Sterol 27-hydroxylase acts on 7-ketocholesterol in Human Atherosclerotic Lesions and Macrophages in Culture.

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Running title: Oxysterol metabolism by 27-hydroxylase in atherosclerosis
SUMMARY

27-Hydroxycholesterol (27OH) is the major oxysterol in human atherosclerotic lesions, followed by 7-ketocholesterol (7K). Whereas 7K probably originates non-enzymically, 27OH arises by the action of sterol 27-hydroxylase, a cytochrome P450 enzyme expressed at particularly high levels in the macrophage and proposed to represent an important pathway by which macrophages eliminate excess cholesterol. We hypothesized and here show that 27-hydroxylated 7-ketocholesterol (27OH-7K) is present in human lesions, probably generated by the action of sterol 27-hydroxylase on 7K. Moreover, [³H]-27OH-7K was produced by human monocyte-derived macrophages (HMDMs) supplied with [³H]-7K but not in HMDMs from a patient with cerebrotendinous xanthomatosis (CTX) shown to have a splice-junction mutation of sterol 27-hydroxylase. While [³H]-27OH-7K was predominantly secreted into the media, [³H]-27OH formed from [³H]-cholesterol was mostly cell-associated. The majority of supplied [³H]-7K was metabolized beyond 27OH-7K to aqueous soluble products (apparently bile acids derived from the sterol 27-hydroxylase pathway). Metabolism to aqueous soluble products was ablated by a sterol 27-hydroxylase inhibitor and absent in CTX cells. Sterol 27-hydroxylase therefore appears to represent an important pathway by which macrophages eliminate not only cholesterol but also oxysterols such as 7K. The fact that 7K (and cholesterol) still accumulates in lesions and foam cells indicates that this pathway may be perturbed in atherosclerosis and affords new opportunity for the development of therapeutic strategies to regress atherosclerotic lesions.
INTRODUCTION

Cholesterol 7α-hydroxylase was previously considered to be the first and rate-limiting enzyme in hepatic bile acid synthesis. Subsequently, an alternate pathway has been identified involving conversion of cholesterol to 27-hydroxycholesterol (27OH)\(^1\) by sterol 27-hydroxylase in the liver, followed by 7α-hydroxylation of 27OH by one or more distinct oxysterol 7α-hydroxylases (1-3). Additional to its role in hepatic bile acid synthesis, there is strong evidence that sterol 27-hydroxylase is involved in the elimination of cholesterol from extrahepatic cells (4-6). This mitochondrial cytochrome P450 enzyme is widely distributed in most organs and tissues, but is expressed at particularly high levels in macrophages (7). It co-localizes with macrophages in human carotid lesions (8) and its product, 27OH, is a major oxysterol in lesions and macrophage-derived foam cells (9). This enzyme sequentially oxidizes the same methyl group to eventually form 3β-hydroxy-5-cholestenoic acid (10). 27OH and cholestenoic acid are synthesized by cultured macrophages in response to cholesterol loading (6) and are more readily excreted by the cells than cholesterol (6,7). Cholesterol-engorged foam cells, derived from macrophages, are the major cell-type in early atherosclerotic lesions and their ability to export sterols to extracellular acceptors (e.g. HDL) is considered crucial for controlling cholesterol accumulation in the artery wall (11). But the conversion of 27OH to cholestenoic acid may also be physiologically important since this sterol can be exported from macrophages to albumin, whereas cholesterol and 27OH export relies on lipoprotein acceptors which may be limiting at sites of atherosclerosis (7). In plasma, most 27OH is associated with high-density lipoprotein, whereas all of the cholestenoic acid is found in the lipoprotein-free fraction (12). Furthermore, the sterol 27-

\(^1\) Abbreviations: 27OH, 27-hydroxycholesterol; 27OH-7K, 27-hydroxylated 7-ketocholesterol; 7K, 7-ketocholesterol; 7βOH, 7β-hydroxycholesterol; ACAT, acyl coenzyme A: cholesterol O-acyl transferase; BSA, bovine-serum albumin fraction V; CTX, cerebrotendinous xanthomatosis; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; HMDM, human monocyte-derived macrophage; HPLC, high performance liquid chromatography; LDL, low-density lipoprotein; mRNA, messenger ribonucleic acid; PBS, phosphate buffered saline; UV, ultraviolet
hydroxylase pathway in peripheral tissues in healthy humans constitutes a small but significant proportion of total bile acid synthesis providing additional in vivo evidence for this being an important pathway for reverse sterol transport (6,13). Moreover, patients with cerebrotendinous xanthomatosis (CTX) lack this enzyme and develop atherosclerosis prematurely, despite having normal circulating levels of cholesterol (14).

