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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

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 ($LOO^{\cdot}$) and alkoxyl radicals ($LO^{\cdot}$) – 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

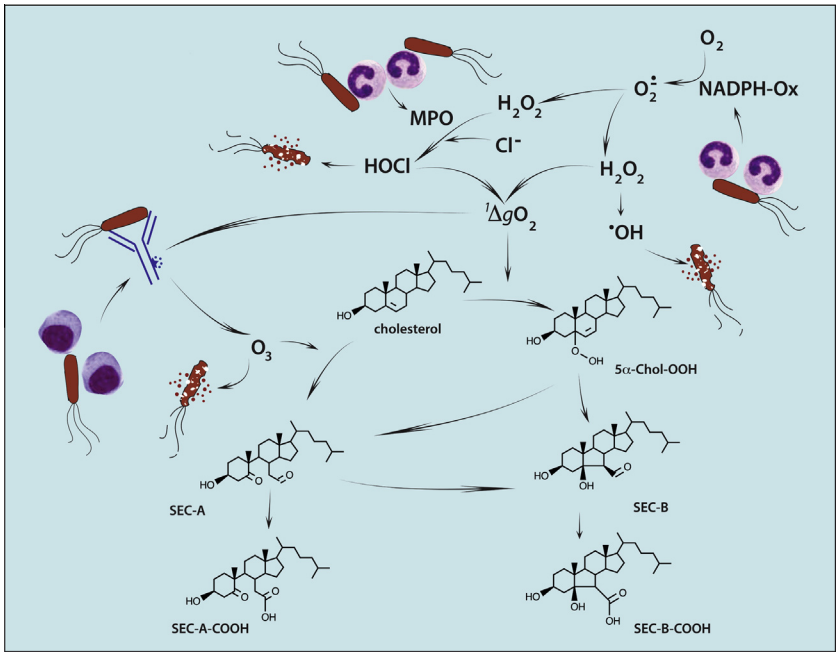

Fig. 1. Schematic representation of secosterols formation during the inflammatory response. Phagocytic cells recruited after a bacterial insult, are activated to produce highly reactive oxygen species through two enzymatic systems, i.e. NADPH-oxidase (NADPH-ox) and Myeloperoxidase (MPO) that contribute to bacterial killing. The formation of ozone by an antibody catalyzed reaction that uses singlet oxygen as substrate, has been proposed by Wentworth et al. [32,70,71] as an ancestral primary immune-response mechanism additional to the well-known superoxide-hydrogen peroxide-HOCl system. Ozone (O_3) and singlet oxygen ($^1\Delta gO_2$) may catalyze the formation of secosterols by promoting cholesterol autoxidation.

