Impairment of Macrophage Cholesterol Efflux by Cholesterol Hydroperoxide Trafficking

Implications for Atherogenesis Under Oxidative Stress

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Objective—Oxidative stress associated with cardiovascular disease can produce various oxidized lipids, including cholesterol oxides, such as 7-hydroperoxide (7-OOH), 7-hydroxide (7-OH), and 7-ketone (7=O). Unlike 7=O and 7-OH, 7-OOH is redox active, giving rise to the others via potentially toxic-free radical reactions. We tested the novel hypothesis that under oxidative stress conditions, steroidogenic acute regulatory (StAR) family proteins not only deliver cholesterol to/into mitochondria of vascular macrophages, but also 7-OOH, which induces peroxidative damage that impairs early stage reverse cholesterol transport.

Approach and Results—Stimulation of human monocyte-derived THP-1 macrophages with dibutyryl-cAMP resulted in substantial upregulation of StarD1 and ATP-binding cassette (ABC) transporter, ABCA1. Small interfering RNA–induced StarD1 knockdown before stimulation had no effect on StarD4, but reduced ABCA1 upregulation, linking the latter to StarD1 functionality. Mitochondria in stimulated StarD1-knockdown cells internalized 7-OOH slower than nonstimulated controls and underwent less 7-OOH–induced lipid peroxidation and membrane depolarization, as probed with C11-BODIPY (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) and JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide), respectively. Major functional consequences of 7-OOH exposure were (1) loss of mitochondrial CYP27A1 activity, (2) reduced 27-hydroxycholesterol (27-OH) output, and (3) downregulation of cholesterol-exporting ABCA1 and ABCG1. Consistently, 7-OOH–challenged macrophages exported less cholesterol to apoA-I or high-density lipoprotein than did nonchallenged controls. StarD1-mediated 7-OOH transport was also found to be highly cytotoxic, whereas 7=O and 7-OH were minimally toxic.

Conclusions—This study describes a previously unrecognized mechanism by which macrophage cholesterol efflux can be incapacitated under oxidative stress–linked disorders, such as chronic obesity and hypertension. Our findings provide new insights into the role of macrophage redox damage/dysfunction in atherogenesis. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.306210.)

Key Words: atherosclerosis ■ cholesterol ■ cholesterol hydroperoxide ■ oxidative stress ■ StAR protein

Vascular macrophages express scavenger receptors, such as CD36 and SR-BI, which can bind and internalize oxidatively modified low-density lipoprotein (oxLDL) arising under oxidative stress conditions associated with chronic obesity, hypertension, and atherosclerosis. Unlike expression of normal LDL receptors that of scavenger counterparts is not regulated by sterol negative feedback. Consequently, macrophages may accumulate large amounts of choles terol esters, nonesterified cholesterol, and other lipids from oxLDL potentially leading to atherogenic foam cell formation. Cholesterol efflux is a key early step in reverse cholesterol transport (RCT) whereby macrophages attempt to maintain cholesterol homeostasis by exporting the sterol when it reaches excessive levels. These cells express several proteins besides CD36 and SR-BI that play important roles in inward and outward cholesterol trafficking. Of special interest are proteins of the steroidogenic acute regulatory (StAR) family, which bind and transport pre-existing or incoming cholesterol and deliver it to/into mitochondria for conversion to 27-hydroxycholesterol (27-OH) by 27-hydroxylase (CYP27A1) on the inner membrane. 27-OH is a prominent agonist of the liver-X receptor transcription factors, which...
control expression of the ATP-binding cassette (ABC) transporters, ABCA1 and ABCG1.13,16 These plasma membrane proteins are known to play key roles in excess cholesterol export to extracellular acceptors, most notably apoA-I and high-density lipoprotein (HDL), the former mainly recognizing ABCA1 and the latter ABCG1.15,16 It is important to point out that a backup mode of cholesterol export exists whereby 27-OH itself or an acid derivative is released to an acceptor, such as HDL, when the intracellular sterol load exceeds the capacity of ABCA1/G1.12,17 Direct export of 27-OH might become more important if ABCA1/G1-mediated cholesterol efflux is incapacitated because of genetic mutations or protein damage/dysfunction.

In addition to oxidized phospholipids and a modified apoB-100 protein component, oxLDL contains several esterified and nonesterified oxysterols, including 7α- and 7β-hydroxycholesterol (7α/7β-OOH), 7-ketocholesterol (7=O), 7α- and 7β-hydroxycholesterol (7α/7β-OH), and 5,6-epoxycholesterol (5-O-6).18–23 Some of these oxysterols can be generated enzymatically.21 However, most of them derive mainly from free radical reactions triggered by reactive oxidants in the LDL lipid milieu, 7α/7β-OOH being generated at early stages and the others as secondary products.18–21 Unlike the other cholesterol oxides mentioned, 7α- and 7β-OOH are redox active and can undergo iron- or copper-catalyzed one-electron reduction to free radical species that propagate damage/dysfunction.21,24 In the process, 7α/7β-OOH gives rise to 7α/7β-OH, 7=O, and 5-O-6.20,21,24 Despite their relative instability, significant levels of 7α/7β-OOH (particularly 7β-OOH) have been detected in atherosclerotic plaques18,20 and in plasma and tissue samples from animals or humans chronically exposed to increased oxidative pressure.25,26 The substantially greater amounts of 7α-OH and 7α/7β-OOH found in plaques21–23 are attributed mainly to one-electron reductive turnover of 7α/7β-OOH,20,21,24 although this has not been specifically assessed for the subendothelial space, where most of the LDL oxidation is known to occur.1 Based on this information and our recent evidence that the StAR homologue, StarD4, transports 7α-OOH to isolated mitochondria with deleterious effects,27 we present the following hypothesis: under pathophysiological conditions associated with oxidative stress, oxLDL-derived 7-OOHs will be caught up in StAR protein–mediated cholesterol trafficking to/into mitochondria in vascular macrophages, thereby causing site-specific structural/functional damage that impairs RCT at its early stages. In a brief recent report,29 we provided the first supporting evidence for this hypothesis by showing that 7α-OOH uptake by mitochondria of cAMP-activated mouse RAW264.7 macrophages was StarD1-dependent and induced lipid peroxidation, membrane depolarization, and cell killing. For greater cardiovascular relevance, we have extended these findings to human THP-1 monocyte-derived macrophages in this study and have shown that 7α-OOH inactivation of CYP27A1, with reduced 27-OH might become more important if ABCA1/G1-mediated cholesterol efflux is incapacitated because of genetic mutations or protein damage/dysfunction.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Protein Expression in Dibutyryl cAMP–Stimulated THP-1 Macrophages: Effects of StarD1 Knockdown