7-ketocholesterol (7K) is the major non-enzymically formed oxysterol in human atherosclerotic lesions and accumulates in macrophage-foam cells relative to whole lesion (9). Moreover, 7K displays many potent effects in vitro that could implicate it in the initiation and/or development of atherosclerosis (15). Given that 7K can serve as a substrate for sterol 27-hydroxylase in human fibroblasts (16) and HepG2 cells we hypothesized and here show that the 27-hydroxylated product of 7K (27OH-7K) is found in human lesions. We further hypothesized that sterol 27-hydroxylase may be an important pathway by which macrophages eliminate not only cholesterol but also oxysterols such as 7K. This hypothesis was tested by investigating the ability of macrophages from normal subjects and a patient with CTX to metabolize 7K.

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EXPERIMENTAL PROCEDURES

Reagents

All solvents were high performance liquid chromatography (HPLC) grade (EM Science or Mallinckrodt). Other chemicals and reagents used are listed with supplier: bovine-serum albumin fraction V (BSA; Sigma-Aldrich); chenodeoxycholic acid (Sigma-Aldrich); cholic acid (ICN); [1α,2β(n)-3H]-cholesterol (Amersham, specific activity: 49 Ci/mmol); [1,2,6-3H]-7-ketocholesterol (5-cholesten-3β-ol-7-one) (American Radiolabeled Chemicals, specific activity: 50 Ci/mmol; 99% pure); RPMI 1640 (Trace Biosciences); OptiPrep™ density-gradient media (Nycomed Pharma); ursodeoxycholic acid (Sigma-Aldrich). [14C]- and non-labeled 27OH-7K standards were prepared as described in Lyons et al. S58-035 and GW273297x were generous gifts from Sandoz and Glaxo-Wellcome, respectively.

Atherosclerotic artery samples

Samples of human atherosclerotic lesions were obtained from 10 patients undergoing a carotid endarterectomy. Patients were mostly male (8/10) and ranged in age from 52 to 78 years. This study was approved by the Central Sydney Area Health Service Ethics Review Committee and full and informed written consent was obtained from the patients. Within one hour of excision, samples were placed into Chelex-treated and argon-flushed phosphate-buffered saline (PBS) containing EDTA (1 mmol/L), butylated hydroxytoluene (0.1 mmol/L), penicillin (100 U/mL) and streptomycin (100 µg/mL). Samples were freeze-dried, extracted with acetone and subjected to ice-cold alkaline saponification as detailed previously, using 19-hydroxycholesterol as an internal standard (17). Four of the extracts were stored for two to three years (-80°C) while another six extracts were freshly-prepared at the time of analysis.
CTX patient

A 39-year old woman presented with classical clinical features of CTX: tendon xanthomata, myopathic facies, cataracts, and neurological abnormalities including low IQ. While displaying a normal electrocardiograph, carotid ultrasonography revealed increased intima-medial thickness and plaques at both carotid bifurcations. Plasma lipids were normal on presentation (Total cholesterol, 5.1 mmol/L; LDL-cholesterol, 3.0 mmol/L; HDL-cholesterol, 1.0 mmol/L; plasma triglycerides, 2.5 mmol/L), but plasma cholestanol levels (59 µmol/L) were elevated four-fold above the upper reference limit (2.6 - 15 µmol/L). The patient was genotyped and a previously described mutation (18) was found in the splice-junction of exon 4 and intron 4 (Burnett, Moses, Croft, Brown, Lietersdorf, Watts, manuscript in preparation). A G to A transition at cDNA position 865+1 (intron 4) results in skipping of exon 4 which leads to a loss of 198 base pairs in the mRNA and 66 amino acid residues from the enzyme (18). A similar splice-junction mutation has been reported to result in complete absence of sterol 27-hydroxase activity (19). Samples of whole blood (100 ml) collected in EDTA (4 µmol/mL) from the CTX patient and from a control subject were transported together at ambient temperature from Perth to Sydney (Australia) where monocytes were isolated and plated within 28 h of collection.

