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Research paper

P2X7-pannexin-1 and amyloid β-induced oxysterol input in human retinal cell: Role in age-related macular degeneration?

Elodie Olivier [a, b], Mélody Dutot [a, c], Anne Regazzetti [a], Teddy Leguillier [a], Delphine Dargère [a], Nicolas Auzeil [a], Olivier Laprévote [a], Patrice Rat [a, *]

[a] UMR CNRS 8638-Chimie Toxicologie Analytique et Cellulaire, Université Paris Descartes, Sorbonne Paris Cité, Faculté de Pharmacie de Paris, 4 Avenue de l'Observatoire, 75006 Paris, France
[b] Soliance-Givaudan, Route de Bazancourt, 51110 Pomacle, France
[c] Recherche et Développement, Laboratoire d'Evaluation Physiologique, Yslab, 2 Rue Félix le Dantec, 29000 Quimper, France

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ABSTRACT

Age-related macular degeneration (AMD) is the most common cause of severe vision loss worldwide. Amyloid β involvement in degenerative diseases such as AMD is well known and its toxicity has been related to P2X7 receptor-pannexin-1. Recently, oxysterols (oxidized derivatives of cholesterol) have been implicated in AMD pathogenesis. The aim of our study was to highlight amyloid β/oxysterols relationship and to describe P2X7 receptor-pannexin-1 role in oxysterols toxicity. Using retinal epithelial cells, we first quantified sterols levels after amyloid β incubation and second we investigated the cytotoxic effects induced by oxysterols. For the first time, our results showed that amyloid β induced oxysterols formation in human retinal pigmented epithelial cells. We showed that oxysterol toxicity is mediated by P2X7 receptor activation. This activation was dependent on pannexin-1 with 25-hydroxycholesterol whereas P2X7 receptor signaling pathway was pannexin-1-independent for 7-ketocholesterol. Taken together our data suggest a pivotal role of P2X7 receptor-pannexin-1 in oxysterols toxicity in retinal cells which could be an important target to develop new treatments for AMD

1. Introduction

Age-related macular degeneration (AMD) is the most common cause of severe vision loss worldwide with 25–30 million affected persons [1]. The prevalence of this disease increases with age and lifespan extension tends to worsen this issue [2]. Consequently, this disease is considered as a major public health challenge.

One of the clinical signs of this multifactorial disease is the accumulation of drusen in the retina between retinal pigmented epithelium (RPE) and Bruch's membrane [3]. Drusen are extracellular proteolipidic deposits, which contain amyloid β peptide as the main component. Moreover, other components such as 7-ketocholesterol (7-KC), an auto-oxidized form of cholesterol (oxysterol) have been detected in human drusen [4,5]; consequently, other oxysterols are suspected to be associated to AMD [4,6,7]. In an interesting manner, cholesterol is the only precursor of oxysterols and hypercholesterolaemia was described as a risk factor to develop AMD [8]. Therefore, it is reasonable to expect high levels of oxysterols in case of hypercholesterolaemia and then AMD. In the literature, the product of cholesterol autoxidation 7-βhydroxycholesterol (7-β) and the cholesterol enzymatically derived 25-hydroxycholesterol (25-OH) and 27-hydroxycholesterol (27-OH) are known for their association to AMD [9,10]. Therefore, these compounds are more and more described as putative biomarkers of degenerative diseases [11,12].

The main component in drusen, amyloid β aggregated form, is well-known as an inducer of oxidative stress, cell death and apoptosis [13–15]. Moreover, P2X7, a cell death purinoreceptor, has been related to amyloid β peptide toxicity in Alzheimer's disease models [16,17] and AMD models [18]. Interestingly, oxysterols are involved in apoptosis [19,20] but no data have been reported regarding the relationship between oxysterols and P2X7 receptor.

Abbreviations: 7-KC, 7-ketocholesterol; 7-β, 7-βhydroxycholesterol; 24-OH, 24-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; 27-OH, 27-hydroxycholesterol; AMD, Age-related Macular Degeneration; HRMS, High Resolution Mass Spectrometry; ROS, Reactive Oxygen Species; RPE, Retinal Pigmented Epithelium; UPLC, Ultra-Performance Liquid Chromatography.

* Corresponding author.
E-mail address: patrice.rat@parisdescartes.fr (P. Rat).

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Activation of P2X7 receptor triggers the formation of large nonselective membranes pores which results in inflammation through the inflammasome, oxidative stress and, ultimately, cell death especially by apoptosis [21,22]. The formation of these death pores probably require other components and it has been described that pannexin-1, previously associated to amyloid β peptide toxicity [23], could be one of the involved proteins [24].

As amyloid β is a key factor in AMD pathogenesis and oxysterols are thought to be biomarkers of this pathology, the aim of our study was first to highlight the amyloid β/oxysterols relationship and second to describe P2X7-pannexin-1 receptor involvement in oxysterols toxicity on RPE cells.

2. Materials and methods

2.1. Preparation of aggregated amyloid β (1–42) peptide

Amyloid β (Bachem, Bubendorf, Switzerland) was dissolved in double-distilled water to a final concentration of 500 μM. Amyloid β fibrillation was performed by heating at 37 °C this solution for 6 days in a waterbath according to Perini's protocol [25]. Aggregation of amyloid β was checked using the thioflavin T binding assay [26] (data not shown).

