Cholesterol

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Cholesterol Hydroperoxides as Substrates for Cholesterol-Metabolizing Cytochrome P450 Enzymes and Alternative Sources of 25-Hydroxycholesterol and other Oxysterols

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Abstract: *The interaction of the primary autoxidation products of cholesterol, namely 25- and 20ξ-hydroperoxides, with the four principal cholesterol-metabolizing cytochrome P450 enzymes is reported. Addition of cholesterol 25-hydroperoxide to the enzymes CYP27A1 and CYP11A1 induced well-defined spectral changes while generating 25-hydroxycholesterol as the major product. The 20ξ-hydroperoxides induced spectral shifts in CYP27A1 and CYP11A1 but glycol metabolites were detected only with CYP11A1. CYP7A1 and CYP46A1 failed to give metabolites with any of the hydroperoxides. A P450 hydroperoxide-shunt reaction is proposed, where the hydroperoxides serve as both donor for reduced oxygen and substrate. CYP27A1 was shown to mediate the reduction of cholesterol 25-hydroperoxide to 25-hydroxycholesterol, a role of potential significance for cholesterol-rich tissues with high oxidative stress. CYP27A1 may participate in the removal of harmful autoxidation products in these tissues, while providing a complementary source of 25-hydroxycholesterol, a modulator of immune cell function and mediator of viral cell entry.*

Cholesterol plays a major role in many biological processes that are considered central to the well-being of living organisms. Apart from being the precursor of steroid hormones and bile acids, cholesterol plays a structural role and is an important component of cell membranes. Cholesterol excess in many tissues is prevented in part through the action of different cytochrome P450 enzymes that hydroxylate cholesterol at specific positions to facilitate its elimination.[1] These enzymes include the 7-, 24- and 27-hydroxylases CYP7A1, CYP46A1, and CYP27A1, as well as the cholesterol side-chain cleavage enzyme CYP11A1.[1,2] CYP7A1 and CYP46A1 are microsomal enzymes, whereas CYP27A1 and CYP11A1 are mitochondrial monooxygenases. CYP7A1 is expressed only in the liver, CYP46A1 is mainly found in the neurons, CYP27A1 is ubiquitous, and CYP11A1 is the main cholesterol hydroxylase in steroidogenic tissues. For cholesterol to be metabolized by these P450s, they need NADPH as a source of reducing equivalents. They also need protein(s) that transfer electrons from NADPH to the P450: cytochrome P450 oxidoreductase for microsomal CYP7A1 and CYP46A1, and ferredoxin oxidoreductase/ferredoxin for mitochondrial CYP27A1 and CYP11A1.[3] The principal intermediate in P450-mediated hydroxylations has been identified as Compound I (Cpd I), the highly reactive heme-Fe^{IV}=$O^{+\cdot}$ species.[4] In addition to the P450 hydroxylases, various tissues express cholesterol 25-hydroxylase (CH25H), an endoplasmic reticulum (ER)-bound protein and a member of the non-heme lipid hydroxylases that use diiron–oxygen as a cofactor.[5] The past few years have seen important advances in understanding the function of 25-hydroxycholesterol (**4**) as a key modulator of immune cell function and an inhibitor of viral entry.[6] This oxysterol may also be produced in cholesterol-rich tissues via autooxidation,[7] a possibility that has received far less attention.

