# Macrophage cholesteryl ester hydrolases and hormone-sensitive lipase prefer specifically oxidized cholesteryl esters as substrates over their non-oxidized counterparts

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The oxidative modification of low-density lipoprotein (LDL) has been implicated as a pro-atherogenic process in the pathogenesis of atherosclerosis. Macrophages rapidly take up oxidized LDL via scavenger-receptor-mediated pathways and thereby develop into lipid-laden foam cells. The uptake mechanism has been studied extensively and several types of scavenger receptors have been identified. In contrast, the intracellular fate of oxidized LDL lipids is less well investigated. We studied the degradation of specifically oxidized cholesteryl esters by murine macrophages using an HPLC-based assay, and found that oxidized substrates are hydrolysed preferentially from a 1:1 molar mixture of oxidized and non-oxidized cholesteryl esters. This effect was observed at both neutral and acidic pH. Similar results were obtained with lysates of human monocytes and with pure recombinant human hormone-sensitive lipase. These data suggest that the intracellular oxidation of cholesteryl esters may facilitate intracellular cholesteryl ester hydrolysis, and thus may represent an anti-atherogenic process.

Key words: atherogenesis, hydroxy fatty acids, LDL modification, lipid peroxidation.

## INTRODUCTION

A characteristic feature of early atherogenesis is the formation of lipid-laden foam cells, which develop from monocyte/macrophages or special subtypes of smooth muscle cells [1,2] due to the uptake of modified low-density lipoprotein (LDL). Although the mechanism of LDL modification *in vivo* is still unclear, there is a substantial body of experimental evidence suggesting the importance of LDL oxidation [3,4]. *In vitro* experiments have indicated that oxidized LDL is taken up rapidly by macrophages via scavenger-receptor-mediated pathways [5]. Initial intracellular metabolism of LDL lipids occurs in the lysosomes, where cholesteryl esters are hydrolysed by acidic cholesteryl ester hydrolases; the resulting free cholesterol is delivered to the cytosol. Cytosolic acyl-CoA:cholesterol acyltransferase (ACAT) re-esterifies free cholesterol, and the resulting cholesteryl esters may be deposited as cytosolic lipid droplets. These intracellular lipid stores are in a dynamic steady state [6] and undergo a continuous hydrolysis/re-esterification cycle involving neutral cholesteryl ester hydrolases and ACAT. In the presence of exogenous cholesterol acceptors, such as high-density lipoprotein or albumin, free cholesterol resulting from the cytosolic cholesteryl ester hydrolase reaction may be exported from the cell to enter reverse cholesterol transport [7].

In macrophages, cholesteryl ester deposition is a result of an imbalance between cholesterol uptake and cholesterol efflux. Both of these processes are tightly regulated. The extent of cholesterol efflux depends mainly on the availability of extracellular cholesterol acceptors [8]. However, other regulatory elements which may impact the rates of cholesteryl ester hydrolysis and/or re-esterification may also be important. For instance, the activity of ACAT is regulated by progesterone [6]. For a long time it was believed that the metabolic counterpart of the ACAT reaction, the hydrolysis of cholesteryl esters, takes place only in the lysosomes. However, experiments with chloroquine suggest that cholesteryl esters do not have to re-enter the lysosomes for hydrolysis, but that they can be hydrolysed in the cytosol by neutral cholesteryl ester hydrolase(s) [9]. Acetyl-LDL was shown to impair the neutral cholesteryl ester hydrolase activity in human monocyte-derived macrophages [10]. In THP-1 cells (a human monocytic cell line) 25-hydroxycholesterol and modified LDL down-regulated the activity of neutral cholesteryl ester hydrolase(s), resulting in increased resistance of the lipid stores to reverse cholesterol transport [11]. On the other hand [12], the activities of neutral and acidic cholesteryl ester hydrolases were up-regulated when monocyte/macrophages were cultured in the presence of macrophage colony-stimulating factor. Intravenous administration of this factor to Watanabe rabbits augmented the neutral cholesteryl esterase/ACAT activity ratio and caused a strong inhibition of lipid deposition in the arterial wall [12]. These data suggested that the hydrolysis of cytosolic cholesteryl esters may slow down the formation of atherosclerotic plaques.

One reason for our relative lack of knowledge with regard to the regulation of macrophage neutral cholesteryl ester hydrolases is the fact that the identity of the enzyme(s) involved has been a matter of discussion for many years. Activation of macrophage neutral cholesteryl ester hydrolase(s) by cAMP [13] and prostaglandin $E_2$ [14] suggested a close relationship of these enzyme(s) with the hormone-sensitive lipase (HSL) of adipose tissue [15,16].

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; HSL, hormone-sensitive lipase; LDL, low-density lipoprotein; LOX, lipoxygenase; RP-HPLC, reverse-phase HPLC.

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Subsequently, HSL protein and mRNA have been detected in macrophages and several macrophage-like cell lines [17–20], and antibodies against HSL have been found to inhibit the neutral cholesteryl ester hydrolase activity of macrophage lysates [17]. With regard to human macrophages, studies reporting the presence of HSL [18,21] are contradicted by other reports showing undetectable HSL mRNA levels in monocyte-derived macrophages [22,23]. The low expression levels (2–3 % of adipocyte expression) and differences in the culture conditions or in the differentiation state may account for these discrepancies. Enzymological characterization of purified HSL has shown that it exhibits a cholesteryl ester hydrolase activity which is approximately twice its triacylglycerol lipase activity [24,25]. These data are consistent with the suggested physiological role of the enzyme as a combined triacylglycerol lipase/cholesteryl ester hydrolase.

Given the fact that oxidized LDL, which contains an increased proportion of oxidized cholesteryl esters [26], is strongly proatherogenic, an impaired rate of hydrolysis of oxidized cholesteryl esters may be expected. However, previous investigations on macrophage lipid turnover have suggested that hydroxy fatty acids containing ester lipids appear to be more rapidly hydrolysed than their non-oxidized counterparts, and it was suggested that cholesteryl esters may provide a transient storage pool for bioactive lipoxygenase (LOX) products [27,28]. We observed recently in an *in vitro* foam cell assay that murine macrophages that have been transfected with pig leucocyte type 12-LOX accumulate less intracellular lipids than their mock-transfected counterparts (H. Kühn and C. Gerth, unpublished work). To search for possible reasons for this unexpected finding, we investigated the metabolism of oxidized and non-oxidized cholesteryl esters by macrophage cholesteryl ester hydrolase(s), and found that these enzyme(s) preferentially cleave oxidized cholesteryl esters.

