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Effects of cholesterol oxides on cell death induction and calcium increase in human neuronal cells (SK-N-BE) and evaluation of the protective effects of docosahexaenoic acid (DHA; C22:6 n-3)

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ABSTRACT

Some oxysterols are associated with neurodegenerative diseases. Their lipotoxicity is characterized by an oxidative stress and induction of apoptosis. To evaluate the capacity of these molecules to trigger cellular modifications involved in neurodegeneration, human neuronal cells SK-N-BE were treated with 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, 6α- and 6β-hydroxycholesterol, 4α- and 4β-hydroxycholesterol, 24(S)-hydroxycholesterol and 27-hydroxycholesterol (50–100 μM, 24 h) without or with docosahexaenoic acid (50 μM). The effects of these compounds on mitochondrial activity, cell growth, production of reactive oxygen species (ROS) and superoxide anions ($O_2^{\cdot-}$), catalase and superoxide dismutase activities were determined. The ability of the oxysterols to induce increases in Ca^{2+} was measured after 10 min and 24 h of treatment using fura-2 videomicroscopy and Von Kossa staining, respectively. Cholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, and 24(S)-hydroxycholesterol (100 μM) induced mitochondrial dysfunction, cell growth inhibition, ROS overproduction and cell death. A slight increase in the percentage of cells with condensed and/or fragmented nuclei, characteristic of apoptotic cells, was detected. With 27-hydroxycholesterol, a marked increase of $O_2^{\cdot-}$ was observed. Increases in intracellular Ca^{2+} were only found with 7-ketocholesterol, 7β-hydroxycholesterol, 24(S)-hydroxycholesterol and 27-hydroxycholesterol. Pre-treatment with docosahexaenoic acid showed some protective effects depending on the oxysterol considered. According to the present data, 7-ketocholesterol, 7β-hydroxycholesterol, 24(S)-hydroxycholesterol and 27-hydroxycholesterol could favor neurodegeneration by their abilities to induce mitochondrial dysfunctions, oxidative stress and/or cell death associated or not with increases in cytosolic calcium levels.

1. Introduction

Atherosclerosis and neurodegenerative diseases, in particular Alzheimer's disease (AD), are the most prevalent pathologies in elderly. In addition to extracellular deposition of Aβ in senile plaques and intracellular accumulation of hyperphosphorylated Tau (Tubulin Associated Unit) in neurofibrillary tangles (NFT) [1], AD is characterized by neuron degeneration and synaptic loss [2,3]. Induction of cell death via generation of oxidative stress is a strongly supported hypothesis explaining neuronal loss. It is tempting to speculate that oxysterols, derived either from autoxidation or enzymatic oxidation of cholesterol, could play critical roles during this process [4]. Autoxidation of cholesterol on the B hydrocarbon ring leads to the formation of 7β-hydroxycholesterol (7β-OHC), 7α-hydroxycholesterol (7α-OHC) and 7-ketocholesterol (7KC). The microsomal cytochrome P450 system is responsible for the enzymatic generation of endogenous oxysterols, including hepatic 7α-OHC (catalyzed by CYP7A1), 24(S)-hydroxycholesterol (24S-OHC) in brain and retina (catalyzed by CYP46 A1) [5], and 27-hydroxycholesterol (27-OHC) in most tissues (catalyzed by CYP27A1). 4β-hydroxycholesterol (4β-OHC) is formed through

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CYP3A4 and CYP3A5 activity [6]. An enzymatic conversion of 7α-OHC into 7KC has also been reported in hamster and chicken microsome preparations [7]. 6α-hydroxycholesterol (6α-OHC) and 6β-hydroxycholesterol (6β-OHC) are derivatives in the reaction of cholesterol with singlet oxygen via ene addition [8].

It is well established that some oxysterols, especially those oxidized on the lateral chain, are involved in the regulation of cholesterol homeostasis [9]. Some of them, mainly those oxidized on C7, have been demonstrated to be cytotoxic and to have pro-inflammatory activities [10]. Currently, there is increasing evidence supporting the involvement of 24S-OHC, 27-OHC, 7KC and 7β-OHC in numerous degenerative diseases such as AD. An increase in both 27-OHC and 24S-OHC has been shown in the frontal cortex of AD brains [11]. A growing interest in oxysterol-induced toxicity is remarked over the last decades with the purpose of elucidating the exact mechanism of oxysterol cytotoxicity. Treatment of neuroblastoma cells with 27-OHC and 24S-OHC, at concentrations detected in AD and normal brain, demonstrated an up-regulation of Amyloid Precursor Protein (APP), β-secretase expression and activity, as well as a marked increase in production of Aβ1-42 [11,12].

Early modifications of the cytoplasmic membrane, calcium influx, and overproduction of oxygen radicals seem to play important roles in oxysterol-induced cell death. In fact, it has been described that treatment with 7KC and 7β-OHC induce important oxidative processes on various cell types [9,13,14]. Thus, in 7KC-treated U937 cells, a decrease of reduced glutathione associated with an increased production of Reactive Oxygen Species (ROS) has been reported [15]. In human aortic smooth muscle cells, 7KC also induces oxidative stress and it has been demonstrated that this specific effect of 7KC is mediated by an up-regulation of Nox-4, an homologue of NAD(P)H oxidase responsible of ROS-generation [14].

