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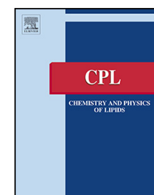

25-Hydroxycholesterol induces both P2X7-dependent pyroptosis and caspase-dependent apoptosis in human skin model: New insights into degenerative pathways

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ABSTRACT

Degenerative diseases are characterized by both cell death and inflammation, which involve different pathways such as apoptosis and pyroptosis. Oxysterols, oxidized derivatives of cholesterol, are known to act as key actors in degenerative disorders such as skin photoaging. We hypothesize that oxysterols could be implicated in either apoptosis or pyroptosis, or both. The aim of our study was first to quantify oxysterol levels in keratinocytes as a function of aging and UV irradiation. Second, we evaluated the effects of 25-OH oxysterol on apoptosis and pyroptosis hallmarks in keratinocytes. Our results showed that 25-OH exhibited an increasing after UV irradiation, highlighting the pivotal role of this oxysterol in skin degeneration. In our model, 25-OH induced not only caspases-dependent apoptosis associated to granzyme B release but also P2X7 receptor-dependent pyroptosis in skin cells. 25-OH seems to be at the origin of the main toxic pathways responsible for degenerative disorders; therefore, it could be the target of antidegenerative treatments, opening new potential therapeutic strategies.
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1. Introduction

In degenerative diseases, the disintegration of cells can be due to different pathways such as apoptosis, pyroptosis or necroptosis as it is evidenced by both cell death and inflammation hallmarks (Ardeljan et al., 2014). Inappropriate apoptotic processes are observed in many degenerative diseases (Elmore, 2007): neuronal cell apoptosis in neurodegenerative process (Mattson, 2000; Rohn, 2010), retinal pigmented epithelial cell apoptosis in age-related macular degeneration (Dunaief et al., 2002), endothelial cell apoptosis in atherosclerosis (Mitchinson et al., 1996) and melanocyte apoptosis in vitiligo (Ruiz-Argüelles et al., 2007). Apoptosis is known as one of the major cell death pathways (Duprez et al., 2009); the underlying mechanisms are highly complex and can involve numerous proteases such as the well-known cysteine proteases of the caspase family and/or the serine protease granzyme B (Froelich et al., 2004). The initiation of apoptosis can be triggered by cell death receptors including the P2X7 cell death receptor, which plays a pivotal role in degenerative disorders (Le Feuvre et al., 2002; Marcellino et al., 2010; Olivier et al., 2016b; Reichenbach and Bringmann, 2016; Wakx et al., 2015). Contrary to apoptosis, pyroptosis is a cell death pathway linked to inflammation (Cookson and Brennan, 2001). Pyroptosis depends on the activation of caspase-1 (Fink and Cookson, 2005), one of the key components of the inflammasome, which directly interacts with the P2X7 receptor (Di Virgilio, 2007). Formation of the inflammasome has been observed during the pathogenesis of Alzheimer disease (Liu and Chan, 2014), age-related macular degeneration (Celkova et al., 2015) and vitiligo (Marie et al., 2014), along with the release of several cytokines such as IL-1α, IL-6, IL-8 and MIF (Cascella et al., 2014; Hüll et al., 1996; Li et al., 2015a; Nassar et al., 2015; Yu et al., 1997). Necroptosis is a sort of necrosis but mediated by programmed caspase-independent pathways.

Abbreviations: 27-OH, 27-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; 24-OH, 24-hydroxycholesterol; 7β-OH, 7-βhydroxycholesterol; 7-KC, 7-ketocholesterol; CHO, cholesterol; DMEM, Dulbecco's modified Eagle's medium; ESI, electrospray ionization; HRMS, high resolution mass spectrometry; MIF, macrophage migration inhibitory factor; PBS, phosphate buffered saline; UPLC, ultra-performance liquid chromatography.

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Necroptosis has been proposed as a major mechanism for retinal pigmented epithelial cell death in age-related macular degeneration (Hanus et al., 2015; Murakami et al., 2014).

Oxysterols, derived from cholesterol oxidation, play a key role in several degenerative diseases in the brain, the eye and the blood circulation: Alzheimer disease, Parkinson disease, age-related macular degeneration, atherosclerosis (Björkhem et al., 2009, 2013; Brown and Jessup, 1999; Lordan et al., 2009; Olivier et al., 2016b; Rodríguez and Larrayoz, 2010; Zarrouk et al., 2014). In the skin, Bellei et al. demonstrated that vitiligo melanocytes had higher amounts of oxysterols, in particular 7-beta-hydroxycholesterol and 7-ketocholesterol (Bellei et al., 2013) and we previously showed on a cell-based model that the level of 25-hydroxycholesterol is increased after UV irradiation (Olivier et al., 2016a). As a result, we hypothesize that oxysterols could be related to skin degenerative disorders such as photoaging or vitiligo.

The aim of our study was to assess the changes in sterol levels in keratinocytes from young and elderly donors irradiated or not and further to evaluate the cellular effects of 25-OH oxysterol on apoptosis and pyroptosis hallmarks on human keratinocytes.

