Biochimica et Biophysica Acta 1833 (2013) 2244–2253

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Calpain 1 induce lysosomal permeabilization by cleavage of lysosomal associated membrane protein 2

Gloria E. Villalpando Rodriguez [a,b], Alicia Torriglia [a,b,c,*]

[a] INSERM U872 Eq. 17, Centre de Recherches des Cordeliers, Paris, France
[b] Université Pierre et Marie Curie Paris 6, France
[c] Université Paris Descartes, France

ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form 16 May 2013
Accepted 20 May 2013
Available online 5 June 2013

Keywords:
Calpain
Lysosome
Cathepsin D
LAMP 2
Endonuclease
Protease

ABSTRACT

In light induced retinal degeneration (LIRD) photoreceptor cell death is mediated by caspase independent mechanisms. The activation of LEI/L-DNase II pathway in this model, is due to cathepsin D release from lysosomes, although the underlying mechanism remains poorly understood. In this paper we studied the involvement of calpains in lysosomal permeabilization. We investigated, for the first time, the calpain targets at lysosomal membrane level. We found that calpain 1 is responsible for lysosomal permeabilization by cleavage of the lysosomal associated membrane protein 2 (LAMP 2). Moreover, LAMP 2 degradation and lysosomal permeabilization were rescued by calpain inhibition and the use of MEF$^{-/-}$ *lamp 2* cells indicates that the cleavage of LAMP 2A is essential for this permeabilization. Finally, we found that LAMP 2 is cleaved in LIRD, suggesting that the mechanism of calpain induced lysosomal permeabilization is not exclusive of a single cell death model. Overall, these data shed new light on understanding the mechanisms of lysosomal and caspase-independent cell death and point to the original targets for development of the new therapeutic protocols.

1. Introduction

Neuron cell death is central to all degenerative diseases. For example, the Alzheimer disease, described in 1906, is characterized by cholinergic neuron death while in Parkinson and Huntington's disease dopaminergic neurons undergo cell death [1]. Neurodegeneration also occurs in the retina as, for example, in retinitis pigmentosa or in age-related macular degeneration, where photoreceptors degenerate due to cell death [2].

According to morphological criteria, Clarke described in 1990 three types of cell death. This nomenclature was updated in 2007 and 2012 by the NCCD [3,4].

Type I programmed cell death, or apoptosis, is characterized by rounding-up of the cell, retraction of pseudopodia and cellular volume, chromatin condensation, and nuclear fragmentation. There is little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing and maintenance of an intact plasma membrane until late stages of the process.

Type II or autophagic cell death occurs without chromatin condensation and is accompanied by massive autophagic vacuolization (LC3 positive vacuoles) of the cytoplasm.

Type III or necrosis shows cytoplasmic swelling, mechanical rupture of the plasma membrane, dilation of cytoplasmic organelles, as well as moderate chromatin condensation.

Many other types of cell death have been described during the last years, so that the NCCD in 2012 enlarged the classification to 13 different types of cell death according to biochemical features [4].

Of these different types of cell death, apoptosis is largely involved in the degenerative diseases of the central nervous system. Its molecular pathways have been studied for several years. Nevertheless, these studies have been concentrated on the role of caspases in apoptotic demise. However, many studies have shown the induction of caspase-independent cell death mechanisms involving other proteases as apoptotic effectors like calpains or cathepsins.

For example, studies of Momoni and Kanje [5,6] show that adult spinal cord slices from mice exhibited apoptotic features in motor neurons after excision and that leupeptin, calpain inhibitor XI and calpain inhibitor VI, but not Z-VAD.fmk, inhibit apoptosis of these neurons. Another study [7] shows that cortical neurons treated with 250 μM HOCl exhibited several biochemical markers of apoptosis in the absence of caspase activation. In contrast, calpain inhibitors protect cortical

