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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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 and 2b) 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-workers[1] have shown that Hock-cleavage of 5α-hydroperoxycholesterol (**4a**), which can arise from the singlet oxygen ene reaction with cholesterol (**3**), occurs facilely 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 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 proatherogenic[5] and induce protein misfolding and amyloidogenesis[6] in a number of biologically-relevant proteins.[2,7] Given the pathological effects of **1a** and **1b**, we are striving to understand their chemical origin *in vivo*. Herein we have studied the formation of **1a** and **1b**, analyzed as their 2,4-DNP hydrazones (**2a** and **2b**), during the oxidation of cholesterol (**3**) in biologically-relevant formats—human low-density lipoprotein (hLDL); human high-density lipoprotein (hHDL); and liposomes using cellular, biological and chemical oxidant systems in aqueous buffer (Table 1 and ESI†).

The 2,4-DNP hydrazone of atheronal-B (**2b**) is observed at levels above untreated hLDL (~0.01% of **3**), after oxidation by superoxide anion (Table 1, entries 1 and 2), by singlet oxygen generating systems (Table 1, entries 6 and 7), and by activated human polymorphonuclear leukocytes (hPMNs, Table 1, entries 8 and 9). In contrast, no elevation in **2b** above untreated samples is observed after hLDL treatment with H_2O_2, 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, is proportional to cell density, is consistently higher in lipid extracts of hLDL rather than the PMN cell membranes and is elevated in D_2O relative to H_2O-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 $^{\bullet}O_2^{-}/HO_2^{\bullet}$ and $^1O_2$ (Table 1, entries 8 and 9 and ESI,† Tables S1–S3).

The photosensitized and hPMN-mediated oxidation of cholesterol in hHDL in aqueous buffer (PBS, pH 7.4) parallels the observations with hLDL in that the 2,4-DNP hydrazone of cholesterol secosterol **2b** is elevated above untreated levels and

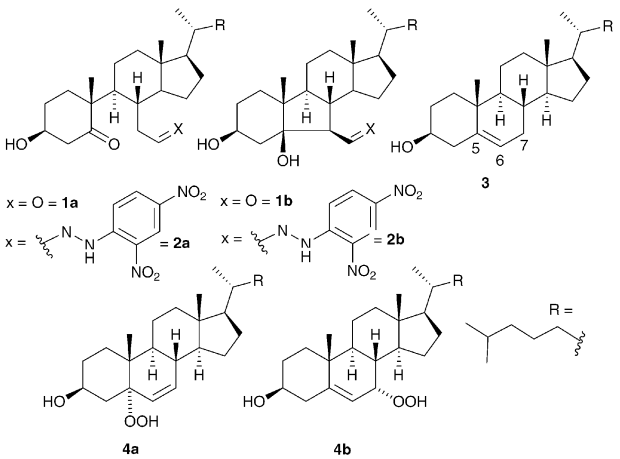

Fig. 1 Chemical routes from cholesterol **3** into the cholesterol secosterols **1a** and **1b** are being sought.

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

Entry	Oxidant system	Reactive species	Products[b] (% of **3**)	
			2a	**2b**
1	Xan/XO[c]	$^{\bullet}O_2^-/HO_2^{\bullet}$	n.d.	0.02
2	Acet/XO[d]	$^{\bullet}O_2^-/HO_2^{\bullet}$	n.d.	0.05
3	Glc/GlcOx[e]	H_2O_2	n.d.	>0.01
4	Glc/GlcOx/MPO[f]	H_2O_2/HOCl	n.d.	>0.01
5	Glc/GlcOx/$FeCl_2$[g]	$H_2O_2/HO^{\bullet}$	n.d.	>0.01
6	HPIX/$h\nu$[h]	$^1O_2$	n.d.	2.30
7	Glc/GlcOx/HPIX/$h\nu$	$H_2O_2/^1O_2$	n.d.	2.61
8	hPMNs[i]	Multiple	n.d.	0.30
9	hPMNs/D_2O	Multiple	n.d.	0.35
10	O_3/O_2[j]	O_3	0.40	0.10

[a] For full details see the ESI;† lipid extracts were derivatized with 2,4-DNP hydrazine (2 mM) in isopropanol–ethanol (9 : 1) containing HCl (0.08%) [b] Products 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 $t = 0$. [c] Xanthine (Xan, 0.5 mM)/xanthine oxidase (XO, 0.4 mg ml^{-1}). [d] Acetaldehyde (Acet, 10 mM) incubated for 30 min. [e] Glucose (Glc, 2 mM)/glucose oxidase (GlcOx, 0.1 mg ml^{-1}) incubated for 14 h at 37 °C. [f] Same as e with myeloperoxidase (MPO, 0.2 μM). [g] Same as e with $FeCl_2$ (20 μM). [h] Hematoporphyrin IX (HPIX, 40 μM), $h\nu$ (400–700 nm), 14 h incubation. [i] hPMNs, (5.4×10^7 cells per ml) activated with PMA (0.1 mg ml^{-1}) in PB or PB in D_2O. [j] A stream of O_3/O_2 gas (5 ml min^{-1}) was passed over a solution of hLDL in PBS (pH 7.4) for 40 s.

no **2a** is observed (ESI,† Tables S4 and S5). 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 $^1O_2$ pathway to **2b**, because the measured levels of **2b** are elevated in PBS containing D_2O, relative to H_2O (ESI,† Table S6).