Isolation and Culture of Human monocyte-derived macrophages

Human monocytes were isolated from white cell concentrates using centrifugal elutriation (20) and cultured as described (21). Monocytes were isolated from the blood of the CTX patient and the contemporaneous control subject by the method of Boyum (22) using OptiPrep™ solution of density 1.068 g/ml and a solution of density 1.063 g/ml to remove platelets. Purified monocytes were differentiated to human monocyte-derived macrophages (HMDM) by plating at a density of 1-2 x10^6 cells/22-mm diameter culture well (Falcon) in RPMI 1640 containing penicillin G and
streptomycin (50 U/mL and 50 µg/mL, respectively), L-glutamine (2 mM) and 10% (v/v) heat-inactivated whole human serum for 9 days. Following differentiation, some cells were washed and incubated with RPMI 1640 containing lipoprotein-deficient serum (10% v/v, final protein concentration 2.5 mg/mL) and acetylated low-density lipoprotein (50 µg/ml) (23) for four days to increase cellular free and esterified cholesterol. After cholesterol-loading or immediately after differentiation (non-loaded), cells were incubated for 24 h with RPMI 1640 containing bovine serum albumin (1 mg/mL) plus [3H]-7K or [3H]-cholesterol (1 µCi/culture) delivered in ethanol (0.1% w/v). In specified experiments, cells were simultaneously incubated with an inhibitor of sterol 27-hydroxylase (GW273297x; 1 µmol/L) or an inhibitor of acyl coenzyme A:cholesterol acyl transferase (ACAT) (Sandoz 58-053; 0.5 µg/mL) (23). Medium was then removed from each culture well and centrifuged (16,000 x g, 4°C, 10 min) to remove any detached cells. Cell monolayers were washed three times in PBS and lysed in 600 µL Triton-X100 (0.1% v/v). Medium and cell lysates were extracted with chloroform/methanol (2:1 v/v) (24).

Sterol analysis

The method of analysis of 27OH-7K in human lesion and cell culture samples was modified from Brown et al. (17) and involved normal-phase HPLC. Separation used a silica column (0.46 x 25 cm) with a 3 cm guard column (Ultremex; 3 µm particle size; Phenomenex). The mobile phase of hexane/isopropanol/acetonitrile (94.5/3.9/0.6 v/v) was run at 1.0 mL/min. Typical retention times of cholesterol, 7K, and 27OH-7K were 5.3, 15.2, and 46 min, respectively. For lesion analysis, the HPLC system (Shimadzu) comprised a SIL-10A autosampler, pump (LC-10AT), and diode array detector (SPD-M10AVP). The detection limit for 27OH-7K was 15 pmol/injection. For experiments including [3H]-sterols, the HPLC system comprised a manual injector (Rheodyne), pump (LK Bromma 2150), variable wavelength monitor (LK Bromma 2151) at 210
nm followed in series by a radiometric detector (Canberra-Packard Series A-100 Radiomatic Flowone Beta Radio-Chromatography Detector) using Ultima-Flo M scintillant (Canberra-Packard) at scintillant to effluent ratio of 2.

**Analysis of standard 27OH-7K and a co-eluting peak from human lesions by GC-MS**

27OH-7K was synthesized and analyzed by GC-MS as described previously. The samples were analyzed as trimethylsilyl (TMS) ether derivatives. Each of the 27OH-7K samples was injected in a volume of 2 µl with the purge valve closed for 1 min. Selected ion monitoring of the molecular ion (m/z 560) and fragment ions (m/z 545, 470, 174, 129) was used for the analysis.