In an initial experiment, fully differentiated THP-1 macrophages were analyzed for StarD1, StarD4, CYP27A1, and ABCA1 protein levels during incubation with dibutyryl cAMP (Bu2cAMP). As shown by the Western blots in Figure 1A, all 4 proteins were detected in cells after 4-hour incubation in medium lacking Bu2cAMP; the level in each case was not significantly different from that observed before incubation (not shown). However, when Bu2cAMP (200 µM/L) was present during incubation, the StarD1 and ABCA1 levels at 4 hours were ≈2.5-fold and ≈2-fold, respectively, than their control levels (Figure 1A). In contrast, there was no significant change in StarD4 level compared with that in the 4-hour control and only a small (probably insignificant) increase in CYP27A1.

A small interfering RNA approach was used to deplete StarD1 and assess the effects of this on expression of the other selected proteins. As shown in Figure 1B (columns 3 and 4), StarD1 knockdown (kd) by ≈60% relative to a scrambled vector control not only blunted StarD1 upregulation by Bu2cAMP (≈70% reduction) but also ABCA1 upregulation (≈25% reduction). There was no change in StarD4 and a small increase (≈15%) in CYP27A1, which is regarded as insignificant (Figure 1B).

StarD1-Dependent 7α-OOH Uptake by THP-1 Macrophages: Whole Cell Versus Mitochondrial Fraction

We found that cholesterol uptake by whole macrophages, as well as the mitochondrial fraction, was substantially increased after Bu2cAMP stimulation (results not shown), and similar results were obtained for 7α-OOH. As shown in Figure 1C, stimulated macrophages that had been transfected with a scrambled small interfering RNA construct (S/Ser) took up...
small unilamellar vesicle (SUV)-borne $[^{14}C]7\alpha$-OOH more rapidly than nonstimulated controls (NS/Scr), resulting in ≈70% more radioactivity in the former after 5 hours of incubation. A more rapid uptake of $[^{14}C]7\alpha$-OOH after cell stimulation was also observed when mitochondrial fractions were examined, the S/Scr level being ≈80% greater than the NS/Scr level after 5 hours of incubation (Figure 1D). However, in stimulated cells with StarD1 kd, $[^{14}C]7\alpha$-OOH uptake was substantially reduced in the total cellular compartment (Figure 1C) and the mitochondrial fraction (Figure 1D). The StarD1 kd uptake values approached those of the NS/Scr samples in each case. These results clearly established that StarD1 was required for Bu$_2$cAMP-enhanced $7\alpha$-OOH uptake and delivery to mitochondria, just as it is for cholesterol.8–12

Of added importance is our finding that the uptake of specific radioactivity (CPM/µg protein) for the mitochondrial fraction (Figure 1D) was much greater than that for whole cells, for example, nearly 3x greater in S/Scr samples after 5 hours (Figure 1C). Because mitochondria comprise on average only 20% to 25% of cellular mass,29 this result highlights the ability of these organelles to selectively import $7\alpha$-OOH (as they do cholesterol) via StarD1.

Figure 1. Effect of dibutyryl cAMP (Bu$_2$cAMP) stimulation and StarD1 knockdown (kd) on protein expression in THP-1 macrophages. A, THP-1 monocytes were differentiated to macrophages by incubating with 150 nmol/L phorbol 12-myristate 13-acetate in 10% FBS/RPMI medium for 3 days. The macrophages at ≈70% confluence were then treated with 1% FBS/RPMI medium and incubated in the absence (not stimulated, NS) or presence (stimulated, S) of 0.2 nmol/L Bu$_2$cAMP for 4 hours. Cells were then recovered, lysed, and subjected to Western blot analysis for StarD1, StarD4, CYP27A1, ABCA1, and β-actin, the latter serving as a loading standard. B, StarD1-kd cells and scrambled controls (Scr) were either stimulated (S) or not stimulated (NS), then Western analyzed for the indicated proteins. Protein load per lane in (A) and (B): 50 µg. Numbers below lanes represent densitometrically determined band intensities normalized to β-actin and relative to values in nonstimulated wild-type cells. Also shown is effect of StarD1 kd on $7\alpha$-OOH uptake by THP-1 macrophages. StarD1-kd cells and Scr controls in serum-free RPMI medium were either stimulated with 0.2 mmol/L Bu$_2$cAMP for 4 hours or not stimulated (Scr-only) and then treated with 1-palmitoyl-2-sn-glycero-3-phosphocholine/Ch/$[^{14}C]7\alpha$-OOH (2:1:1 by mol) small unilamellar vesicles. After incubation at 37°C for the indicated times, radioactivity in whole cells (C) and in mitochondrial fractions (D) isolated by differential centrifugation of cell homogenates was measured by scintillation counting. Plotted values in (C) and (D) are means±SE (n=3 in each case). ABC indicates ABCA1.

7α-OOH-Induced Lipid Peroxidation and ∆Ψ mitochondria: Role of StarD1

C11-BODIPY (4,4-difluoro-5-(4-phenyl-1,3-butanediyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) is a membrane-localizing ratiometric probe that can be used to detect free radical-mediated lipid peroxidation occurring in its membrane surroundings.30 We used this fluorophore to assess the extent to which $7\alpha$-OOH redox activity would induce damaging lipid peroxidation in THP-1 mitochondria. As shown in Figure 2A, Bu$_2$cAMP-stimulated StarD1-kd macrophages exhibited substantially less green than red fluorescence after SUV-$7\alpha$-OOH exposure than scrambled controls, indicating more extensive probe oxidation in the latter. The more extensive peroxidation in control cells, which was virtually identical to that in wild-type cells (not shown), was localized mainly in punctate perinuclear zones (Figure 2A). These hot spots were most likely mitochondria, in keeping with the data shown in Figure 2.