## EXPERIMENTAL

### Materials

P-388D1 cells (a murine macrophage cell line) were obtained from the German Collection of Microorganisms and Cells (Braunschweig, Germany). The chemicals used were from the following sources: $^{14}$C-labelled cholesteryl linoleate (labelled at the carboxylic carbon of linoleic acid; specific radioactivity 2 MBq/$\mu$mol) was a gift from A. Habenicht (University of Heidelberg, Germany); (9*Z*,12*Z*)-octadeca-9,12-dienoic acid (linoleic acid) and unlabelled cholesteryl linoleate were from Serva (Heidelberg, Germany); sodium borohydride was from Aldrich (Steinheim, Germany); triphenylphosphine and EDTA were from Merck (Darmstadt, Germany); BSA was from Sigma (Deisenhofen, Germany). All solvents were of HPLC grade and were purchased from Baker (Deventer, The Netherlands).

### Preparations

Recombinant HSL was expressed in the baculovirus/insect cell system and purified to homogeneity from lysates of infected cells [29]. Briefly, *Spodoptera frugiperda* (Sf9) cells were infected with recombinant baculovirus encoding full-length human HSL. At 60 h post-infection, cells were harvested and homogenized. After detergent solubilization, HSL was purified by anion-exchange chromatography on a Q-Sepharose column (Pharmacia), followed by hydrophobic interaction chromatography on phenyl-Sepharose (Pharmacia). The final enzyme preparation was $>$ 95 % pure (by SDS/PAGE) and exhibited a specific activity of 100 units/mg of protein. Pure HSL was stored at $-$70 °C at a concentration of 0.12 mg/ml in 5 mM sodium phosphate buffer, pH 7.4, containing 1 mM dithioerythritol, 0.2 % $C_{13}E_{12}$ (alkyl polyoxyethylene ether detergent) and 50 % glycerol.

P-388D1 cells were grown at 37 °C in RPMI 1640 medium containing 20 % (v/v) horse serum, 3.5 g/l glucose, 0.03 % L-glutamine and antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin). After washing with $Ca^{2+}$/$Mg^{2+}$-free PBS, the cells were harvested by scraping and were resuspended in 5 vol. of 25 mM Tris/HCl buffer, pH 7.4, containing 1 mM EDTA and 20 % (v/v) glycerol. The cells were disrupted by sonication using a microtip Labsonic 2000 U sonicator (Braun-Melsungen, Germany) for 10 s on ice. The freshly prepared lysates were used as a crude preparation of neutral cholesteryl ester hydrolase. For selected experiments, the lysates were centrifuged for 5 min at 500 ***g*** to remove cell debris and the supernatant was used as the enzyme source.

Human peripheral monocytes were prepared from samples from healthy volunteers by Ficoll density-gradient centrifugation and adherence to plastic dishes [30]. To allow macrophage differentiation, the cells were kept in culture for 8 days. Just before the experiments the cells were scraped from the dishes and washed once with PBS. Cell disruption and activity assays were carried out as described for the P-388D1 cells.

Radioactively labelled oxidized cholesteryl esters were prepared from cholesteryl linoleate using the rabbit reticulocyte 15-LOX. A 5 $\mu$l portion of a 0.2 M cholesteryl linoleate solution was mixed with 520 kBq of [$^{14}$C]cholesteryl linoleate. The solvent was evaporated and the residue was reconstituted in 200 $\mu$l of 10 % (w/v) aqueous sodium cholate solution. Then 9.8 ml of 0.1 M sodium phosphate buffer, pH 7.4, was added and the sample was sonicated with a tip-sonifier until an opalescent suspension was obtained. Oxygenation of cholesteryl linoleate was started by the addition of 10 nmol of pure rabbit reticulocyte 15-LOX. After 30 min at room temperature, the lipids were extracted [31], the hydroperoxides were reduced to the corresponding hydroxy compounds (addition of a molar excess of sodium borohydride) and the oxidized cholesteryl esters were prepared by reverse-phase HPLC (RP-HPLC) [32]. The final preparation of oxidized cholesteryl esters was stored as ethanolic stock solution at $-$40 °C under an argon atmosphere.

### Activity assays

Cholesteryl ester hydrolase activity was assayed by HPLC quantification of the liberation of radioactively labelled linoleate derivatives from the substrate cholesteryl [1-$^{14}$C]linoleate. For this purpose, aliquots of methanolic stock solutions (oxidized and non-oxidized cholesteryl esters) were mixed with a 5 mM aqueous EDTA solution to give a final volume of 0.4 ml (substrate solution). Then 0.2 ml of 200 mM sodium phosphate buffer, pH 7.4, and 0.1 ml of a 2.5 % (w/v) BSA solution were added and the mixture was vortexed vigorously. The final ethanol concentration in the assay system was adjusted to 5 %, but no additional emulsifiers or detergent were added. For measurements of the acidic cholesteryl ester hydrolase activity, a 200 mM sodium acetate buffer, pH 3.9, was used instead of the phosphate buffer. The cholesteryl ester hydrolase reaction was started by the addition of 0.3 ml of cell lysate, and the mixture was incubated for various time periods. The reaction was stopped by the addition of 1 ml of methanol and the lipids were extracted [31]. The organic phase was recovered, the solvent was evaporated and the remaining lipids were reconstituted in 0.4 ml of chloroform. Cholesteryl ester hydrolase activity was measured by quantifying (by HPLC) either the disappearance of cholesteryl esters and/or the liberation of free linoleate derivatives. Although we did not routinely determine the specific radioactivity of the

metabolites, we found in several experiments that endogenous substrates did not influence the metabolism of exogenous cholesteryl esters. This may be partly be due to the fact that the endogenous cholesteryl ester concentration of P-388D1 cells is rather low.