**Analysis for bile acid-like compounds**

Media from human macrophages incubated with [3H]-7K were extracted by the Folch method (24). Replicates were pooled (from two experiments for control cells and from one experiment for CTX cells), concentrated using solid phase extraction (C18 Sep-Pak Vac RC cartridge, Waters) and the methanol eluent reduced under vacuum. Samples were dissolved in mobile-phase (methanol/acetonitrile/20 mmol/L sodium acetate, pH 4.3: 60/20/20 v/v/v) and injected onto the HPLC system described above with 210 nm and radiometric detection in series. Separation was achieved by an Ultrasphere ODS column (0.46 x 15 cm) with a 3 cm guard column (Beckman; 5 µm particle size) (25) at a flow-rate of 1.0 mL/min.

**Protein determination**

The protein content of the cell extracts and LDL preparations was measured using the bicinchoninic acid method (Pierce) using BSA as standard. Standards were prepared in Triton-
X100 (0.1 % v/v) or PBS for the acetylated LDL preparations. After incubation (60 °C, 60 min), absorbance was measured at 562 nm.

Statistics and presentation of data

Data from multiple experiments are given as mean ± standard error of the mean (SEM) and data from a single experiment (in triplicate) are given as mean ± standard deviation (SD). Correlations between lesion sterol levels were obtained by linear regression. Data were compared using unpaired t-tests with equal variance. A p-value <0.05 (two-tailed) was considered statistically significant.

RESULTS

27OH-7K in human carotid lesions

Routinely, 27OH-7K was below the limits of detection in non-saponified samples of human carotid lesion extracts when 1 mg of lipid was analyzed. Hence, analysis was scaled-up five- to ten-fold for two samples (equivalent to 0.17 nmol of total 27OH-7K per sample). Unesterified oxysterols (including 19-hydroxycholesterol as internal standard) were isolated by silica thin-layer chromatography before analysis by normal-phase HPLC. The difference in 27OH-7K levels measured in samples with and without saponification allowed estimation of the proportion of esterified 27OH-7K. This comprised 96% and 61% of total 27OH-7K, respectively in the two samples. Thus, 27OH-7K was predominantly esterified.

After cold alkaline saponification of lipid extracts from carotid lesions, a peak was found to co-elute with standard 27OH-7K both on gas chromatography and normal-phase HPLC (retention time relative to 7K: 1.29 and 3.0, respectively). This peak also had an identical UV spectrum and mass spectrum to the authentic standard (Fig.1), confirming its identification as 27OH-7K. A
preliminary investigation of four stored extracts indicated the presence of 27OH-7K (mean ± SD: 37.1 ± 25.5 mmol/mol 7K). Subsequent analysis of 6 freshly processed samples yielded comparable results (33.9 ± 11.3 mmol/mol 7K), suggesting that 27OH-7K does not arise artefactually from oxidation of 27OH during storage. When the data were pooled (n=10) and expressed on a cholesterol-standardized basis, it was evident that 27OH-7K is a minor component of lesions (77 ± 92 µmol/mol cholesterol, n=10). The levels of 27OH-7K were not correlated with 27OH but were strongly associated with 7K levels (r=0.90; p=0.001) (Fig. 2). This indicates that 27OH-7K in lesions is likely to arise mainly by the action of sterol 27-hydroxylase on 7K, rather than by non-enzymic oxidation of 27OH.

Production of 27OH-7K by human macrophages in culture

Monocytes were isolated from blood obtained from an apparently healthy donor and a typical CTX patient that lacked functional sterol 27-hydroxylase. Cells were matured into macrophages for 9 d in human serum and incubated with [3H]-7K or [3H]-cholesterol for 24 h. Separate Folch extractions of the cells and their respective media yielded lipid fractions containing residual, unaltered added radiosterol (7K or cholesterol) and lipophilic products (including 27OH-7K or 27OH). When control HMDMs were incubated with [3H]-7K, formation of [3H]-27OH-7K (Fig. 3) was 2.7 ± 0.9 pmol/mg cell protein (mean ± SEM from 3 independent experiments). 27OH-7K was absent in cultures derived from monocytes isolated from a CTX patient. As detailed below, most of the 27OH-7K was secreted to the media by control HMDMs. A much smaller amount of 27OH-7K present in cell extracts from control cells but completely absent from CTX cells (not shown). In media from control cells (Fig. 3B), there was a large, unidentified peak (retention time relative to 7K = 1.34) which corresponds to peak X observed in media from HepG2 cells that had been incubated with [3H]-7K2. This product was inhibitable by
GW273297x (not shown). In media from CTX cells (Fig. 3C), there was a small peak (relative retention time = 1.44) which is more likely to be 7β-hydroxycholesterol (7βOH) formed by reduction of 7K, since CTX cells contained a large amount of this co-eluting oxysterol compared to control cells (8.0 vs. 0.04 pmol/mg of cell protein).