2.2. Cell culture

The human RPE cell-line ARPE-19 (ATCC® CRL-2302™), widely used in AMD *in vitro* studies, was grown in DMEM/F12 (volumetric ratio of 1:1, Eurobio, Courtaboeuf, France) supplemented with 10% fetal calf serum, 2 mM of glutamine, 50 IU/mL of penicillin and 50 IU/mL of streptomycin (Eurobio) in a 37 °C incubator with 5% CO_2. When cells reached confluency, cell dispersion was done using trypsin (Eurobio) and cells were counted. The cellular suspension was diluted and seeded in flasks to reach subconfluency for mass spectrometry analysis or seeded either in 96-well or in 6-well microplates to reach subconfluency for cytometric assays. In the first experiment, cells were incubated with aggregated amyloid β at 25 μM, as previous studies used micromolar amyloid β concentrations in ARPE-19 and MIO-M1 retinal cell lines to mimic *in vitro* AMD [15,18]. In further experiments, cells were incubated with 7-ketocholesterol (7-KC, Avanti Polar Lipids, Alabaster, AL), 27-hydroxycholesterol (27-OH, Avanti Polar Lipids, Alabaster, AL) or 25-hydroxycholesterol (25-OH, Sigma-Aldrich, Saint Louis, MO) at final concentrations ranging from 1 μM to 100 μM for 48 h, as previous experiments used micromolar concentrations to study oxysterols cellular effects on ARPE-19 cells [10,27]. For stock solutions, oxysterols were dissolved in absolute ethanol. Probenecid (Sigma-Aldrich), a pannexin-1 inhibitor [28], was used at a final concentration of 1 mM in oxysterol solution for 48 h.

2.3. Cell necrosis evaluation

Cell necrosis was evaluated through lactate dehydrogenase (LDH) quantitation. LDH is a cytoplasmic enzyme, which is released in the extracellular compartment in case of membrane damage. The extracellular rate of lactate dehydrogenase has been correlated with cell death [29]. LDH mixture was prepared according to manufacturer instructions (Sigma-Aldrich). 50 μL of cell supernatant were added to 50 μL of LDH mixture, and plate was incubated under agitation for 30 min at room temperature. Reaction was stopped with 10 μL HCL 1N and absorbance was read at 490 nm (λref = 690 nm) using a cytometer (Safire, Tecan, Männedorf, Switzerland).

2.4. Sterols quantification

Quantification of sterols in human RPE cells was performed according to the method developed by Ayciriex et al. [30] using ultra-performance liquid chromatography–high resolution mass spectrometry analysis (UPLC-HRMS) after a special derivation by isocyanate to improve electrospray ionization (ESI).

ARPE-19 cells were incubated with, or without, 25 μM amyloid β in flasks. After 48 h, cells were dispersed with trypsin and rinsed. Cell pellets were dissolved in 600 μL double-distilled water, vortexed for 30 s and sonicated for 5 min. Sterols were extracted with hexane/methanol mixture (7:1, v/v) under agitation for 40 min and dried under reduce pressure. Sterols were derivatized into carbamate using a solution of 4-(dimethylamino) phenyl isocyanate in dichloromethane. Dichloromethane was evaporated under reduced pressure, derivatized sterols were resuspended in an acetonitrile/isopropanol mixture (1:1, v/v) and analyzed using UPLC-ESI-HRMS on a Synapt™ G2 HDMS™ mass spectrometer (Waters MS Technologies, Manchester, UK). Oxysterols levels were normalized to protein content measured by BCA method.

2.5. Cell morphology observation

After a 48-h incubation time with oxysterols, non-fixed cells were observed under optical microscope (Leica DMIRB, Wetzlar, Germany). Representative pictures were taken using Nikon Coolpix camera (Nikon, Shinjuku, Japan).

2.6. P2X7 pore formation assessment

YO-PRO-1 was first used to discriminate cells dying by apoptosis versus necrosis with flow cytometry [31]. The YO-PRO-1 probe only enters into cells after P2X7 receptor activation-induced pore opening, and binds to DNA emitting fluorescence. A 1 mM YO-PRO-1 (Life Technologies, Saint Aubin, France) stock solution was diluted at 1/500 in PBS just before use to be distributed in each well (200 μL/well). After a 10-min incubation period at room temperature, fluorescence signal was read (λex = 491 nm, λem = 509 nm) using a cytofluorometer (Safire, Tecan) [32,33].

2.7. Chromatin condensation evaluation

The UV fluorescent probe Hoechst 33342 (Life Technologies) enters living and apoptotic cells, intercalating into DNA. The fluorescent signal is proportional to chromatin condensation as in apoptosis. A 10 μg/mL Hoechst 33342 solution was distributed into wells (200 μL/well). After a 30-min incubation period at room temperature, the fluorescence signal was read (λex = 350 nm, λem = 450 nm) using a cytofluorometer (Safire, Tecan) [34].

2.8. Cell oxidative stress study

2.8.1. Reactive oxygen species (ROS) production: H2DCF-DA assay

Intracellular ROS were measured using 2′,7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Life Technologies) [35], which is hydrolyzed by cell esterases in 2′,7'-dichlorodihydrofluorescein and oxidized by ROS in highly fluorescent 2′,7'-dichlorofluorescein. A 10 μM solution of H2DCF-DA was distributed into wells (200 μL/well). After a 20-min incubation period at 37 °C, the fluorescence signal was read (λex = 485 nm, λem = 535 nm) using a cytofluorometer (Safire, Tecan) [36].

2.8.2. Lipid peroxidation evaluation: thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation was evaluated using TBARS assay: