Brimonidine tartrate effect on retinal spreading depression depends on Müller cells

O efeito do tartarato de brimonidina sobre a depressão alastrante retiniana depende das células de Müller

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ABSTRACT

Objective: Demonstrate the Brimonidine effect over Retinal Spreading Depression (SD). Brimonidine is an alpha-2–adrenergic receptor agonist, used in the management of glaucoma. Alpha2-agonists have been shown to be neuroprotective in various experimental models, however the molecular and cellular targets leading to these actions are still poorly defined. The SD of neuronal electric activity is a wave of cellular massive sustained depolarization that damages the nervous tissue. Local trauma, pressure, ischemic injuries and other chemical agents as high extracellular potassium concentration or glutamate, can trigger SD, leading to exaggerated focal electrical followed by an electrical silence.

Methods: Using chicken retina as model, we performed alpha2-receptor detection by Western Blotting and Immunohistochemistry. After that we obtained electrical signals of SD by microelectrodes on retina in the absence or presence of Brimonidine. For in vivo visualization we observed retina with optical coherence tomography on normal state, with SD passing, and with SD + Brimonidine. Results: Our data demonstrated that: (1) alpha2-adrenergic receptors are present in Müller cells, (2) the treatment with Brimonidine decreases the SD’s velocity as well as the voltage of SD waves and (3) OCT revealed that SD creates a hyper reflectance at inner plexiform layer, but on retinal treatment with brimonidine, SD was not visualized. Conclusion: Our study about brimonidine possible pathways of neuroprotection we observed it reduces SD (a neuronal damage wave), identified a new cellular target – the Müller cells, as well as, firstly demonstrated SD on OCT, showing that the inner plexiform layer is the main optically affected layer on SD.

Keywords: Retina/drug effects; Adrenergic alpha-2 receptor agonists/therapeutic use; Glaucoma

RESUMO

Objetivo: Demonstram o efeito do Tartarato de Brimonidina, um alfa2-agonista usado no manejo do glaucoma, sobre a depressão alastrante (DA) retiniana. Esses agonistas têm demonstrado ser neuroprotetores em vários modelos experimentais, contudo seu alvos celulares e moleculares continuam indefinidos. A DA da atividade elétrica neuronal é uma onda de despolarização celular massiva e sustentada que leva ao dano no tecido nervoso. Trauma local, pressão, isquemia e outros agentes químicos como o aumento do potássio extracelular e o glutamato podem disparar a DA, levando a uma atividade elétrica exagerada seguida de silêncio elétrico.

Métodos: Usando a retina de pinto como modelo, realizamos a detecção do alfa2-receptor por Western Blotting e ensaio Imunohistoquímico. Após isso, obtemos os sinais elétricos da DA através de microeletrôdos inseridos na retina durante sua passagem na presença ou ausência de Brimonidina. Para visualização do tecido utilizamos o tomógrafo de coerência óptica (OCT), analisando como é a retina no seu estado de repouso, durante a passagem da DA, e a DA + brimonidina. Resultados: Nossos dados demonstraram que: (1) os receptores alfa adrenérgicos presentes na retina são do subtipo-2A e estão localizados nas células de Müller; (2) o tratamento com Brimonidina diminui a velocidade e a voltagem da onda de DA; (3) A OCT demonstrou que a DA retiniana possui um sinal óptico de maior reflectância na camada plexiforme interna, fato não observado quando foi associada à Brimonidina. Conclusão: A Brimonidina foi capaz de reduzir a DA (uma onda de lesão neuronal) e identificamos um novo possível alvo celular – a célula de Müller e demonstramos pela primeira vez uma OCT da DA, visualizando a camada plexiforme interna como a mais afetada opticamente pelo fenômeno.

Descritores: Retina/efeito de drogas; Agonistas de receptores adrenérgicos alfa 2/uso terapêutico; Glaucoma

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INTRODUCTION

Glaucoma is an optic nerve disease generally characterized by elevated intraocular pressure (IOP). Besides the elevated IOP, glaucoma is best defined as a neurodegenerative disease characterized by the slow, progressive degeneration of retinal ganglion cells (RGCs), which is manifested initially as visual field loss and, ultimately, irreversible blindness if left untreated. Glaucoma is the second leading cause of blindness in the world, and the first cause of irreversible blindness. It is estimated that there are 60 million people worldwide affected by glaucoma and 8.4 million being bilaterally blind. Even in developed countries, half of glaucoma cases are undiagnosed(1,2).