We used the ratiometric fluorophore JC-1 (5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide) to probe for mitochondrial membrane potential (∆Ψm) in stimulated versus nonstimulated THP-1 macrophages and how this might change under a $7\alpha$-OOH challenge. As anticipated from the $7\alpha$-OOH–induced membrane damage observed in Figure 2A, JC-1–assessed ∆Ψm (represented as red/green fluorescence intensity ratio) decreased more rapidly with increasing $7\alpha$-OOH concentration in stimulated cells than in nonstimulated controls (Figure 2B). For example, the fluorescence intensity ratio value at 30 nmol/L $7\alpha$-OOH was ≈45% lower than that of control cells. Moreover, stimulated StarD1-kd cells exhibited a substantially greater ∆Ψm than fluorescence intensity ratio (Figure 2B inset). These results...
add support to our hypothesis that StarD1 plays a crucial role not only in cholesterol uptake by mitochondria but also 7α-OH uptake, the latter triggering free radical–mediated peroxidative damage resulting in membrane depolarization and metabolic dysfunction.

**Cytotoxic Effects of 7α-OH Compared With Other Cholesterol-7-Oxide Species**

Various studies have shown that oxysterols, such as 7=O and 7-hydroxide (7-OH), which exist in oxLDL and atherosclerotic lesions,20,22 are toxic to vascular endothelial cells and macrophages.19,23 Redox inert 7=O and 7-OH are produced by redox turnover of 7-OH.20,24 However, these 3 different cholesterol-7-oxides have not been compared for toxicity in vascular macrophages. To examine this toxicity, we used ethanol as a delivery vehicle instead of liposomes to ensure rapid and consistent cellular uptake of the 3 7-oxides. Using stimulated cells, we found that 7α-OH was much more cytotoxic than 7=O (LD50 =15 versus ≈120 µmol/L), whereas 7α-OH was nontoxic, even at the highest concentration tested (Figure 3A). 7α-OH’s high lethality is attributed to its robust redox activity (Figure 3A), whereas relatively weak 7=O may operate via some other toxic mechanism(s).22 The results in Figure 3A clearly demonstrate for the first time that 7α-OH is exceedingly deleterious to macrophages compared with its redox products (7-OH, 7=O), which have been more widely studied with regard to cytotoxicity.21

**Role of StarD1 in 7α-OH Cytotoxicity**

As anticipated from the results in Figures 2 and 3, exposing Bu2cAMP-stimulated macrophages to SUV-7α-OH in increasing concentrations ≤100 µmol/L for 24 hours resulted in a progressive loss of viability (Figure S1A in the online-only Data Supplement). In contrast, nonstimulated cells, which expressed far less StarD1 than stimulated counterparts (Figure 1), were less sensitive to 7α-OH toxicity, the LD50 values being ≈50 and ≈18 µmol/L, respectively. We then asked how cell stimulation would affect sensitivity toward another type of redox-active hydroperoxide, viz t-butyl hydroperoxide (t-BuOOH). As shown in Figure S1B in the online-only Data Supplement, t-BuOOH, which did not require a liposome delivery vehicle instead of liposomes to ensure rapid and consistent cellular uptake, as well as to t-BuOOH-stimulated macrophages.19,23 As expected from the results in Figures 2 and 3, we found that t-BuOOH–induced cell killing was confirmed by showing (Figure 3B) that stimulated StarD1-kd cells were substantially more resistant to the hydroperoxide than scrambled construct controls (LD50 =90 versus 40 µmol/L), the latter responding identically to wild-type cells (not shown). However, there was no difference in sensitivity between stimulated and nonstimulated cells, the LD50 for both being ≈170 µmol/L for the indicated incubation time. StarD1 involvement in 7α-OH–induced cell killing was confirmed by showing (Figure 3B) that stimulated StarD1-kd cells were substantially more resistant to the hydroperoxide than scrambled construct controls (LD50 =90 versus 40 µmol/L), the latter responding identically to wild-type cells (not shown). However, there was no difference in sensitivity between stimulated and nonstimulated cells, the LD50 for both being ≈170 µmol/L for the indicated incubation time. StarD1 involvement in 7α-OH–induced cell killing was confirmed by showing (Figure 3B) that stimulated StarD1-kd cells were substantially more resistant to the hydroperoxide than scrambled construct controls (LD50 =90 versus 40 µmol/L), the latter responding identically to wild-type cells (not shown). However, there was no difference in sensitivity between stimulated and nonstimulated cells, the LD50 for both being ≈170 µmol/L for the indicated incubation time.
ABC1 expression.\textsuperscript{11} We measured both mitochondrial CYP27A1 activity and total 27-OH output for stimulated THP-1 macrophages and how each of these was affected by 7α-OOH exposure. As shown in Figure 4A, CYP27A1 activity was reduced by \(\approx 40\%\) after cells were incubated with 20 \(\mu\)mol/L SUV-7α-OOH for 10 hours. The viable cell count at this point was at least 90\%. Overall cellular output of 27-OH was likewise reduced and in a starting 7α-OOH concentration-dependent fashion. Thus, at 10 \(\mu\)mol/L SUV-7α-OOH, 27-OH yield dropped by \(\approx 20\%\), and at twice this concentration, it dropped by \(\approx 40\%\) relative to the control yield (Figure 4B). The decreases in CYP27A1 activity and 27-OH yield are attributed to peroxide-induced mitochondrial free radical damage (Figure 2), resulting in severe metabolic dysfunction.

