Cannabinoid receptors and TRPA1 on neuroprotection in a model of retinal ischemia

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ABSTRACT
Retinal ischemia is a pathological event present in several retinopathies such as diabetic retinopathy and glaucoma, leading to partial or full blindness with no effective treatment available. Since synthetic and endogenous cannabinoids have been studied as modulators of ischemic events in the central nervous system (CNS), the present study aimed to investigate the involvement of cannabinoid system in the cell death induced by ischemia in an avascular (chick) retina. We observed that chick retinal treatment with a combination of WIN 55212-2 and cannabinoid receptor antagonists (either AM251/O-2050 or AM630) decreased the release of lactate dehydrogenase (LDH) induced by retinal ischemia in an oxygen and glucose deprivation (OGD) model. Further, the increased availability of endocannabinoids together with cannabinoid receptor antagonists also had a neuroprotective effect. Surprisingly, retinal exposure to any of these drugs alone did not prevent the release of LDH stimulated by OGD. Since cannabinoids may also activate transient receptor potential (TRP) channels, we investigated the involvement of TRPA1 receptors (TRPA1) in retinal cell death induced by ischemic events. We demonstrated the presence of TRPA1 in the chick retina, and observed an increase in TRPA1 content after OGD, both by western blot and immunohistochemistry. In addition, the selective activation of TRPA1 by mustard oil (MO) did not worsen retinal LDH release induced by OGD, whereas the blockage of TRPA1 completely prevented the extravasation of cellular LDH in ischemic condition. Hence, these results show that during the ischemic event there is an augment of TRPA1, and activation of this receptor is important in cell death induction. The data also indicate that metabotropic cannabinoid receptors, both type 1 and 2, are not involved with the cell death found in the early stages of ischemia. Therefore, the study points to a potential role of TRPA1 as a target for neuroprotective approaches in retinal ischemia.

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1. Introduction

Glaucoma and diabetic retinopathy are among the main causes of blindness in the world (Resnikoff et al., 2004). In addition, retinal ischemia plays an important role in these retinopathies leading to cell death, and partial or total blindness (Osborne et al., 2004; Resnikoff et al., 2004). Ischemia causes a decrease in Na+/K+ ATPase activity, which triggers an alteration of Na+, Cl− and K+ levels, followed by an increase in intracellular Ca2+ and neurotransmitter release (D’Onofrio and Koeverle, 2013). These alterations can lead to cell death.

Several studies have been investigating strategies to reduce tissue damage caused by ischemic events. In this context, the cannabinoid system has emerged as a promising neuroprotective target. There are two types of metabotropic cannabinoid receptors, type 1 (CB1R) and type 2 (CB2R), which are coupled to Gi/o (Devane et al., 1988; Matsuda et al., 1990; Munro et al., 1993). CB1R and CB2R have been described in the central nervous system (CNS) (Gong et al., 2006; Lencic et al., 2011; Onaivi, 2006; Van Sickle et al., 2005) and immune system (Howlett et al., 2002), respectively.
However, it is now known that both receptors are present in the CNS. Their agonists are derived from plants (Cannabis sativa) such as Δ9-THC (Δ9 – tetrahydrocannabinol), while others are endogenous ligands called endocannabinoids such as anandamide (AEA) and 2-arachidonyl glycerol (2-AG). Synthetic ligands are also known such as WIN55212-2 (Devane et al., 1992; Di Marzo and De Petrocellis, 2012). Each of these ligands has specific binding affinities.

These cannabinoid compounds may also have the ability to activate receptors from the superfamily of transient receptor potential (TRPs) Ca2+ channels (Clapham et al., 2001; Ramsey et al., 2006; Wu et al., 2010). For example, anandamide can directly activate TRPV1 receptors (TRPV1) leading to an increase in intracellular Ca2+ (De Petrocellis and Di Marzo, 2009; Zygmunt et al., 1999). Meanwhile, WIN55212-2, cannabino as well as AM251 and AM630 may also activate TRPA1 (Akopian et al., 2008, 2009; Patil et al., 2011).

