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A Novel Calpain Inhibitor for Treatment of Transient Retinal Ischemia in the Rat

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Abstract

Following an acute ischemia/reperfusion of the rat retina, the activation of cytotoxic proteases, including calpain, results in necrosis and apoptosis of retinal ganglion cells resulting in their degeneration. Using a systemically administered calpain inhibitor that crosses the Blood-Retinal-Barrier would provide for novel systemic intervention that protects the retina from acute injury and lost function. Here we study a novel calpain peptide inhibitor, cysteine-leucyl-argininal (CYLA), in an in vivo rat model of retinal ischemia to determine functional protection using electroretinography. The CYLA-prodrug was administered intraperitoneally before and/or after ischemia-reperfusion at concentrations of 20-40 mg/kg. We found that administering 20 mg/kg CYLA even only after ischemia provides significant preservation of retinal function.

Introduction

Ischemia–reperfusion injury involves many signaling mechanisms resulting in necrotic and apoptotic cell death. These injuries are characterized by both retinal ganglion and inner

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*Conflicts of Interest: A. Stracher and L. Kesner are executive members of ProTor Pharma Corp, which is currently developing CYLA for treatment of neurodegenerative diseases.

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nuclear cell death. Evidence suggests that strategies that target multiple cell death pathways are potential therapeutic agents to protect the retina from this progressive neurodegenerative disease [1,2]. It follows that a strategy that targets a final common pathway, such as calpain inhibitors, offers the potential for greater therapeutic benefit for ischemia-reperfusion injury as well as other retinal degenerative diseases.

Calpains belong to a family of calcium-dependant cysteine proteases of which there are at least 14 different members [3]. Aberrant activation of this enzyme promotes the breakdown of kinases, phosphatases, transcription factors and the proteolysis of cytoskeletal proteins including α -spectrin, all of which contribute to neuronal cell death [4].

The role of calpain in neuronal cell death is thought to occur via anti-necrotic and anti-apoptotic mechanisms [5,6]. Calpains may be activated by an increased influx of Ca^{+} ions that occurs during ischemia that contributes to necrotic cell death [7]. Furthermore, activation of calpain is generally associated with DNA degradation rather than condensation, a feature associated with necrosis [8]. Alternatively, calpain is closely associated with apoptosis via the caspase-activation pathway. For example, caspase-3 activation from pro-apoptotic signaling degrades calpastatin, an endogenous inhibitor of calpain [9]. Calpain activates caspase-3, demonstrating that the apoptotic pathway is associated with increased calpain activity [10]. Further studies suggest that calpain acts by activating caspase-12 through direct proteolytic cleavage [11]. Calpain inhibition would therefore provide an ideal treatment due to this final common pathway between the necrotic and apoptotic cell death systems.

A difficulty posed by calpain inhibitors in *in vivo* models is the inability of many of these drugs to penetrate the blood brain barrier (bbb) and the blood retinal barrier (brb) [12]. The calpain inhibitor cysteic-leucyl-argininal (CYLA) prodrug, evaluated in the present study, is synthesized by attaching the carboxyl group of cysteic acid (α -amino- β -sulfo-propionic acid) to leucyl-argininal diethyl acetal. Cysteic acid shares structural similarities with taurine (2-aminoethanesulfonic acid), a β -amino acid that is transported through cell membranes via a Na^{+} and Cl^{-} -dependent transporter (TauT/Slc6a6) [12] that penetrates the bbb, and acts as a competitive inhibitor of the taurine transport mechanism [13]. Furthermore, taurine is the most abundant free amino acid in the retina [14]. It is about 100 times greater in concentration compared to that of the serum [15]. The CYLA prodrug is a diethyl acetal and has been documented as an inactive calpain inhibitor *in vitro* but is thought to be converted to the active inhibitor *in vivo* [16].