Abbreviations: AIF, Apoptosis Inducing Factor; ALLN, N-Acetyl-Leu-Leu-Nle-CHO; AP, Alkaline Phosphatase; BCA, Bicinchoninic Acid; BSA, Bovine Serum Albumin; CAD, Caspase Activated DNase; CA, Cornu Ammonis; CMA, chaperon mediated autophagy; DMEM, Dulbecco's Modified Eagle Medium; HNE, hydroxynonenal; LAMP 2, Lysosomal Associated Membrane Protein 2; LC3, Light Chain 3; LDH, Lactate dehydrogenase; L-DNase II, LEI-derived DNase II; LEI, Leukocyte Elastase Inhibitor; LIRD, Light Induced Retinal Degeneration; MEF, Mouse Embryonic Fibroblasts; MTT, Thiazolil Blue Tetrazolium Bromide; NCCD, Nomenclature Committee of Cell Death; PBS, Phosphate-Buffered Saline; TUNEL, Terminal transferase dUTP Nick End Labeling; WB, Western Blot

* Corresponding author at: INSERM U872 Eq. 17, Centre de Recherches des Cordeliers, Paris, France. Tel.: +33 1 44 27 81 73; fax: +33 1 44 27 81 83.
E-mail address: alicia.torriglia@inserm.fr (A. Torriglia).

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.05.019

neurons from apoptosis. Moreover, this study reported that calpain activation induces lysosomal disruption and that stabilization of lysosomes and inhibition of cathepsins protect cells from cell death.

Although the studies of Momoni and Kanje [5,6], Yap [7] and others have shown the implication of calpains in cell death, little is known about the precise molecular mechanisms of calpain mediated cell death.

In 1998, Yamashima proposed the "calpain–cathepsin hypothesis" [8] which suggests that lysosomal membrane of post-ischemic Cornu Ammonis (CA) 1 neurons is disrupted by activation of calpain 1 (μ-calpain) which causes the release of lysosomal proteases, in particular cathepsin B. This release causes post-ischemic CA 1 neuronal death in primates which is prevented with CA-074, a specific inhibitor of cathepsin B. This hypothesis has been used by other authors, as Ceccariglia in 2010 [9] to explain the effects of a calpain inhibitor on cathepsin D activation in CA 3 hippocampal neurons. Tsukada also used this hypothesis to explain the release of lysosomal DNase II in ischemic neuronal necrosis and its role on cell death [10]. This hypothesis has been also cited in the 2004 review of Syntichaki and Tavernarakis as part of the machinery of necrosis [11]. Yamashima built his hypothesis on the observation that calpain 1 is activated in lysosomal membrane of CA 1 neurons 20 min after ischemia [12]. Until 2010 this hypothesis has been accepted without knowing the mechanism by which calpain 1 destabilizes lysosomal membrane. Recently, Sahara and Yamashima found that activated calpain 1 colocalizes with the heat shock protein 70.1 (Hsp70.1) at the lysosomal membrane of the post-ischemic CA 1 neurons after the ischemia–reperfusion insult. They also reported that in CA 1 neurons Hsp 70.1 is cleaved, in vitro, in the presence of calpain 1, a cleavage that is inhibited with N-Acetyl-Leu-Leu-Nle-CHO (ALLN) and is more important in presence of Hydroxynonenal (HNE) or H_2O_2 [13].

In another model such as Light Induced Retinal Degeneration (LIRD), we found that photoreceptors of Fisher rats exposed to continuous light undergo caspase-independent cell death with an activation of LEI/L-DNase II. Two proteases were found activated: calpains and cathepsin D. We previously showed that cathepsin D, but not calpains [14], is responsible for the cleavage of LEI into L-DNase II. Inhibition of this protease protects the photoreceptors from cell death [15]. However, the precise molecular mechanism behind the calpains involvement in this model is still unknown. Yamashima found in his ischemia–reperfusion model, an activation of calpain 1 and a release of cathepsin B from lysosomes. He also showed that inhibition of calpain was neuroprotective. In agreement, we found that inhibition of cathepsin D, in our LIRD model, is also protective. In this work we further tested the Yamashima's hypothesis which proposes that, calpain 1 is responsible for lysosomal permeabilization. We thus investigated the calpain's targets at the lysosomal membrane during permeabilization by using a cellular model presenting lysosomal permeabilization and L-DNase II activation, such as treatment of HeLa cells with ciprofloxacin.

