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The ratio of cholesterol 5,6-secosterols formed from ozone and singlet oxygen offers insight into the oxidation of cholesterol *in vivo*

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Abstract

Ongoing efforts to unravel the origins of the cholesterol 5,6-secosterols (**1a** and **1b**) in biological systems have revealed that the two known chemical routes to these oxysterols; ozonolysis of cholesterol (**3**) and Hock-cleavage of 5- α -hydroperoxycholesterol (**4a**), are distinguishable based upon the ratio of the hydrazone derivatives (**2a–b**) formed in each case and this ratio offers an insight into the chemical origin of the secosterols *in vivo*.

In a recent report, Pratt and co-workers¹ have shown that Hock-cleavage of 5- α -hydroperoxycholesterol (**4a**), that can arise from the singlet oxygen ene reaction with cholesterol (**3**), occurs facily under acidic conditions in organic solvents leading to the formation of primarily cholesterol 5,6-secosterol atheronal-B (**1b**). Atheronal-A (**1a**) is either not formed at all, or is a minor component in participating solvents such as ethanol (Fig. 1).

We discovered the cholesterol 5,6-secosterols **1a** and **1b** within human atherosclerotic plaque material *in vivo*² and surmised that, based upon the wealth of literature in the field of chemical-, biological- and auto-oxidation of cholesterol at the time,^{3, 4} that only ozone was capable of generating **1a** from cholesterol (**3**).² In fact, we considered the presence of **1a** [measured as **2a** after extraction and derivatization with 2,4-dinitrophenyl (2,4-DNP) hydrazine] within inflammatory arteries as indirect evidence that an oxidant with the chemical signature of ozone may be being generated during atherosclerosis progression. We also showed that **1a** undergoes an almost instantaneous aldolization process to form **1b** in whole blood and therefore the cholesterol 5,6-secosterols **1a** and **1b** were both potential signature molecules for cholesterol ozonolysis *in vivo*.²

The oxysterols **1a** and **1b** are proatherogenic⁵ and induce protein misfolding and amyloidogenesis⁶ in a number of biologically-relevant proteins.^{2, 7} Given the pathological

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effects of **1a** and **1b**, we are striving to understand their chemical origin *in vivo*. To do this we have studied the oxidation of cholesterol (**3**) in biologically-relevant formats; low-density lipoprotein (hLDL); high-density lipoprotein (hHDL) and liposomes using cellular, biological and chemical oxidant systems, that include singlet oxygen generating systems and ozone, in aqueous buffer (Table 1 and ESI-Tables 1–6) and analyzed the cholesterol *secosterols* formed as their 2,4-DNP hydrazones (**2a–b**).

We find that the 2,4-DNP hydrazone of atheronal-B (**2b**) is observed at levels above untreated hLDL (~0.01 % of **3**), after 2,4-DNP hydrazine derivatization of hLDL that has been oxidized in aqueous buffer by superoxide anion (Table 1, entries 1–2) and by singlet oxygen generating systems (Table 1, entries 6–7): and by activated human polymorphonuclear leukocytes (hPMNs, Table 1, entries 8–9). In contrast, no elevation in **2b** above untreated samples, is observed after hLDL treatment with H₂O₂, HOCl or hydroxyl radical generating systems (Table 1, entries 3–5). The generation of **2b** during hLDL oxidation by phorbol myristate acetate (PMA)-activated hPMNs and is proportional to cell density, is consistently higher in cholesterol extracted from hLDL rather than the PMN cell membranes and is elevated in D₂O relative to H₂O-containing buffer. These combined data suggest that the oxidant responsible for the generation of **2b** is both sufficiently long-lived to be released into the medium, and its life-time is prolonged in deuterated buffers, hinting at roles for both •O₂⁻/HO₂• and ¹O₂ (Table 1, entries 8–9 and ESI-Tables 1–3).

