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Review

Implications of cholesterol autoxidation products in the pathogenesis of inflammatory diseases

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ABSTRACT

There is rising interest in non-enzymatic cholesterol oxidation because the resulting oxysterols have biological activity and can be used as non-invasive markers of oxidative stress *in vivo*. The preferential site of oxidation of cholesterol by highly reactive species is at C_7 having a relatively weak carbon–hydrogen bond. Cholesterol autoxidation is known to proceed via two distinct pathways, a free radical pathway driven by a chain reaction mechanism (type I autoxidation) and a non-free radical pathway (type II autoxidation). Oxysterols arising from type II autoxidation of cholesterol have no enzymatic correlates, and singlet oxygen ($^1\Delta gO_2$) and ozone (O_3) are the non-radical molecules involved in the mechanism. Four primary derivatives are possible in the reaction of cholesterol with singlet oxygen via ene addition and the formation of 5α-, 5β-, 6α- and 6β-hydroxycholesterol preceded by their respective hydroperoxyde intermediates. The reaction of ozone with cholesterol is very fast and gives rise to a complex array of oxysterols. The site of the initial ozone reaction is at the $\Delta_{5,6}$ –double bond and yields 1,2,3-trioxolane, a compound that rapidly decomposes into a series of unstable intermediates and end products. The downstream product 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (sec-A, also called 5,6-secosterol), resulting from cleavage of the B ring, and its aldolization product (sec-B) have been proposed as a specific marker of ozone-associated tissue damage and ozone production *in vivo*. The relevance of specific ozone-modified cholesterol products is, however, hampered by the fact sec-A and sec-B can also arise from singlet oxygen via Hock cleavage of 5α-hydroperoxycholesterol or via a dioxietane intermediate. Whatever the mechanism may be, sec-A and sec-B have no enzymatic route of production *in vivo* and are reportedly bioactive, rendering them attractive biomarkers to elucidate oxidative stress-associated pathophysiological pathways and to develop pharmacological agents.

Contents

1. Introduction . . . 00
2. Cholesterol autoxidation . . . 00
3. Cholesterol aldehydes: ozone or not ozone? . . . 00
4. Biological activity of secosterols . . . 00
5. *In vivo* detection . . . 00
Acknowledgments . . . 00
References . . . 00

Abbreviations: Aβ, amyloid-β; Chol-OOHs, cholesterol hydroperoxides; C27 3β -HSD, 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase; DNPH, dinitrophenyl hydrazine; DH, dansyl hydrazine; GP, Girard P; GC/MS, gas chromatography/mass spectrometry; HMP, 2-hydrazino-1-methylpyridine; LC/MS, liquid chromatography/mass spectrometry; LOD, limit of detection; $LOO^{\cdot}$, lipid peroxyl radicals; $LO^{\cdot}$, lipid alkoxyl radicals; MBP, myelin basic protein; MPO, myeloperoxidase; PBH, pyrenebutyric hydrazine; PHGPx, phospholipid-hydroperoxide glutathione peroxidase; sec-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secA-COOH, 3β-hydroxy-5-oxo-secocholestan-6-oic acid; sec-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde; secB-COOH, 3β-hydroxy-5β-hydroxy-B-norcholestane-6-oic acid; 5α-Chol-OOH, 5α-cholesterol-hydroperoxide; 5β-Chol-OOH, 5β-cholesterol-hydroperoxide; 6α-Chol-OOH, 6α-cholesterol-hydroperoxide; 6β-Chol-OOH, 6β-cholesterol-hydroperoxide; 7α-OHC, 7α-hydroxycholesterol; 7α-Chol-OOH, 7α-cholesterol-hydroperoxide; 7β-Chol-OOH, 7β-cholesterol-hydroperoxide; 24-OHC, 24-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol.

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1. Introduction

Oxysterols are derivatives of cholesterol containing one or more oxygen atoms, other than the OH group on C_3, as hydroxyl, keto, epoxide or peroxyde group – that is mounted on the A and B ring or on the side chain. Oxysterols can be generated either enzymatically, mainly by the group of cytochrome (CYP) P450 family, or by autoxidation [1]. In brief, in biological systems oxygenation on side-chain is almost exclusively enzymatic, while that on the A and B ring can occur both enzymatically and by autoxidation.

Oxysterols arising from enzymatic synthesis can be used as markers of their respective cytochrome activity. Circulating 7α-hydroxycholesterol (7α-OHC), a starting intermediate in the biosynthesis of bile acids [2], correlates with the activity of CYP7A1 [3], 7α-hydroxy-4-cholesten-3-one, a conversion product of 7α-OHC is formed by the microsomal 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase (C27 3β-HSD) [4], 4β-hydroxycholesterol can be used as an endogenous marker of CYP3A4 and CYP3A5 activity [5], 24S-hydroxycholesterol (24-OHC) is the product of the brain-specific cholesterol 24-hydroxylase (CYP46A1) [6,7], 27-hydroxycholesterol (27-OHC) is formed by the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1), which is widely distributed in tissues [8,9]. Examples of oxysterols forming enzymes different than the cyt450 family are cholesterol 25-hydroxylase [10] and oxidosqualene cyclase [11], which produce 25-hydroxycholesterol and 24(S),25-epoxycholesterol, respectively, and cholesterol epoxide hydrolase that converts 5,6-epoxydes into cholesterol-triol [12].

