Lipid synthesis in macrophages derived from the human cell line THP-1: modulation of the effects of native and oxidized chylomicron-remnant-like particles by oestrogen

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ABSTRACT

The effects of native and oxidized chylomicron remnants on the synthesis of cholesteryl ester and triacylglycerol in macrophages, and the way that this is influenced by exposure of the cells to oestrogen, was investigated using the human monocyte cell line THP-1 and chylomicron-remnant-like particles containing human apolipoprotein E (CRLPs). Synthesis of the lipids was measured by the incorporation of [3H]oleate into cholesteryl ester and triacylglycerol. CRLPs (5–40 µg of cholesterol/ml) containing either trilinolein or triolein as the triacylglycerol component caused a dose-dependent decrease in cholesteryl ester formation, while triacylglycerol production was unchanged. After oxidation of the CRLPs, the level of thiobarbituric acid-reactive substances was increased by 6.3-fold and 2.2-fold in particles containing trilinolein and triolein respectively. Furthermore, CRLPs containing oxidized trilinolein lost their ability to down-regulate cholesterol esterification, while CRLPs containing oxidized triolein did not. Both types of oxidized CRLPs decreased triacylglycerol synthesis. Treatment of the macrophages with 17β-oestradiol caused increases of approx. 94% and 34% in the synthesis of cholesteryl ester and triacylglycerol respectively in the absence of CRLPs. The differences between control and oestrogen-treated cells were abolished, however, when CRLPs (40 µg of cholesterol/ml) were added to the incubations. In addition, in contrast with their lack of effect in control cells, CRLPs containing oxidized trilinolein decreased cholesterol esterification in oestrogen-treated cells by approx. 48%. These findings with CRLPs suggest that chylomicron remnants have significant effects on cholesteryl ester and triacylglycerol synthesis in macrophages, which may be modulated both by the oxidation state of the particles and by oestrogen.

INTRODUCTION

Arterial lesions in atherosclerosis begin with the appearance of fatty streaks, which are initiated after the invasion of the blood vessel wall by monocyte-macrophages. These cells scavenge lipoproteins in the sub-endothelial space and store the lipid intracellularly, eventually accumulating so much that they become foam cells [1]. The lipid in foam cells is often in the form of cholesteryl ester, but in some circumstances triacyl-
glycerol may also accumulate [2], and a recent report has suggested that, in addition to cholesterol esterification, triacylglycerol synthesis may also be important in the formation of macrophage-derived foam cells [3]. Low-density lipoprotein (LDL) is known to be involved in foam cell formation, and its role in the process has been studied extensively [4,5]. In recent years, however, it has become clear that lipoproteins of dietary origin are also atherogenic [6], and there is increasing interest in their effects on cells in the artery wall.

Fat and cholesterol from the diet are carried by chylomicron remnants to the liver for processing [7], and these triacylglycerol-enriched lipoproteins have been shown to be taken up by the aorta [6,8–10] as efficiently as LDL [6,11]. Furthermore, triacylglycerol-rich lipoproteins have been isolated from human aortic intima and atherosclerotic plaque [12,13]. It is clear from many studies that LDL must be modified by oxidation before it is capable of causing the accumulation of cholesteryl ester in macrophages and the formation of foam cells [5]. In contrast, triacylglycerol-rich lipoproteins such as very-low-density lipoprotein (VLDL) have been shown to induce foam cell formation without prior modification, although in this case the lipid accumulated is triacylglycerol, rather than cholesteryl ester [2,14]. Studies by Yu and Mamo [15] have demonstrated that chylomicron remnants can also induce foam cell formation in human monocyte-derived macrophages without prior modification, although the type of lipid accumulated was not reported in that study.

Little information is available about the effects of chylomicron remnants on cholesteryl ester or triacylglycerol synthesis in macrophages. Triacylglycerol-rich lipoproteins from patients with hypertriglyceridaemia, which consist of a mixture of chylomicrons, chylomicron remnants and VLDL, have been found to increase cholesterol esterification in human monocyte-derived macrophages [16], and chylomicron remnants have been reported to have a similar effect in primary rabbit macrophages [17]. The effects of oxidation of the particles on macrophage lipid metabolism, however, have not been investigated previously.

Evidence from epidemiological, experimental and clinical studies indicates that oestrogen has important anti-atherogenic effects [18,19]. The hormone has a beneficial effect on plasma lipids, but as this only accounts for part of its protective action against cardiovascular disease [18–20], it is thought that oestrogen also plays a direct role in the events in the artery wall that lead to lesion development, and macrophage foam cell formation has been identified as an important site of oestrogen action [19]. Oestrogen has been found to prevent the accumulation of lipid in atherosclerotic lesions [21] and to reduce lipid accumulation after treatment with acetylated LDL in human monocyte-derived and mouse peritoneal macrophages [19,22,23] and in macrophages derived from the human cell line THP-1 [23]. In addition, the increase in cholesterol esterification induced by acetylated LDL in human monocyte-derived macrophages has been reported to be reduced by 17β-oestradiol [23].