Diminished StarD1, ABCA1, and ABCG1 Expression in Cells Exposed to 7α-OOH

As a direct follow-up to the results in Figures 2–4, we asked how 7α-OOH exposure might affect StarD1 and ABCA1 expression in THP-1 macrophages. As shown by the Western blots in Figure 5A, the level of StarD1 protein in nonstimulated cells did not change during 6 hours of incubation with 100 \(\mu\)mol/L SUV-7α-OOH, whereas in stimulated cells, it decreased by \(\approx 48\%\) relative to the nonchallenged control. As anticipated from the results in Figure 4, ABCA1 protein also underwent a steady decline in stimulated cells, reaching \(\approx 50\%\) of its control level after 6 hours. Parallel RT-PCR analysis revealed that the steady state level of ABCA1 mRNA dropped by \(\approx 80\%\) after 6 hours of cell exposure to 100 \(\mu\)mol/L SUV-7α-OOH (Figure 5B). The observed decrease in ABCA1 protein as well as mRNA implies that transcription was impaired by 7α-OOH, which is consistent with the observed 27-OH shortfall (Figure 4B). Some oxidative modification of ABCA1 protein with possible loss of function and antibody recognition may also have occurred and cannot be ruled out at present. Western analysis indicated that the level of ABCG1 protein in stimulated cells also fell during SUV-7α-OOH treatment, reaching \(\approx 30\%\) of the control level after 6 hours (Figure 5A).

Negative Effect of 7α-OOH on Cholesterol Export

Of special interest at this point was how early stage RCT in macrophages would be affected by 7α-OOH trafficking. Such interest would address the functional significance of the findings in Figures 2–6. THP-1 cells preloaded with \([3H]\)cholesterol were stimulated and exposed to SUVs either lacking or containing 7α-OOH, after which ABCA1-mediated \([3H]\)cholesterol export to apoA-I in the medium was assessed. As shown in Figure 6A, \([3H]\)cholesterol efflux at 6 hours was significantly reduced in stimulated cells that had been exposed to 7α-OOH, whereas no difference was observed in nonstimulated controls. The diminished efflux reflects mitochondrial injury inflicted by 7α-OOH that was imported by upregulated StarD1. A small decrease in \([3H]\)cholesterol efflux was also observed after 3 hours of exposure to 7α-OOH, but it was proved to be insignificant (results not shown). We also determined how 7α-OOH import would affect ABCG1-mediated \([3H]\)cholesterol efflux to HDL in the medium. As shown in Figure 6B, \([3H]\)cholesterol efflux from stimulated cells to HDL after a SUV-7α-OOH challenge was significantly reduced relative to a SUV-cholesterol control under identical reaction conditions. A much smaller effect of 7α-OOH was observed for nonstimulated cells, which proved to be statistically insignificant. These findings provide even more compelling support for our hypothesis that 7α/7β-OOH, an oxysterol often overlooked up to now because of its low content in atherosclerotic lesions, could be a primary driving force in RCT impairment associated with oxidative stress disorders.

Discussion

Scavenger receptor–expressing macrophages normally resist harmful buildup of cholesterol from endogenous or exogenous sources by activating transport processes, whereby cholesterol is delivered to mitochondria for CYP27A1-mediated conversion to 27-OH.\textsuperscript{8–12} The latter stimulates liver-X
In the case of ABCG1.15,16 In addition to scavenger receptors, α7 in of the medium were analyzed for [14C]27-OH by HPTLC-PI. Each cholesterol in Me-CD for 12 hours. Lipid extracts from samples βOOH for 10 hours followed by washing and exposure to [14C]-µmol/L SUV-7

These proteins include the cholesterol side-chain cleavage enzyme (CYP11A1) on the inner membrane, StarD1 on the outer membrane, and at least one cytosolic homologue, such as StarD4 or StarD5. It is becoming increasingly clear that the STAR transporters act cooperatively in delivering cholesterol to the inner membrane for CYP11A1-catalyzed formation of pregnenolone, the first step in steroid hormone biosynthesis.31-33 In a recent study,34 we showed that Bu2cAMP-stimulated MA-10 cells expressed higher levels of StarD1 and StarD4 proteins than nonstimulated controls, and also (1) channeled more liposomal 7α-OOH as well as cholesterol to/into mitochondria, (2) underwent a greater loss of ∆Ψm and progesterone output during 7α-OOH exposure, and (3) underwent more extensive intrinsic apoptosis. These findings strongly support the notion that under oxidative stress conditions, steroidogenic cells may deliver not only cholesterol to mitochondrial compartments but also cholesterol hydroperoxides (ChOOHs), such as 7α/7β-OOH, thereby setting the stage for free radical damage, metabolic dysfunction, and even apoptotic cell death.

An analogous mechanism of mitochondria-targeted oxidative damage/dysfunction is described for vascular macrophages in this study. As also demonstrated for murine RAW 264.7 macrophages recently,28 human THP-1 macrophages robustly overexpressed StarD1 and ABCA1 on stimulation with Bu2cAMP. Mitochondria in stimulated cells imported liposomal 7α-OOH and cholesterol at significantly higher rates than in nonstimulated controls, and StarD1 kd blunted this, thus establishing this protein’s involvement in the uptake. Although no significant upregulation of StarD4 was observed after Bu2cAMP treatment, this protein was expressed at fairly high constitutive levels in THP-1 cells and this may have been sufficient for a trafficking role along with StarD1. Although StarD4 involvement in 7α-OOH delivery was not specifically assessed by a knockdown approach, we showed previously that recombinant StarD4 was fully capable of transporting 7α-OOH from liposomes to isolated mitochondria, which caused peroxidative damage and membrane depolarization.27 No such effect was observed with a phospholipid hydroperoxide, consistent with the known sterol specificity of StarD4 and most other StAR proteins.27 In this study, we observed a StarD1-dependent uptake of 7α-OOH by mitochondria of stimulated THP-1 macrophages. This uptake resulted in more rapid induction of free radical–mediated lipid peroxidation and membrane depolarization than observed in nonstimulated controls. These deleterious effects were significantly diminished by StarD1 knockdown, again consistent with its involvement in selective 7α-OOH targeting to mitochondria. t-BuOOH also caused macrophage damage/dysfunction, but this occurred independently of Bu2cAMP stimulation or StarD1 knockdown. Because t-BuOOH lacks the structural requirements of a StAR ligand,25 this result confirmed StarD1 binding/trafficking specificity for 7α-OOH.

