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7-Ketocholesterol and 5,6-secosterol induce human endothelial cell dysfunction by differential mechanisms

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ABSTRACT

7-Ketocholesterol and 5,6-secosterol are cholesterol autoxidation products generated under oxidative stress by two distinct mechanisms. They are present in atherosclerotic plaques and are candidate players in the disease initiation and progression. While 7-ketocholesterol effects at cellular level, in particular apoptosis, are well known and reported on diverse cell lines, 5,6-secosterol is a recently discovered oxysterol with relatively few reports on the potential to affect endothelial cell functions. Endothelial cells have a central role in cardiovascular disease as they provide the barrier between blood and the vessel wall where atherosclerosis starts and progresses. Insults to endothelial cells provoke their dysfunction favoring pro-atherogenic and pro-thrombotic effects.

In the present work, we tested 7-ketocholesterol and 5,6-secosterol on endothelial cells – focusing on apoptosis and the associated mitochondrial/lysosome alterations – and on endothelial function using the in vitro model of arterial relaxation of aortic rings. Our data provide evidence that 7-ketocholesterol and 5,6-secosterol are efficient instigators of apoptosis, which for 5,6-secosterol is associated to PKC and p53 up-regulation. In addition 5,6-secosterol is a potent inhibitor of endothelial-dependent arterial relaxation through PKC-dependent mechanisms. This may contribute to pro-atherogenic and pro-thrombotic mechanisms of 5,6-secosterol and highlights the role of cholesterol autoxidation in cardiovascular disease.

1. Introduction

Oxysterols are oxidized derivatives of cholesterol generated either by enzymatic or autoxidation mechanisms [1]. Cholesterol autoxidation proceeds via two distinct pathways, a free radical pathway driven by a chain reaction mechanism (type I) and a non-free radical pathway (type II), which is driven stoichiometrically by highly reactive oxygen species [2].

A number of studies have identified autoxidation-type I oxysterols in human atherosclerotic plaques, including 7-ketocholesterol (7-KC) and 7β-hydroxycholesterol [3,4]. Oxysterols have been demonstrated to have a number of biological actions that may be important for the development of atherosclerosis, for example they perturb cholesterol biosynthesis and are cytotoxic or induce apoptosis in endothelial cells, smooth muscle cells, macrophages and lymphocytes [3], and impair endothelial function, including endothelial-dependent arterial relaxation [5].

In the last decade, considerable interest has emerged in autoxidation-type II oxysterols because these compounds have been shown formed *in vivo*, including in the atherosclerotic plaque [6], and are bioactive [2]. The most studied compound arising from type II cholesterol autoxidation, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al, namely, 5,6-secosterol (SECO) – has been shown to alter the biophysical properties of phosphatidyethanolamine membranes, which is suggested to result in pathological damage to cell membranes [7], induce chemotaxis of cultured macrophages, up-regulate E-selectin on endothelial cells [8], induce apoptosis in cardiomyblasts [9], and modulate stress-activated mitogen-activated protein kinases in liver cells [10].

Considering that the prototypical type I and type II cholesterol autoxidation products in the atherosclerotic plaque, i.e., 7-KC and

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SECO, are structurally different and are expressed in different concentration in plaque – 7-KC, 17–117 nmol/g vs. SECO, 6.8–61.3 nmol/g [6,11] – we hypothesized that the two compound have different mechanism of action and potency. In the present study, we used human endothelial cells and the model of perfused aortic rings to compare the effects of 7-KC and SECO on apoptosis and function of endothelial cells.

2. Materials and methods

2.1. Materials

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (LGC Standards, Milan, Italy). AnnexinV (Annex-V)/propidium iodide (PI) kit was from Immunostep (Salamanca, Spain). MitoTracker Red CMXRos (MTRC), LysoTracker green (LTG) were from Molecular Probes (Eugene, OR). FITC-conjugated antip53 mAb (clone DO-7) and Cytofix/Cytoperm were from BD Pharmingen (Milan, Italy), anti-PKC α mAb (clone Y143) was from Novus Biologicals (Cambridge, UK), anti-phospho ERK and anti-phospo-p38 were from Cell Signalling Technology (Leiden, The Netherlands). Horseradish-peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad (Milan, Italy). 7-ketocholesterol, chelerythrine, 10-nonyl acridine orange (NAO), Nile Red (NR) were purchased from Sigma–Aldrich (Milan, Italy). FITC goat-antimouse IgG was from Chemicon–Millipore (Vimodrone, Italy). 5,6-Secosterol (3β-hydroxy-5-oxo-5,6-secocholestan-6-al) was synthesized by cholesterol ozonization according to Wang [12]. Identy was verified by mass spectrometry on a HCT plus instrument (Bruker Daltonics, Bremen, Germany). 5,6-secosterol is actually a mixture of 5,6-secosterol and its aldol condensation product, 3β-hydroxy-5α-hydroxy-B-norcholestan-6β-carboxaldehyde. All other reagents were of the highest grade available from Sigma–Aldrich (Milan, Italy).