Many oxysterols found in living organisms are also present in air-aged cholesterol, which led to the speculation that some of them may have fundamental roles in mammalian cells.[8] Although secondary autoxidation products have been detected in cholesterol-rich human tissues, the 24-hydroxycholesterol in human brain[9] and 27(26)-hydroxycholesterol in human atherosclerotic plaques[10] are mainly of enzymatic origin. Most oxysterols found in nature are derived from singlet-oxygen attack at the cholesterol Δ5 double bond to give the 5- and 7-hydroperoxide derivatives and their decomposition products.[11] In contrast, ground-state biradical triplet oxygen attacks at tertiary side-chain carbons to give cholesterol 25-hydroperoxide (**1**) as a major product, together with small amounts of the epimeric 20ξ-hydroperoxides **2** and **3** (Scheme 1).[7] Minute amounts of cholesterol 26(27)- and 24-hydroperoxides have also been isolated from air-aged cholesterol.[12] Decomposition of these hydroperoxides accounts for the formation of a spate of secondary rearrangement products, including the reduced hydroxy analogues,[13] as well as a complex mixture of volatile products.[14] Thermal decomposition of the isomeric 20α- and 20β-hydroperoxides **2** and **3** yields pregnenolone as a major product, while mitochondrial CYP11 A1 converts **2** and **3** to 20α,22R-dihydroxycholesterol (**7**) and 20β,21-dihydroxy-20-*iso*-cholesterol (**8**), respectively.[15] Even though **7** is a natural intermediate in the CYP11A1-mediated cholesterol side-chain cleavage reaction, the 20α-hydroperoxide **2** is not. Instead, the enzymatic conversion of cholesterol into pregnenolone is believed to proceed via three consecutive oxidation steps to give 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol as enzyme-bound intermediates[16] prior to cleavage of

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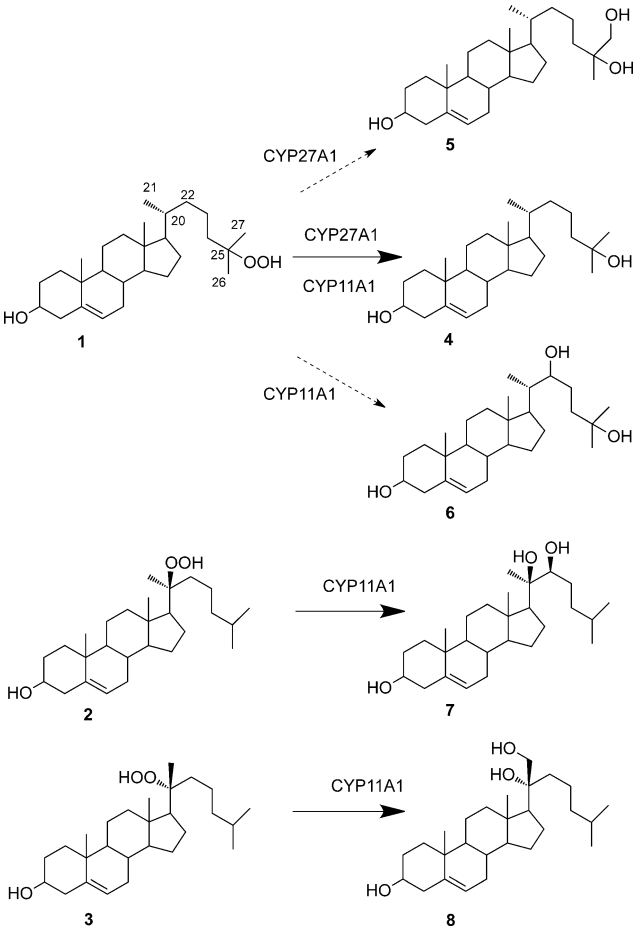


Scheme 1. Structures of cholesterol 25-hydroperoxide (**1**), cholesterol 20α-hydroperoxide (**2**), and 20-*iso*-cholesterol 20β-hydroperoxide (**3**) and their metabolites. Incubation of **1** with either CYP27A1 or CYP11A1 gave 25-hydroxycholesterol (**4**) as the major product. Secondary products include 25,26-dihydroxycholesterol (**5**) from CYP27A1 and 25,22-dihydroxycholesterol (**6**) from CYP11A1. Incubation of **2** with CYP11A1 gave 20α,22R-dihydroxycholesterol (**7**), whereas the epimeric 20β-hydroperoxide **3** gave 20β,21-dihydroxy-20-*iso*-cholesterol (**8**) as single product.