One of the most significant of our observed functional consequences of StarD1-mediated 7α-OOH transfer to

Figure 4. Impact of 7α-hydroperoxide (7α-OOH) uptake on CYP27A1 activity and 27-hydroxycholesterol (27-OH) yield. A, Dibutyryl cAMP-stimulated THP-1 macrophages were incubated with 0 or 20 µmol/L SUV-7α-OOH for 10 hours, then washed, recovered, and homogenized, after which mitochondria were isolated by differential centrifugation. After protein determination, CYP27A1 activity was measured by incubating the mitochondria with [1,2,6,7-]cholesterol in methyl-β-cyclodextrin (Me-β-CD) vehicle for 90 minutes, followed by lipid extraction and high-performance thin-layer chromatography with phosphor-imaging detection (HPTLC-PI) analysis of [1,2,6,7]27-OH. B, Stimulated macrophages were incubated with 0, 10, or 20 µmol/L SUV-7α-OOH for 10 hours followed by washing and exposure to [3H]cholesterol in Me-β-CD for 12 hours. Lipid extracts from samples of the medium were analyzed for [1,2,6,7]27-OH by HPTLC-PI. Each of the sample bands shown represents 105 cells. Potted values in A and B are means±SE (n=3); *P<0.05 compared with 0 µmol/L 7α-OOH. SUV indicates small unilamellar vesicle.
mitochondria of stimulated THP-1 cells was the large decrease in 27-OH production. This decrease was dependent on both hydroperoxide dose and contact time. We chose to measure 27-OH in the medium because these cells can release considerable amounts of it as biosynthesis progresses.\textsuperscript{15} The decline in 27-OH yield was accompanied by reduced expression of ABCA1 and ABCG1. The latter finding was not unexpected, given that 27-OH is a strong natural agonist of nuclear liver-X receptor, which stimulates ABCA1 and ABCG1 transcription.\textsuperscript{16,36} The 27-OH decline would have resulted from the observed loss of mitochondrial CYP27A1 activity (Figure 4A). The latter is attributed to 7α-OOH–induced redox damage either directly to the enzyme itself or indirectly to its inner membrane surroundings. Diminished 27-OH output and ABCA1 expression after 7α-OOH exposure, most likely explains the impaired early stage RCT of these cells, that is, reduced ability to export cholesterol to apoA-I (Figure 6A). An analogous explanation would apply to their reduced cholesterol export became over-whelmed or incapacitated. A similar explanation may apply for the gradual 7α-OOH–induced decline in StarD1 that we observed.

Most prior studies on oxysterol involvement in atherogenesis have concentrated on major secondary products of free radical–mediated cholesterol oxidation in LDL before and after it enters macrophages in vascular subendothelial spaces. Considerable attention has been directed to 7=O and 27-OH, which are the most abundant oxysterols in atherosclerotic plaques and at sufficiently high levels, can induce apoptosis in fibroblasts, endothelial cells, and macrophages.\textsuperscript{19,21–23,37} Although not considered in this light recently, primary 7-OHs, which arise from free radical reactions,\textsuperscript{21,24} are the major sources of 7=O and 7-OH via redox turnover. This can account for the low levels of 7-OH detected in oxLDL and arterial plaques.\textsuperscript{16,20,21} In this study, we compared 7α- and 7β-OOH with 7α-OOH for cytotoxic potency and found that the hydroperoxide was much more toxic to stimulated THP-1 macrophages than the others, for example, at 50 μmol/L it produced a near complete loss of viability, whereas 7α- and 7β-OOH were essentially nontoxic at this concentration. We used ethanol as a 7-oxide vehicle in this experiment (Figure 3A) in attempts to standardize delivery rate of the three 7-oxides to cells. When SUVs were used as delivery vehicles, LD\textsubscript{50} for 7α-OOH was >100 μmol/L (Figure 3B), whereas that for 7=O, which is much less hydrophilic than 7α or 7β-OOH,\textsuperscript{19} could not be determined, evidently because it remained tightly associated with the liposomes, even after 24-hour incubation with cells. It is reasonable to assume that if oxLDL were the 7-oxide vehicle into macrophages, results similar to those in Figure 3A would be obtained, highlighting the toxic importance of 7α-OOH (and its 7β-epimer) relative to the other 7-oxides. This is the first time that these three 7-oxides have been compared in this fashion using vascular macrophages as target cells. Most prior studies used higher concentrations of oxysterols (7=O, 7-OH, and 27-OH) delivered in organic solvents (ethanol and isopropanol) to other cell types, for example, fibroblasts, endothelial, and smooth muscle cells.\textsuperscript{19,38,39} Only one of these studies\textsuperscript{39} included a 7β-OOH, which was detected at relatively high levels in LDL oxidized in vitro. 7β-OOH was found to be far more toxic to dermal fibroblasts than 7=O, 7β-OH, 5-O-6, or 25-OH.\textsuperscript{19} It was concluded that 7β-OOH was the 7-oxide most likely to be

Figure 5. Effect of 7α-hydroperoxide (7α-OOH) on StarD1, ABCA1, and ABCG1 expression. A, Wild-type THP-1 macrophages at ≥60% confluency in serum-free RPMI medium were either not stimulated or stimulated with 0.2 mmol/L dibutyryl cAMP for 10 hours, then washed, overlaid with fresh medium, and either analyzed before (0 hour) or after incubating with 1-palmitoyl-2-sn-glycero-3-phosphocholine/Ch/7α-OOH (2:1:1 by mol) small unilamellar vesicles (SUVs) for 2, 4, and 6 hours, the starting overall concentration of 7α-OOH being 100 μmol/L in each case. At the indicated times, cells were washed, lysed, and samples of predetermined total protein were subjected to Western analysis for StarD1, ABCA1, and ABCG1. Numbers below protein bands in the nonstimulated (NS) and stimulated (S) groups indicate band intensities relative to 0-time NS and normalized to β-actin. B, RT-PCR–assessed ABCA1 mRNA levels in stimulated cells that had been incubated with 100 μmol/L SUV-7α-OOH for 0, 2, 4, and 6 hours; SUV composition was as described in A. Numbers below bands indicate band intensities relative to 0-time and normalized to GAPDH. Data in (A) and (B) are from 1 experiment in each case, which is representative of at least 3 with similar results. ABC indicates ATP-binding cassette.