2. Materials and methods

2.1. Cell culture

2.1.1. Primary cell culture

Normal Human Epidermal Keratinocytes (NHEK) from young and elderly donors were purchased from PromoCell (Heidelberg, Germany) and Tebu bio (Le Perray en Yvelines, France). Primary keratinocytes were isolated from foreskin of two young donors (2 and 3 years old) and from back and face skin of three elderly donors (between 64 and 83 years old). Primary cells were cultured in Keratinocyte Growth Medium 2 (PromoCell) supplemented with 50 IU/mL of penicillin and 50 IU/mL of streptomycin (Eurobio, Courtaboeuf, France). NHEK cultures were maintained in a cell culture incubator (37 °C, 95% humidity, 5% CO_2). When NHEK reached 70–80% confluency, they were dispersed using trypsin, counted and seeded at 200,000 cells/mL in flasks for sterol quantitation and incubated for a week. Every three days, culture medium was changed. Primary cultures were used at passages under 6.

2.1.2. HaCaT cell culture

HaCaT cells, spontaneously transformed human keratinocytes, were obtained from Cell lines service (Cell lines service-CLS-Germany). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Eurobio) supplemented with 10% fetal calf serum, 2 mM of glutamine, 50 IU/mL of penicillin and 50 IU/mL of streptomycin (Eurobio). HaCaT cultures were maintained in a cell culture incubator (37 °C, 95% humidity, 5% CO_2). When the HaCaT cells reached confluency, they were dispersed using trypsin and counted. The cellular suspension was diluted and seeded in 96-well microplates at a cellular density of 80,000 cells/mL and incubated for 24 h.

2.2. UV irradiation experiments

UV irradiation was performed on primary keratinocytes (NHEK) with a solar light simulator Suntest CPS+ (Atlas, Mount Prospect, IL, USA). This simulator equipped with a xenon arc lamp and special glass filters restricting transmission of light below 290 nm provides irradiance that approximates sunlight. NHEK were seeded in flasks and irradiated at a dose of 2.5 J/cm^2 as we previously described (Olivier et al., 2016a). The cells were subsequently rinsed and incubated for 24 h in culture medium. Non-irradiated cells were used as control.

2.3. Sterols quantitation

Quantitation of sterols in human primary keratinocytes (NHEK) was performed according to the method developed by Ayciriex et al. (2012) using ultra-performance liquid chromatography–high resolution mass spectrometry analysis (UPLC-HRMS). After cell dispersion using trypsin and centrifugation, cell pellets were dissolved in 600 μL double-distilled water, vortexed for 30 s and sonicated for 5 min. Sterols were extracted with a hexane/methanol mixture (7:1, v/v) under agitation for 40 min and dried under reduced 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 dissolved in an acetonitrile/isopropanol mixture (1:1, v/v) and analyzed using UPLC-ESI-HRMS on a Synapt^{TM} G2 HDMS^{TM} mass spectrometer (Waters MS Technologies, Manchester, UK). Oxysterol levels were normalized to protein content measured by BCA method.

2.4. Keratinocytes incubation with 25-hydroxycholesterol

HaCaT cells were incubated with 25-hydroxycholesterol (25-OH, Sigma-Aldrich, Saint Louis, MO, USA) for 48 h. 25-OH was dissolved in absolute ethanol to obtain a 40 mM stock solution. The stock solution was sonicated to solubilize oxysterol. 25-OH was diluted in culture medium to obtain targeted concentrations ranging from 5 to 40 μM. These concentrations were chosen according to our previous results (Olivier et al., 2016a).

2.5. Apoptosis

2.5.1. Chromatin condensation

Chromatin condensation was evaluated using the Hoechst 33342 assay (ThermoFisher Scientific, Illkirch, France). Hoechst 33342 UV fluorescent probe enters and intercalates into DNA in living and apoptotic cells. The fluorescent signal is proportional to chromatin condensation. A 10 μg/mL Hoechst 33342 solution was distributed into the wells of the microplate and the fluorescence signal was read after a 30-min incubation time at room temperature (λex = 350 nm, λem = 450 nm) using Safire cytofluorometer (Tecan, Männedorf, Switzerland).

2.5.2. Caspases 3 and 8 activity

Caspases 3 and 8 activities were respectively determined using CASP3F and CASP8F fluorimetric assay kits (Sigma-Aldrich). According to manufacturer's instructions, the cells were incubated with lysis buffer on ice for 20 min. Then, Ac-DEVD-AMC substrate for caspase 3 detection or Ac-IETD-AMC substrate for caspase 8 detection was added in each well. After a 1-h incubation at room temperature, fluorescence was read (λex = 360 nm, λem = 460 nm) using Safire cytofluorometer (Tecan).

2.5.3. Granzyme B release

Granzyme B release in cell supernatants was quantified using an enzyme-linked immunoassay (Human Granzyme B ELISA kit, Abcam, Cambridge, United Kingdom). Manufacturer's instructions were followed to perform the assay. Absorbance was scanned (λabs = 450 nm, λref = 620 nm) using Safire cytometer (Tecan).

2.5.4. P2X7 receptor activation

P2X7 cell death receptor activation was evaluated using the YO-PRO-1 assay. YO-PRO-1 probe only enters into cells after P2X7 receptor activation-induced pore opening, and binds to DNA,

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