As observed in different areas of the CNS, metabolotropic cannabinoid (Leonelli et al., 2005; Straiker et al., 1999; Yazulla et al., 1999, 2000; Zabouri et al., 2011) and TRP (GiLi and Wensel, 2011; Martínez-García et al., 2013) receptors are also present in the retina. Both CB1R and CB2R are widespread in the retina of different species. CB1R is present in rat and chicken ganglion cell layer; in human, rat and chicken inner plexiform layer; in rat and chicken inner nuclear layer; in human, rat and goldfish outer plexiform layer, and in rat outer nuclear layer (Cécyre et al., 2013; Leonelli et al., 2005; Yazulla et al., 1999, 2000; Zabouri et al., 2011). CB2R is present in rat, mouse and monkey ganglion cell layer, inner nuclear layer and in photoreceptors (Rouskila et al., 2013; Cécyre et al., 2013). Although the gene expression of almost all TRP receptors, including TRPA1 and TRPV1, is detected in rat retina (GiLi and Wensel, 2011), the retinal localization and role of these receptors are only established for drosophila (Lev et al., 2012).

Models of toxicity have been used to study the neuroprotective effects of both metabolotropic cannabinoid receptors (Kokona and Thermos, 2015; Nucci et al., 2007; Opere et al., 2006; Pinar-Sueiro et al., 2013; Song and Slowey, 2000; Yang et al., 2013; Zhang et al., 2008) and TRP receptors (Butenko et al., 2012; Miller et al., 2014; Sasaki et al., 2014). However, the role of metabolotropic cannabinoid receptors and TRP receptors in the early stages of retinal ischemia has not been investigated.

Therefore, we aimed to investigate the neuroprotective role of CB1R, CB2R and TRPA1 receptors in the cell death induced by ischemia in chick retina. The use of an avascular retina enabled the evaluation of cannabinoid effects directly on retinal cells without vascular influence.

2. Experimental procedures

2.1. Animals

Fertilized White Leghorn eggs were obtained from a local hatchery. We used chicks between 1 and 7 days post-hatch. At this stage the retina has adopted its mature configuration (Finnegan et al., 2008; Prada et al., 1991; Thanos and Mey, 2001). Animal procedures were approved by the Ethics Committee for Animal Research of the CEPA/PROPP, Fluminense Federal University (protocol number 197/2012), and were in accordance to the National Institute of Health Guide for the Care and Use of Laboratory Animals (8th Edition, 2011).

2.2. Oxygen and glucose deprivation (OGD)

Retinal oxygen and glucose deprivation for 50 min was used as a model of ischemia. After enucleation, the posterior half of the eye containing the retina was used for the experiments. The chick retina is relatively large and this allowed us to perform more experimental conditions using the same animal (for a review, see Vergara and Canto-Soler, 2012). Each retina could be divided into four pieces. These retinal segments were randomly assigned to the control condition or each experimental condition.

To perform the experiments, Normoxia condition comprised control medium (in mM: 120.0 NaCl; 3.0 KCl; 30.0 NaHCO3; 1.0 NaH2PO4; 1.0 CaCl2; 1.0 MgCl2·6H2O; 10.0 glucose) saturated with 95%O2/5%CO2 (White Martins Praxair Inc.). The ischemic condition (OGD) had the same medium composition as the control, except for the absence of glucose, and it was saturated with 95%N2/5%CO2 (White Martins Praxair Inc.). The pH of these solutions was adjusted to 7.2–7.4, and they were used immediately.

Retinal pieces were immersed in 1 ml of solution when using ½ retina, or in 0.5 ml when using ¼ retina. Normoxia or OGD conditions were perfused with the respective gaseous solutions during the entire experimental period. The retinas remained for 50 min in Normoxia or OGD, called here as incubation period.