### Analysis

HPLC was carried out on a Shimadzu SPD-M10AVP instrument coupled with a diode array detector and a CTO-10A column oven. Oxidized and non-oxidized cholesteryl linoleate were analysed by RP-HPLC at 40 °C using a Nucleosil C-18 column (Macherey/Nagel, Düren, Germany; KS-system, 250 mm × 4 mm, 5 $\mu$m particle size) and the solvent system acetonitrile/propan-2-ol (3:1, v/v), at a flow rate of 1 ml/min. The absorbancies at 235 nm (detection of oxidized cholesteryl esters) and at 210 nm (detection of non-oxidized cholesteryl esters) were recorded simultaneously. Non-esterified fatty acid derivatives were analysed by RP-HPLC (Nucleosil C-18 column, Macherey/Nagel; KS-system, 250 mm × 4 mm, 5 $\mu$m particle size) with a solvent system of methanol/water/acetic acid (80:20:0.1, by vol.) at a flow rate of 1 ml/min. Here again the absorbancies at 235 nm (detection of oxidized fatty acids) and 210 nm (detection of non-oxidized polyenoic fatty acids) were recorded. HPLC fractions containing the oxidized and/or non-oxidized substrates and/or products were fractionated, and radioactivity was quantified by liquid scintillation counting (Wallac 1410; Pharmacia). Protein concentration were determined using the Bio-Rad detection kit.

## RESULTS

### Oxygenated cholesteryl esters are hydrolysed more rapidly by macrophage cholesteryl ester hydrolases

Macrophages contain two major classes of cholesteryl ester hydrolases: (i) acidic isoforms located in the lysosomes, and (ii) cytosolic neutral cholesteryl ester hydrolases. To assay the cellular cholesteryl ester hydrolase activity, we developed an HPLC-based assay system which quantifies the consumption of labelled cholesteryl linoleate and/or the formation of labelled non-esterified fatty acid derivatives (Figure 1). The two methods yielded almost identical results in several series of experiments (results not shown). However, we found that quantification of cholesteryl ester hydrolase activity was more accurate when the formation of non-esterified fatty acid derivatives was measured, because of the low background radioactivity in the controls (0 min of incubation time). Under such conditions, even a small increase in the formation of non-esterified fatty acid derivatives could be detected (Figure 1A). In contrast, when the decrease in cholesteryl ester concentration is assayed (Figure 1B), one starts at high levels of radioactivity (0 min of incubation) and thus a small decrease in substrate concentration may easily escape detection. Therefore we selected the quantification of non-esterified fatty acid derivatives as the method of choice for the determination of cellular cholesteryl ester hydrolase activities in subsequent experiments.

When we compared the rates of cholesteryl linoleate hydrolysis by P-388D1 cell homogenates at neutral (7.4) and acidic (3.9) pH, we observed a higher rate at pH 7.4 (Figure 2). It should be stressed that these *in vitro* data do not necessarily reflect the situation *in vivo*. Cell lysis dilutes the lysosomal isoenzymes much more than the cytosolic isoforms, and one has to consider that our assay systems have not been optimized completely for the two isoforms. In a second set of experiments we compared the rates of hydrolysis of oxidized and non-oxidized cholesteryl

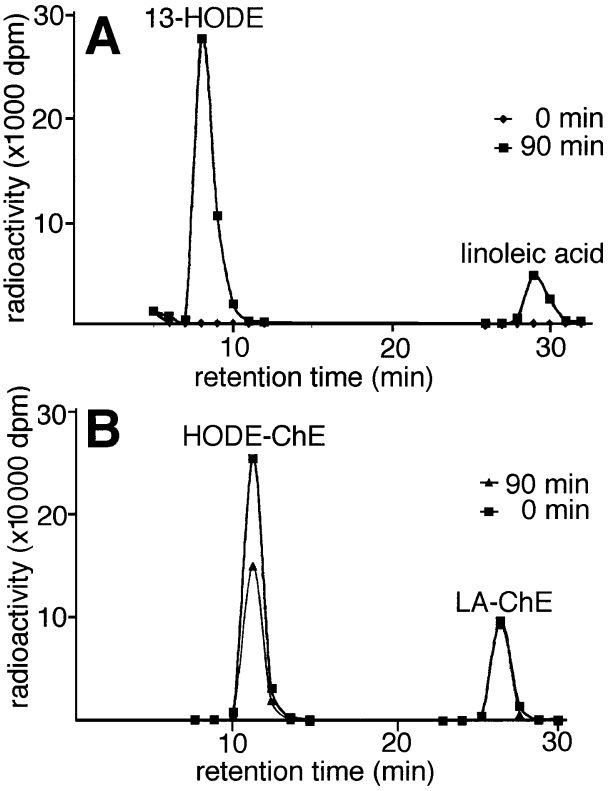


**Figure 1 HPLC quantification of cholesteryl ester hydrolase**

Cholesteryl ester hydrolase activity was measured by quantifying the liberation of non-esterified linoleic acid derivatives (**A**) and the consumption of cholesteryl esters by radio-RP-HPLC (**B**). A 0.3 ml sample of a cell lysate of P-388D1 cells was incubated with a 1:1 molar mixture of oxidized and non-oxidized cholesteryl linoleate (total cholesteryl ester concentration = 40 $\mu$M). Incubation conditions, sample preparation and HPLC analysis were carried out as described in the Experimental section. Fractions (1 ml) were collected and radioactivity was counted. Abbreviations: 13-HODE, 13-hydroxylinoleic acid (13-hydroxy-9*Z*,11*E*-octadecadienoic acid); HODE-ChE, cholesteryl hydroxylinoleate; LA-ChE, cholesteryl linoleate

linoleate. From Figure 2 it can be seen that, under both neutral and acidic conditions, the oxidized cholesteryl esters were metabolized more rapidly than their non-oxidized counterparts. Similar results were obtained in three independent experiments with enzyme preparations from different cell batches. In control incubations (results not shown) using heat-denatured cell lysates, no cholesteryl ester hydrolysis was observed with either oxidized or non-oxidized substrates.