Metabolism of 7K and cholesterol to aqueous soluble products by human macrophages in culture

Aqueous soluble [3H]-products in the aqueous phase of the Folch extracted media (from the same experiments described above) comprised >90% of the total [3H]-7K or [3H]-cholesterol-derived polar products; cells contained negligible aqueous soluble counts (<15 dpm/µg cell protein). Aqueous products were observed in media after 24h incubation with [3H]-7K or [3H]-cholesterol (Table 1). Co- or pre-incubation of control HMDMs with GW273297x resulted in near ablation of the release of aqueous [3H]-products from cells incubated with [3H]-7K (Fig. 4a) and also reduced metabolism of [3H]-cholesterol substantially (Fig. 4b). Metabolism of either sterol to aqueous metabolites was absent in CTX cells with or without the GW273297x inhibitor, confirming that these products are metabolites of sterol 27-hydroxylase.

Preliminary characterization of aqueous soluble products derived from 7K by human macrophages

Concentrated, pooled aqueous extracts of media from non-loaded macrophages that had been incubated with 7K were analyzed using a reverse-phase HPLC method that separates the major primary unconjugated bile acid products of cholesterol (25). The radioactivity was quantitatively recovered in two broad peaks. The major peak eluted with standard chenodeoxycholic acid while the minor peak eluted in the vicinity of ursodeoxycholic acid, the 7β-hydroxy-epimer of chenodeoxycholic acid (Fig. 5). Chenodeoxycholic acid is a major product of the sterol 27-
hydroxylase pathway while ursodeoxycholic acid is subsequently formed from this bile acid. Although chenodeoxycholic acid can also be formed by the classical (neutral) pathway, which is initially catalysed by cholesterol 7α-hydroxylase, no formation of cholic acid, produced only by this classical pathway, was found. Moreover, negligible radioactivity was detected in the aqueous extract from the media of CTX macrophages incubated with [3H]-7K (Fig.5c). Thus formation of these bile acid-like products is dependent on the action of sterol 27-hydroxylase on 7K.

Effect of cholesterol-loading and inhibition of sterol 27-hydroxylase or ACAT on metabolism of 7K by human macrophages in culture

Cholesterol-loading of macrophages reduced (~40%) the metabolism of 7K but not cholesterol to aqueous soluble products (despite isotope dilution, in the case of cholesterol) (Fig. 6). Metabolism of both 7K and cholesterol to aqueous soluble products could be abolished by co-incubation with the inhibitor of sterol 27-hydroxylase. Since sterol 27-hydroxylase is located on the inner mitochondrial membrane (26) only unesterified sterol can serve as a substrate for this enzyme. As cellular 7K tends to be predominantly esterified (27), an ACAT inhibitor was included in some incubations to investigate how manipulation of sterol esterification affected metabolism of 7K by HMDMs. The addition of the ACAT inhibitor during uptake of [1H]-7K reduced 7K esterification from 7.7 to 1.7 pmol/mg of cell protein and significantly increased metabolism of 7K (from 22.3 ± 1.5 to 29.8 ± 3.9 pmol/mg of cell protein; p=0.035 by paired t-test). Approximately two-fold more of the primary 27-hydroxylated products of 7K and cholesterol were formed in the presence of the ACAT inhibitor and there was an increase of ~20% and ~65% in aqueous soluble products formed from 7K and cholesterol, respectively. For both 7K and cholesterol, the ratio of the 27-hydroxylated intermediate to aqueous soluble
products increased by increasing the levels of a competing substrate (with cholesterol-loading) or with increased availability of substrate (with ACAT inhibition) (7K: 0.03, 0.11, 0.22; cholesterol: 0.05, 0.61, 0.99, respectively for non-loaded, cholesterol-loaded and cholesterol-loaded with ACAT inhibition), suggesting that the subsequent metabolism of the 27-hydroxylated intermediate is the rate-limiting step. Even though both 27OH and 27OH-7K are exclusively lipid-soluble (by Folch extraction), 27OH-7K was predominantly secreted into the media, whereas 27OH remained mostly cell-associated.