2.3. Drugs

The drugs used were: the non-selective cannabinoid and TRP receptor agonist, WIN55212-2 (Cayman Chemical Company, Ann Arbor, MI, USA) (1 and 10 μM); CB2R antagonists, AM251 (Cayman Chemical Company, Ann Arbor, MI, USA) (1 and 10 μM) and O-2050 (Tocris, Bristol, UK) (1 and 10 μM); CB2R antagonist, AM630 (Cayman Chemical Company, Ann Arbor, MI, USA) (1 and 10 μM); TRPA1 agonist, mustard oil (MO) (Sigma Aldrich, St Louis, MO, USA) (25; 50; 75; 100; 150, and 200 μM); TRPA1 antagonist, HC-030031 (Cayman Chemical Company, Ann Arbor, MI, USA) (30 μM); the inhibitor of fatty acid amide hydrolase (FAAH), URB597 (Cayman Chemical Company, Ann Arbor, MI, USA) (0.1 μM) and the inhibitor of monoacylglycerol lipase (MAGL), URB602 (Cayman Chemical Company, Ann Arbor, MI, USA) (10 μM).

Retinal pieces were exposed to the antagonists diluted in Normoxia medium 10 min before the incubation period. The 10 min pre-incubation was also performed in the experimental groups exposed only to agonists or inhibitor of degradation enzymes. Subsequently, drugs (agonists or inhibitors of the degradation enzymes) were added to the Normoxia or OGD medium together with the antagonists, remaining until the end of the 50 min OGD.

2.4. Cell viability assay

To evaluate cell death, we measured the release of lactate dehydrogenase (LDH), which occurs after the disruption of cell membranes caused by necrosis. At the end of the experiments, samples were processed as follows: retinal pieces (corresponding to the intracellular fraction) were incubated in lysis solution (Triton X-100, 0,9%) for 30 min at room temperature, and subsequently frozen at –20 °C. The respective solution (extracellular fraction) was also collected and frozen. Before submitting such fraction to analysis, samples were defrosted, homogenized, and centrifuged at 2000 rpm at 25 °C for 4 min.

To evaluate cell viability, a colorimetric commercial kit for identification and quantification of LDH was used (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega). Measurements were performed in duplicates for each intra and extracellular fraction, and performed according to the manufacturer’s instructions. Quantification was performed using a microplate reader (Mark, BioRad, Philadelphia, USA) with the wavelength corresponding to 490 nm. Thus, it was possible to determine the amount of LDH present in the intracellular compartment and extracellular media of each sample. The percentage of the LDH released

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corresponds to the fraction found in the extracellular medium normalized by the total LDH (intracellular plus extracellular).

2.5. Western blot

Retinas were dissected in a calcium and magnesium free medium (CMF) (in mM: 131.00 NaCl; 4.09 KCl; 0.92 Na2HPO4(2H2O); 0.45 KH2PO4; 12.20 glucose; 9.40 NaHCO3; 0.0282 phenosulfophthalein), and placed in 200 µL RIPA buffer (150.0 mM NaCl; 50.0 mM Tris-base; 5.0 mM EGTA; 1% Triton X-100; 0.5% DOC; 0.1% SDS) containing 1.0 mM DTT (Sigma Aldrich, St Louis, MO, USA). After homogenization, the samples were agitated for 1 h at 4 °C. Then, they were centrifuged at 12,000 rpm for 10 min at 4 °C, the supernatants were collected, and stored at −20 °C.