isolation of cholesteryl-9-oxononanoate and the subsequent appearance of both the fatty acid and cholesteryl moiety oxidation products, i.e. 9-oxononanoyl-sec-A and 9-oxononanoyl-sec-B [28]. These compounds derived from cholesterol/cholesterol esters ozonolysis exert potent biological activities including the denaturation of proteins and strong cytotoxicity in different cells lines (see below). High levels of sec-A and sec-B have been detected in human atherosclerotic plaques [29] and tissues samples of brains affected by neurodegeneration, such as Alzheimer's disease and Lewy body dementia [30,31], suggesting that increased formation of these compounds may be associated with inflammation-related diseases. Wentworth et al. [29] conducted pioneering works on secosterols and advised sec-A and sec-B as potential diagnostic markers of endogenous ozone production. They proposed a novel mechanisms for the unprecedented formation of ozone *in vivo* consisting of reactive oxygen species cascade (Fig. 1): (a) superoxide generation by activated neutrophil, (b) dismutation into hydrogen peroxide, (c) hypochlorous acid (HOCl) formation by myeloperoxidase (MPO), (d) singlet oxygen generation by the reaction of HOCl and hydrogen peroxide, and afterwards (e) formation of ozone from the singlet oxygen in an antibody-catalyzed water oxidation pathway [32,33]. A similar mechanism for the production of ozone-like reactive species from singlet oxygen in an amino acids-catalyzed water oxidation pathway was also reported [34]. However others have argued against the ozone-dependent mechanism of sec-A formation *in vivo* [35–37], and pointed out an alternative pathway for the formation of sec-A and sec-B. Uemi et al. [38] proposed a mechanism based on the Hock cleavage of 5α-Chol-OOH or a Chol-1,2-dioxetane intermediate formed by the reaction of cholesterol with singlet oxygen. Others provided evidence for sec-A and sec-B generation by the reaction of cholesterol with singlet oxygen produced by 1-methylnaphthalene-4-endoperoxide in phosphate buffer [39], and by the MPO–H_2O_2–Cl system [39]. Taken together these data point to a duplicate mechanism of sec-A and sec-B formation involving either ozone or singlet oxygen. Garner et al. [40] recently reported the development of a specific fluorogenic probes for ozone detections that, eventually, may help to investigate the role of ozone in pathophysiology. Being sec-B the predominant species formed during singlet oxygen-mediated cholesterol oxidation (sec-B is about 5–10 times higher than sec-A), the ratio sec-A to sec-B has been proposed as a surrogate measure to decipher the ozone-dependent and independent oxidation of cholesterol [39]. On the other hand, sec-A occurs as the dominant species formed by ozone in aqueous buffer system [41], and by phorbol-12-myristate-3-acetate-activated neutrophil in culture [27]. In addition, we were able to observe a time-dependent elevation of sec-A and sec-B in plasma after injecting lipopolysaccaride to C57BL/6j mice, but not in MPO-deficient mice. Besides, basal levels of sec-A and sec-B in the plasma of MPO-deficient mice were lower than the value found in wild type mice, but sec-A was barely detectable [27]. Sec-B was shown to be formed by aldolization of sec-A and, also, in an ozone-independent pathway via 5α-OOH-Chol or cholesterol-1,2-dioxetane [38]. Sec-B detected in the plasma of MPO-deficient mice, therefore, could be formed by the reaction of cholesterol with singlet oxygen generated *in vivo*, although its exact origin is currently unknown. Taken together these findings advise the occurrence of ozone-mediated reaction *in vivo* even if no conclusive evidences so far could be drawn for ozone production *in vivo*.

4. Biological activity of secosterols

The aldehydic function of secosterols is highly reactive and efficiently forms Schiff bases with ε- or N-terminal amino groups of proteins and with phosphatidylethanolamine, relevantly connected with atherosclerosis and a number of diseases associated with protein misfolding.

Wentworth et al. [29] reported that incubation of human LDL with either sec-A or sec-B led to time-dependent changes in the circular dichroism spectra of apoB-100, consistent with an altered