The photosensitized and hPMN-mediated oxidation of cholesterol in hHDL in aqueous buffer (PBS, pH 7.4) parallel the observations with hLDL in that the 2,4-DNP hydrazone of cholesterol *secosterol* **2b** is elevated above untreated levels and no **2a** is observed (ESI Tables 4–5). Similarly, the photosensitized oxidation of **3** in liposomes (DOPC 80 mol %; **3** 5 mol %; PIP-2 15 mol %) in PBS (pH 7.4) implicates a ¹O₂ pathway to **2b**, because the measured levels of **2b** are elevated in PBS containing D₂O, relative to H₂O (ESI Table 6).

One of the clearest observations of these aqueous hLDL oxidation studies is that the 2,4-DNP hydrazone of atheronal-A (**2a**) is only observed after ozonolysis (Table 1). Thus, passage of an ozone/oxygen mixture over the surface of an hLDL solution containing catalase in aqueous buffer (pH 7.4), followed by 2,4-DNP hydrazine derivatization of the lipid extract yields **2a** and **2b** in a ratio of ~ 4:1 (Table 1, entry 10, ESI Fig. 1). To validate this observation cholesterol **3**, that had been exposed to ozone in aluminium oxide (Al₂O₃)-treated CDCl₃, was shaken in aqueous buffers at a range of physiological pHs (5.8, 6.5, 7.0 and 7.4) in the presence or absence of a reductant (Zn dust), and the resultant products were assessed by ¹H NMR (ESI Fig. 2). This analysis reveals that, in all cases tested, only cholesterol *secosterol* **1a** is generated in this aqueous treatment of ozonolyzed **3**. *Secosterol* **1b** is not formed by this route as confirmed by 2,4-DNP hydrazine derivitization of the ¹H NMR samples.

The oxidation of **3** by ¹O₂ is a well-understood process that was first reported by Schenck.⁸ In brief, the 5α-hydroperoxide **4a** is formed as the major product, which can rearrange to the 7α-hydroperoxide **4b** under certain conditions.⁹ Therefore, the observation that ¹O₂ and and superoxide anion-mediated biological- and chemical-oxidations of hLDL, hHDL and liposome-derived **3** in aqueous buffers results in elevated levels of **2b**, could be a result of the following possibilities: 1. Conversion of **4a** or **4b** into **1a** and subsequent aldolization of **1a** into **1b in aqua** (followed by 2,4-DNP hydrazine derivatization after extraction); 2. conversion of **4a** or **4b** directly into **1b in aqua** (followed by 2,4-DNP hydrazine derivatization after extraction); or 2. conversion of **4a** or **4b** into **2b** during the 2,4-DNP hydrazine derivatization process.

To summarize the experiments to deconvolute these possibilities; when authentic **4a** is incubated in aqueous buffers for 20 h (pH 5.8–7.5) the allylic hydroperoxide undergoes a low

percentage isomerization into **4b**, but there is no formation of **1a** or **1b** (ESI Table 7 and Fig. 3); we have shown previously that authentic **1a** does not undergo measurable aldolization to **1b** under the conditions of our studies¹. Pratt and co-workers⁷ have recently observed that Hock cleavage of **4a** occurs in the presence of 2,4-DNP hydrazine to give **2b** with a trace of **2a** being formed. Using authentic **4a**, we find that this hydroperoxide undergoes Hock-cleavage during the hydrazine derivatization process utilized in this study (20 mM 2,4-DNP hydrazine, 0.8 % HCl in *iso*-propanol) to yield quantitative amounts of **2b** (no **2a** is detected, ESI Fig. 4). In addition, 7 α -hydroperoxide **4b** does not undergo conversion into either **2a** or **2b** during the 2,4-DNP hydrazine derivitization process *vide supra*. Therefore, the **2b** that arises after $\bullet\text{O}_2^-$, $^1\text{O}_2$ and PMA-activated hPMN-mediated oxidation of hLDL, hHDL and liposomes (Table 1) is most likely a result of cholesterol peroxidation to generate **4a**, followed by Hock cleavage of **4a** during the 2,4-DNP hydrazine derivitization process.