Protein concentration was assessed by BCA assay (Thermo Scientific, Rockford, IL, USA), and 30 µg of protein separated in 8% polyacrylamide gel. Then, proteins were transferred to a PVDF membrane (GE Healthcare, Pittsburgh, PA, USA) using a semi-dry system (Trans-Blot SD semi-dry electrophoretic transfer cell, Bio-Rad, Hercules, CA, USA). Membranes were washed with TBS-T (20.0 mM Tris, 200.0 mM NaCl, 0.1% Tween-20), blocked with 5% non-fat milk for 2 h, and incubated with 1:1000 anti-TRPA1 polyclonal antibody overnight (Abcam, Cambridge, MA, USA; ab58844). After incubation with the primary antibody, membranes were washed 3 times with TBS-T, and incubated with 1: 10,000 HRP-conjugated anti-rabbit antibody (GE Healthcare Life Sciences, Piscataway, NJ, USA) for 2 h. As loading control, we used 1: 100,000 α-tubulin antibody (Sigma Aldrich, St Louis, MO, USA; T5168). Finally, membranes were washed with TBS-T, exposed to enhanced chemiluminescence (ECL) prime Western Blotting Detection (GE Healthcare, Pittsburgh, PA, USA), and revealed using an imaging system (ChemIDoc, BioRad, Hercules, CA, USA).

2.6. Immunohistochemistry

After the experiment, retinas were fixed in 4% paraformaldehyde for 2 h, and then washed in phosphate buffer (0.16 M, pH 7.2). After, the retinas were cryoprotected with a sucrose gradient, frozen and sectioned in a cryostat (12 µm). Normoxia and OGD retinal sections were collected on the same slide, so that they could be processed equally. Sections were incubated with 5% bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO, USA) for 1 h, and then with 1: 1000 anti-TRPA1 polyclonal antibody overnight (Abcam, Cambridge, MA, USA; ab58844). Subsequently, retinal sections were incubated with 1:200 goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) for 2 h, and then incubated with 1:50 avidin-biotin complex (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) for 90 min. Followed by an incubation with 0.05% 3,3’-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) and 0.01% hydrogen peroxide for 10 min.

2.7. Analysis

Measurements of cell viability was performed by LDH release assay were plotted as percentage of control (mean ± SEM). Data were analyzed with Mann-Whitney or Kruskal-Wallis tests followed by Dunn’s or Student Newman Keuls post hoc test to ascertain statistical differences. Means were considered statistically different when p < 0.05. Data were statistically analyzed using GraphPad Prism 6.0 (GraphPad, San Diego, CA).

Digital images from Western Blots were acquired with an imaging system (ChemIDoc, BioRad, Hercules, CA, USA), and they were analyzed using Imagej software (NIH). Data were analyzed by Mann-Whitney test.

3. Results

3.1. OGD increase LDH release from retinas

Initially, the effect of 50 min of an ischemic insult (evoked by OGD) in the viability of retinal tissue was evaluated by LDH content in the extracellular compartment in comparison to the total (intracellular plus extracellular). We observed an increase of 2.7 fold (274.2 ± 19.62%, n = 27) in the release of LDH, suggesting that retinal cell death was induced by OGD (Fig. 1).

3.2. The combination of WIN55212-2 with cannabinoid receptor antagonists was effective in reducing LDH release induced by OGD

In order to evaluate the role of cannabinoids in the early stages of retinal ischemia, we initially treated retinal pieces with WIN55212-2 (10 µM) or CB1R antagonists, AM251 (10 µM) or O-2050 (10 µM), 10 min prior to OGD insult. Then, these retinal tissues were additionally submitted to 50 min of OGD, still in the presence of the drugs. However, neither WIN55212-2 nor the antagonists alone changed LDH release induced by OGD (Fig. 2A–C). Nevertheless, when retinas were incubated with WIN55212-2 (10 µM) in the presence of AM251 (10 µM) a significant decrease in OGD-dependent LDH release was found (Fig. 2A). A similar effect was observed with the other selective CB1R antagonist O-2050 (10 µM) (Fig. 2B). Furthermore, treatment with CB2R antagonist AM630 (10 µM), in the presence of WIN55212-2 (10 µM), attenuated the release of LDH induced by OGD, even though it was not statistically significant (Fig. 2C). On the other hand, the exposure to AM630 alone did not modify the release of LDH stimulated by OGD (Fig. 2C).