2. Materials and methods

2.1. Cell lines and culture conditions

HeLa cells (S3 clone) were cultured in DMEM (Dulbecco's Modified Eagle Medium) with GlutaMAX™ I, supplemented with 4.5 g/L (25 mM) D-glucose, 0.11 g/L (1 mM) sodium pyruvate, 10% fetal bovine serum, 0.5% penicillin/streptomycin at 37 °C under 5% CO_2 and 90% of relative humidity.

Wild type Mouse Embryonic Fibroblasts (MEF) and MEF *Lamp 2*$^{-/-}$ cells (a generous gift from Dr. Alex Schneede) were cultured in DMEM (Dulbecco's modified eagle medium) supplemented with 4.5 g/L (25 mM) D-glucose, 0.58 g/L (3.97 mM) L-glutamine 10% fetal bovine serum and 0.5% penicillin/streptomycin at 37 °C under 5% CO_2 and 90% of relative humidity.

2.2. Cell death induction and proteases inhibition

For cell death induction different concentrations of ciprofloxacin (Santa Cruz) were used to evaluate the appropriated dose.

Calpains were inhibited by using Calpain VI inhibitor (Calbiochem ref. 208745) prepared in DMSO at a concentration of 78 nM.

Cathepsin D was inhibited with Pepstatin A-BODIPY® (Invitrogen) 100 nM alone or in combination with calpain inhibitor VI.

2.3. MTT reduction assay

1×10^5 HeLa cells were seeded in 24-wells plate. After 48 h, cells were treated with ciprofloxacin with or without inhibitors, alone or in combination as mentioned before. Thiazolil Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich) was diluted in phosphate-buffered saline (PBS) solution at a concentration of 1 mg/mL. Twenty-four hours after treatment, culture medium was removed and 250 μL of MTT was added to each well. The plate was kept for 1 h at 37 °C. Cells were then lysed with 250 μL of isopropanol. MTT reduction in each sample was subsequently assessed by measuring absorption at 570 nm versus 630 nm using a microplate reader (BioRad). The survival rate was expressed as the percentage of the untreated cells.

2.4. Cytotoxicity assay

1×10^5 HeLa cells were seeded in 24-wells plate and treated as mentioned above, and the cytotoxicity was measured 24 h after the treatment by measuring lactate dehydrogenase (LDH) activity using a LDH kit (Roche). Medium of each condition was incubated with 100 μL LDH reaction mixture for 30 min at room temperature. Cells treated with 0.2% Triton X-100 constituted positive control representing 100% of cytotoxicity. Each sample was subsequently assessed by measuring absorption at 490 nm versus 630 nm using a microplate reader (BioRad).

2.5. Clonogenic test

HeLa cells were seeded in 1 mL of complete medium at the density of 6×10^4 cells/well in 24-well plate and treated 24 h later with 350 μg/mL ciprofloxacin alone or in combination with 78 nM calpain inhibitor VI. At the end of the treatment cells were washed with PBS and trypsinized. For each condition, 10^4 cells were seeded into 6-well plate and grown with complete medium. Ten days later, medium was removed and cells were carefully washed with PBS, fixed and stained with 2 mL of 6% glutaraldehyde and 5% cresyl violet. After 30 min, staining solution was removed; plates were washed with tap water, and dried in normal air at room temperature. The surface of the plate occupied by the colonies was calculated as follows: pictures of each plate were taken in constant conditions of distance between the camera and the plate, source of light, ISO, zoom, exposition time and aperture of diaphragm. The area of analysis for the wells on the plate was selected (the radius was the same for all the wells). Using image analysis software, every image was binarized and the rate between the area occupied by colonies and the total area of the well was calculated.

2.6. Terminal transferase dUTP nick end labeling (TUNEL)

1×10^5 HeLa cells/mL were seeded in wells of lab-tek lips (Nunc). After 48 h they were treated with 350 μg/mL of ciprofloxacin and 24 h after treatment they were air dried and fixed in 4% paraformaldehyde 15 min at room temperature and then washed twice with PBS. The cells were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4 °C. After washing once with PBS, half of the sections were incubated for 30 min with calf intestinal alkaline phosphatase 6.66 U/well (Invitrogen) at 37 °C. After phosphatase incubation sections were rinsed once with PBS and incubated 1 h