We have studied the singlet oxygen route to the cholesterol *secosterols* further; photochemical oxidation of **3** in Alox-treated CDCl_3 with *in situ* acid (TFA, 1 % v/v), leads to a good yield of **1b** (> 69 %) with a low yield of **1a** being formed (~ 3.5 %). Inclusion of participating solvents, such as methanol, or increasing acid concentration do not significantly increase the yield of isolated *secosterol* **1a** (ESI Table 8). When the concentration of TFA is lowered suffiecently (0.02 % v/v), the progress of Hock-cleavage of **4a** to form **1b** can be followed by ^1H NMR, reinforcing that **1a** is not formed even transiently, during this process on a time-scale where allylic isomerization of **4a** to **4b** competes measurably (ESI Table 9 and Fig. 5). Furthermore, when authentic **4a** is incubated in neat IPA for 3 h, ^1H NMR reveals a complex mixture of **1b** and **1b**-derived dehydration and acetal products but no **1a** or **1a**-derived products. Derivatization of this **4b**-derived mixture with 2,4-DNP hydrazine then yields **2b** and **1b**-derived dehydration products with no measurable **2a** or **2a**-derived dehydration or acetal products in either case (ESI Fig. 6). Thus, the summary of this singlet oxygen/Hock cleavage in route to the cholesterol *secosterols* in organic solvents is that in all the systems we have studied, **1b** is formed in vast excess over **1a**.

The $^1\text{O}_2$ route to *secosterol* hydrazone **2b** has a number of implications for our quantification method of atheronals in biological systems. Analytical methods to quantify cholesterol *secosterols* from biological extracts must be able to deconvolute the 5 α -hydroperoxycholesterol **4a** from the cholesterol *secosterol* **1b** (the 2,4-DNP hydrazine method does not). To this end, we have developed an isotope dilution (ID) GC-MS assay, which measures the levels of **1b** in biological extracts after treatment with bis (trimethylsilyl) trifluoroacetamide (BSTFA) in pyridine, conditions where **4a** is completely stable (ESI Table 9, ESI Figs. 9–10). A comparison of this IDGCMS assay with the 2,4-DNP hydrazine method in a plasma analysis of **1b**-derivatives in the apoE $^{-/-}$ mouse model of atherosclerosis reveals that the measured levels are not significantly different [GCMS method, **1b** = 7.5 ± 1.3 nM ($n=4$); 2,4-DNP hydrazine method, **2b** = 9.3 ± 2.4 nM ($n=6$); $p>0.05$], suggesting that **4a** is not contributing significantly to the measured amounts of **2b** in this system (ESI Fig. 9).

Analysis of human arterial plaque extracts by IDGCMS reveals a mean [**1a(b)**] of 32 ± 15 pmol/mg of tissue which is similar to levels we previously reported for **2b** in plaque extracts² (ESI Figs. 10–12). Interestingly, treatment of these arterial plaque extracts with Zn/acetic acid prior to lipid extraction results in an increase in the measured **1a(1b)** levels suggesting that either **4a** or cholesterol ozonides are present in these atheroma samples and are being converted into **1a** and **1b** during tissue processing. We are currently investigating this issue (ESI Figs. 11–12).

We are also interested in understanding to what extent the acid-catalyzed rearrangement of **4a** or a chemical oxidant with the chemical signature of ozone may be contributing to cholesterol *secosterol* biogenesis *in vivo*. As described *vide supra* authentic **4a** is stable at