Since we observed that the co-exposure of WIN55212-2 and CB1R antagonists, but not CB2R antagonist, significantly decreased the release of LDH induced by OGD (Fig. 2A–C), we asked if the co-administration of both cannabinoid receptor antagonists, 10 µM AM251 and 10 µM AM630, could modulate the release of LDH induced by OGD. We observed that the concomitant exposure of retinas to CB1R and CB2R antagonists during OGD did not affect LDH release induced by OGD (Fig. 2D). Again, only the addition of 10 µM WIN55212-2 together with 10 µM AM251 and 10 µM AM630 seemed to decrease LDH release induced by OGD (Fig. 2D), similarly to what has been previously shown in Fig. 2A. Together, these data suggest that metabotropic cannabinoid receptors CB1 and CB2 do not protect cells during early stages of ischemia in the chick retina. In addition, the findings raised the possibility that WIN55212-2 (10 µM) could act on other molecular targets.

![Fig. 1. Retinas exposed to OGD release LDH.](image-url)
3.3. Increased availability of endocannabinoids is not sufficient to prevent the cell death observed during acute ischemia

In order to examine the effect of endocannabinoids, inhibitors of the degradation enzymes FAAH (URB597) or MAGL (URB602) were used to increase the availability of anandamide and 2-AG, respectively. Treatment with URB597 (0.1 μM) had no effect in the LDH release induced by OGD (Fig. 3A-B). However, similar to results obtained with WIN55212-2, simultaneous treatment of the retina with URB597 (0.1 μM) and CB1R or CB2R antagonists (10 μM AM251 or AM630) resulted in a significant decrease in LDH release induced by OGD (Fig. 3A-B). Again, the treatment with CB1R or CB2R antagonists alone had no effect in the release of LDH induced by OGD (Fig. 3A-B). In a similar way, URB602 had no effect in the LDH release induced by OGD, whereas we have also found a protective effect using URB602 (10 μM) in combination with 10 μM AM251 or 10 μM AM630 (Fig. 3C-D), but not with the antagonists alone (Fig. 3C-D). Thus, these data suggest that treatment with synthetic cannabinoid receptor antagonists together with increased endocannabinoids availability may exert a protective effect on ischemia, probably by acting on targets other than metabotropic cannabinoid receptors.

3.4. The combination of cannabinoid receptor agonist and antagonist at lower concentrations has also a neuroprotective effect

We also studied the effect of WIN55212-2 in a concentration considered selective (1 μM) to activate only metabotropic cannabinoid receptors (Akopian et al., 2009). We observed that, at this lower concentration, neither WIN55212-2 nor AM251 (1 μM) or AM630 (1 μM) alone were able to affect LDH release induced by OGD (Fig. 4). These results were similar to those obtained with 10 μM WIN55212-2, a higher and non-selective concentration for CBs receptors, and this reinforces the idea that metabotropic cannabinoid receptors CB1 and CB2 have no role in cell death in the early stages of ischemia. In addition, the combination of 1 μM CB1R or 1 μM CB2R antagonists with 1 μM WIN55212-2 resulted in a significant decrease in OGD-induced LDH release (Fig. 4).
addition, treatment with 1 μM WIN55212-2 in Normoxic conditions was not able to significantly change LDH release (CTR = 100 ± 28.82% n = 4; WIN55212-2 = 144.8 ± 5.95% n = 3, p = 0.6426). Since these drugs can also bind and activate TRPA1, we decided to investigate the involvement of TRPA1 in ischemic events in the chick retina.