**DISCUSSION**

In this paper, we have demonstrated the occurrence of a novel oxysterol, 27OH-7K, in human carotid lesions. This oxysterol is likely to be formed by the action of sterol 27-hydroxylase rather than non-enzymic oxidation of 27OH, considering that levels in lesions correlated with levels of 7K but not with levels of 27OH. Moreover, human macrophages in culture, known to express high levels of sterol 27-hydroxylase (7), produced 27OH-7K when incubated with 7K. Formation of 27OH-7K by these cells was ablated by pre-incubation with a potent, specific inhibitor of sterol 27-hydroxylase and was absent in cells isolated from a CTX patient lacking a functional enzyme.

Sterol 27-hydroxylase has been proposed as an important pathway by which macrophages eliminate excess cholesterol (4,6). This enzyme acts initially to form 27OH and can then oxidize this intermediate further to the carboxylic acid derivative, which is polar enough to be released from the cell without the requirement of lipoprotein acceptors. This carboxylic acid, like free fatty acids, is believed to bind to albumin for transport back to the liver for further catabolism and eventual excretion. In the present study, 27OH mostly remained cell-associated in agreement with a previous report (7). In contrast, the primary 27-hydroxylation product of 7K, 27OH-7K,
was predominantly found in the medium, consistent with the concept that sterol 27-hydroxylase catalyzes the elimination of sterols other than cholesterol from macrophages. Moreover, the bulk of metabolized 7K was detected in the medium as more aqueous-soluble products. Preliminary HPLC characterization suggests that these aqueous-soluble products may in fact be downstream metabolites of the alternative (acidic) bile acid pathway catalyzed by sterol 27-hydroxylase. That formation of these products is dependent on sterol 27-hydroxylase activity is further substantiated by their absence in media from CTX cells. It appears that the subsequent action of sterol 27-hydroxylase on 27OH-7K may be the rate-limiting step in the formation of more aqueous-soluble metabolites since a ‘bottleneck’ is created either by increasing the availability of substrate (with ACAT inhibition) or by increasing the levels of a competing substrate (with cholesterol-loading).

This is the first study to demonstrate metabolism of 7K by macrophages. For the same amount of added sterol, 7K was metabolized to a much greater extent than cholesterol. 7K was also metabolized at a far greater rate than we observed in a human hepatoma cell-line\(^2\) (~50 vs. ~2 pmol/mg of cell protein/24 h, respectively). The fact that this metabolism is not ultimately successful in atherosclerotic lesions where these two sterols accumulate raises several issues. Firstly, radiotracer levels of 7K were employed in this study and the amount of 7K metabolized by the HMDMs was three to four orders of magnitude lower than levels in authentic human foam cells (28) or cellular levels shown to inhibit cholesterol export from cholesterol-loaded HMDMs (Cell Biology Group, HRI, unpublished observations). It could be argued that sterol 27-hydroxylase activity is insufficient to catabolize the relatively large amounts of 7K found in lesion foam cells. But it could also be argued that 7K accumulation in lesion macrophages would be gradual, with cells initially exposed to low levels of oxysterol, comparable to the radiotracer levels used herein. This latter possibility would suggest a perturbation in sterol 27-hydroxylase-mediated clearance of 7K in foam cells.
Possible perturbations may involve cholesterol-loading and esterification of 7K, since we have shown that both influence the extent of 7K metabolism by human macrophages in culture. Cholesterol-loading reduced the amount of 7K metabolized to aqueous soluble products probably through substrate competition by cholesterol, as shown for the action of a reconstituted enzyme system on 27OH (10). Thus, cholesterol-loading, which defines the macrophage-foam cell, may limit the ability of this cell-type to metabolize 7K.