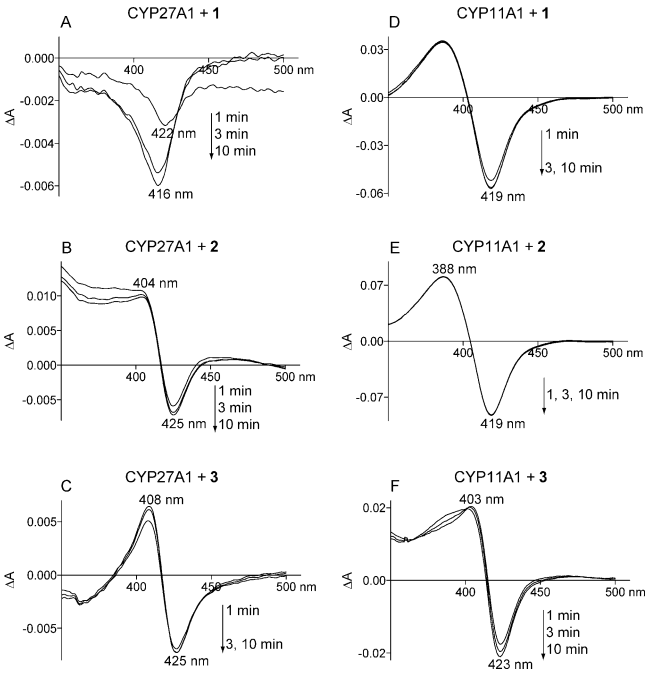


Figure 1. Difference spectra developed within 1–10 min after the addition of cholesterol 25-hydroperoxide (**1**), cholesterol 20α-hydroperoxide (**2**) or 20-*iso*-cholesterol 20β-hydroperoxide (**3**) to CYP27A1 and CYP11A1. A) CYP27A1 and **1**, B) CYP27A1 and **2**, C) CYP27A1 and **3**, D) CYP11A1 and **1**, E) CYP11A1 and **2**, F) CYP11A1 and **3**.

the C20–C22 bond to release pregnenolone from the enzyme.[17]

Spectral changes in CYP27A1 upon the addition of 25-hydroperoxide **1** gave a difference spectrum with a trough at 416 nm that shifts within 10 min to 422 nm (Figure 1 A). Thin-layer chromatography (TLC) of the $MeCl_2$ extract showed that most of **1** had been reduced to the corresponding 25-hydroxycholesterol (**4**) as confirmed by identical gas chromatography mass spectrometry (GC–MS) properties of the TMS derivative as compared to authentic **4**. A small polar spot on the TLC suggested the presence of a triol product that correlated to a new peak in the GC–MS profile of the TMS derivative (< 30 % yield relative to **2**), to which we assign the 25,27-dihydroxycholesterol (**5**) structure (Scheme 1) based on its characteristic GC–MS fragmentation pattern. Addition of 20α-hydroperoxide **2** to CYP27A1 induced a difference spectrum with a maximum at 404 nm and a minimum at 425 nm (Figure 1 B), a spectral response that does not seem to correspond to any of the reported types of P450 responses.[18] TLC analysis showed a small brown spot in the triol region and a small peak (< 10 %) in GC–MS analysis, with an MS fragmentation pattern consistent with 20α,27-dihydroxycholesterol. Addition of the 20β-hydroperoxide **3** to CYP27A1 also induced a difference spectrum similar to that elicited by the 20α-hydroperoxide **2**, with a maximum at 408 nm and a minimum at 425 nm (Figure 1 C). However, no metabolites were detected in the $MeCl_2$ extracts as indicated by TLC and GC–MS analyses. Likewise, addition of hydroperoxides **1**, **2**, or **3** to CYP7A1 or CYP46A1 did not produce major reaction products as observed by TLC and GC–MS analysis. Spectral changes in the difference spectra of CYP7A1 suggested significant light scattering as indicated by a lack of well-defined peaks or troughs. However, there was no visible protein precipitation in the cuvette, which could be a reason for the increased solution light scattering. The light scattering was smallest upon the addition of 25-hydroperoxide **1** and produced a difference spectrum with a maximum at 384 nm and a minimum at 426 nm, parameters close to those of a type 1 spectral response (maximum at 380–390 nm and minimum at 415–420 nm).[19]