secondary structure, and increased atherogenicity, e.g. the secosterol-modified LDL was avidly taken up by macrophage leading to foam cell formation. Sec-A was shown to randomly modify the 6 different Lys residues of ApoC-II, as well as apolipoprotein that in the absence of lipids has conformational instability and undergoes fibrillization [42]. Sec-A accelerated ApoC-II polymerization with concurrent increase in thioflavin fluorescence [42], a signature of amyloidogenesis [43]. Interestingly, secA-COOH, which lacks the aldehydic functionality and is unable to form Schiff bases, was also able to accelerate ApoC-II fibril formation, albeit at a lesser extent, suggesting that non-covalent mechanisms may support secosterol-dependent ApoC-II amyloidogenesis [42]. These findings are relevant to the mechanisms of atherosclerosis because amyloid deposits are present in 50–60% of atherosclerotic lesions [44] and ApoC-II is a prominent component of these deposits [45]. Concentrations of secosterols are reportedly elevated in the cortex of patients with Lewy body dementia [31], a disease associated with intra-neuronal accumulation of α-synuclein in the form of amyloid fibrils or Lewy bodies. Sec-A, sec-B, and secoA-COOH have been shown to accelerate α-synuclein aggregation *in vitro*, and more interestingly secA-COOH was even more potent in forwarding the process [31]. Amyloidogenicity of amyloid-β (Aβ) is considered a crucial player of Alzheimer disease but an open question is the 2–3 order of magnitude disparity between the critical concentration to induce aggregation, which is in the micromolar range, and the actual concentration of Aβ at tissue level, which is in the nanomolar range [46]. Secosterols have been shown to effectively reduce below 100 nM the critical concentration of Aβ to aggregate [30,47]. Among the Aβ adducts with secosterol, Lys-16 Aβ modification formed amorphous aggregates fast and at very low concentrations of Aβ (20 nM), followed by the Lys-28 and Asp-1. Besides, the aggregates resulting from Lys-secosterols adducts were more toxic to primary rat cortical neuron [48]. Sec-A and sec-B in brain samples of patients affected by neurodegenerative disease approach concentrations of up 1 μM [30,49] that are suitable to covalently modify Aβ and increase its amyloidogenicity [30,31,47,50,51]. Sec-A and sec-B have been reported to induce structural change to myelin basic protein (MBP) relevant to the context of demyelinating diseases [52]. MBP accounts for approximately 30% of the total myelin protein, and is responsible for adhesion and stabilization of the intracellular surfaces of myelin layers. By reacting with MBP, secosterols have been shown to increase the surface exposure of the immunodominant epitope, decrease the surface exposure of the cathepsin D binding, and reduce the size and structural stability of MBP-induced aggregates. As a consequence of these alterations in the structure and function, MBP is unable to maintain the integrity of the myelin sheath and becomes vulnerable to autoimmune attack. In line to that which is observed with secosterol-initiated misfolding of Aβ and α-synuclein, sec-A and sec-B have been reported to induce misfolding of wild-type p53 [53]. The tumor suppressor protein p53 functions to maintain the integrity of the genome, and its activation in response to DNA damage promotes cell-cycle arrest in G1 phase or apoptosis. Upon incubation with secosterols, p53 undergoes polymerization that anticipates the formation of amyloid fibrillary aggregates. This misfolding renders p53 unable to bind to DNA and to induce transactivation of p21 [53]. Given that inflammation is the fuel for secosterols formation and that inflammation functions in all stages of tumor development, secosterols provide a chemical link to understand cancer carrying inactive p53.

Light-chain deposition disease is a severe, often fatal, clinical condition in which amyloid or amorphous deposits, as a consequence of antibody light chain aggregation, accumulate in the heart and/or kidney. Sec-A and sec-B have been reported to accelerate aggregation of human antibody kappa and lambda light chains *in vitro* under physiologically relevant conditions, causing an amorphous-type aggregation that is thioflavin and Congo red negative for both the kappa and lambda light chains [54]. Given the inflammatory microenvironment of secosterol production and its association with antibodies, the secosterol-induced protein misfolding is consistent with a pathophysiological role in light-chain deposition disease.

While the above reported studies show secosterols as playing deleterious roles by promoting misfolding of varied proteins, sec-B has unexpectedly been shown to inhibit the misfolding of a truncated murine mutant prion protein. Incubation of sec-B with a murine prion protein, paradoxically, induced stabilization of the native form of the prion and inhibited the generation of the disease-causing scrapie form [55]. The inhibition was specific for sec-B, where structural analogues were ineffective, offering a promising tool to develop new pharmacological active compounds to treat prion disease.