A clear finding of the hLDL oxidation studies detailed in Table 1 is that the 2,4-DNP hydrazone of atheronal-A (**2a**) is only generated 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. S1). To validate this result, cholesterol **3**, which had been exposed to ozone in aluminium oxide (Al_2O_3)-treated $CDCl_3$, 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. S2). 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 derivatization of the ^1H NMR samples.

The oxidation of **3** by $^1O_2$ 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 $^1O_2$ and superoxide anion-mediated biological- and chemical-oxidations of hLDL, hHDL and liposome-derived **3** in aqueous buffers result 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 (3) conversion of **4a** or **4b** into **2b** during the 2,4-DNP hydrazine derivatization process.

A number of experiments have been performed that help to deconvolute these alternatives. 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 S7 and Fig. S3). In addition, we have shown previously that authentic **1a** does not undergo measurable aldolization to **1b** under the conditions of these studies.[7] Finally, Pratt and co-workers[1] 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 (2 mM 2,4-DNP hydrazine, 0.08% HCl in isopropanol–ethanol (9 : 1)) to yield quantitative amounts of **2b** (no **2a** is detected, ESI,† Fig. S4). In addition, 7α-hydroperoxide **4b** does not undergo conversion into either **2a** or **2b** during the 2,4-DNP hydrazine derivatization process *vide supra*. Therefore, the **2b** that arises after $^{\bullet}O_2^-$, $^1O_2$ 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 derivatization process.

We have studied the singlet oxygen route to the cholesterol secosterols further; photochemical oxidation of **3** in Al_2O_3-treated $CDCl_3$ with *in situ* acid (TFA, 1% v/v) leads to a good yield of **1b** (>69%), and surprisingly, albeit in low yield, of **1a** (~3.5%). Inclusion of participating solvents, such as methanol, or increasing acid concentration does not significantly increase the yield of isolated secosterol **1a** (ESI,† Table S8). When the concentration of TFA is lowered sufficiently (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 as a discrete intermediate, during this process on a time-scale where allylic isomerization of **4a** to **4b** competes measurably (ESI,† Table S9 and Fig. S5). Furthermore, when authentic **4a** is incubated in isopropanol–ethanol (9 : 1) with 0.08% HCl 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 **4a**-derived mixture with 2,4-DNP hydrazine then yields **2b** and hydrazones of **1b**-derived dehydration products with no measurable **2a** or **2a**-derived dehydration or acetal products in either case (ESI,† Fig. S6). Thus, the summary of this singlet oxygen/Hock-cleavage 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 $^1O_2$ 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 5α-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 S10, Fig. S7-S8)). 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 = 7.5 ± 1.3 nM (n = 4); 2,4-DNP hydrazine method = 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. S9).

Analysis of human arterial plaque extracts by IDGCMS reveals a mean [**1a(b)**] of 32 ± 15 pmol mg^{-1} of tissue which is similar to levels we previously reported for **2b** in plaque extracts[2] (ESI,†Fig. S10–S12). 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 finding (ESI,† Fig. S11 and S12).

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 5α-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, does not, at least in part, arise from this route.

Pratt and co-workers[1] have shown that Hock-cleavage of authentic **4a** in organic solvents 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. 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 over a range of pH values, temperatures and durations of reaction and found that the ratio **1a** : **1b** varies from ~15 : 1 to ~4 : 1, but in all cases, the level of atheronal-A is greater than atheronal-B (ESI,† Table S12). An analysis of our original report of the levels of cholesterol secosterols (measured as their hydrazones **2a** and **2b**) in human arterial plaque samples reveals that the ratio of **2a** : **2b** varies greatly (61.3 : 1 to 0.06 : 1, n = 28) (ESI,† Table S11). However, the ratio of **2a** : **2b** is >1 in 20 of the 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, is **1a**.[2] Based upon our new understanding of the ozone and singlet oxygen pathways from **3** into **2a** and **2b** (after 2,4-DNP hydrazine derivatization), this clinical data offers support for an oxidant with the chemical signature of ozone playing a composite role in arterial plaque cholesterol secosterol **1a** biogenesis.

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. The terminal products from cholesterol ozonolysis, **1a** and **1b**, may not discriminate the pathways to their formation under all conditions, however, a ratio of **1a** to **1b** of >1 is strong support for oxidation of **3** by an oxidant with the chemical signature of ozone. 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.

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