3.5. Presence of TRPA1 receptor in the chick retina

To verify whether TRPA1 was present in the chick retina, Western Blot was performed with retinal samples. We found a band in the predicted molecular weight (130 kDa) from both Normoxia and OGD retinal samples (Fig. 5A). Densitometry quantification of the bands indicated an increase in TRPA1 content in OGD group compared to Normoxia (Fig. 5B). The presence of TRPA1 was also evaluated by immunohistochemistry. TRPA1 was found throughout the retina in cells of the ganglion cell layer, inner and outer nuclear layer as well as in the plexiform layers (Fig. 5C). Interestingly, the cell population pattern of TRPA1 immunolabeling in OGD retina was similar to Normoxia. However, the intensity of TRPA1 immunoreactivity in OGD was clearly higher than Normoxia, strengthening the western blot results.

Since TRPA1 are present in the chick retina, and its content increased in retinas that undergone OGD, we further evaluate their involvement with cell death induced by acute ischemia.

3.6. Retinal exposure to TRPA1 agonist does not enhance LDH release observed in OGD

Higher concentrations of WIN55212-2 (more than 1 μM) as well as AM251 and AM630 can activate other targets, besides metabotropic cannabinoid receptors. One possibility is that TRPA1 could be modulated by these drugs, which could be responsible for the decrease of LDH release induced by OGD. To test this hypothesis, we first investigated the activation of TRPA1 receptor by its agonist mustard oil (MO). We performed a concentration-curve using 25, 50, 75, 100, 150 and 200 μM of MO during an ischemic insult. However, none of TRPA1 agonist concentrations used was able to intensify the release of LDH already seen in OGD (Fig. 6A). In addition, treatment with 200 μM MO in Normoxic conditions was
not able to increase LDH release (CTR = 100 ± 28.82% n = 4; MO = 120.80 ± 17.97% n = 4; p > 0.9999).

Interestingly, 25 µM MO in the presence of 1 µM WIN55212-2 further increased LDH release compared to OGD (Fig. 6B). Accordingly, when ischemic retinas were exposed simultaneously to 25 µM MO and CBR antagonists, 1 µM AM251 or 1 µM AM630, a decrease in LDH release was observed, although significance was found only for AM251 treatment (Fig. 6B). Furthermore, the exposure to metabotropic CBR antagonists with the TRPA1 agonist MO and/or WIN55212-2 induced a significant decrease of LDH release induced by OGD (Fig. 6B). Thus, this group of results suggests that cannabinoid drugs are acting as modulators of TRPA1 receptor-mediated responses during chick retinal ischemia.

3.7. Blockade of TRPA1 receptor prevents LDH release induced by ischemia

Since TRPA1 is present in the retina, its content is increased in OGD, it is permeable to calcium, and its agonist alone did not intensify LDH release induced by OGD, the question that arises is whether TRPA1 was involved in the cell death induced by ischemia. To test this possibility, the effect of TRPA1 receptor antagonist (30 µM HC-030031) in ischemia was investigated. Remarkably, the increase in LDH release induced by OGD was totally blocked whenever TRPA1 was inhibited by HC-030031 (Fig. 7A-B). Thus, these results point to TRPA1 receptors as a key receptor involved in retinal cell death found in early stages of ischemia.

Fig. 4. The combined exposure to a selective concentration of agonist and antagonists of cannabinoid receptor prevents the release of LDH induced by OGD. The exposure to 1 µM WIN55212-2 associated with 1 µM CB1R (AM251) and/or CB2R (AM630) receptor antagonist block the increase in LDH release found in OGD condition. White bars represent data from Normoxia condition. #p < 0.05 compared to OGD.

Fig. 5. TRPA1 receptor identification in chick retina. A. Representative immunoblot of TRPA1 and α-tubulin of retinal samples from Normoxia and OGD conditions. B. Quantification of TRPA1 using α-tubulin as the reference protein (Normoxia = 0.35 ± 15.08% n = 7; OGD = 0.53 ± 9.36% n = 7; p = 0.053). C. Immunohistochemistry of TRPA1 receptor in retina submitted to OGD and Normoxia. The TRPA1 immunolabeling was visualized in cells of the ganglion cell layer, inner and outer nuclear layer as well as in the plexiform layers.
4. Discussion

Retinal ischemia is a pathological condition present in several ocular diseases such as diabetic retinopathy and glaucoma, leading to partial or full blindness (Resnikoff et al., 2004). Hence, studies to understand the mechanisms of cell death triggered by ischemia and to establish methods to minimize or reverse such damage are important.