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**Figure 4.** Time course of 7α-hydroperoxide (7α-OOH)–induced decline in StarD1, ABCA1, and ABCG1 expression. A, Western analysis: Top, 100 μmol/L SUV-7α-OOH for 0, 2, 4, and 6 hours; bottom, 200 μmol/L SUV-7α-OOH for 0, 2, 4, and 6 hours; and middle, 200 μmol/L SUV-7α-OOH for 0, 2, 4, and 6 hours. B, RT-PCR: A, B, and C, 0, 2, 4, and 6 hours; A, B, and C, 0, 2, 4, and 6 hours. Note that in both cases, the intensity of ABCA1 and ABCG1 bands decreases over time, while StarD1 bands increase over time. This suggests that the loss of StarD1 may be responsible for the decrease in ABCA1 and ABCG1 expression. ABC indicates ATP-binding cassette.
This model depicts a type of stealthy delivery of 7-OOH via a natural trafficking pathway, which normally delivers cholesterol to mitochondria to initiate export signaling when its level become excessive. Because 7-OOHs are structurally similar to cholesterol, they can be recognized by StAR proteins and trafficked to mitochondria alongside cholesterol. We postulate that significant levels of 7-OOHs and other 7-oxides exist in oxLDL because it enters macrophages in the vascular wall. Most of the 7-OOHs would have arisen during free radical–mediated oxLDL formation in subendothelial spaces. At the early stages of this process, the steady levels of 7-OOH would be relatively high, but then decline as chain lipid peroxidation progresses; meanwhile, there would be a build-up of redox–inert products of 7-OOHs, namely 7-α-OH and 7=O. All of these 7-oxides, including those derived from hydrolysis of oxidized cholesteryl esters, would be recognized by the StAR trafficking network for distribution to intracellular sites, including mitochondria, but only 7α/β-OOH would be capable of the redox damage/dysfunction observed in this study, viz chain lipid peroxidation, membrane depolarization, reduced ABCA1 expression, and RCT impairment. Such effects would be more serious at early stages of LDL oxidation, when 7-OOH levels are relatively high, than at late stages. These are new mechanistic insights into oxidative stress–linked atherogenesis which warrant more extensive study at the in vitro and in vivo level.

It is worthwhile to consider how vascular macrophages might cope with stress-inducing 7-OOH using some natural antioxidant modality. The only antioxidant enzyme known to be capable of catalyzing direct inactivation of 7-OOHs and other ChOOHs is glutathione peroxidase type-4 (GPx4), a selenoenzyme that converts these peroxides to redox-inactive alcohols at the expense of reduced glutathione.40 GPx4 is known to be expressed in mitochondria and other compartments of mammalian cells. ChOOHs as a group are detoxified much more slowly by GPx4 than phospholipid counterparts (PLOOHs), making ChOOHs potentially more damaging than PLOOHs.41,42 Release of incoming ChOOHs with subsequent induction of free radical peroxidation is assumed to occur in the vicinity of the mitochondrial inner membrane because StarD1 delivers cholesterol at/near this site for processing by the mitochondrial ChOOHs, leading to mitochon-

drial damage and impairment of RCT at its earliest stages. These are new mechanistic insights into oxidative stress–linked atherogenesis which warrant more extensive study at the in vitro and in vivo level.

In summary, we have demonstrated for the first time how a natural transport pathway for cholesterol in macrophages can be co-opted by a redox-active ChOOH, leading to mitochondrial damage and impairment of RCT at its earliest stages. This work provides new insights into how oxLDL may initiate atherogenesis and sets the stage for more comprehensive studies involving 7-OOHs borne by LDL itself.
Cholesterol Peroxide-Impaired RCT in Macrophages

Acknowledgments
We thank D.M. Stocco, D. Rodriguez-Agudo, and W.M. Pandak for helpful information regarding cholesterol trafficking by STARD family proteins.

Sources of Funding
This work was supported by National Institutes of Health grants HL85677 (Dr Girotti) and HL58012 (D. Sahoo), and by Polish National Science Center grant 11011/B/NZ/2012167 (Dr Korytowski). Support of American Heart Association Fellowship 14PRE185800221 (A.C. Chadwick) is also acknowledged.

Disclosures
None.

References

Significance

An elevated level of oxidized low-density lipoprotein in the circulation (associated with disorders such as chronic inflammation and diabetes mellitus) is a known risk factor in atherogenesis. In the artery wall, macrophages bind and internalize oxidized low-density lipoprotein as a source of cholesterol and other lipids. Macrophages can also release cholesterol by reverse cholesterol transport. If cholesterol import exceeds export, macrophages become overloaded with cholesterol and accumulate in zones called atherosclerotic plaques, which obstruct blood flow and can eventually lead to heart attacks. In this study, we describe a previously unrecognized mechanism by which reverse cholesterol transport can be incapacitated in oxidative disorders, namely through delivery of redox-active cholesterol hydroperoxides to mitochondria via a natural cholesterol trafficking pathway. Our findings are highly significant because they provide new insights into the role of macrophage redox damage in atherogenesis. Moreover, our findings suggest that mitochondria-targeted antioxidant drugs can be used to prevent this damage.
Impairment of Macrophage Cholesterol Efflux by Cholesterol Hydroperoxide Trafficking: Implications for Atherogenesis Under Oxidative Stress

Witold Korytowski, Katarzyna Wawak, Pawel Pabisz, Jared C. Schmitt, Alexandra C. Chadwick, Daisy Sahoo and Albert W. Girotti

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Materials and Methods

Ch, 7=O, PMA, Bu₂cAMP, MTT, JC-1, t-BuOOH, methyl-β-cyclodextrin (Me-β-CD), fatty acid-free bovine serum albumin (BSA), and fetal bovine serum were obtained from Sigma-Aldrich. Lipoprotein-deficient serum was from GE-Healthcare-HyClone. C11-BODIPY and MitoTracker Deep Red were from Molecular Probes-Life Technologies, and POPC, 27-OH and 7α-OH from Avanti Polar Lipids. Boehringer Mannheim supplied the Complete-Mini mixture of protease inhibitors. Primary antibodies against human StarD1, StarD4, CYP27A1, ABCA1, ABCG1, β-actin, and tubulin were obtained from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated IgG secondary antibodies were from Cell Signaling Technology. Amersham Biosciences supplied the [4-¹⁴C]cholesterol (~50 mCi/ml). [³H]Cholesterol was from Perkin-Elmer. Recombinant apoA-I was kindly provided by Dr. Mary Sorci-Thomas as a research gift. Human HDL was obtained from Biomedical Technologies, Inc. 7α-OOH was prepared by dye-sensitized photoperoxidation of cholesterol; isolation of the hydroperoxide and structural verification by proton-NMR were as described previously.¹² [¹⁴C]7α-OOH was prepared and isolated similarly, using [4-¹⁴C]cholesterol.