The human monocyte cell line THP-1 can be induced to differentiate into macrophage-like cells by treatment with a phorbol ester [24]. It has the advantage of providing a homogeneous population of cells and has been widely used as an experimental model for foam cell formation [24–26]. In the present study, we have used THP-1 cells to compare the effects of native and oxidized chylomicron remnants on cholesteryl ester and triacylglycerol synthesis in macrophages, and to investigate how these processes are influenced by prior exposure of the cells to oestrogen. As it is difficult to separate chylomicron remnants from chylomicrons and VLDL in human blood, artificial chylomicron-like lipid particles containing human apolipoprotein E (apoE) (CRLPs), with a size, density and lipid composition similar to that of physiological remnants, were used [27].

Previous experiments in our laboratory have shown that the fatty acid composition of chylomicron remnants differs, depending on the type of dietary fat from which they were derived [28], and that these differences influence the metabolism of the particles in vivo [29,30]. Furthermore, it is known that polyunsaturated fatty acids in lipoproteins are more susceptible to oxidation than are mono-unsaturated or saturated fatty acids [31]. It is possible, therefore, that the effect of remnants on macrophage lipid metabolism, and the way that this is altered by their exposure to oxidizing conditions, may be influenced by the type of fatty acid predominating in the particles. In order to investigate this possibility, two different types of CRLPs were used for this study, prepared from either triolein (containing oleic acid; monounsaturated; C18:1) or trilinolein (containing linoleic acid; n-6 polyunsaturated; C18:2).

**MATERIALS AND METHODS**

**Materials**

RPMI 1640 medium (with or without Phenol Red), fetal bovine serum, glutamine (Glutamax), penicillin/streptomycin and β-mercaptoethanol were obtained from Gibco (Paisley, Scotland, U.K.). Fetal bovine serum was heat-inactivated by incubation at 56 °C, and any steroids were removed by incubation with activated charcoal as described previously [32]. Trypan Blue (0.4 % solution), PMA, fatty-acid-free BSA, 1,1,3,3-tetraethoxypropane and 17β-oestradiol were supplied by Sigma (Poole, Dorset, U.K.), and [9,10(n)-3H]oleate and cholesteryl [1-14C]oleate were purchased from Amersham International.
Preparation and oxidation of CRLPs

CRLPs were prepared by a method based on that described by Diard et al. [27]. A lipid mixture containing (by wt.) 70% trilinolein or trilinolein, 2% cholesterol, 3% cholesteryl ester and 25% phospholipids (comprising 72.5% phosphatidylcholine, 11% phosphatidylethanolamine, 6.9% lysophosphatidylcholine, 6.5% sphingomyelin, 2.6% phosphatidylinositol and 2.6% phosphatidylserine) was sonicated at 22–24 μm in 0.9% NaCl in Tricine buffer (20 mM, pH 7.4) (4.25 ml/50 mg of lipid) for 20 min at 56 °C. The density of the resulting emulsion was increased to 1.21 g/ml with KBr, layered under a stepwise density gradient as described previously [33] and centrifuged at 17000 g in a swing-out rotor (SW-40) for 20 min at 20 °C. The upper layer was discarded and replaced by an equal volume of 0.9% NaCl (density 1.006 g/ml), the centrifugation was repeated at 70000 g for 1 h at 20 °C, and CRLPs (without apoE) were harvested from the top layer.

Human apoE was bound to the CRLPs by incubation with human plasma. The plasma was dialysed extensively against 0.9% NaCl, then centrifuged in a fixed-angle rotor at 85000 g for 16 h at 4 °C. The top layer containing lipoproteins of density < 1.006 g/ml (chylomicrons, chylomicron remnants and VLDL) was discarded, and the bottom layer containing apoE was dialysed against 0.9% NaCl for 18 h at 4 °C. Aliquots were then frozen and stored at −20 °C until required. This procedure allowed a single batch of plasma to be used to prepare all the CRLPs for this study. CRLPs (without apoE) were added to the dialysed plasma in a ratio of 1:2.5 (v/v) and incubated at 37 °C with shaking for 18 h. After this time, the mixture was layered under 0.9% NaCl (density 1.006 g/ml) and centrifuged at 45000 g for 18 h at 12 °C. CRLPs containing apoE were harvested from the top 1.0–1.5 cm of the tubes and stored under nitrogen until required (all preparations were used within 1 week). Analysis of the lipid composition of the particles before and after the incubation with plasma showed that the triacylglycerol/total cholesterol ratio was not changed by the procedure. In addition, we have found that CRLPs prepared in this way contain apoE and no other apolipoproteins, and are of a size consistent with that reported for chylomicron remnants in vivo [34].