Only free 7K (not esterified 7K) can access sterol-27 hydroxylase in the inner mitochondrial membrane (26). Esterification of 7K is therefore another important consideration. We demonstrated that blocking esterification of 7K increased its metabolism by sterol 27-hydroxylase. In human carotid lesions, approximately 80% of 7K is esterified (17) and preliminary analyses suggest that 7K in human lesion foam cells is also predominantly esterified (van Reyk and Brown, unpublished observations). While 7K esterified to normal (unoxidized) fatty acids can be hydrolyzed in murine (27,29,30) and human (Cell Biology Group, HRI, unpublished observations) macrophages, ester hydrolysis may be rate-limiting in atherosclerosis, as in some foam cell models (31-33). 7K ester hydrolysis will clearly influence the availability of unesterified 7K for metabolism by sterol 27-hydroxylase.

Using an immunohistochemical approach, Crisby et al. (8) have demonstrated the presence of sterol 27-hydroxylase protein in human carotid lesions, co-localizing with macrophages. How active sterol 27-hydroxylase is in lesions remains to be established. In preliminary experiments (Brown, unpublished observations), we have found metabolism of [\(^{1}H\)]-7K to aqueous-soluble products was greater in normal versus atherosclerotic intima isolated from the same freshly-excized carotid artery (mean ± range: 90.6 ± 4.2 and 10.1 ± 0.5 dpm/mg wet tissue/24 h; n=2 sections). Thus, not all of the sterol 27-hydroxylase protein detected in human lesions may be active.
In this study, we have presented four lines of evidence indicating a critical role for sterol 27-hydroxylase in the metabolism of 7K in human macrophages. Firstly, the 27-hydroxylated product of 7K forms when human macrophages are incubated with 7K. Secondly, metabolism of 7K to aqueous soluble products can be ablated by a selective inhibitor of sterol 27-hydroxylase. Thirdly, the aqueous soluble metabolites produced may correspond to bile acids formed by the sterol 27-hydroxylase pathway, and finally metabolism of 7K is absent in cells isolated from a CTX patient lacking a functional enzyme. The relevance of this pathway in vivo is indicated by the presence of 27OH-7K in human atherosclerotic lesions. We propose that this reflects an attempt by arterial cells, notably macrophages, to eliminate 7K. Sterol 27-hydroxylase activity has already been proposed as an important mechanism by which macrophages eliminate excess cholesterol (4,6) and this work extends this proposal to oxysterols. The presence of advanced carotid atherosclerosis in a CTX patient in the absence of recognized cardiovascular risk factors supports the contention that a defect in this pathway may be atherogenic. Given that 7K is a major oxysterol found in human lesions and foam cells and has been ascribed many potentially atherogenic properties, such as being cytotoxic, apoptotic and perturbing cellular sterol homeostasis (reviewed in (9)), the action of sterol 27-hydroxylase in metabolizing 7K offers an additional means by which this enzyme system may ameliorate the atherogenic process. The fact that 7K (and cholesterol) still accumulates in lesions and foam cells indicates that this pathway may be perturbed in atherosclerosis. Stimulating sterol 27-hydroxylase activity may afford a novel strategy for regressing atherosclerotic lesions.
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_Atherosclerosis_ **120**, 135-145
FIGURE LEGENDS

Figure 1. Identification of 27OH-7K in pooled extracts from human carotid lesions.
Standard 27OH-7K was analyzed by GC-MS (chromatogram A). An extract of human carotid lesions was analyzed and purified by HPLC (not shown) and was subsequently analyzed by GC-MS (chromatogram B). Using selected ion monitoring, the mass spectrum was collected for both standard 27OH-7K (C) and the co-eluting peak found in human carotid lesions (D). The molecular ion is at m/z 560. In addition the UV spectra of standard 27OH-7K and the co-eluting lesion peak were collected during HPLC analyses using a diode array detector. Procedures are as described in the Methods.

Figure 2. Levels of 27OH-7K in human carotid lesions are significantly correlated with levels of 7K (A) but not with 27OH (B).
Oxysterols were analyzed by normal-phase HPLC with UV detection at 234 nm as described in the Methods. In panel A, the line of best-fit is y = 0.020 x + 0.015 (n=9; r=0.90; p=0.001). When an outlier was included the correlation was still statistically significant: y = 0.054 x + 0.031 (n=10; r=0.78; p<0.01). In panel B, there was no significant correlation (r=-0.28; p=0.44).