Methods

Preparation and application of CYLA

The prodrug of CYLA, a diethyl acetal, was a gift from Dr. Alfred Stracher and its activation *in vitro* was previously described [16]. The CYLA prodrug was stored in the cold and prior to use, it was dissolved in PBS and was administered intraperitoneally (I.P.) in 0.33 mL PBS per rat regardless of the quantity of drug. The doses were administered 1 hour prior to the ischemia and 3 hours post ischemia, or only 3 hours post ischemia. The quantity of drug used in this study was 3 mg (20mg/kg) and 6 mg (40mg/kg) of CYLA prodrug (ie: 20 mg/kg or 40 mg/kg of CYLA prodrug were dissolved in 0.33mL of PBS). These doses of drug were chosen based on correlations to previous studies using the same drug in an *in-vivo* model [13]. Leupeptin was administered at 40 mg/kg in 0.33mL of PBS and acted as a control to CYLA prodrug due to its active calpain inhibitor effects but lack of CNS-barrier permeability [16].

Retinal Ischemia

All animals were treated in accordance with State University of New York (SUNY) Downstate Medical Center Animal Care and Use Committee (Protocol Number 06-307-10). Male Sprague-Dawley rats weighing 150-175g (Taconics) were subjected to high intraocular pressure that produces retinal ischemia [2,17,19]. Briefly, rats are deeply anesthetized with an intramuscular injection of ketamine (40 mg/kg) and xylazine (2.5 mg/kg), the anterior chamber of the right eye was cannulated using a 27 gauge needle and attached via silastic tubing to an infusion of sterile 0.9% saline. Under direct vision, the needle tip was placed within the anterior chamber and the corneal puncture site sealed with cyanoacrylate cement. Intraocular pressure (IOP) was raised to 120 mmHg by elevating the saline container, in order to exceed systemic arterial blood pressure, for a duration of 45 minutes, a time point found previously to provide ischemia that causes physiological irreversible cell damage[1,2]. Animals were kept normothermic at 37°C ±0.5°C with a rectal probe and heating pad during the procedure. Whitening of the iris and loss of the red reflex of the retina confirmed retinal ischemia. Following completion of the target period of ischemia, the needle was withdrawn and the intraocular pressure normalized. The contralateral eye of each animal served as a non-ischemic control. One drop of gentamicin ophthalmic solution was applied topically to the right eye before and after cannulating the anterior chamber.

Electroretinography

The procedures used were those we have previously reported [2,18] Briefly, animals were anesthetized and their pupils dilated with tropicamide and cyclomydril. Platinum electroencephalographic electrodes were placed on the corneas, and the a-wave and b-wave were identified after presenting full-field stroboscopic flashes at the rate of 1.0/s at a distance of 15 cm. Electroretinograms (ERGs) were taken before the ischemic insult to determine the baseline and again before enucleation 7 days post ischemia. ERGs of the right and left eyes were always taken together, with the left eye serving as the nonischemic control.

All data are expressed as mean ± SEM. ERG data were analyzed with ANOVA followed by Dunnett's test. For all comparisons, $p < 0.05$ was regarded as statistically significant.

For all studies, investigators were blinded to treatment during surgery, data acquisition, and analysis.

Results

ERG

Previous studies have demonstrated that maximum changes in ERG occur 7 days post-ischemia [1,19]. The present ERG data was collected 7 days following a 45 minutes of retinal ischemia. CYLA prodrug given I.P. significantly preserved retinal function compared with the vehicle treated rats as there was significant preservation of the b-waves in the treated animals (figure 1). We observed that compared to the PBS control which exhibited only a 17% preservation of retinal function (SEM±3.0), as represented by the b-wave, CYLA prodrug preserved 43.0% of function (SEM±6.2, $p < 0.05$) when administered 1 dose (20 mg/kg) of drug 3 hours post ischemia. We also observed that when the drug was administered twice at the same dosage (20 mg/kg), 1 hour prior to the ischemia and 3 hours post ischemia, there was 39.4% preservation of function represented by the b-wave was preserved (SEM±8.6, $p < 0.05$). Furthermore, there appeared to be little difference between the animals treated with 2 doses of drug (20 mg/kg), one hour pre-ischemia and 3 hours post ischemia, compared to the animals treated with one dose of drug (20 mg/kg) 3 hours post ischemia. Although the groups treated with 40 mg/kg of drug did not show statistically

significant benefit when compared to the control group ($p>0.05$), there still appeared to be little difference in retinal preservation of function compared with the animals treated with 20 mg/kg of drug ($p>0.05$). Leupeptin, a calpain inhibitor that is unable to cross the bbb, appeared to not protect the retinal tissue against ischemia as it preserved retinal function by only 21.8% ($SEM \pm 3.3$, $p>0.05$) when compared to the control group (figure 2).