For the experiments shown in Figure 2, oxidized and non-oxidized cholesteryl linoleate were used as the substrate in separate incubation samples. To find out whether cholesteryl ester hydrolases also prefer oxidized cholesteryl esters when both substrates are available, we used a 1:1 molar mixture of the two substrates and quantified the liberation of oxidized and non-oxidized linoleic acid in a single incubation mixture. From Figure 3 it can be seen that, over the entire time course of the experiment, oxidized cholesteryl esters were preferentially cleaved by neutral cholesteryl ester hydrolases. This preference leads to a selective removal of oxidized cholesteryl esters from the incubation mixture, and thus the overall level of oxidation of the remaining substrate mixture is decreased. Under our experimental conditions the degree of substrate oxidation (percentage of oxidized cholesteryl esters) was lowered from 50% (1:1 mixture of oxidized and non-oxidized cholesteryl linoleate) at the

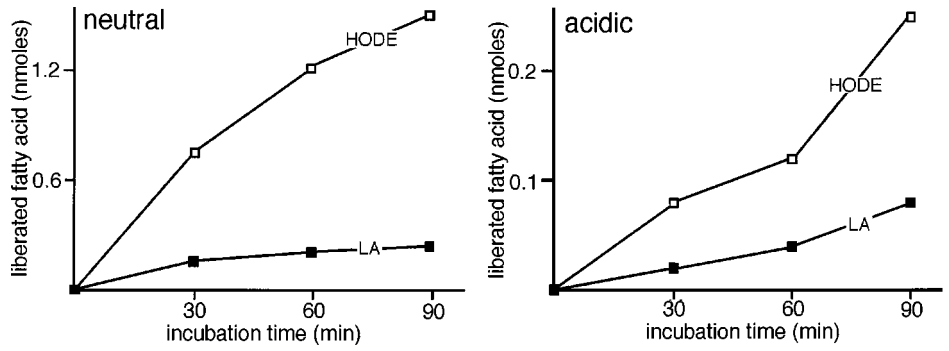


**Figure 2 Preferential cleavage of oxidized cholesteryl esters**

Lysates of P-388D1 cells were incubated at pH 7.4 (neutral cholesteryl ester hydrolase) or pH 3.9 (acidic cholesteryl ester hydrolase) with oxidized or non-oxidized cholesteryl linoleate (40 $\mu$M total substrate concentration). Aliquots were taken at the time points indicated and the release of free linoleate derivatives was quantified by RP-HPLC. This experiment was carried out three times with enzyme preparations obtained from three different cell batches, and in all cases we observed a higher rate of hydrolysis of the oxidized substrates. Abbreviations: HODE, liberated hydroxylinoleic acid; LA, liberated linoleic acid.

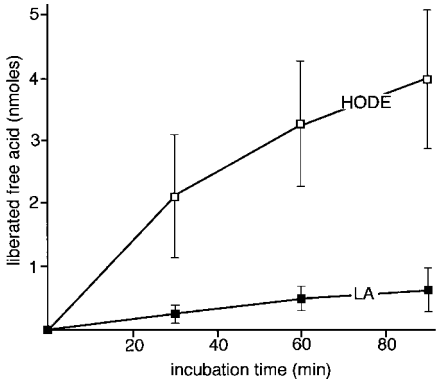


**Figure 3 Preferential cleavage of oxidized cholesteryl linoleate by macrophage neutral cholesteryl ester hydrolase**

Lysates of P-388D1 cells were incubated at pH 7.4 with a 1:1 molar mixture of oxidized and non-oxidized cholesteryl linoleate (total cholesteryl ester concentration = 40 $\mu$M). At the times indicated, aliquots were taken and the release of non-esterified fatty acid derivatives was quantified by RP-HPLC ($n = 4$). Abbreviations: HODE, liberated hydroxylinoleic acid; LA, liberated linoleic acid.

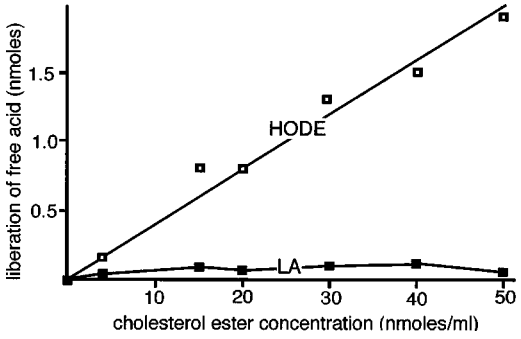


**Figure 4 Dependence of cholesteryl ester hydrolysis rate on substrate concentration**

Lysates of P-388D1 cells were incubated at pH 7.4 with different concentrations of either oxidized or non-oxidized cholesteryl linoleate for 30 min at 30 °C (separate samples for each substrate). At the times indicated, aliquots were taken and the release of non-esterified fatty acid derivatives was quantified by measuring the radioactivity after RP-HPLC preparation of the metabolites. Abbreviations: HODE, liberated hydroxylinoleic acid; LA, liberated linoleic acid.

beginning of the experiment to approx. 40 % after 90 min of incubation ($n = 4$).

The neutral cholesteryl ester hydrolase activity of macrophages varied considerably between different batches of cells. On averaging the results from experiments carried out with oxidized cholesteryl linoleate as substrate, a specific activity of the enzyme of $0.69 \pm 0.45$ nmol of substrate hydrolysis/30 min per mg of lysate protein was calculated ($n = 12$). The corresponding value for the hydrolysis of non-oxidized cholesteryl linoleate was $0.09 \pm 0.08$ nmol/30 min per mg ($n = 5$).

The specific activity of our cholesteryl ester hydrolase preparation was lower than that reported in the literature [14,33]. We did not investigate the reasons for this discrepancy, but the following points may contribute. (i) Usually neutral cholesteryl ester hydrolase activity is assayed in the cytosol, whereas we used the total cell lysate. Since cell lysates contain more 'foreign proteins' than cytosolic preparations, the specific activity is expected to be lower. In addition, it may be possible that centrifugation of the lysate removes endogenous inhibitors of the cholesteryl ester hydrolase [33], so that the specific activity would be increased. (ii) In previous papers, cholesteryl oleate has commonly been used as substrate. In contrast, we assayed the activity with cholesteryl linoleate, although we were aware of the fact that cholesteryl linoleate may not be a good substrate for the enzyme [34]. (iii) The specific activity of neutral cholesteryl ester hydrolase varies depending on the cellular source, and also perhaps on the degree of cell differentiation. An enzyme activity was measured in arterial cells [35] which was one order of magnitude lower than that determined in the present study.