Although the precise cause of RGCs death in glaucoma is unknown, several mechanisms have been proposed. These include: mechanical compression due to elevated IOP, neurotransmitter deprivation, excitotoxicity, ischemia, hypoxia and oxidative stress. Because conventional treatment to reduce IOP does not always prevent progression of glaucomatous neurodegeneration, recent research in glaucoma has been focused on the alternative treatment strategies as the neuroprotection(2).

The concept of ocular neuroprotection has been advanced to target the primary problem in glaucoma, which is the neuronal death, a common feature to all optic neuropathies. Neuroprotection is the name given to treatment directed for prevention of neuronal death. Treatment may be prophylactic or delivered as early as possible when an insult has taken place. By definition, neuroprotection in glaucoma is considered to be independent of pressure lowering.

Brimonidine is a drug used in the treatment of glaucoma throughout the world and is a modern alpha(2)-adrenergic receptor agonist available(3,4). Alpha(2)-adrenergic receptor agonists have been shown to be neuroprotective in various experimental models, but the molecular and cellular targets leading to these actions are still poorly defined(5,6).

Spreading depression (SD), is a phenomenon initially described in the central nervous system (CNS) by Leão(7) and it is a wave of sustained depolarization that spreads cell by cell causing a severe disruption of neuronal activity and causing a silent state with no action potential firing(8). SD results in a transient collapse of transmembrane ionic gradients and membrane potential(9,10). In addition, proinflammatory compounds are released, often accompanied by edema and extravasation of blood proteins(11,12). Repeated episodes of SD increases neuronal loss and has been implicated as a pathway of cellular injury on many of CNS pathologies, including trauma and cerebral vascular disease(10,11).

Experimental evidence has demonstrated that brimonidine is a potential neuroprotective agent, independent of IOP lowering, although the mechanism of the neuroprotective effect of brimonidine remains unknown(10).

The aim of this study was to investigate the cellular target of brimonidine and its effect over SD using the chicken retina as a model and the optical coherence tomography.

METHODS

Animals

Experiments were performed in compliance with Brazilian laws for the use of animals and approved by the commission of animal care of Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (UFRJ). Fertilized white Leghorn eggs were obtained from a local hatchery and kept in an appropriately incubator under 12 hours light and dark cycles until the day of use. Post-hatched animals were maintained in a free running condition with water and food ad libitum. Before tissue excision the animals were euthanized by decapitation. Brimonidine were obtained from Allergan (Irvine, CA).

Protein detection by Western blot

Retinas of post-hatched chickens were dissected out in a calcium magnesium free solution (CMF) and homogenized with a tissue grinder in a solution composed of 20mM Tris base, 10 mM MgCl₂, 600 µM CaCl₂, 500 µM EGTA, 1mM DTT, 1mM PSMF, 5 µg/ml aprotinin, 2 µg/ml leupeptin, and 0.05% Triton X-100. Protein concentration was determined with the Bradford method. Samples were treated as described elsewhere(14) (Western blots were developed with Immobilon Western Chemiluminescent HRP according to manufacturer’s instructions).

Immunohistochemistry

Immediately after enucleation, eyeballs were hemisected with a razor blade and the posterior half fixed by immersion in 4% paraformaldehyde (PA) in 0.16M phosphate buffer solution (PBS), pH 7.2, for 2h. The tissue was then rinsed with PB and then cryoprotected in increased gradient of sucrose (10%, 20% and 30%). After 24 hours, retinas were mounted in OCT embedding medium (Sakura Finetek, Torrance, CA), frozen and cryosectioned. Sections perpendiculart to the vitreal surface (12µm) were collected on gelatinized slides and finally, stored at -20°C. Immunoreactivity was analyzed in alternate perpendicu- lar sections of the retina. We performed double labelning experiments for alpha-2A-adrenergic receptor and 2M6 (a specific glial marker of the chicken retina). First, non-specific binding sites were blocked with PBS containing 5% BSA and 0.25% Triton X-100 for 3 hours. Sections were incubated overnight with a mixture of primary antibodies against alpha(2A)-adrenergic receptor (1:100, Abcam cat# 65388, raised in rabbit) and 2M6 (1:1000, kindly provided by Dr Schlosshauer, raised in mouse) (Schlosshauer et al., 1991) diluted in PBS containing Triton X-100. Sections were then rinsed in PBS and incubated in a mixture of secondary fluorescent antibodies donkey anti-mouse Alexa 488 (Life Technology cat# A11029) and donkey anti-rabbit Alexa 555 (Life Technology cat# A21249), both at 1:500 in PBS plus 0.25% Triton-X100, for 2h. After washing the sections were mounted using a saturated solution of n-propyl-galate in PBS and analyzed in Axiophot (serial# 451889) microscope. Figures were mounted with Adobe Photoshop 7.0. Manipulation of the images was restricted to threshold and brightness adjustments.