2.2. Cell culture and treatment

Cells were cultured in 25 cm^2 flasks in, EndoGRO-LS (Millipore, USA), supplemented with low serum medium for human endothelial cells, L-Glutamine (100 mM) and 1% antibiotics (penicillin, streptomycin). The cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C. At 50% confluence cells were detached with trypsin–EDTA, washed and sub-cultivated in new flasks for 1–2 days before the experiments.

7-KC and SECO were dissolved in ethanol. Cells were incubated at 37 °C with different concentrations of 5,6 secosterol (SECO) and 7-ketocholesterol (1, 5, 10, 20 μM f.c.) or vehicle ethanol (0.1% f.c.) for 6, 12, or 24 h. At the end of the incubation time, cells were washed with PBS and stained with flurophores or resuspended with RIPA buffer for protein extraction.

2.3. Assessment of apoptosis

Viable, apoptotic and necrotic cells were evaluated by using the fluorescent probes AnnexinV (Annex-V) and propidium iodide (PI) [13]. Annex-V fluorescence is a standard method for measuring the amount of phosphatidylserine exposed on the outer face of the plasma membrane, following caspases activation, as an indicator of intermediate events of apoptosis. Fluorescence of the nucleic acid dye PI is a measure of most advanced stages of apoptosis and even necrosis, since it is not permeant to live cells. Briefly, cells (1×10^6/ml) were resuspended in binding buffer (1×) and stained with Annex-V Fitc and PI according to the manufacturer's instructions. Cytometric experiments were carried out with a FaCScalibur flow cytometer (Becton Dickinson, Palo Alto, CA) equipped with an argon laser (Ex 488 nm). Analyses were performed with a CellQuestTM (BD Biosciences, Milan, Italy) and DiVA software.

2.4. Analyses of mitochondria network

Mitochondria were studied by fluorescence labeling with 10-nonyl-acridine orange (NAO) and MitoTracker Red CMXRos (MTRC). NAO uptake, which binds to mitochondrial membrane-bound cardiolipin, reflects total mitochondrial mass independently of function [14]. MTRC passively diffuse across the plasma membrane of live cells and accumulates in active mitochondria [15]. Cells (1×10^6) were directly labeled in six well plate with NAO (100 nM, f.c.) for 30 min at 37 °C in 5% CO_2. At the end of incubation, cells were trypsinized, washed, and subjected to flow cytometry analysis. For mitochondria confocal live imaging, cells were grown on MatTek glass bottom chambers (MatTek corporation, Ashland, MA), stained with MTRC (500 nM, f.c.) and incubated 20 min at 37 °C. Red (FL3) fluorescence emission (Ex 543 nm) was measured with a Leica TCS SP5 II (Leica Microsystem, Milan, Italy) instrument.

2.5. Labeling of lysosomes

The acidotropic dye LysoTracker green (LTG) is freely permeant to cell membranes and typically concentrate with high selectivity in lysosomes [16]. LTG was diluted in EndoGRO-L to obtain a working concentration of 50 nM. After specific treatments with oxysterols, cells were incubated with LTG containing medium for 45 min at 37 °C. At the end of incubation, the cells were rinsed and re-suspended in fresh pre-warmed medium. LTG fluorescence of 10,000 cells per sample was determined by flow cytometry using the FL1 channel. For confocal analysis cells were grown on MatTek glass bottom chambers and labeled with LTG (500 nM, f.c.) [17]. Green (FL1) fluorescence emission (Ex 488 nm) was measured with a Leica TCS SP5 II (Leica Microsystem, Milan, Italy) instrument.

2.6. Nile Red and flow cytometric analysis of lipid content

Nile Red (NR) is a phenoxazine dye used on live cells to localize and quantify neutral and polar lipids. NR stains neutral lipids yellow (emission > 528) and polar lipids orange–red (emission > 590) when excited at 488 nm [18,19]. NR was diluted in dimethylsulfoxide to obtain 100 μg/ml stock solution. After the specific cell treatment, NR was added to the culture medium (0.1 μg/ml working solution) and incubated with 10^6 cells/ml for 15 min at 37 °C. After detaching, labeled cells were evaluated by flow cytometry. The fluorescence of 10,000 cells per sample was evaluated using FL2 and FL3 channels. For confocal live imaging of lipids, cells were grown on MatTek glass bottom chambers, stained with NR (0.1 μg/ml, f.c.) and incubated 15 min at 37 °C. Yellow (FL2) and red (FL3) fluorescence emission (Ex 488 nm) was measured on a Leica TCS SP5 II (Leica Microsystem, Milan, Italy) instrument.

2.7. Intracellular detection of p53 and PKCα

HUVECs cells were washed in PBS, re-suspended in 250 μl of Cytofix reagent and incubated at 4 °C for 30 min. Cells were then washed with permeabilization buffer, re-suspended in 250 μl of Cytoperm and stained with FITC conjugated anti-p53 mAb (1:10) at 4 °C for 30 min. To evaluate spurious fluorescence to subtract from anti-p53 fluorescence, control samples were labeled with a FITC conjugated isotype control. For each fixed/permeabilized sample, we performed a p53 labeling (FITC-conjugated antip53 mAb). For the unconjugated PKC-α staining cells were harvested, resuspended in 1 ml of PBS and centrifuged at 5 min at 300*g*. Cells were

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