The activity of macrophage neutral cholesteryl ester hydrolase with oxidized cholesteryl linoleate as substrate depended strongly on substrate concentration (Figure 4). In the concentration range 5–50 $\mu$M there was an almost linear increase in the rate of hydrolysis, indicating that the enzyme(s) was not saturated. In contrast, the rate of hydrolysis of non-oxidized cholesteryl linoleate was not augmented further when the substrate concentration was raised above 15 $\mu$M. Here again we observed more rapid hydrolysis of the oxidized substrates over the entire concentration range. In fact, at higher concentrations the

**Table 1 Neutral cholesteryl ester hydrolase activity of human monocyte-derived macrophages and P-388D1 cells**

| Substrate | Cholesteryl ester hydrolase activity (pmol of substrate/30 min per mg of protein) | |
|---|---|---|
| | P-388D1 cells | Human macrophages |
| Oxidized cholesteryl linoleate | 800 | 15.1 |
| Non-oxidized cholesteryl linoleate | 90 | 5.8 |

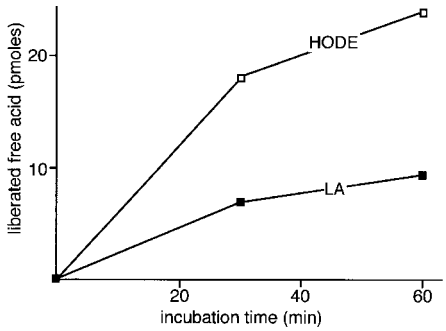


**Figure 5 Cleavage of oxidized and non-oxidized cholesteryl esters by neutral cholesteryl ester hydrolase of human monocyte/macrophages**

Lysates of monocyte-derived human macrophages were incubated at pH 7.4 with a 1:1 molar mixture of oxidized and non-oxidized cholesteryl linoleate (total cholesteryl ester concentration = 40 $\mu$M). At the times indicated, aliquots were taken and the release of non-esterified fatty acid derivatives was quantified by radio RP-HPLC. This experiment was repeated twice, with similar results, with macrophages prepared from buffy coats from two different donors. Abbreviations: HODE, liberated hydroxylinoleic acid; LA, liberated linoleic acid.

differences between the rates of hydrolysis of oxidized and non-oxidized substrates were even more pronounced.

### Cholesteryl ester hydrolysis by human monocyte-derived macrophages

To confirm the finding of preferential cleavage of oxidized cholesteryl esters for human monocyte-derived macrophages, we prepared peripheral monocytes from healthy volunteers and cultured the cells for 8 days in the presence of human AB serum to induce macrophage differentiation. After harvesting, the cells were washed and lysed by sonication, and the lysates were incubated for different time periods with a 1:1 molar mixture of oxidized and non-oxidized cholesteryl linoleate. On measuring the liberation of non-esterified fatty acids, we found that the neutral cholesteryl ester hydrolase activity of human monocyte-derived macrophages was lower than that of P-388D1 cells (Table 1). However, we again observed preferential cleavage of oxidized cholesteryl esters over the entire time course of the experiment (Figure 5). These results were confirmed in two independent experiments with monocytes from two different donors.

### Water solubility may not be a major reason for the different rates of hydrolysis of oxidized and non-oxidized cholesteryl esters

In general, lipid substrates exhibit limited water solubility, and kinetic effects (such as an augmented reaction rate or an impaired

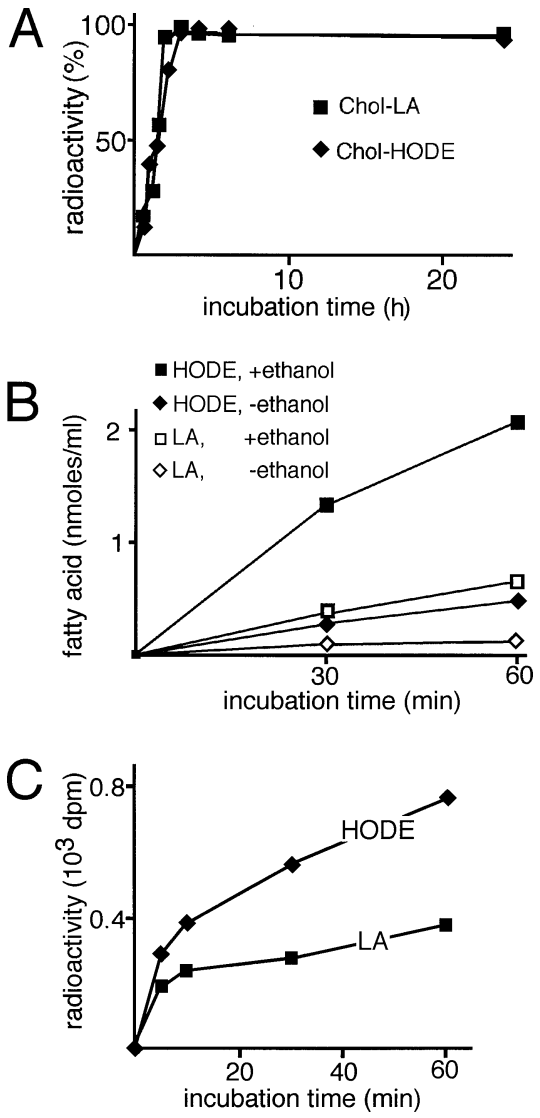


**Figure 6 Substrate availability is not a major reason for the different rates of hydrolysis of oxidized and non-oxidized cholesteryl esters**

