutasteride (1 μM) were dissolved in aCSF at the time of experiment and administered by bath perfusion. Eyecup preparations were treated with these drugs for 1 h at 30 °C before pressure loading. For pressure loading, the 95% O_2–5% CO_2 gas mixture was infused until the pressure reading given by a manometer reached the appropriate level. The pressure was then locked by adjusting the control dial of the effusion valve, and monitored continuously for 24 h at 30 °C. After maintaining the chamber at the set pressure (10, 35, 75 mmHg) for the indicated time, the pressure inside the chamber was carefully decreased by opening the effusion valve.

2.2. Quantitative real-time RT–PCR

We quantified *tspo* and *srd5a* mRNA expression in pressure-loaded eyecups incubated at 10, 35, and 75 mmHg for 24 h. At the end of each experiment, the retina of the empty eyecup was sampled, and immersed in RNAlater solution (Qiagen, Hilden, Germany), and frozen at −80 °C. Total RNA was extracted by using RNeasy kit (Qiagen) from the retinal samples and used for cDNA synthesis. Aliquots (1 μg) of total RNA were reverse transcribed into first-strand cDNA by using a PrimeScrip® RT reagent kit (Takara Bio Inc. Shiga, Japan) and a thermal cycler (Takara PCR Thermal Cycler MP, Takara). Real-time reverse transcription (RT)–PCR reaction was carried out with a Thermal Cycler Dice® Real Time System (Takara). According to the manufacturer's instructions, the RT-PCR reaction was conducted in 25 μl of reaction buffer containing 12.5 μl SYBR® Premix Ex Taq II (Takara Bio Inc), 1 μl of 10 μM forward and reverse primers, 2 μl cDNA and 9.5 μl water. The RNA expression levels were normalized to the level of *gapdh* expression. Table 1 summarizes the primers used in the present study. The primers were designed using the Perfect Real Time® Support System (Takara). Quantitative real-time RT–PCR curves were analyzed by the crossing-point standard curve method.

In the present study, six independent experiments were performed for each condition. All PCR reactions were repeated in duplicate, and the average values were used for statistical analysis. The RNA expression levels were normalized to *gapdh* expression. These data were then evaluated by Dunnett's multiple comparison test to determine whether the expression rates at 35 mmHg and 75 mmHg were significantly higher than control rates at 10 mmHg.

2.3. ELISA

Seven eyes were examined by ELISA in each condition. After pressure loading, the retinal samples were rapidly homogenized in PBS followed by centrifugation at 4 °C for 15 min at 3,000 g. The supernatants were used to measure the concentrations of TSPO, 5aRD type I (5aRD1), 5aRD type II (5aRD2), and 5aRD type III (5aRD3) using corresponding ELISA kits (TSPO ELISA kit, Cat#CSB-EL025168RA, Cusabio, Wuhan, China; 5α-reductase I ELISA Kit, Cat#MBS700746-48TEST, MyBioSourc Inc., San Diego, CA; 5α -reductase II ELISA Kit, Cat#SEM285RA, Uscn Life Science, Houston, TX; 5α-reductase III ELISA Kit, Cat#MBS9320507, MyBioSourc Inc.). According to the manufacturer's instructions, the absorbance was