Spectral changes in CYP11 A1 upon addition of the 25-hydroperoxide **1** developed within 3 min to give a stable spectrum with a maximum at 388 nm and a minimum at 419 nm (Figure 1 D). This type of a response is indicative of water displacement from the coordination sphere of the heme iron and is usually elicited by P450 substrates.[20] Analysis of the $MeCl_2$ extract showed that most of **1** had been reduced to the 25-hydroxy analogue **4**, which was in all respects identical

to authentic **4** (Scheme 1). TLC analysis of the $MeCl_2$ extract and GC–MS analysis of the TMS derivatives revealed the presence of a minor product to which we assign the 22,25-dihydroxycholesterol (**6**) structure based on its characteristic MS fragmentation pattern (Scheme 1). The positon of the second hydroxy group in **5** and **6** corresponds to the natural cholesterol hydroxylation sites of CYP27A1 and CYP11A1, that is, C27 and C22, respectively, thus suggesting specific binding of the 25-hydroperoxide **1** in the P450 heme pockets. Addition of the 20α-hydroperoxide **2** to CYP11 A1 induced a classical type 1 difference spectrum with a maximum at 388 nm and a minimum at 419 nm (Figure 1 E) within 1 min. The isomeric 20β-hydroperoxide **3** gave a similar type of response, except that the maximum was shifted to 403 nm and the minimum to 423 nm and it took longer (ca. 10 min) for the spectrum to develop (Figure 1 F). Extraction of the incubation mixtures with $MeCl_2$ followed by TLC analysis showed that each isomer (**2** and **3**) was transformed into a single glycol product identified as the 20α,22R-glycol **7** and the 20β,21-glycol **8**, respectively (Scheme 1). Assigned structures were confirmed by comparison of the GC–MS fragmentation pattern to those of authentic samples.[12c]

The stereospecific 20ξ-hydroperoxide–glycol reactions can be envisaged by substituting 22R-hydroxycholesterol with 20α-hydroperoxide **2** or 20β-hydroperoxide **3** in the active site of the previously determined 22R-hydroxycholesterol/CYP11 A1 co-complex[2b] (Figure 2). The model shows the hydroperoxide oxygen of **2** at a distances of 2.7 Å from the Fe^{III} heme iron, which enables formation of a transient heme-Fe^{IV}=$O^{+\cdot}$ species (Cpd I) that can subsequently oxidize the adjacent 22R-position of **2** (4 Å from the heme iron) to yield the 20α,22R-glycol **7**. Changing the configuration about C20 to the 20β-isomer **3** places the hydroperoxide oxygen 3.1 Å from the heme iron and the 21-methyl 3.5 Å from the heme iron, thereby favoring the formation of the 20β,21-glycol **8** (Figure 2). The involvement of a reactive transient intermediate (Cpd I) in the hydroperoxide–glycol conversion was previously proposed for adrenal bovine mitochondrial CYP11A1 and supported by intermediate product analysis,[21] stopped-flow UV/Vis spectroscopy, and electron paramagnetic resonance measurements.[22]

Our data conform a reaction mechanism (Scheme 2) whereby the cholesterol hydroperoxide substrates **1**–**3** bind in the CYP27A1 or CYP11A1 heme pocket and provide a reduced oxygen atom to support a hydroperoxide-shunt reaction to yield the intermediate heme-Fe^{IV}=$O^{+\cdot}$ species (Cpd I). This shunt does not require CYP27A1 redox partners or endogenous NADPH as a source of reducing equivalents. Depending on the proximity of the reactive oxygen of Cpd I to the cholesterol side chain, the latter may be hydroxylated to give dihydroxycholesterols or may dissipate while the reduced hydroxy compound is released. This shunt is well established for xenobiotic-metabolizing cytochrome P450 enzymes,[3] but is here shown to be operative for CYP27A1 and CYP11A1 for the first time. The metabolism of 25-hydroperoxide **1** by CYP27A1, a mitochondrial enzyme, may complement 25-hydroxycholesterol (**4**) production from cholesterol by C25H, a microsomal enzyme, and thus provide a source of a biologically active oxysterol to different cellular compartments. Metabolism of 25-hydroperoxide (**1**) by