(**A**) Distribution of oxidized (cholesteryl hydroxylinoleate; Chol-HODE) and non-oxidized (cholesteryl linoleate; Chol-LA) cholesteryl esters between hexane and water. The percentage of radioactivity present in the hexane phase is shown. Oxidized and non-oxidized cholesteryl esters (4 nmol each) were dissolved in 0.5 ml of sodium phosphate buffer, pH 7.4, containing 5% (v/v) ethanol. This solution was overlaid with 0.5 ml of hexane and the mixture was shaken gently. At the times indicated, aliquots were taken from both the water and the hexane phases, and radioactivity was measured by liquid scintillation counting. (**B**) Addition of ethanol did not influence the difference in hydrolysis rate between oxidized and non-oxidized cholesteryl esters. Lysates of P-388D1 cells were incubated at pH 7.4 with a 1:1 molar mixture of oxidized and non-oxidized cholesteryl esters (total cholesteryl ester concentration = 40 $\mu$M) in the absence and presence of 5% ethanol. At the times indicated, aliquots were taken and the release of non-esterified fatty acid derivatives was quantified by RP-HPLC. Abbreviations: HODE, hydroxylinoleic acid; LA, linoleic acid. (**C**) Oxidized cholesteryl esters are hydrolysed more rapidly than non-oxidized ones when incorporated in phospholipid micelles. An ethanolic solution (10 $\mu$l) of radioactively labelled cholesteryl esters (20 nmol each of oxidized and non-oxidized derivatives) was mixed with an ethanolic solution (10 $\mu$l) of dipalmitoyl phosphatidylcholine (59 $\mu$g). Then 30 $\mu$l of ethanol and 0.95 ml of assay buffer were added and the mixture was vortexed. The P-388D1 cell lysate was incubated with this mixture, and aliquots were taken at the time points indicated. The release of non-esterified fatty acid derivatives was quantified by RP-HPLC as indicated in the legend to Figure 1. Abbreviations: HODE, hydroxylinoleic acid; LA, linoleic acid.

substrate affinity) may be due to differences in substrate availability. We addressed the question of whether the increased rate of hydrolysis of oxidized cholesteryl esters may be due to better

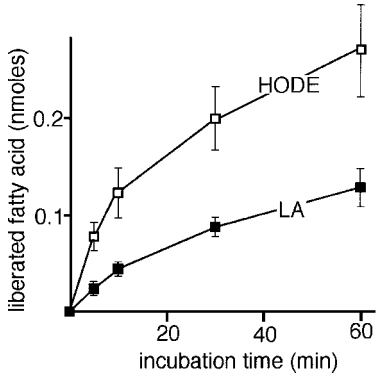


**Figure 7 Cholesteryl ester hydrolase activity of purified recombinant human HSL**

Human HSL was expressed in the baculovirus insect cell system and purified to homogeneity as described previously [25]. Aliquots of this preparation (5 ng/ml of incubation sample) were incubated for 60 min at 30 °C with a 1:1 molar mixture of oxidized and non-oxidized cholesteryl linoleate (total cholesteryl ester concentration = 40 $\mu$M). Aliquots of 250 $\mu$l were taken and the release of non-esterified fatty acid derivatives was quantified by radio RP-HPLC. Abbreviations: HODE, hydroxylinoleic acid; LA, linoleic acid.

water solubility of the substrate, using three different approaches. First, we compared the water/hexane distribution coefficient of oxidized and non-oxidized cholesteryl esters as a measure of the water solubility of the lipophilic substrates, but did not find major differences between the two substrates (Figure 6A). Secondly, we improved the substrate solubility by the addition of organic solvents. From Figure 6(B) it can be seen that ethanol enhanced the rates of hydrolysis of the cholesteryl esters, suggesting that water solubility in fact may be a limiting factor. However, since this enhancement was observed to a similar extent for both oxidized and non-oxidized cholesteryl esters, water solubility may not be a major reason for the different rates of hydrolysis of the two substrates. Thirdly, we attempted to prevent the difference in hydrolysis rates by presenting the substrate for hydrolysis in phospholipid micelles. The rationale behind this series of experiments was to incorporate the cholesteryl esters into micelles and thereby present oxidized and non-oxidized cholesteryl esters to the enzyme in a similar form. Here again, we observed a higher rate of hydrolysis for the oxidized cholesteryl esters (Figure 6C). Although the data presented in Figure 6 suggest that water solubility may not be a major reason for the increased rate of hydrolysis of oxidized cholesteryl esters, the possibility cannot be ruled out that a more suitable presentation of the oxidized substrates may contribute to the observed effects (see Discussion).

### Recombinant HSL also prefers oxidized cholesteryl ester

The cellular cholesteryl ester hydrolase activity of macrophages may be regarded as the result of several enzymic processes to which various lipid-hydrolysing enzymes (phospholipases, triacylglycerol lipases) may contribute. For macrophages it has been suggested that the majority of the neutral cholesteryl ester hydrolase activity may be due to HSL [36], but this hypothesis is still under discussion. To investigate whether a purified preparation of this enzyme may also prefer oxidized cholesteryl linoleate over the non-oxidized counterpart, the human enzyme was overexpressed in the baculovirus/insect cell system and purified to homogeneity. When incubated with a 1:1 molar mixture of oxidized and non-oxidized cholesteryl linoleate, the oxidized substrate was cleaved preferentially over the entire incubation period (Figure 7). It should, however, be mentioned that the differences in hydrolysis rates with oxidized and non-oxidized cholesteryl esters were more pronounced with macrophage cytosol (Figure 3) than with human recombinant HSL (Figure 7) as enzyme source.

**Table 2 Kinetic constants for cholesteryl ester hydrolysis by pure recombinant HSL**

Purified HSL was incubated for 5 min at different substrate concentrations (4–50 $\mu$M) and the liberation of non-esterified fatty acid derivatives was measured. The constants were extracted from Lineweaver–Burk plots and represent six single measurements for each substrate as well as for the 1:1 molar mixture of oxidized and non-oxidized cholesteryl ester. In the latter case, the liberation of linoleic acid (non-oxidized substrate share) and of hydroxylinoleic acid (oxidized substrate share) was quantified separately. The values given represent the means of two independent series of experiments. The $K_m$ values determined here are in the same range as for the rat enzyme [59].

| Parameter | Single substrates | Mixture of substrates |
|---|---|---|
| $K_m$ ($\mu$M) | | |
| Oxidized cholesteryl linoleate | 4.0 | 7.21 |
| Non-oxidized cholesteryl linoleate | 1.27 | 0.72 |
| $V_{max}$ ($s^{-1}$) | | |
| Oxidized cholesteryl linoleate | 6.39 | 8.78 |
| Non-oxidized cholesteryl linoleate | 6.34 | 3.26 |