Figure 3. 27OH-7K is secreted into the medium of normal human macrophages supplied with 7K but not in CTX macrophages.
Panel A shows standard [3H]-27OH-7K purified from Hep G2 mitochondria which had been incubated with [3H]-7K. Panels B and C show lipid extracts from the media of human monocyte-derived macrophages incubated with [3H]-7K for 24 h using normal (B) or CTX (C)
cells. The media were extracted (Folch method (24)) and oxysterols were analyzed by normal-phase HPLC with radiometric detection as described in the Methods.

**Figure 4.** Metabolism of radiolabeled 7K by human macrophages to aqueous soluble products is ablated by an inhibitor of sterol 27-hydroxylase (GW273297x) and absent in CTX.

Monocytes simultaneously isolated from blood from a control subject and a CTX patient, were differentiated for 9 d before incubation for 24 h with [³H]-7K (A) or [³H]-cholesterol (B) (1 µCi/culture). The media were extracted by the Folch method (24) and radioactivity in the upper, aqueous-phase measured by β-scintillation counting as described in the Methods. Values are mean ± standard deviation for triplicate cultures. The same superscript denotes a statistically significant difference (p<0.05) using an unpaired t-test for control cells.

**Figure 5.** Human monocyte-derived macrophages metabolize 7K to bile acid-like products.

Soluble products were analyzed by reverse-phase HPLC with 210 nm (A) or radiometric detection (B, C). The detectors were in series and the time-scales of Panels C and D have been corrected for the one minute delay from UV to radiometric detection. Panel A shows authentic bile acid standards (40 µg of each): UDCA, ursodeoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid. Panel B and C shows a concentrated pooled aqueous extract from the media of human monocyte-derived macrophages incubated for 24 h with [³H]-7K, using normal (B) or CTX (C) cells.
Figure 6. Metabolism of radiolabeled 7K by human monocyte-derived macrophages to 27OH-7K (A) and aqueous soluble products (B) is influenced by cholesterol-loading and the extent of esterification.

Human monocyte-derived macrophages were incubated for 24 h with [3H]-7K (A, B) or [3H]-cholesterol (C, D) (1 µCi/culture) with or without cholesterol-loading with acetylated LDL (50 µg/ml; 4 d). In some conditions, cholesterol-loaded cells were also incubated with an inhibitor of sterol 27-hydroxylase (GW273297x) or an inhibitor of ACAT (Sandoz 58-053) added at the same time as the radiolabeled sterol. Panels A and C indicate generation of the primary 27-hydroxylated product from 7K (A) or cholesterol (C). Lightly shaded bars represent cell associated products while dark bars represent products recovered from the media. For panels A and C, triplicate cultures were pooled and extracted (Folch method (24)) before analysis by normal-phase HPLC with radiometric detection as described in the Methods. Panels B and D represent the generation of aqueous soluble products by cells exposed to 7K and cholesterol, respectively. Values are mean + standard deviation for triplicate cultures. The same superscript denotes a statistically significant difference (p<0.05) using an unpaired t-test. Typically >85% of added radioactivity was recovered as sterols (by radio-HPLC) and aqueous soluble products (by scintillation counting).
Table 1. Metabolism of radiolabeled 7K and cholesterol by human monocyte-derived macrophages to aqueous soluble products

Human monocyte-derived macrophages were incubated for 24 h with \(^3\)H-7K or \(^3\)H-cholesterol (1 µCi/culture). The media were extracted by the Folch method (24) and radioactivity in the upper, aqueous-phase measured by \(\beta\)-scintillation counting as described in the Methods. Values are means ± SEM from 3 independent experiments.

<table>
<thead>
<tr>
<th>Units</th>
<th>(^3)H-7K</th>
<th>(^3)H-cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of added radioactivity</td>
<td>17.0 ± 4.6</td>
<td>0.49 ± 0.6</td>
</tr>
<tr>
<td>dpm/1000 cells</td>
<td>168 ± 47</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>dpm/µg of cell protein</td>
<td>4800 ± 1100</td>
<td>76 ± 36</td>
</tr>
<tr>
<td>pmol/mg cell protein</td>
<td>43 ± 10</td>
<td>0.70 ± 0.06</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
Sterol 27-hydroxylase acts on 7-ketocholesterol in human atherosclerotic lesions and macrophages in culture

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