Retinal SD electrical measurements mount

The experiments were performed on retina of post-hatched (1-2 weeks) chickens. After decapitation the eyeballs were removed and the posterior half, containing the retina was transferred to a recording chamber and perfused with a modified Ringer solution (RS), containing 100mM NaCl, 4mM KCl, 1mM CaCl₂, 1mM MgCl₂, 30mM NaHCO₃, 1mM NaH₂PO₄, and 20mM glucose (pH 7.5). The RS was prewarmed and perfused at 1.2 ml/min.

Once in the chamber, microelectrodes were positioned by
a micromanipulator on the inner plexiform layer by entering 150µm on retina. After 1 hour with RS perfusion, SD was obtained by mechanical stimulus by a sharp tungstenium needle. SD voltage variations were measured with a WPI electrometer(15).

To measure BT effect over SD, brimonidine (0.1% and 0.2%) were added to RS and a new mechanical stimulus made after 1h perfusion. Data are presented as mean ± standard error of at least three independent experiments. The null hypothesis was rejected at p < 0.05.

Optical Coherence Tomography (OCT)

OCT was performed on chicken retina as it was mounted on a chamber filled with BSS. Once only the posterior half of the eye was used to document SD, a positive 78D lens was used between OCT and retina in order to focus infra-red rays over retina. A Heidelberg Spectralis OCT was used on this experiment.

RESULTS

Identification of alpha-2-adrenergic receptor type in chicken retina

Molecular cloning has led to the identification of three structurally and pharmacologically distinct alpha-2-adrenergic receptor subtype, termed a2A, a2B and a2C. All the three alpha-2-adrenergic receptor are widely distributed in the nervous system, although the a2A and a2C subtypes appear to predominate in CNS(16).

Our first step was the identification of alpha-2-adrenergic receptor subtypes present in chicken retina using western blot (figure 2).

As shown in figure 2 only alpha(2)-adrenergic receptor subtype A could be detected in chicken retinal tissue. This data suggest that brimonidine neuroprotective effects might be mediated by alpha(2)-adrenergic receptor subtype A in retina.

Localization of alpha(2)-adrenergic receptor in retinal tissue

Several animal studies demonstrate the presence of alpha(2)-adrenergic receptors in the retina, laying the foundation for the neuroprotective role of brimonidine(17-19).

In order to detect the cellular localization of alpha(2)-adrenergic receptor subtype A in chicken retina we performed a immunohistochemistry. As shown in figure 2A alpha(2)-adrenergic receptor subtype A reside within Müller cells. Double labeling experiments were performed to confirm the glial localization of adrenergic receptor in chicken retina. As shown in figure 3C both markers are seen in Müller cells.

This data suggest that pharmacological action of brimonidine is mediated by Müller cells. Neuroprotective strategies are Müller cells-dependent and may involve the supply of neurotrophins or induce endogenous expression of trophic factor as well as antiapoptotic strategies or inflammation modulation.
According to its physiochemical properties, the CNS is an excitable medium, which consequently exhibits propagation waves. We have investigated the retinal SD as an experimental tool to collect information about tissue viability (figure 4).

As shown in figure 4 the treatment with brimonidine promoted the increase of the time lapse between the passage through the two electrodes, meaning a decrease of SD velocity. We also observed a decrease of voltage amplitude, both at 0.1% BT. At 0.2% BT, SD was blocked.

**Spreading depression optical coherence tomography**

As shown on figure 5 there is a good colocalization of chick retina on OCT and histology. As seen on humans, inner plexiform layer is normally hyper-reflectant.