### Basic kinetic characterization of the cholesteryl ester hydrolase activity of purified HSL

Since our HSL preparation was electrophoretically pure, we carried out basic kinetic characterization of its cholesteryl ester hydrolase activity using oxidized or non-oxidized cholesteryl esters as substrate, as well as a 1:1 molar mixture of both. The kinetic constants summarized in Table 2 indicate that the enzyme binds non-oxidized cholesteryl esters with a higher affinity. As a possible reason for this observation, one may hypothesize that burial of the hydrophilic hydroxy group of the fatty acid moiety in the hydrophobic environment of the enzyme's active site is hindered energetically, and that this energetic barrier is reflected by the higher $K_m$. A similar effect was reported recently for rabbit 15-LOX, another lipid-metabolizing enzyme [37]. The $V_{max}$ values for the hydrolysis of oxidized and non-oxidized cholesteryl esters did not differ significantly when the substrates were hydrolysed separately (6.39 $s^{-1}$ and 6.34 $s^{-1}$ for oxidized and non-oxidized cholesteryl linoleate respectively). However, when the substrates were applied as 1:1 molar mixture of oxidized and non-oxidized cholesteryl esters, the oxidized substrate was cleaved with a higher $V_{max}$ (8.78 $s^{-1}$ compared with 3.26 $s^{-1}$). It should be stressed at this point that the kinetic constants are rather descriptive and that their mechanistic interpretation is problematic. The major difficulty for the kinetic characterization of lipid-metabolizing enzymes is the physico-chemical state of the substrate. Owing to the limited water solubility, there is always a steady state of different substrate forms (e.g. substrate monomers, dimers, micelles), and this steady state is influenced by pH, temperature, overall substrate concentration, presence or absence of emulsifiers, etc. In this respect, $K_m$ and $V_{max}$ may be considered as apparent parameters, which may vary considerably when determined under different conditions.

During our kinetic studies on HSL, we noticed that the enzyme loses activity during the time course of the hydrolysis

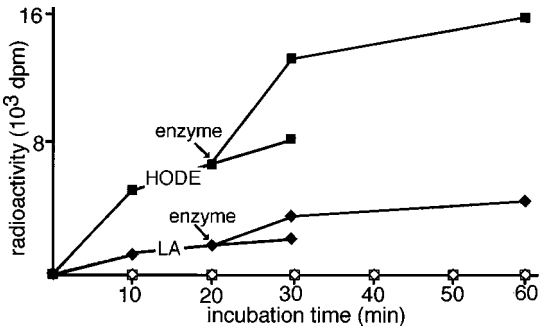


**Figure 8 Inactivation of recombinant HSL during the hydrolysis reaction**

A 1 ml aliquot of the HSL preparation was heated to 80 °C for 5 min. Aliquots (50 $\mu$l) of native (solid symbols) and denatured (open symbols) enzyme preparations (5 ng/ml of incubation sample) were incubated for 60 min at 30 °C with a 1:1 molar mixture of oxidized and non-oxidized cholesteryl linoleate (total cholesteryl ester concentration = 10 $\mu$M). Aliquots were taken at the times indicated and the release of non-esterified fatty acid derivatives was quantified by radio RP-HPLC. After 20 min of incubation, the incubation sample was divided into two halves. Another aliquot (50 $\mu$l) of the enzyme preparation was added to one half of the sample, as indicated, whereas the other half was used as a control. Abbreviations: HODE, hydroxylinoleic acid; LA, linoleic acid. Open symbols are overlaid.

reaction (Figure 7). Possible reasons for such kinetic behaviour include depletion of the substrate and enzyme inactivation. Although the substrate concentration in our standard assay was chosen to be high enough to avoid overall substrate depletion, the system may have run out of accessible substrate. In order to investigate this problem in more detail, the following experiment was designed. HSL was allowed to react with oxidized or non-oxidized cholesteryl esters for 20 min, and then the incubation samples were divided. One portion was used as the control, and new HSL was added to the second portion. Then the hydrolysis activity was assayed 10 and 40 min after the second addition of enzyme. As shown in Figure 8, addition of new enzyme increased the hydrolysis rate for both oxidized and non-oxidized cholesteryl esters, to give a rate almost identical to that at the very beginning of the reaction. These data indicate that substrate limitation may not be the major reason for the slowing down of the hydrolysis reaction during its time course. In contrast, it appears likely that the enzyme may be inactivated during the incubation period (suicidal inactivation), but the mechanistic reasons behind this process remain to be established.

## DISCUSSION

Atherosclerosis is a complex disease, which involves dysfunction of local lipoprotein metabolism at the site of lesion formation. An early key event in atherogenesis is the formation of lipid-laden foam cells, which accumulate in the vascular subendothelial space to form fatty streaks that are generally accepted to be early atherosclerotic lesions. Although the detailed mechanism of foam cell formation is far from clear, oxidative modification of LDL and intracellular hydrolysis of cholesteryl esters appear to be involved. Extracellular LDL oxidation is believed to be a pro-atherogenic process, and there is evidence for the *in vivo* involvement of enzymic and non-enzymic oxidation reactions [3]. In recent years several studies have suggested a role for various LOX isoforms in systemic LDL oxidation. (i) *In vitro*, LOXs are capable of oxidizing LDL to an atherogenic form. This reaction may proceed via a direct attack on the LDL ester lipids [38,39] or indirectly, via oxygenation of non-esterified fatty acids which have been liberated previously from the ester lipids [40,41]. (ii) Zymosan-stimulated macrophages prepared from mice in which the gene encoding the leucocyte-type 12-LOX was disrupted exhibited impaired LDL-oxidizing capability [42]. In addition, fibroblasts transfected with human 15-LOX cDNA have a higher LDL-oxidizing capacity [43]. (iii) 12/15-LOXs are expressed as functional enzymes in atherosclerotic lesions [44–46], and somatic gene transfer of 15-LOX to rabbit iliac arteries led to the appearance of oxidized LDL epitopes in the transfected areas [47]. (iv) A 15-LOX inhibitor, which apparently lacks major antioxidative properties, prevented the formation of atherosclerotic lesions in cholesterol-fed rabbits [48] by slowing down monocyte/macrophage enrichment in the vessel wall [49]. (v) Functional disruption of the murine macrophage 12-LOX gene significantly reduced lesion formation when 12/15-LOX knockout mice were crossed with apolipoprotein E-deficient animals [50]. These data suggest a pro-atherogenic role for 12/15-LOXs, but the detailed mechanism of action remains to be investigated [51].