Discussion

In this study, we have used a blood-CNS barrier-permeable calpain inhibitor, CYLA, in an *in vivo* model to demonstrate that post-ischemic inhibition of calpain significantly reduces retinal cell death and improved retinal function after an acute ischemia-reperfusion. The significant protective effect of this inhibitor provides evidence supporting a role for calpain in retinal dysfunction following retinal ischemia.

In retinal ischemia, cell death is in part due to (i) an increase in glutamate from damaged synapses activating NMDA and AMPA/Kainate receptor activation, as well as (ii) the activation of voltage-dependent calcium channels causing a Ca^{++} influx and activation of Ca^{++} -dependent processes [20,21]. Calpain has been implicated in the proteolysis of the Na^{+}/Ca^{+} exchanger resulting in a reduced expulsion of the Ca^{++} ions resulting in an ion imbalance that favors a Ca^{+} influx resulting in the activation of Ca^{++} -dependent calpain [22]. Aberrant activation of this enzyme promotes the breakdown of kinases, phosphatases, transcription factors and the proteolysis of cytoskeletal proteins all of which contribute to neuronal cell death [3]. Thus, using calpain-inhibitors can prevent many processes involved in neuronal cell homeostasis, integrity, plasticity and repair.

Of note is the fact that the CYLA prodrug was effective when it was administered 3 hours post ischemia. An additional dose administered pre-ischemia provided no additional benefit. This further supports that the mechanism of action of calpain is of post ischemic inhibition of proteolysis. This too is of clinical importance as calpain has been shown to protect neuronal cells in brain tissue [21] as well and may therefore be of importance in conditions such as stroke and traumatic brain injury. Further studies will have to be conducted to determine how long after an ischemic event the calpain inhibitor would still be effective. In addition, the dose response curve should be determined.

Most literature that has used calpain inhibitors have focused either on single organ *in vitro* studies [23] or used a calpain inhibitor infusion over time [24,25]. The present study focuses preservation of neuronal cell death following an acute ischemic after a single injection of a calpain inhibitor. Of interest is that fact that Leupeptin, an active calpain inhibitor, showed no functional preservation when administered I.P. while the inactivated calpain inhibitor, CYLA prodrug, showed significant functional preservation suggesting that the prodrug is converted to the active drug *in vivo* as was seen on other *in vivo* studies [16] and emphasizes the clinical importance of such a drug.

Conclusion

This study demonstrates that a calpain-inhibitor can improve functional outcome following retinal ischemia. Additionally, the fact that the drug was administered systemically and was able to penetrate the blood-CNS barrier and protect the retina from damage is of clinical relevance.

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Figure 1. Representative ERGs at pre-ischemic baseline and 7 days subsequent to 45 minute ischemia. Note the preservation of function after administration of 20 mg/kg of CYLA prodrug I.P. 3 hours post ischemia. Mean $\pm$ SEM are provided. * $P < 0.05$ by ANOVA ($n > 6$ per group).

Figure 2.

Measurements of ERG b-wave amplitudes as a percentage of normal baseline 7 days after 45 minutes of ischemia. Injections were given as follows: I.P. injection of i. 0.33mL PBS per rat 1 hour pre-ischemia and 3 hours post-ischemia or ii. CYLA prodrug (20 mg/kg or 40 mg/kg of drug dissolved in 0.33mL PBS per rat) 1 hour pre-ischemia and 3 hours post-ischemia. A second set of experiments used the same doses however the vehicle or drug were administered only 3 hours post-ischemia. * $P < 0.05$ by ANOVA followed by Dunnett's test ($n > 6$ per group).