In the present study, we observed that OGD increase LDH release by 270% compared to Normoxia, demonstrating that OGD induces cell death. The cannabinoid system has been associated with neuroprotection in ischemic events in different neuronal tissues (Eljaschewitsch et al., 2006; Pellegrini-Giampietro et al., 2009; Shohami et al., 1993; Sinor et al., 2000), including the retina (Nucci et al., 2007; Pinar-Sueiro et al., 2013; Song and Slowey, 2000). In the retina, one suggested beneficial effects of cannabinoids could be due to a vascular action, for example, by inducing a vasorelaxation (Macintyre et al., 2014). Thus, we investigated the role of the cannabinoid system in an avascular retinal model to evaluate the effects of cannabinoids directly on retinal cells, minimizing interference of the vasculature.

Therefore, we treated retinas with the non-selective cannabinoid receptor agonist WIN55212-2 in the absence or presence of the antagonists of CB1R and CB2R. Since neither WIN55212-2 nor AM251/02050 nor AM630 alone altered the release of LDH induced by OGD, the results suggested that both metabotropic cannabinoid receptors (CB1R and CB2R) are not directly involved with, or at least are not critical to, the cell death observed in early ischemia. However, when retinas were co-exposed to WIN55212-2 and CB1R antagonists (AM251 or O-2050), a reduction in LDH release induced
by OGD was observed. Therefore, a combination of targets is possibly being modulated by these drugs, resulting in the prevention of cell death. The CB1R antagonist AM251 has recently been described as a GPR55 agonist (Kapur et al., 2009; Ryberg et al., 2007). Nevertheless, we found that both AM251 and O-2050 (the last, a selective CB1R antagonist) have similar effects suggesting that, probably, the main target of AM251 in the retina during ischemic condition is not the GPR55 receptor.

The absence of neuroprotection by direct activation or blockage of CB1R/CB2R during early ischemia was unexpected. As mentioned above there are many studies showing cannabinoid receptors have a role in ischemic damage. In a model of cerebral ischemia-reperfusion in mice, neuroprotection was induced by both blockade of CB1R or by activation of CB2R (Zhang et al., 2008). Zhang et al. (2008) also observed an intensification of neuroprotective effect when CB1R antagonist was applied together with CB2R agonist. Nagayama et al. (1999) have also showed that WIN55212-2, in a CB1R-dependent manner, can prevent ischemic injury. Further evidence of cannabinoid receptors neuroprotection role was that ischemia causes a larger brain infarct size in the knockout for CB1R than in wild type animals (Parmentier-Batteur et al., 2002). CB1R activation also promotes neuroprotection in a model of retinal toxicity induced by AMPA (Kokona and Thermos, 2015). The different results obtained by these studies, in comparison to our data probably reside in the different models used (AMPA toxicity, ischemia/reperfusion and acute ischemia). It is difficult to directly compare data from studies using only one pharmacological cytoxic agent (NMDA or AMPA agonist, for example) as a model of study with data obtained with an ischemic model, because the last probably involves many more mediators and alteration in several intracellular pathways. Moreover, cannabinoid drugs have distinct pharmacological properties and this complexity was found in the present study. Finally, we cannot rule out the possibility that the lack of vasculature has contributed to the non-protective effect of metabotropic cannabinoid receptors.