CRLPs were oxidized by incubation with CuSO₄ (10 mM) for 18 h at 37 °C followed by dialysis, as described previously [10]. The extent of oxidation was determined by measuring the level of thiobarbituric acid-reactive substances (TBARS) in the preparations before dialysis by the method of Steinbrecher et al. [35]. Tetraethoxypropane, which is converted into malondialdehyde in the assay, was used as standard.

Culture of THP-1 cells and determination of lipid synthesis

THP-1 monocytes in RPMI 1640 culture medium containing 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin and 50 mM β-mercaptoethanol were maintained in suspension in 25 cm² filter top flasks at a density of between 3 × 10⁴ and 9 × 10⁴ cells/ml until needed for the experiments. Cell viability was determined by Trypan Blue exclusion, and was > 95% in all experiments.

The cells were induced to differentiate into macrophages by incubation in RPMI 1640 medium without Phenol Red and supplemented as above, except that steroid-free fetal bovine serum was used (culture medium) with PMA (200 ng/ml; added in 1 μl/ml ethanol) in 24-well plates (5 × 10⁵ cells/well in 0.5 ml) for 72 h at 37 °C in 5% CO₂/95% air. At this time, differentiated cells adhered to the plates, and any remaining monocytes and the medium containing PMA were removed. The cells were washed with pre-warmed PBS (2 × 1 ml), culture medium (0.5 ml/well) containing 17β-oestradiol (10 μM; added in 2 μl/ml ethanol) or an equal volume of ethanol only was added, and the plates were incubated for a further 72 h. Duplicate wells were prepared for each treatment. The medium was then removed, the cells were washed with pre-warmed PBS (2 × 1 ml) and the formation of cholesteryl ester, triacylglycerol and phospholipid in the presence or absence of CRLPs was measured by the incorporation of [³H]oleate into the three lipid classes. Culture medium (0.5 ml/well) containing 0–40 μg of CRLP or oxidized CRLP cholesterol/ml, [³H]oleate (100 nmol; 74 kBq/well) and 2% (w/v) fatty acid-free BSA was added, and the cells were incubated for 4 h at 37 °C in 5% CO₂/95% air. The morphology of the cells, as judged by light microscopy, was unaffected by treatment with CRLPs or oxidized CRLPs at the end of the incubation period. The medium was then removed, the cells were washed with PBS (3 × 1 ml) and the lipids were extracted with hexane/isopropanol alcohol (3:2, v/v) in the presence of cholesteryl [1-¹⁴C]oleate (50 Bq) as an internal standard. The lipid classes were separated by TLC on silica gel using hexane/diethyl ether/formic acid (80:20:2, by vol.) and the bands corresponding to cholesteryl ester and triacylglycerol (identified using appropriate standards) were scraped into scintillation vials and assayed for radioactivity by liquid scintillation counting. After lipid extraction, the cells were solubilized in 1 M NaOH for protein determination.
Analytical methods
Total cholesterol and triacylglycerol were measured enzymically using commercially available kits (Boehringer Mannheim, Mannheim, Germany) and protein was determined by the method of Lowry et al. [36]. Significance limits were calculated using Student’s t test (paired).

RESULTS
Lipid composition and extent of oxidation of CRLPs
All CRLP preparations had a similar triacylglycerol/total cholesterol ratio [5.4 ± 0.5; n = 12], which was comparable with the value of 5.6:1 found for rat chylomicron remnants prepared in vivo by the method described by Lambert et al. [28]. There were no significant differences between the ratios found for native and oxidized CRLPs, or CRLPs containing either triolein or trilinolein.

Oxidation of the CRLPs was measured by the levels of TBARS present after incubation with CuSO₄. TBARS levels were significantly higher in CuSO₄-treated than in untreated particles [nmol of malondialdehyde/μmol of triacylglycerol (means ± S.E.M.; n = 3): trilinolein CRLPs, untreated 6.7 ± 1.3, CuSO₄-treated 42.1 ± 12.1; triolein CRLPs, untreated 4.0 ± 0.6, CuSO₄-treated 8.9 ± 1.3]. As expected, the extent of the increase was greater in CRLPs containing trilinolein (6.3-fold) than in CRLPs containing triolein (2.2-fold).

Effects of CRLPs on cholesteryl ester and triacylglycerol synthesis in control and oestrogen-treated THP-1 macrophages
The addition of 17β-oestradiol to THP-1 macrophages in the absence of CRLPs led to significant increases in the incorporation of [3H]oleate into both cholesteryl ester and triacylglycerol (approx. 1.9- and 1.3-fold respectively) (Table 1).