Cell culture and transfection conditions

Human THP-1 monocytes obtained from the American Type Culture Collection were cultured in humidified 5% CO₂, 95% air at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Prior to an experiment, cells were differentiated to macrophages by treating with 30 nM phorbol 12-myristate 13-acetate (PMA) for 3 days. These cells at ~70% confluency were incubated for an additional 24 h in fresh medium without PMA, and then for up to 12 h in 1% serum-RPMI 1640 medium either lacking or containing 0.2 mM Bu₂cAMP. After the latter incubation (and where indicated), cells were washed again, then incubated with POPC/Ch/7α-OOH (2:1:1 by mol) or POPC/Ch/7α-OH (2:1:1 by mol) SUVs in RPMI 1640 medium. POPC/Ch (3:1 by mol) SUVs served as a control for the other systems, total cholesterol content remaining constant. The bulk phase concentration of SUV lipid in the presence of cells maximized at ~1 mM, which was found to be innocuous.

Silencer (si) RNAs for human StarD1 and scrambled oligonucleotide pairs were purchased from Santa Cruz Biotechnology. Single transfections of duplex mixtures were carried out in OPTI-MEM medium (Gibco-Life Technologies) for 6 h, using Lipofectamine LTX (18 µl/ml) as advised by the supplier. Following transfection, cells were switched to full growth medium without siRNA or Lipofectamine for a 36-48 h recovery period, then washed, overlaid with RPMI 1640 medium, and stimulated with 0.2 mM Bu₂cAMP for 8-12 h. After this, they were either challenged with SUV-7α-OOH or scraped into ice-cold protease inhibitor-containing PBS and analyzed for StarD1, StarD4, CYP27A1, and ABCA1 expression by Western blotting.

Liposome preparation

Small unilamellar liposomes (50 nm SUVs) composed of POPC/Ch (3:1 by mol), POPC/Ch/7α-OOH (2:1:1 by mol), or POPC/Ch/7α-OH (2:1:1 by mol), for example, were prepared in Chelex-treated PBS by an extrusion process.³ A chloroform solution of chosen lipids was dried under argon and then held under a vacuum for several hours at room temperature. After hydration in Chelex-treated PBS followed by five cycles of freezing in liquid nitrogen and thawing, the vesicle suspension was passed 10 times through two stacked 50-nm pore polycarbonate filters in an Extruder apparatus (Lipex Biomembranes, Vancouver, British Columbia). The resulting SUVs (~10 mM total lipid in bulk suspension) were stored under argon at 4 °C and used in experiments within three days after preparation.

Western blot analyses
Macrophage lysates were prepared in RIPA buffer containing Complete™ protease inhibitor cocktail (Roche Diagnostics). After determination of total protein content, electrophoretic separation by NuPAGE SDS-PAGE (Life Technologies) was carried out, using 4-12 % gels for StarD1, StarD4, CYP27A1, and 4-15 % gels for ABCA1 and ABCG1. Separated proteins were transferred to a polyvinylidene difluoride membrane and probed, using supplier-recommended dilutions of polyclonal antibodies against StarD1, StarD4, CYP27A1, ABCA1, ABCG1, and β-actin or tubulin. After membrane treatment with a horseradish peroxidase-linked IgG secondary antibody, protein bands were visualized using a SuperSignal WestPico detection kit from Thermo Scientific. A LabWorks system was used for band imaging and UVP software for quantification. Other details were as described previously.4,5

RT-PCR analyses
Total RNA was isolated from THP-1 macrophages using a GeneMATRIX DNA/RNA/Protein Purification Kit (EURx), and reverse-transcribed to cDNA using oligo(dT)23 and MMLV reverse transcriptase (Sigma-Aldrich). Specific mRNA levels were determined by semi-quantitative RT-PCR as described,6 using oligonucleotide primers designed by means of PrimerBLAST software and with the following sequences: ABCA1 (forward: CTAGTCCCGGCAAAAACCCC; reverse: GCTGGTCATTAACTGTTTTCCACT; accession number NM_005502.3), and GAPDH (forward: GGCCACTAGGGCGCTCAC; reverse: GCCCAATACGACCAAATCCGT; accession number NM_001289746.1).

Determination of 7α-OOH uptake
For determining 7α-OOH uptake, non-stimulated and Bu2cAMP-stimulated THP-1 macrophages were exposed to liposomal [14C]7α-OOH (0.2 µCi/ml) at an initial concentration of 50 µM or 100 µM, using a stock suspension of POPC/Ch/[14C]7α-OOH (2:1:1 by mol) SUVs. After incubation at 37 °C for increasing time periods up to 5 h, cells were washed twice, detached, and recovered by centrifugation. For assessing uptake by mitochondria, incubated cells were homogenized and mitochondria isolated by differential centrifugation.4,5 Total cell samples and Mito fractions were analyzed for incorporated radioactivity by means of scintillation counting. The effect of StarD1 knockdown on 7α-OOH uptake by mitochondria in stimulated cells was also assessed, the general approach being similar to that described above for wild type cells.