According to Nucci et al. (2007), activity of FAAH is augmented after ischemia, induced by a transient increase in intraocular pressure, leading to cell death. This effect is partially inhibited by treatment with URB597 or CB1R activation by an AEA analog. Interestingly, during acute ischemia in the present study, the increased availability of AEA or 2-AG, by exposure to URB597 or URB602, were not sufficient to protect retinal cells. We cannot exclude the possibility that this difference relies on specificities of the different animal models studied. However, the increased availability of AEA or 2-AG during acute ischemia protected cells from death when cannabinoid metabotropic receptors were blocked. These data corroborate the hypothesis that there is another signaling system involved in neuroprotection. Since both AEA and 2-AG can activate TRP receptors (Zygmunst and Högestätt, 2014) it became a promising target to further investigation.

Co-treatment with 1 μM of cannabinoid receptor agonist and antagonists, considered selective for CB2R and CB1R, reduced LDH release induced by OGD. This protection was also significant for CB2R, which was not seen in non-selective concentrations for CB1R. This result was also different from other studies that suggest that CB1R is neuroprotective (Zhang et al., 2008; Contartese et al., 2012). Such apparent contradictions could be a consequence of differences in the evaluation period of ischemia.

Here, we also identified the presence and the cell population pattern of expression of TRPA1 in the chick retina, and the results demonstrated a skewed distribution of TRPA1 occurring in all retinal layers. The blockage of TRPA1 completely prevented cell death induced by ischemia. Koch et al. (2011) also raised the possibility that TRPA1 activation could induce cell death, in a similar way as in our findings. However, direct activation of TRPA1, by its agonist, did not induce cell death under Normoxic conditions neither intensified the LDH release induced by OGD. The involvement of TRPA1 in the cell death observed during acute ischemia could be related to molecular changes induced by ischemia, such as the observed increase in TRPA1 retinal content. It is also important to mention that during ischemia there is an increase in reactive oxidative species which can directly activate the TRPA1 receptor (Trevisani et al., 2007; 2014, 2016). Therefore, the activation of TRPA1, by its agonist, does not necessarily mimic the activation pattern of this receptor promoted by OGD. In addition, TRPA1 agonist 25 μM MO with 1 μM WIN55212-2 during OGD dramatically increased the release of LDH, even though WIN55212-2 alone or MO did not induce cell death, either in Normoxic or ischemic conditions. Interestingly, treatment with AM251 or AM630 prevented the cell death induced by WIN55212-2 + MO. Thus, the changes induced by ischemia probably contributed to the harmful effect of TRPA1. Several mechanisms could be related to these findings. There is evidence that TRPs could form heteromultimers, with members of their own family, which modulates the magnitude of agonist-activated current. The threshold of activation of the native TRPM8 in dorsal ganglion root neurons, for example, is five degrees Celsius higher than in heterologous expression systems (Reid, 2008); the magnitude of AM1241-activated current in TRPA1-expressing CHO cells is 10-fold smaller than in sensory neurons or TRPA1/TRPV1-coexpressing CHO cells (Akopian et al., 2008); the TRPA1 activated-current is higher, and desensitization is smaller, in neurons expressing TRPV1 compared to those from TRPV1 null mutant mice (Akopian et al., 2007). Then, it is possible that TRPA1 forms heteromultimer(s) with other TRPs family members in the retina, which modulate its response during ischemia. TRP receptors could also be regulated by metabotropic cannabinoid receptors, via activation of PLC pathway (Kim et al., 2003) or phosphorylation (Govea et al., 2016). Therefore, several molecular changes could occur during ischemia leading to an increase in TRPA1 activation associated to cell death.

5. Conclusion

The results show that cannabinoid system and TRP receptors could be potential targets to study neuroprotection in models of retinal ischemia. We suggest that CB1R and CB2R do not influence cell death found in early ischemia even though they probably have a crucial contribution in the later phases. The data presented herein also bring new information regarding the mechanism of cell death in early stages of ischemia since TRPA1 seems to be a key element to induce cell death. Therefore, the participation of TRPA1 in cell death induced by ischemia, and the consequences of its inhibition in neuroprotection should be further elucidated.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2016.11.015.