Additionally, secosterols have been reported to affect membrane and enzyme function. It was shown that secosterols bound phosphatidylethanolamine and phosphatidylserine via Schiff base formation, and also reduced biophysical parameters of membrane stability, which could be associated with various pathogenic insults [56–58]. Recently Genaro-Mattos and co-workers [59] reported that sec-B covalently bound and anchored cytochrome c to mitochondrial mimetic membranes, although its physiological role is still under investigation. Sec-A, but not sec-B, reportedly inhibited endothelial- and neuronal-type of nitric oxide synthase (NOS) activities, probably mediated by adduct formations with lysine residues on these enzymes [60]. The biochemical and biophysical properties of secosterols could be associated with their noxious activity on cells. Several studies have found that sec-A and sec-B induce cell death in various cell lines, including human B-lymphocytes (WI-L2), T-lymphocytes (Jurkat), vascular smooth muscle cells (VSMC), abdominal aorta endothelial cells (HAEC), murine tissue macrophages (J774.1), and an alveolar macrophage cell line (MH-S) [29]. Sathishkumar et al. [61] reported that sec-A exerted about 2-fold higher cytotoxicity than 5,6β-epoxy-Chol in hypothalamic neuron GT1-7 cells. Several pathways have been postulated for secosterol-triggered cell death, including the caspase-3/7-dependent pathway and the mitochondrial and death receptor pathway in cardiomyocyte H9c2 cells [62,63], the reactive oxygen species-dependent pathway in hypothalamic neuron GT1-7 cells [61,64], a mitochondrial death pathway in macrophage J774 cells, and the mitogen-activated protein kinase pathway in hepatocarcinoma HepG2 and Huh7 cells [65]. Moreover, secA-COOH and secB-COOH showed strong cytotoxic activities in human acute promyelocytic leukemia HL-60 cells [66]. Recently, it has been reported that 9-oxononanoyl-secA and 9-oxononanoyl-secB – ozonolysis products of cholesteryl-oleate and cholesteryl-linoleate present in human LDL – exert potent cytotoxicity towards HL-60 cells [28]. Their activity is stronger than other cytotoxic oxysterols, exhibiting EC50s of 10–20 μM, which were very similar against various cell lines tested.

5. *In vivo* detection

An overview of methods, biological samples investigated and levels of secosterols reported to date in the literature is shown in Table 1. For the analysis of sec-A and sec-B in biological or clinical samples, HPLC separation with UV, fluorescence, or MS detection have been widely employed. In general, lipid extracts of blood or tissue samples containing sec-A and sec-B are derivatized with hydrazine derivatives, such as dinitrophenylhydrazine (DNPH) [29,67,72]. To perform higher sensitivity detection, derivatization with dansyl hydrazine (DH, LOD = 1 fmol in [27,31,39]), 1-pyrenebutyric hydrazine (PBH; LOD = 10 fmol in [68]), Girard P (GP) hydra-

Table 1
Detections of sec-A and sec-B in biological samples.

Tissues/fluids	Species	Forms	Equipments	Concentrations	N	Ref
Lung	Rat (SD) exposed to ozone	Sec-A-DNPH	HPLC-UV	ND		72
		Sec-B-DNPH		ND		
Atherosclerotic plaque	Human	Sec-A-DNPH	HPLC-UV and LC-MS	6.8 - 61.3 pmol/mg	n=11	29
Plasma	Human (atherosclerotic patients)	Sec-B-DNPH		70 - 1690 nM	n=8	
Brain	Human (Alzheimer patients)	Sec-A-DNPH + Sec-B-DNPH	HPLC-UV and LC-MS	0.44 pmol/mg	n=4	30
	Human (Control)	Sec-A-DNPH + Sec-B-DNPH		0.35 pmol/mg	n=7	
Brain	Human (Lewy body dementia)	Sec-A-DNSL + Sec-B-DNSL	HPLC-F	0.21 uM	n=15	31
	Age-matched control	Sec-A-DNSL + Sec-B-DNSL		0.09 uM	n=18	
Brain	Rat	Sec-A-GP	LC-MS	~100 pg/mg		49
		Sec-B-GP		~300 pg/mg		
	Human	Sec-A-GP + Sec-B-GP		150 pg/mg		
Plasma	Mouse (C57BL/6)	Sec-A-DNSL	LC-MS/MS with IS (^{13}C-sec-A + ^{13}C-sec-B)	0.5 ± 0.2 nM	n=8	27
		Sec-B-DNSL		1.1 ± 0.3 nM	n=8	
	Mouse (C57BL/6 MPO-KO)	Sec-A-DNSL		0.03 ± 0.1 nM	n=7	
		Sec-B-DNSL		0.5 ± 0.4 nM	n=7	
Liver	Mouse (C57BL/6)	Sec-B-DNSL		126.0 ± 42.7 pmol/g	n=8	
	Mouse (C57BL/6 MPO-KO)	Sec-B-DNSL		62.2 ± 21.6 pmol/g	n=7	
Plasma	Human (healthy volunteers)	Sec-A-HMP	LC-MS/MS with IS (^{13}C-sec-A + ^{13}C-sec-B)	23.6 ± 16.6 nM	n=10	69
		Sec-B-HMP		27.3 ± 41.0 nM	n=10	
	Mouse (C57BL/6)	Sec-A-HMP		1.4 ± 0.7 pmol/g	n=3	
		Sec-B-HMP		4.3 ± 0.8 pmol/g	n=3	
Brain		Sec-A-HMP		10.4 ± 16.3 pmol/g	n=3	
		Sec-B-HMP		110.9 ± 10.6 pmol/g	n=3	
Liver		Sec-A-HMP		34.1 ± 21.6 pmol/g	n=3	
		Sec-B-HMP		161.5 ± 56.3 pmol/g	n=3	
Lung		Sec-A-HMP		29.1 ± 1.3 pmol/g	n=3	
		Sec-B-HMP		80.4 ± 1.4 pmol/g	n=3	