On figure 6A, the yellow ellipse highlight the wavefront of SD. On figure 6B from top image to bottom image, SD spreads from right to left through inner plexiform layer. It is seen as a hyper reflectance signal.

On figure 7, OCT revealed that in the presence of BT, SD cannot be seen.

**DISCUSSION**

During the past decade, Brimonidine has gained attention for its role in the initial and long term treatment of glaucoma. Although several clinical studies document its safety and efficacy (20-22) more recent experimental and animal models suggest a neuroprotective effect of Brimonidine.

The alpha-2-adrenergic receptor is likely to be involved in
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modulating cellular resistance and/or adaptation to stress/injury. Brimonidine shows neuroprotective ability in animal models and adequate concentrations of the drug can reach the retina(23).

The existence of alpha-2-adrenergic receptors in the retina has been described(24-25). Although the major expressing sites are the inner plexiform and ganglion cell layers of the inner retina(19), they are also present in the outer retina, in the photoreceptors(26). Some studies has suggested that neuroprotective effects of adrenergic agonists are achieved by activation of alpha(2)-adrenergic receptors expressed within RGCs, against optic nerve crush injury(27) and transient retinal ischemia(28). Moreover, the addition of noradrenaline (NA), an adrenergic receptor agonist, was able to increase Brain-derived neurotrophic factor (BDNF) levels in retinal Müller glia in vitro(29), and the systemic administration of alpha-2-adrenergic agonist elicited second messenger activation selectively in Müller cells(30).

Several studies focusing on elucidate the possible neuroprotective mechanisms of brimonidine against RGCs death have been performed. The N-methyl-D-aspartate (NMDA) receptors are highly permeable to Ca++ ions, and an intracellular Ca++ overload can lead to excitotoxicity and neuronal cell death. Dong and collaborators (2008) has shown that Brimonidine preserves RGCs by blocking NMDA receptors(31). In addition, Brimonidine has been shown to up-regulate endogenous BDNF expression in RGCs, a potent neuroprotective factor that promotes RGC survival following optic nerve crush injury(32). However, there is little information about the effects of brimonidine in Müller cells(33-35).

Previous studies have shown that alpha-2-adrenergic receptor subtype A could mediate signals to regulate Müller cell functions through activation of mitogen-activated protein kinase (MAPK) pathway by extracellular signal-activated kinase (ERK) phosphorylation, triggering to cytoplasmic signals transduction into transcriptional activation in the nucleus(36). The MAPK pathway is known to trigger the appropriate responses to chemical and physical stresses, and play a key role in cell survival and adaptation(37). Thus, the modulation of MAPK activation could play a possible role in interrupting pathways that stimulate apoptosis, thereby shifting the balance in retinal cells toward survival.

In this work, we demonstrate that Brimonidine has increased the time lapse and decreased the amplitude of SD wave, playing a possible role in neuroprotection. This effect could be mediated by the activation of alpha(2)-adrenergic receptor subtype A, expressed only within Müller cells in chicken retina. The activation of alpha-2-adrenergic receptor subtype A might lead to ERK phosphorylation, triggering cellular responses to promote Müller cell survival and to stimulate secretion of neurotrophic factors. Simultaneously, these cellular signaling might act down-regulating the glial reactivity and stimulating neuronal resistance, protecting retina from further damage after an injury. Moreover, Müller cells are undamaged in animal models of glaucoma and diabetic retinopathy. The discovery of agents that promote activation of alpha-2-adrenergic receptor subtype A in Müller cells would provide a potential tool in treatment of these pathological conditions.

Although the main BT pathway actions still not well known, it is clear its powerful effect. The recent association of SD with many traumatic disorders of the central nervous tissue, leads us to wonder if SD is not a pathway of neuronal damage that is common to traumatic/high pressure induced damage. How BT blocks SD? Possibly acting as a neuro-protector drug. The observed dose dependent effect of it, lead us to think what could be its possible acting pathway. G protein mediated response, down regulating cAMP is a strong possibility. Its action mediated via alpha 2 adrenergic receptor/Muller cells once again gives us a significant neuroprotector effect of these cell that once were thought to be only structural cells. There is no doubt that further studies are necessary to explain the pathophysiology of the SD and its connections to neuronal diseases.

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Figure 7: OCT of stimulated retina with BT; no SD is seen


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