Measurement of lipid peroxidation in 7α-OOH-challenged cells
Wild type and StarD1-kd macrophages grown on Starstedt FlexiPERM® slides in glass-bottom dishes were stimulated for 8 h with 0.2 mM Bu2cAMP, then treated with 2 µM C11-BODIPY, a lipid peroxidation probe,7 for 30 min at 37 °C. Non-stimulated control cells were treated alongside. All cells were then incubated with 25 µM SUV-7α-OOH for 4 h or 50 µM SUV-7α-OOH for 2.5 h, then washed, overlaid with RPMI medium, and examined by confocal fluorescence microscopy, using a Leica TCS SESII instrument with 488 nm excitation and 607-680 nm (red) or 500-585 nm (green) emission to observe unoxidized and oxidized C11-BODIPY, respectively.7

Measurement of mitochondrial membrane potential (ΔΨm)
Wild type and StarD1-kd macrophages in black-wall 96-well plates were either untreated or stimulated with 0.2 mM Bu2cAMP in 1% FBS/RPMI medium for 8 h, and exposed to SUV-7α-OOH in either increasing concentrations (10-100 µM) for a fixed time (5 h), or 100 µM SUV-7α-OOH for increasing times up to 7 h. After peroxide exposure, cells were washed, incubated with the ΔΨm probe JC-1 (5 µg/ml) for 30 min, washed again, and analyzed using a BioTek Synergy-MX fluorescence plate reader. Settings were as follows: red (λex 560 nm; λem 595 nm); green (λex 485 nm; λem 535 nm). Other details were as reported previously.4,5
Determination of mitochondrial CYP27A1 activity
CYP27A1 activity in mitochondrial fractions from SUV-7α-OOH-treated cells was determined according to published procedures with slight modifications. Each reaction mixture (500 µl) contained 0.4 mg of mitochondrial protein, 40 nmol of cholesterol dissolved in 10 µl of 45% Me-β-CD, 0.5 μCi of [14C]cholesterol, 0.2 mM EDTA, 1 mM dithiothreitol, 5.0 mM trisodium isocitrate, 0.2 units of isocitrate dehydrogenase, and 100 mM sodium phosphate (pH 7.5). Reactions were started by adding 60 µl of 1 mM β-NADPH and incubating at 37 °C with gentle mixing. Each reaction was stopped after 90 min by adding 40 µl of 5N NaOH. After neutralization with HCl, sterols were extracted with 4.3 ml of dichloromethane-ethanol (5:1, v/v) plus 1.2 ml of H2O. Sample extracts, along with authentic 27-OH as a standard, were applied to a silica gel-60 HPTLC plate in a hairline nitrogen stream using a Camag Linomat-IV applicator, and chromatographed using hexane/ethyl acetate (1:1, v/v) as the mobile phase. After development and drying, the plate was analyzed for [14C]27-OH level by means of phosphorimaging. Other details of the HPTLC-PI technique used were described previously.

Determination of 27-hydroxycholesterol production
Wild type THP-1 macrophages (~2 x 10^6 in 35 mm dishes) were either untreated or stimulated with 0.2 mM Bu_{2}cAMP in 1% FBS/RPMI medium for 4 h and then exposed to SUV-7α-OOH in increasing concentrations up to 20 µM for 10 h, 20 µM SUV-Ch serving as a control. Cells were then washed and incubated for 12 h with a [14C]cholesterol/Me-β-CD complex, as described. After incubation, 1 ml of each culture medium was mixed with 3 ml of methanol plus 1 ml of saturated KCl solution and extracted twice with 6 ml of hexane. The organic layers were combined, evaporated under nitrogen, and each residue was dissolved in 100 µl of 5% isopropanol in hexane. Sample aliquots of 20 µl were applied to a high-performance silica gel thin layer chromatography plate and chromatographed, using toluene/ethyl acetate (2:3, v/v) as the mobile phase. The [14C]27-OH band was visualized and quantified using a phosphorimaging system; identification was based on co-migration of an authentic 27-OH standard.

Comparative cytotoxicity of cholesterol-7-oxides
THP-1 macrophages stimulated with 200 µM Bu_{2}cAMP for 8 h were washed, overlaid with serum-free RPMI medium, and treated with 7α-OOH, 7α-OH, or 7-OH in ethanolic solution, the final concentration of each 7-oxide ranging from 0 to 100 µM. Each compound, having limited aqueous solubility, was rapidly dispersed into the medium to allow uniform and reproducible contact with cells. After 24 h of incubation at 37 °C, cell viability was determined using the MTT (thiazolyl blue) assay, as described. In another approach, stimulated StarD1-kd macrophages and their scrambled controls were incubated in the presence of 0-100 µM SUV-7α-OOH in 1% FCS/RPMI medium for 24 h, then recovered for viability measurement by MTT assay.

Determination of cholesterol efflux from 7α-OOH-challenged vs. control macrophages
THP-1 macrophages (~1 x 10^6 cells/well) in 12-well plates were differentiated using 30 nM phorbol-12-myristate-13-acetate in 10% FBS/RPMI medium for 3 days. After differentiation, cells were incubated in fresh RPMI/10% FBS overnight. Cells were then washed, and treated with 1.5 mM Me-β-CD / 20 µM [3H]cholesterol (0.4 μCi/ml) overnight in serum-free medium. Cells were then washed and either stimulated with Bu_{2}cAMP (for ABCA1-mediated efflux) or T0901317 (for ABCG1-mediated efflux), or not stimulated, using RPMI medium containing 0.5% BSA. After an overnight incubation, the cells were washed and treated with POPC/Ch (3:1 by mol) SUVs or POPC/Ch/7α-OOH (2:1:1 by mol) SUVs overnight in serum-free medium. After washing, the cells were overlaid with apoA-I (20 µg/ml for ABCA1-mediated efflux) or HDL (50 µg/ml for ABCG1-mediated efflux) in 0.5% BSA/RPMI medium, and incubated for up to 6 h. Exported [3H]cholesterol as a percentage of total cellular [3H]cholesterol was determined by liquid scintillation counting. Other details were as described previously.
Statistics
All experiments were carried out at least in triplicate, with representative results shown in the case of Western blot analyses. Means ± SE of experimental values are shown. The two-tailed Student’s t-test was used for determining the significance of apparent differences between experimental values, with P>0.05 considered statistically insignificant. Data from cholesterol efflux experiments were analyzed by one-way ANOVA with Bonferroni post-test for all groups.

References
Figure SI. Cytotoxic effects of 7α-OOH and t-BuOOH on stimulated vs. non-stimulated macrophages. THP-1 macrophages in 1% FBS/RPMI medium were stimulated (●) by treating with 0.2 mM Bu₂cAMP for 4 h. Non-stimulated controls (○) were carried alongside. The cells were then incubated for 24 h with SUV-7α-OOH in increasing concentrations up to 100 µM (A) or for 8 h with t-BuOOH in increasing concentrations up to 400 µM (B), after which viability was determined by MTT assay. Means ± SE of values from three replicate experiments are plotted in (A) and (B).