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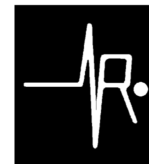

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Original Contribution

Relevance and mechanism of oxysterol stereospecifity in coronary artery disease

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Abstract

Cholesterol oxidation products (oxysterols) are markers for in vitro LDL oxidation. They are potent inducers of programmed cell death and are also found in high concentrations inside atherosclerotic lesions. Among physiologically occurring oxysterols, 7β-OH-cholesterol suggests an increase of lipid peroxidation in vivo. In the underlying study, we quantified free plasma oxysterols by means of gas chromatography in patients with stable coronary artery disease (CAD). Total free plasma oxysterols were elevated more than 2-fold in patients with stable CAD (233 ± 49 vs 108 ± 19 ng/ml, $n = 22$, $P < 0.05$) compared to a control group ($n = 20$) with similar atherogenic risk profile and angiographically normal coronary arteries. We found that 7-ketocholesterol, as well as the β-isomers of epoxide (25.7 ± 10.0 vs 7.3 ± 1.4 ng/ml, $P = 0.07$) and 7β-OH-cholesterol (65.1 ± 15.7 vs 19.4 ± 8.9 ng/ml, $P < 0.01$), was mainly responsible for this increase. To elucidate a potential relevance of oxysterol stereospecificity in regard to endothelial damage, we further conducted in vitro experiments using human arterial endothelial cells (HAECs). Surprisingly, β-isomers exerted an up to 10-fold higher amount of cell death in equivalent doses when compared to α-isomers. The greater cytotoxic potential of β-isomers was due to increased apoptosis, preceded by mitochondrial release of cytochrome *c* with subsequent caspase-3 activation. Stereospecific release of cytochrome *c* depended on the presence of an intact cytoplasmic membrane, hinting at the existence of a putative oxysterol receptor or a direct stereospecific effect on membrane biology. Finally, both isoforms of oxysterols directly released cytochrome *c* only in conjunction with protein containing cytosol and endoplasmatic reticulum. Free plasma oxysterol levels, particularly 7-ketocholesterol, β-epoxide and 7β-OH-cholesterol, are elevated in patients with stable CAD, independent of their LDL cholesterol levels. Due to the highly increased cytotoxicity of oxysterol β-isomers in vitro, they may represent important atherogenic risk factors.
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Keywords: Atherosclerosis; Apoptosis; Human endothelial cells; Oxysterols; Stereospecificity; Cytochrome *c*; Free radicals

Abbreviations: HAEC, human arterial endothelial cell; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2*H*-tetrazolium; α-CE, cholesterol-5α,6α-epoxide; β-CE, cholesterol-5β,6β-epoxide; 7-KC, 7-ketocholesterol; 7α-OH-C, 7α-hydroxycholesterol; 7β-OH-C, 7β-hydroxycholesterol; 25-OH-C, 25-hydroxycholesterol; $InsP_3$, inositol-1,4,5-trisphosphate; HDL, high-density lipoproteins; LDL, low-density lipoproteins; oxLDL, oxidized LDL; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; CAD, coronary artery disease; PCI, percutaneous coronary intervention; MI, myocardial infarction; FCS, fetal calf serum; PMS, phenazine methosulfate; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; Pipes, 1,4-piperazinebis (ethanesulfonic acid); DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; TMS, trimethylsilyl.

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Introduction

Atherosclerosis is the most common cause of death in the Western world, contributing to about 50% of all deaths. Many environmental and genetic risk factors have been identified, including high-fat diet, smoking, lack of exercise, and infectious agents as well as elevated low-density lipoprotein (LDL) or reduced levels of high-density lipoprotein (HDL) serum levels, high blood pressure, metabolic syndrome, and others [1]. The development of atherosclerosis requires several changes in the microenvironment of the arterial wall, such as the entrapment of lipoproteins in the subendothelial layers, the migration of monocytes into the intima, and the formation of foam cells, which represent the very first stage of the atherosclerotic plaque [1–3].