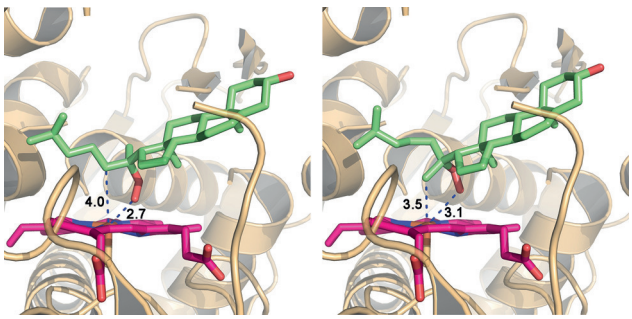
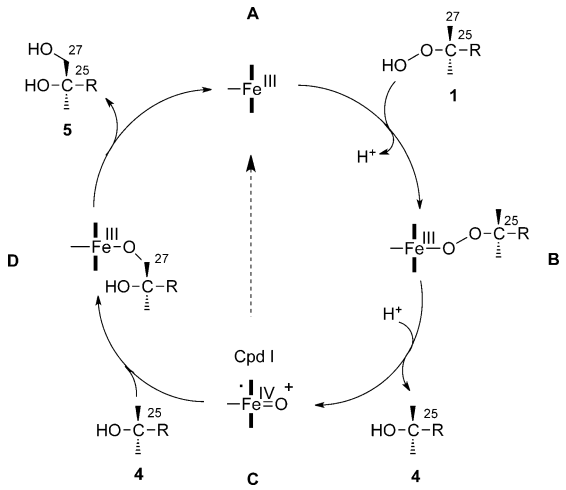


Figure 2. Putative positions of cholesterol 20α-hydroperoxide (**2**; left panel) and 20-*iso*-cholesterol 20β-hydroperoxide (**3**; right panel) in the active site of CYP11A1. Steroids were placed into the crystal structure of the previously determined 22R-hydroxycholesterol-CYP11A1 co-complex instead of 22R-hydroxycholesterol.[2b] The model shows the 20α-hydroperoxide oxygen atom of **2** at a distances of 2.7 Å from the Fe^{III} heme iron, which permits formation of the transient heme-Fe^{IV}=$O^{+\cdot}$ species (Cmp I) that subsequently hydroxylates the adjacent 22R-position of **2** (4 Å from the heme iron) to yield the 20α,22R-glycol **7**. In the case of the 20β-isomer **3**, the hydroperoxide oxygen is located 3.1 Å from the heme iron and the 21-methyl group is located 3.5 Å the Fe^{III} heme iron, thus resulting in the formation of the 20β,21-glycol **8** (Scheme 1).



Scheme 2. Hydroperoxide-shunt reaction in the catalysis of cholesterol 25-hydroperoxide (**1**) by CYP27A1, showing the proposed intermediates (adapted from Ref. [3]). The 25-hydroperoxide **1** binds to the oxidized (Fe^{III}) heme iron of CYP27A1 (**A**) with the release of a hydrogen ion (H^+) to give intermediate (**B**). Heterolysis of the O-O hydroperoxide bond and H^+ addition gives Cpd I (**C**) and 25-hydroxycholesterol (**4**), which is either released with the dissipation of Cpd I (dashed arrow) or the latter reacts with the nearby C27 carbon of **4** to give complex **D**, from which 25,27-dihydroxycholesterol (**5**) is released. In the case of the reaction of **1** and CYP11A1 the secondary triol product is 22,25-dihydroxycholesterol (**6**). Both CYP27A1 and CYP11A1 give 25-hydroxycholesterol (**4**) as the principal product. In the case of the interaction of the isomeric 20ξ-hydroperoxides **2** and **3** and CYP11A1, specific binding in the heme pocket (Figure 2) results in the formation of triols **7** and **8**, respectively, without release of the intermediate 20-hydroxy analogues.