zine (LOD = 2.7 fmol in [49]), or 2-hydrazino-1-methylpyridine (HMP; LOD = 10–50 amol in [69]). Using these derivatizing reagents, sec-A and sec-B present in blood or tissues were detectable as secosterol–hydrazone derivatives by HPLC-fluorescence detector and LC–MS (Table 1). Griffiths and co-workers reported levels of sec-A and sec-B in rat brain of ~100 pg/mg (240 pmol/g) and ~300 pg/mg (720 pmol/g), respectively, determined after derivatization with GP hydrazine [49]. Sec-A and sec-B in human brain were also analyzed by HPLC-UV or LC–MS after derivatization with DNPH resulting in levels of (sec-A + sec-B) 0.44 pmol/mg in Alzheimer's patients ($n = 4$) and 0.35 pmol/mg in control subjects ($n = 7$) [30]. In addition, increased levels of secosterols (sec-A + sec-B) were observed in the cortex of brain affected by Lewy body dementia (0.213 μM, $n = 15$) compared to those of age-matched controls (0.093 μM, $n = 18$) in analysis done by HPLC-fluorescence detector and LC–MS after DH derivatization [31]. Wentworth and co-worker analyzed DNPH-derivatives of sec-A and sec-B in organic extracts of human atherosclerotic plaque by LC–MS, and found them in the ranges of 6.8–61.3 pmol/mg plaque [29]. Elevated levels of sec-B were also observed in the plasma of these patients (70–1690 nM) compared to those of controls subjects [29]. We have recently developed a highly sensitive isotope dilution method to detect sec-A and sec-B as HMP derivatives by LC–ESI–MS/MS, and using 3,4-^{13}C-sec-A and 3,4-^{13}C-sec-B as internal standards [69]. We found levels of sec-A and sec-B of 23.6 ± 16.6 nM and 27.3 ± 41.0 nM, respectively, in human plasma ($n = 10$). The levels of sec-A and sec-B were respectively 1.4 ± 0.7 and 4.3 ± 0.8 nM in the plasma, 10.4 ± 16.3 and 110.9 ± 10.6 pmol/g in the brain, 34.1 ± 21.6 and 161.5 ± 56.3 pmol/g in the liver and 29.1 ± 1.3, and 80.4 ± 1.4 pmol/g in the lung of C57BL/6j mice ($n = 3$). In addition, ozonolysis products of cholesteryl-oleate and cholesteryl-linoleate, 9-oxononanoyl-sec-A and 9-oxononanoyl-sec-B, were found in human LDL at levels of 16.5 ± 5.4 and 11.3 ± 3.9 pmol/mg LDL protein, respectively [28]. Notably, the values of cholesterol aldehydes in biological samples differ widely among the different laboratories. As sec-A is very unstable, at least the use of stable-isotope labeled internal standards in secosterol analysis is mandatory.

Although formation mechanisms of secosterols are not still fully unveiled, elevated levels of secosterols have been observed in various tissues collected from different inflammatory diseases. Sec-A, sec-B, and other related compounds including secA-COOH, secB-COOH, and 9-oxononanoyl secosterols exert strong biological activities compared to other oxysterols. Further studies are warranted to elucidate the mechanisms of secosterols formation *in vivo* and their pathological roles in relation to pathogenesis of several inflammatory diseases.

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