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Calpains are activated by light but their inhibition has no neuroprotective effect against light-damage

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ABSTRACT

Calpain had been shown to be highly activated at one day after exposure to the damaging light (Perche et al. (2007) *Caspase-dependent apoptosis in light-induced retinal degeneration. Invest Ophthalmol Vis Sci 48:2753–2759.*), suggesting that they might play a critical role in photoreceptor apoptosis induced by light. Therefore in the present study we investigate the role of calpain in light-induced photoreceptor cell death. In a first set of experiments, untreated albino Wistar rats were sacrificed at 0, 2, 4, 6, 12, 24 h of light exposure and at one day after the light was turned off (D1) to measure retinal calpain activity and to study calpain expression. In a second set of experiments, after control electroretinograms (ERGs), rats were uninjected or injected intravitreally with DMSO or the calpain inhibitor Mu-Phe-hPhe-FMK, before being exposed to the damaging light for 24 h. ERGs were then recorded at one day (D1) and fifteen days (D15) after the end of light exposure. Rats were sacrificed at D1 for apoptotic cell detection or D15 for histological analysis (ONL thickness). Calpain activity and expression significantly increased in Untreated retinas, from 0 h to D1. DMSO has no effect on calpain activity. Mu-Phe-hPhe-FMK significantly inhibited retinal calpain activity by 85% at 2 h of light exposure and still 48% at D1. However, Mu-Phe-hPhe-FMK has no effect on light-induced retinal degeneration as evidence by equivalent loss of function, equivalent loss of photoreceptor cells and an equivalent number of apoptotic cells in Mu-Phe-hPhe-FMK and DMSO retinas. Therefore, calpains are up-regulated by light stress but they do not have a pivotal role in photoreceptor apoptosis.

1. Introduction

Calpains are Ca^{2+}-dependent intracellular cystein proteases (Sorimachi et al., 1997). There are fourteen calpain isoforms (Suzuki et al., 2004). The two major isoforms (Elce et al., 1995; Sorimachi et al., 1997; Goll et al., 2003), μ-calpain (or calpain-1) and m-calpain (or calpain-2), are ubiquitous and are distinguished by the optimal Ca^{2+}-concentration for maximal activity (Cong et al., 1989; Croall and DeMartino, 1991). Calpains are synthesized as inactive proenzyme. Under the right Ca^{2+}-concentration, they are cleaved to generate an active calpains composed of an 80 kDa "catalytic" subunit and a 30 kDa "regulatory" subunit (Suzuki et al., 1992). Once activated, calpains cleave a variety of key proteins leading the cell to death (Vanags et al., 1996). Several calpain substrates have been identified including p53 (Kubbutat and Vousden, 1997), IkBa (Han et al., 1999), calpastatin (Porn-Ares et al., 1998) or caspases (Chua et al., 2000; Gafni et al., 2009). It has been shown that calpains can positively or negatively regulate apoptosis. For example, cleavage of caspase-9 by calpain leads to the inhibition of dATP and cytochrome-c-dependent caspase-3 activation (Chua et al., 2000). More recently, it has been shown that calpain-1 induces apoptosis by cleaving and activating caspase-7 (Gafni et al., 2009). Unlike caspases that require specific amino acid sequences at the cleavage sites, calpains show no apparent sequence preferences. It is believed that the site of calpain cleavage is more structure-dependent (Sorimachi et al., 1997).

Uncontrolled and prolonged calpain-mediated proteolysis has been suggested in the pathogenesis of neuronal cell death such as in Alzheimer's and Parkinson's diseases (Nixon, 2003). In recent years, the activation of calpain has been shown to be involved in retinal degeneration in several different animal models (Donovan and Cotter, 2002; Doonan et al., 2003; Azuma et al., 2004; Paquet-Durand et al., 2007). Indirect evidence for activation of calpain in *rd1* retina came from Doonan et al. (2003), who found that beginning at postnatal day 11 (PN11), the enzyme poly-ADP-ribosepolymerase (PARP) was cleaved in a way typical for calpain, giving rise to a 40 kDa fragment. This idea is supported by findings with Ca^{2+}-channel blockers which delay photoreceptor degeneration *rd1* mouse (Frasson et al., 1999; Takano et al., 2004). Paquet-Durand demonstrated that the increased in calpain activity in the *rd1* is localized in

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the photoreceptor (Paquet-Durand et al., 2007). In WBN/Kob rats, a spontaneously diabetic rat, retinal degeneration is accompanied by an increase in calpain-1 and 2 activity and mRNA levels (Azuma et al., 2004). Calpain activity was lower in the retina of the neonatal RCS rat, before the onset of any morphological deterioration, and it preceded any other detected abnormalities (Azarian and Williams, 1995). In a mouse model of light-induced retinal degeneration, Donovan and Cotter (2002) showed that calpain activation is associated with Ca^{2+}-influx and that the L-type Ca^{2+}-channel blocker Diltiazem protects photoreceptors from light-damage.