CYP27A1 may also serve the purpose of elimination of a highly reactive hydroperoxide that may further oxidize other lipids and thereby damage the cells where it is produced.

In summary, the isolation of cholesterol 25-hydroperoxide (**1**) and the epimeric 20-hydroperoxides **2** and **3** from air-aged cholesterol, as well as the availability of all four purified recombinant cholesterol-metabolizing cytochrome P450 enzymes, provided us with the unique opportunity to evaluate their interactions by using spectroscopy and metabolite analysis. Herein, we have shown for the first time that both CYP27A1 and CYP11A1 reduce cholesterol 25-hydroperoxide (**1**) to 25-hydroxycholesterol (**4**) while generating small amounts of dihydroxycholesterols. The epimeric 20ξ-hydroperoxides **2** and **3** reacted with CYP11A1 only to give characteristic glycol products. A reaction mechanism involving a P450 hydroperoxide-shunt reaction is proposed, whereby the sterol hydroperoxides provide a reduced oxygen atom to generate Cpd I and the corresponding reduced hydroxysterol, while simultaneously serving as substrate for further hydroxylation. Our results provide the first in vitro evidence for a role of CYP27A1 in cholesterol-rich tissues with high oxidative stress. In this context, it could function as a catalyst for the reduction of cholesterol 25-hydroperoxide to 25-hydroxycholesterol, a modulator of immune cell function and mediator of viral cell entry. Elimination of highly reactive cholesterol hydroperoxides by CYP21A1 may prevent the formation of peroxidative chain reactions that can inflict cellular damage.

Experimental Section

The cholesterol 25-hydroperoxide (**1**), cholesterol 20α-hydroperoxide (**2**), and the 20-*iso*-cholesterol 20β-hydroperoxide (**3**), were isolated from a more than 15 year old sample of 500 g cholesterol (Scheme 1).[7,12c] The 20-hydroxycholesterols, 22R-hydroxycholesterol, 25-hydroxycholesterol (**2**), 27-hydroxycholesterol, and pregnenolone were acquired from Steraloid Inc. (Pawling, N.Y.). The 20α,22R-dihydroxycholesterol (**7**) was prepared by established methods.[23] The 20α,21-dihydroxycholesterol and epimeric 20β,21-dihydroxy-20-*iso*-cholesterol (**8**) were prepared from 21-hydroxypregnenolone with addition of the side chain via a Grignard reaction.[12a,d] The assigned configurations were confirmed by X-ray crystallography.

Bovine recombinant CYP11A1 and human recombinant CYP27A1, CYP46A1, and CYP7A1 were expressed and purified as previously described.[2b,24] P450 difference spectra were recorded as described,[25] except that two double, not single, cuvettes were used to compensate for a residual hydroperoxide absorption in the visible region. The concentrations of P450 and cholesterol hydroperoxide were 5 μM and 10 μM, respectively. Incubations were carried out at 24 °C. The incubation buffers were 50 mM potassium phosphate (KP_i), pH 7.2, containing 1 mM EDTA (for CYP11A1 and CYP7A1); 50 mM KP_i, pH 7.2, containing 0.5 M NaCl, 10 % glycerol, and 1 mM EDTA (for CYP27A1); and 50 mM KP_i, pH 7.2, containing 0.1M NaCl (for CYP46A1). For product analyses, P450s were incubated with cholesterol hydroperoxides for 20 min followed by sterol extraction with $MeCl_2$ and analysis by TLC as previously described.[15e] The remaining sample was derivatized with bis-(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane (TMS) and analyzed by GC–MS as described.[26]

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