The susceptibility of cholesterol to non-enzymatic oxidation has generated considerable interest in oxysterols as potential markers for the non-invasive study of oxidative stress *in vivo*. Additional interest in oxysterols stems from the biological activity of many oxysterols that is useful to elucidate pathophysiological pathways in human diseases and for pharmacological purposes [13]. 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 [13,14]. The unique cholesterol double bond between carbons 5 and 6 is the most vulnerable site for oxidation by free radicals and highly reactive species [15].

2. Cholesterol autoxidation

Type I autoxidation involves initiation and propagation reactions. Free radicals provide the initiation step by hydrogen abstraction, formation of a carbon centered radical and subsequent oxygen capture. Afterwards, the process advances through free radical intermediates – including, peroxyl radicals (LOO°) and alkoxyl radicals (LO°) – that in turn recruit additional non-oxidized molecules and provoke the spreading of the process via a chain-reaction, the propagation phase.

Despite the hydrogen bond-dissociation energy of C_7-cholesterol is higher than the hemolytic cleavage of allylic hydrogens in polyunsaturated fatty acids [16], entropic factors determine a predominant role of cholesterol oxidation in cellular membranes [17].

A multitude of oxysterols can be formed upon type I autoxidation, but analytical issues restrain the number of species usable as markers of oxidative stress in biological matrices. The species that actually perform well on GC/MS, which is the gold standard for oxysterols measurement, are: 4α- and 7β-hydoxycholesterol, 5α,6α- and 5β,6β-epoxides, and 7-ketocholesterol [13]. Recent studies from Porter and co-workers have established the product distribution of several oxysterols obtained through the free radical chain oxidation of the cholesterol precursor 7-dehydrocholesterol [18].

In type II autoxidation the main molecules that are involved in cholesterol oxidation are the non-radical species singlet oxygen and ozone. Singlet oxygen is formed by an input of energy, such as photoactivation, the Russell mechanism, based on the decomposition of lipid hydroperoxides, and by the reactions of hypochlorous acid and hydrogen peroxide. The following primary species are possible in the reaction of cholesterol with singlet oxygen via ene addition: 5α-cholesterol-hydroperoxide (5α-Chol-OOH), 5β-cholesterol-hydroperoxide (5β-Chol-OOH), 6α-cholesterol-hydroperoxide (6α-Chol-OOH), 6β-cholesterol-hydroperoxide (6β-Chol-OOH), and Chol-1,2-dioxetane. The formation of 5α-Chol-OOH is highly favored at a rate of approximately one order of magnitude higher than that of 6α-Chol-OOH and 6β-Chol-OOH [19]. Minor products of ozone-driven cholesterol oxidation are 5α,6α- and 5β,6β-epoxides, which have been found to form in ethyl acetate [20], but their participation in a physiological environment is not reported. The 7α- and 7β-Chol-OOH are formed during the reaction of singlet oxygen with cholesterol and generated indirectly by the allylic rearrangement of 5α-Chol-OOH [21], which takes place at high peroxidation levels but is negligible under limited cholesterol oxidation (<5%) [22]. Cholesterol hydroperoxides are susceptible to 1 e^- reduction that gives rise to alkoxyl- and peroxyl-radical intermediates that, in turn, can trigger chain reactions and amplify the free radical cascade of cholesterol oxidation and the oxidative damage. All cholesterol hydroperoxides are expected to be equally susceptible to 1 e^- reduction in the presence of metal catalysts. Similar rate constants have been reported for the reduction of 5α-Chol-OOH and 6α-Chol-OOH formation during incubation with an iron-based redox cycling system in a homogeneous solution in which cholesterol was the only chain-carrying species [19]. The potency of 5α-Chol-OOH and 7α-Chol-OOH as chain initiators is comparable [23]. Cholesterol hydroperoxides (Chol-OOHs) are resistant to direct 2 e^- reduction that is catalyzed by Se-dependent glutathione peroxidase [24]. This means that Chol-OOHs have a potential long half-life in cells. The only enzyme capable of catalyzing the reduction of Chol-OOHs to stable diols, is the phospholipid-hydroperoxide glutathione peroxidase (PHGPx) [25]. However, the reduction of Chol-OOH by PHGPx is 6 times slower compared to the reduction of phospholipid hydroperoxides [26], and shows different rate constants ranging from $0.8 \times 10^2\ min^{-1}$ for 5α-Chol-OOH to $\approx 6 \times 10^2\ min^{-1}$ for 6α-Chol-OOH and 6β-Chol-OOH [19]. Thus, 5α-Chol-OOH results the most abundant product of singlet oxygen reaction with cholesterol, and the least resistant to detoxification via PHGPx. The forward products arising from type-II cholesterol autoxidation are cholesterol aldehydes.

3. Cholesterol aldehydes: ozone or not ozone?

3β-Hydroxy-5-oxo-5,6-secocholestan-6-al (sec-A), the major cholesterol ozonolysis products [20], is unstable in physiological aqueous conditions, such as culture medium containing serum, and is readily converted to its aldolization product 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (sec-B) (Fig. 1) [27]. In part, sec-A and sec-B are further converted to their oxidized forms 3β-hydroxy-5-oxo-secocholestan-6-oic acid (secA-COOH) and 3β-hydroxy-5β-hydroxy-B-norcholestane-6-oic acid (secB-COOH) in culture media and probably *in vivo* [27]. Recently, ozonolysis products of the major cholesteryl fatty acid esters transported in human LDL have been reported [28]. Under a flux of ozone, cholesteryl palmitate gives rise to palmitoyl-sec-A and palmitoyl-sec-B. Instead, ozonolysis of cholesterol esterified with unsaturated fatty acids oleate and linoleate admits the initial

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