On the other hand, there is experimental evidence in the literature for an anti-atherogenic activity of 12/15-LOXs. New Zealand White rabbits that overexpress the human reticulocyte-type 15-LOX specifically in monocyte/macrophages were protected from lesion formation during the time course of cholesterol feeding [52]. When these animals were cross-bred with Watanabe rabbits, a similar effect was seen. More recently, it was reported that induction of experimental anaemia, which induces strong 15-LOX overexpression in rabbits [53], inhibits progression of atherosclerotic lesions in apolipoprotein E knockout mice [54].

For the time being the mechanistic reasons for the conflicting data on the potential role of LOX expression remain unclear. Systemic knockout of 12/15-LOX expression may lead to a decrease in overall LDL-oxidizing potential. In fact, the decreased levels of autoantibodies against oxidized LDL in 12/15-LOX/apolipoprotein E double-knockout mice may be suggestive of such impaired systemic LDL oxidation [50]. Similarly, oral administration of a LOX inhibitor is expected to prevent systemic LDL oxidation [48]. In contrast, cell-specific overexpression of 15-LOX in monocyte/macrophages may not be sufficient to increase systemic LDL oxidation, because of the relatively low number of macrophages. However, intracellular lipid metabolism within the macrophages may well be affected. Our findings that oxidized cholesteryl esters are preferred substrates for macrophage neutral cholesteryl ester hydrolase(s) suggest that specific intracellular lipid peroxidation may accelerate cytosolic cholesteryl ester hydrolysis, providing non-esterified cholesterol for reverse cholesterol transport. Thus intracellular LOX-catalysed oxidation of cholesteryl esters may augment reverse cholesterol transport, which would explain the anti-atherogenic effect of the macrophage-specific overexpression of human 15-LOX. However, in the absence of extracellular cholesterol acceptors, this anti-atherogenic effect may be limited, since the resulting non-esterified cholesterol will re-enter the cytosolic re-esterification/hydrolysis cycle.

Previous studies on the *in vitro* interaction of purified 15-LOXs with human LDL have indicated that the LOX-catalysed oxidation of cholesteryl esters is restricted to their fatty acid moiety. In contrast with non-enzymic lipid peroxidation, we did not observe the formation of significant amounts of oxysterols during the LOX/LDL interaction (H. Kühn, unpublished work). This specificity may be of pathophysiological relevance, since oxysterols, such as 25-hydroxycholesterol, markedly increased ACAT activity in THP-1 cells [11]. Thus the formation of significant amounts of oxysterols would counteract the above-described anti-atherogenic effect of the LOX reaction.

With regard to atherogenesis, it may be of particular importance that the preferential hydrolysis of oxidized cholesteryl esters is more pronounced at higher substrate concentrations (Figure 5). In resting macrophages the intracellular concentration of cholesteryl esters is rather low and, according to our data, there may not be a major difference in the rates of hydrolysis of oxidized and non-oxidized substrates. However, during foam cell formation the intracellular cholesteryl ester concentration is rising, and oxidation of its fatty acid moieties may contribute to increased metabolism of internalized lipids.

Oxidatively modified LDL acts in a pro-atherogenic manner because it is taken up rapidly by macrophages, and there appears to be no feedback control. It should be stressed that in biologically modified LDL the vast majority ($> 90\%$) of cholesteryl esters are present as non-oxidized lipids. We analysed different LDL species that were oxidized in various biological systems and never observed a degree of oxidation of cholesteryl esters of greater than $10\%$. From these data one may conclude that scavenger-receptor-mediated uptake of modified LDL leads mainly to the intracellular accumulation of non-oxidized cholesteryl esters. The LOX-catalysed oxidation of these non-oxidized substrates may be regarded as an integral part of the cholesteryl ester breakdown cascade counteracting intracellular lipid deposition, and thus may prevent foam cell formation.

The role of HSL in atherogenesis has become a matter of discussion. For a long time it was believed that the enzyme might be anti-atherogenic because of its ability to metabolize deposited cholesteryl esters. Macrophage-derived foam cells purified from human and rabbit atherosclerotic lesions express HSL only at very low levels [55]. Stable transfection of murine macrophages with HSL led to the augmented hydrolysis of cholesteryl ester stores in *in vitro* foam cell models [56,57]. However, these data also indicated that an increase in the cholesteryl ester hydrolysing capacity of macrophages alone is not sufficient to protect against lesion development, but needs to be accompanied by a parallel increase in the capacity for efflux of non-esterified cholesterol, requiring a decrease in ACAT activity. Disruption of the HSL gene in mice [58] did not alter the neutral cholesteryl ester hydrolase activity of peritoneal macrophages. These data contradict earlier reports indicating that anti-HSL antibodies are capable of reducing the neutral cholesteryl ester hydrolase activity of macrophage lysates [17]. The reason for this discrepancy is not known, but it should be pointed out that the absolute levels of neutral cholesteryl ester hydrolase activity measured in the HSL knockout animals were very low compared with those in other reports [58]. The phenotype of HSL knockout mice fed an atherogenic diet remains to be studied.

The detailed mechanistic reasons for the preferential cleavage of oxidized cholesteryl esters by macrophage cholesteryl ester hydrolase and HSL remain unclear. Although we found that the water solubility of oxidized and non-oxidized cholesteryl esters may not be dramatically different (Figure 6A) and that emulsifiers, such as ethanol or phospholipids (Figures 6B and 6C), do not prevent the differences in hydrolysis rate, one cannot completely rule out the possibility that a more suitable substrate presentation of the oxidized cholesteryl esters may contribute to the effect. It may be speculated that oxidized cholesteryl esters are clustered at the surface of substrate superstructures (micelles, liposomes) so that they are more easily accessible to the enzyme. However, from the biological point of view, the molecular basis for the increased rate of hydrolysis of oxidized cholesteryl esters is not of major importance. Even if oxidized cholesteryl esters are presented in a more suitable form than their non-oxidized counterparts, the effect may be of biological relevance. It is a general principle in the metabolism of hydrophobic compounds (not just xenobiotics) to decrease hydrophobicity in order to facilitate metabolic breakdown and/or excretion. During evolution entire enzymic systems, such as the cytochrome P450 isoenzyme family, have been developed for this purpose. It may be speculated that LOX may play a similar role in cholesteryl ester turnover in macrophages.

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