physiological pH for > 20 h and the 5 α -hydroperoxide **4a** has been shown to be isolable from biological samples by a number of groups.^{4, 10} However, one cannot discount that the formation of **1b**, which is ubiquitous in all biological samples we have tested thus far,¹ does not at least in part, arise from this route. Perhaps most relevant to the question of the oxidation route of **3** *in vivo* is the difference in the cholesterol 5,6-*secosterols* that occurs by each pathway. Pratt and co-workers⁷ have shown that Hock-cleavage of authentic **4a** *in vitro* leads to levels of **1b**>>**1a** and we herein have shown that **4a**, generated by chemical and biological oxidations of cholesterol in aqueous buffers, generates **2b** (with no measurable **2a**) upon 2,4-DNP hydrazine derivatization. In complete contrast, ozonolysis of **3** in aqueous buffers generates **1a**>>**1b** (at all physiologically-relevant pHs either in the presence or absence of added chemical reductant). We have also studied the ozone-mediated oxidation of hLDL-bound cholesterol in aqueous buffer at a range of pH (5.4–7.4), temperatures and durations of reaction and found that the ratio **1a:1b** varies from ~ 15:1 to ~ 4:1 but the critical point to emphasize is that in all cases measured for ozonolysis, the level of atheronal-A is > than atheronal-B (ESI Table 12). A reinvestigation of our original report of the levels of cholesterol *secosterols* (measured as their hydrazones **2a** and **2b**), reveals that the ratios of **2a:2b** varies greatly between arterial plaque samples (61.3 to 0.06, *n*=28) (ESI Table 11). Interestingly, the ratio of **2a:2b** is > 1 in 20 of 28 samples and > 10 in 10 of the 28 samples. Therefore, the dominant form of cholesterol 5,6-*secosterols* in arterial plaque extracts, where aldolization is minimal, seems to be **1a**.² This fact is important, because based upon our new understanding of the ozone and singlet oxygen pathways from **3** into **2a** and **2b** (after 2,4-DNP hydrazine derivatization), our original clinical data offers support for an oxidant with the chemical signature of ozone playing at least a composite role in arterial plaque cholesterol *secosterol* **1a** biogenesis in these samples.

While the debate as to the relevance of ozone generation in biological systems continues,¹¹ we continue to search for more direct markers of this oxidant in biological extracts. While the terminal products from cholesterol ozonolysis, **1a** and **1b**, may not discriminate the pathways to their formation under all conditions, a ratio of **1a** to **1b** of > 1 is strong support for oxidation of **3** by an oxidant with the chemical signature of ozone. Clearly, the ratio of **1b:1a** is going to be environmentally dependant and so only offer an insight into the composite oxidation processes ongoing within tissues. Thus our current search is aimed at quantifying more direct species that originate as a result of the primary chemical reaction of ozone with biological molecules, such as 1,2,4-trioxolanes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1Oxidation of hLDL-bound cholesterol in aqueous buffer^a

Entry	Oxidant system	Reactive species	Products ^b (% of 3)	
			2a	2b
1	Xan/XO ^c	•O ₂ -/HO ₂ •	n.d.	0.02
2	Acet/XO ^d	•O ₂ -/HO ₂ •	n.d.	0.05
3	Glc/GlcOx ^e	H ₂ O ₂	n.d.	>0.01
4	Glc/GlcOx/MPO ^f	H ₂ O ₂ /HOCl	n.d.	>0.01
5	Glc/GlcOx/FeCl ₂ ^g	H ₂ O ₂ /HO•	n.d.	>0.01
6	HPIX/hv ^h	¹ O ₂	n.d.	2.30
7	Glc/GlcOx/ HPIX/hv	H ₂ O ₂ / ¹ O ₂	n.d.	2.61
8	hPMNs ⁱ	multiple	n.d.	0.30
9	hPMNs/D ₂ O	multiple	n.d.	0.35
10	O ₃ /O ₂ ^j	O ₃	0.40	0.10

^aFor full details see the ESI.^bProducts were analyzed as their 2,4-DNP hydrazones **2a** or **2b** and are reported as the amount of *secosterol* formed as a percentage of the cholesterol in hLDL at *t*=0.^cXanthine (Xan, 0.5 mM)/xanthine oxidase (XO, 0.4 mg/ml).^dAcetaldehyde (Acet, 10 mM) incubated for 30 min.^eGlucose (Glc, 2 mM)/glucose oxidase (GlcOx, 0.1 mg/ml) incubated for 14 h at 37 °C.^fSame as e with myeloperoxidase (MPO, 0.2 μM).^gSame as e with FeCl₂ (20 μM).^hHematoporphyrin IX (HPIX, 40 μM), hv (400–700 nm), 14 h incubation.ⁱhPMNs, (5.4 × 10⁷ cells/ml) activated with PMA (0.1 mg/ml) in PB or PB in D₂O.^jA stream of O₃/O₂ gas (5 ml/min) was passed over a solution of hLDL in PBS (pH 7.4) for 40 s.