Furthermore, oxidative stress induced by 7KC, 7β-OHC, and 27-OHC is associated with cell death on numerous cell types. Oxysterols can induce cell death by necrosis [16,17], necroptosis [18], apoptosis [19] or by apoptosis associated with autophagic characteristics (oxiapoptophagy), as reported in U937 cells, human aortic smooth muscle cells, and 158 N murine oligodendrocytes treated with 7KC [13,20–22]. On the other hand, it has been shown that oxysterols induced apoptosis via the mitochondrial pathway: release of cytochrome c from mitochondria, activation of caspase 9, which subsequently activates caspase 3, leading to internucleosomal DNA fragmentation [23,24]. It has also been reported that oxysterol-induced apoptosis was preceded by intracellular modifications of calcium levels, considered as the first step of apoptosis [25–27]. Increases in calcium levels may activate enzymes such as the calcium dependent endonucleases and calcium dependent proteases, implicated in apoptosis and necrosis, respectively. Furthermore, increased calcium concentrations could cause membrane depolarization and opening of voltage-dependent channels, resulting in increased ROS and cellular death [28]. Supporting evidence for this link between calcium homeostasis and ROS are data showing that cells treated with glutamate had increased levels of DCF fluorescence, which is dependent on NMDA receptor activation and Ca^{2+} entry [29,30].

In the present study, we investigated the different neurotoxic processes elicited by oxysterols produced through enzymatic or autoxidation of cholesterol, on the human neuroblastoma cell line SK-N-BE, in order to evaluate the ability of these molecules to trigger cellular modifications involved in neurodegeneration. We tested the influence of cholesterol, 7KC, 7α-OHC, 7β-OHC, 6α-OHC, 6β-OHC, 4α-OHC, 4β-OHC, 24S-OHC and 27-OHC on SK-N-BE cells; we recorded cell viability, generation of ROS and antioxidant enzymes activities. In addition, we evaluated nuclear morphology, increases in intracellular calcium as well as caspase-3 activation, and cleavage of LC3 to LC3 I and LC3 II, with the aim of determining the type of cell death involved in this process. Reduced intrinsic synthesis of docosahexaenoic acid (DHA, C22:6 n-3) with aging, and its protective effects on the prevention of neuronal degeneration [31,32] lead us to use DHA at a physiological concentration for the purpose of counteracting oxysterol–cytotoxicity.

2. Materials and methods

2.1. Cells cultures, and cell treatments

Human neuronal cells (SK-NB-E) were seeded at 5000–10,000 cells/cm^2 either in 96 well plates or 24-well microplates and cultured in Dulbecco's Modified Eagle Medium with L-glutamine (DMEM) (Lonza) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Pan Biotech) and 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) (Pan Biotech). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and passaged twice a week. At each passage, SK-NB-E cells were trypsinized with a (0.05% trypsin-0.02% EDTA) solution (Pan Biotech).

Initial solutions of oxysterols [(7KC, 7β-OHC, 7α-OHC, and 27-OHC) (Biovalley SA/Research Plus Corporation); (6α-OHC, 6β-OHC, 4α-OHC, 4β-OHC, and 24S-OHC) (provided by Prof. M. Samadi, University of Lorraine, Metz, France); cholesterol (Sigma)] were prepared as previously described [15]. After 24 h of culture, subconfluent SK-N-BE cells were incubated with 7KC, 7β-OHC, 7α-OHC, 6α-OHC, 6β-OHC, 4α-OHC, 4β-OHC, 24S-OHC or 27-OHC (50–100 μM) for 24 h. The concentrations of oxysterols were chosen in the range of those previously shown to induce cell death on numerous cell types [33,34]. When SK-N-BE cells were simultaneously treated with oxysterols in the presence of docosahexaenoic acid (DHA: 50 μM) (Sigma), this fatty acid was added 2 h prior to oxysterols. DHA (Sigma–Aldrich) was solubilized in α-cyclodextrin (Sigma–Aldrich) as described [35]. The final concentration of α-cyclodextrin (vehicle) in the culture medium was 125 μM.

2.2. Cell Counting with Trypan Blue

SK-N-BE cells were seeded 24 h before treatment on 24-well plates. After 24 h of treatment with oxysterols (50–100 μM) with or without DHA (50 μM), the supernatant of each well was collected and the adherent cells collected after trypsinization with a Trypsin/EDTA solution (0.05% trypsin-0.02% EDTA). These suspensions were homogenized. Live and dead cells were counted in a hemacytometer via trypan blue exclusion under an inverted-phase contrast microscope Diaphot (Nikon).

2.3. Evaluation of cell growth and/or mitochondrial activity with the colorimetric MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was carried on SK-N-BE cells using Cell proliferation kit (Roche Applied science). Cells were seeded in 96-well plates and were treated for 24 h with oxysterols (50–100 μM) with or without DHA (50 μM). This assay was used to evaluate the effects of oxysterols on mitochondrial activity and/or cell growth. MTT assay is based on the conversion of MTT to formazan by mitochondrial succinate dehydrogenase in the metabolically active cells [36].The amount of blue color formed was determined by measuring absorbance at 570 nm.

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