In a previous study, whereas we demonstrated that caspases and specifically caspase-3 play a major role in light-induced retinal cell death, we also observed a large increase in calpain activity at one day after exposure to a damaging light (Perche et al., 2007). This observation suggested that beside caspases, calpains play also a role in light-induced retinal degeneration. This hypothesis is conceivable since caspases and calpains have been shown not to be excluding to each other (McGinnis et al., 1999; Chua et al., 2000; Sanges and Marigo, 2006; Del Bello et al., 2007). Therefore, in the present study, we evaluate the role of calpains in light-induced photoreceptor apoptosis. First we study calpain activity and expression during light-induced retinal degeneration. And then, because calpains were highly activated, we have tested the effect of a calpain inhibitor on light-induced retinal degeneration.

2. Methods

2.1. Animals

Albino Wistar rats were raised in dim-cyclic light (12D:12L, <10 lux). They were fed ad libitum and had free access to water. All experiments were conducted in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the regional Animal Care Committee.

2.2. Damaging light

Light exposure was carried out as described previously (Perche et al., 2007). Briefly, rats were dark-adapted overnight before being exposed for 24 h to a white fluorescent light set at 3400 lux (Photometre S350, United Detection Technology, USA). During light exposure, rats had free access to food and water.

2.3. Calpain activity

Total retinal calpain (m-calpain and μ-calpain) activity was measured using a fluorimetric kit (Calpain fluorometric kit, Calbiochem, Strasbourg, France). Briefly, two retinas from one rat were homogenized in the lysis buffer and total protein concentration was determined by BCA method (Pierce, France). Proteins (150 μg) were incubated at 37 °C for 1 h with calpain specific substrate (Suc-Leu-Leu-Val-Tyr-AMC) in reaction buffer. The fluorometric substrate (AMC) is released upon cleavage by calpain and is measured at an excitation wavelength of 360–380 nm and an emission wavelength of 440–460 nm. Results are expressed as fold increase in calpain activity compared to the basal level (0 h, 100%).

2.4. Protein expression

Two retinas from one rat were pooled, homogenized in a lysis buffer [20 mM TrisHCl, pH 7.6, 5 mM EDTA, 5 mM β-Mercaptoethanol pH 8] containing proteinase inhibitors (Complete mini tablet EDTA free; Roche, Indianapolis, IN, USA), and incubated on ice for 10 min before centrifugation (11 000 g for 20 min at 4 °C). The protein content of the supernatant was quantified using BCA protein assay (Pierce, VWR, Starsbourg, France). Thirty micrograms of protein was loaded on SDS–polyacrylamide gels (12%). Then, proteins were blotted onto a nitrocellulose membrane. After blocking (Tris-buffered saline containing 0.1% Tween 20 and 10% Non fat dry milk, TTBS), the membrane was incubated overnight at 4 °C with anti-calpain-1, anti-calpain-2, or anti-calpastatin (kindly provided by M. Ouali, INRA, Theix, France) diluted in TTBS1X at 1:500. Immunopositive signals were detected using an ECL plus chemiluminescence detection kit (Amersham Biosciences, Orsay, France). Then blots were stripped and reprobed with anti-βActin antibody (Santa Cruz Biotechnology, Tebubio, le Perray-en-Yvelines, France). Quantification of immunoreactive bands was done using Image-Pro Plus software (MediaCybernetics). On each blot, there were 2 samples per time and three blots were done per antibody.

3. Treatments

Rats were injected intravitreally (2 μl) with a solution of DMSO 2%, or calpains inhibitor Mu-Phe-hPhe-FMK (1.1 mM in DMSO 2%; Calbiochem, Strasbourg, France). Intravitreal injection was performed as already described (Perche et al., 2007). Briefly, anesthetized rats were injected under microscope with a 30-gauge needle mounted on a Hamilton syringe (VWR, Strasbourg, France). After injection, a drop of antibiotic (Tobrex®, Alcon, France) was applied on the cornea and then rat was placed in the dark for 18–19 h. Both eyes received the same treatment.

3.1. Electroretinography

Electroretinograms (ERG) were recorded as described previously (Ranchon et al., 2003). Briefly, ERGs were recorded through Ag/AgCl electrodes, for 14 stimuli (10 μs flash) of increasing luminance. The b-wave amplitude of the ERG plotted against the luminance of the stimulating flash gives the b-wave sensitivity curve. Each curve was fitted using the software program Microsoft Origin 6.0 (Microcal Software, Northampton, MA) to calculate the maximal b-wave amplitude (B_{max}).

3.2. Histology

Eyes were embedded in paraffin as described previously (Ranchon et al., 2003). Sections of 3 μm were cut along the meridian through the optic nerve. The outer nuclear layer (ONL) thickness was measured every 0.36 mm from the optic nerve to the inferior and to the superior ora serrata (Perche et al., 2007). The curves representing the ONL thicknesses variations in both sides on the retina were integrated using Origin 6.0 program (Microcal Software).

3.3. Apoptotic nuclei detection

Rats were sacrificed and immediately after death, their eyes were enucleated, placed in fixative (4% paraformaldehyde in PBS) at 4 °C for 4 h and embedded in paraffin. Sections of 5 μm were cut along the meridian through the optic nerve. The Apoptag Kit S 7101 (Qbiogen, Ilkirch, France) was used per manufacturer's instructions. Positive cells were counted under microscope at 1.2 mm from the optic nerve in the superior part of the retina on a 0.2 mm section length (Perche et al., 2007).

3.4. Experimental paradigm

In a first set of experiments, calpain activities and expression were analyzed from untreated retinas. Retinas were collected just