There is accumulating evidence that oxidized LDL (oxLDL) plays a major role in the injury of the endothelium. This has been described as the response-to-injury hypothesis [2]. The cytotoxic effects of oxLDL have been widely attributed to its bioactive compounds, which are oxysterols, lysophospholipids, and fatty acid peroxides. They are found in high concentrations in atherosclerotic plaques, with the oxysterol:cholesterol ratio markedly elevated in plaques compared to normal tissues or plasma [4]. Oxysterols are oxygenated derivatives of cholesterol that play an important physiological role in the cholesterol excretion pathways. They are able to pass cell membranes much faster than cholesterol itself [5]. Their structure is based on the double-steran ring system of cholesterol. It is important to note that the polar groups, the hydroxygen or oxygen residues, can be attached to the steran ring in an α- or β-position, respectively. The goal of this study was (1) to investigate whether oxysterol plasma levels could identify patients with coronary artery disease among those with similar atherogenic risk profile and (2) to elucidate the stereospecific intracellular effects of oxysterols.

Methods

Endothelial cell isolation and culture

Human iliac and renal arteries were obtained from organ donors (approved by the local ethics committee). HAECs were isolated and passaged according to techniques described previously [6]. HAECs were subcultured onto collagen-coated plastic culture dishes (Iwaki Glass, Tokyo, Japan) and grown in an endothelial medium kit (EBM2, Clonetics, San Diego, CA) containing VEGF, hEGF, hFGF, R^3-IGF-1, hydrocortisone, 2% FCS, and 1% penicillin/streptomycin.

Subcultured HAECs were characterized by immunocytochemical stainings with polyclonal antibodies against the von Willebrand factor (Boehringer Mannheim, Mannheim, Germany), and contamination with vascular smooth muscle cells was excluded by additional double stainings using antibodies against smooth muscle α-actin. Monolayers showed characteristic cobblestone morphology. Cells were fed every third day and used for experiments in low passages (2 through 4). DAPI staining (Boehringer Mannheim) was performed in order to exclude contamination with mycoplasma.

Chemicals

Chemicals obtained from Sigma are a water-soluble cholesterol preparation (polyoxyethanyl-cholesteryl sebacat, C1145), 7-ketocholesterol (5-cholesten-3β-ol-7-on, C-2394), cholesterol 5α,6α-epoxide (C-2773), cholesterol 5β,6β-epoxide (C-2648), 7β-hydroxycholesterol (H-6891), 25-hydroxycholesterol (H-1015), 26-hydroxycholesterol (C-6570), and cholestan-3β,5α,6β-triol (C-2523). 7α-hydroxycholesterol (C-6420) was obtained from Steraloids (Steraloids Inc., Newport, RI). Oxysterols were solubilized in 100% acetone, except polyoxyethanyl-cholesteryl sebacat, which was dissolved in H_2O.

Patient population

All patients presented with typical symptoms of HCAD and gave informed consent for participation in this study. Blood samples for determination of oxysterol levels were drawn on admission, preceding coronary angiography. Based on the angiographic results, patients were assigned either to a control (completely normal angiogram, $n = 20$) or stable CAD group (at least one coronary artery demonstrating ≥70% diameter stenosis, $n = 22$). Patients with acute coronary syndrome or hemodynamically nonrelevant coronary stenosis were excluded.

Determination of oxysterol serum levels

Patient's blood samples were anticoagulated by EDTA and centrifuged at 3500 rpm for 10 min at 4°C. Probes were stored at –70°C. Synthetical oxysterols (100 ng 6-ketocholestanol) were added as an internal standard prior to lipid extraction for gas chromatography. Oxysterols were extracted by addition of 5 vol hexane/toluol (1/2), aspiration of the upper phase, and subsequent solid phase extraction by column chromatography (Strata Columns, Phenomenex, Torrance, CA). Cholesterol and other neutral lipids were eluted by 2-propanol/hexane (0.5%) and discarded. Oxysterols were eluted by 2-propanol/hexane (25%). For derivatization of oxysterols into trimethylsilyl derivates (oxysterol-TMS) pyridine and BSTFA were added to dried probes, and samples were incubated at 90°C for 30 min. Oxysterol-TMS derivates were analyzed and quantified using a 15-m capillary column (0.25 mm diameter) (Supelco, Bellefonte, PA) for gas chromatography. DB-1 (Poly(dimethylsiloxan)) was used as unpolar phase. Gas chromatography was run under the following conditions: injector, 260°C; detector, 320°C; oven, 1.5 min at 40°C; temperature programming from 40 to 210°C at 70°C/min rise. After an incubation of 15 s temperature was further raised to 275°C at 2.2°C/min. Oxysterol-TMS concentrations

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