Table 1 Effects of oestrogen on the synthesis of cholesteryl ester and triacylglycerol in THP-1 cells
THP-1 macrophages were incubated with 17β-oestradiol (10 μM) for 72 h, and the incorporation of [3H]oleate into cellular cholesteryl ester and triacylglycerol during 4 h of incubation was then determined. Data shown are means ± S.E.M. from three separate experiments. Significance: *P < 0.05, **P < 0.01 compared with control cells.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Control</th>
<th>+ Oestrogen</th>
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<tbody>
<tr>
<td>Cholesteryl ester (pmol·h⁻¹·mg⁻¹ cell protein)</td>
<td>51.3 ± 5.7</td>
<td>98.1 ± 5.8**</td>
</tr>
<tr>
<td>Cholesteryl ester (% of control value)</td>
<td>100</td>
<td>1942 ± 17.7</td>
</tr>
<tr>
<td>Triacylglycerol (nmol·h⁻¹·mg⁻¹ cell protein)</td>
<td>8.7 ± 3.2</td>
<td>10.9 ± 2.8*</td>
</tr>
<tr>
<td>Triacylglycerol (% of control value)</td>
<td>100</td>
<td>1343 ± 12.7</td>
</tr>
</tbody>
</table>

Figure 1 Effects of native trilinolein CRLPs on lipid metabolism in THP-1 macrophages
Control (●) or oestrogen-treated (▲) THP-1 macrophages were incubated in the presence or absence of CRLPs containing trilinolein for 4 h, and the incorporation of [3H]oleate into (A) cholesteryl ester and (B) triacylglycerol was determined. Each point represents the mean from three separate experiments; error bars indicate S.E.M. Mean values corrected for the contribution of CRLP triacylglycerol to the fatty acid substrate pool for triacylglycerol and cholesteryl ester synthesis are also shown: ○, control cells; ▲, oestrogen-treated cells.

The effects of trilinolein CRLPs on the synthesis of cholesteryl ester and triacylglycerol in THP-1 macrophages, and the changes caused by prior treatment of the cells with oestrogen, are shown in Figure 1. In untreated cells, trilinolein CRLPs caused a dose-dependent decrease in cholesteryl ester formation, reaching a maximum of approx. 40% at the highest cholesterol concentration used (40 μg/ml) (Figure 1A; Table 2), but the formation of triacylglycerol was not affected significantly (Figure 1B; Table 2). In order to investigate the possibility that the decrease observed in the incorporation of [3H]oleate into cholesteryl ester was caused by dilution of the label in the substrate pool by fatty acids originating from CRLP triacylglycerol, double-label experiments were carried out using [14C]oleate and CRLPs labelled in triacylglycerol with [3H]oleate. The results from three separate experiments, shown in Table 3, indicated that there was a significant contribution from CRLP triacylglycerol to the substrate pool (24.3 ± 3.1% of the...
control or oestrogen-treated THP-1 macrophages were incubated in the presence or absence of native or oxidized CRLPs (40 μg/ml cholesterol) for 4 h, and the incorporation of \(^{3}\text{H}\)oleate into cellular cholesteryl ester (CE) and triacylglycerol (TG) was determined. Data are expressed as a percentage of the value found in the absence of chylomicron remnants in each incubation type, and are means ± S.E.M. from three separate experiments. Significance of differences: *P < 0.05 compared with control cells; †P < 0.05 compared with native CRLPs in cells with corresponding treatment.

<table>
<thead>
<tr>
<th>CRLP type</th>
<th>Lipid</th>
<th>Native CRLPs</th>
<th>Oestrogen-treated cells</th>
<th>Oxidized CRLPs</th>
<th>Oestrogen-treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control cells</td>
<td>[%]</td>
<td>Concentration</td>
<td>[%]</td>
</tr>
<tr>
<td>Trilinolein</td>
<td>CE</td>
<td>60.9 ± 11.3</td>
<td>35.4 ± 6.2*</td>
<td>118.5 ± 13.8†</td>
<td>52.5 ± 3.9*</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>81.9 ± 28.1</td>
<td>64.3 ± 22.1</td>
<td>48.3 ± 21.7</td>
<td>46.0 ± 10.0</td>
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<tr>
<td>Triolein</td>
<td>CE</td>
<td>52.1 ± 14.6</td>
<td>48.7 ± 2.3</td>
<td>51.8 ± 6.2</td>
<td>31.0 ± 10.6</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>93.9 ± 22.1</td>
<td>74.9 ± 26.1</td>
<td>22.7 ± 4.6†</td>
<td>19.5 ± 3.2†</td>
</tr>
</tbody>
</table>

**Table 3** Contribution of CRLP triacylglycerol to the fatty acid substrate pool for triacylglycerol and cholesteryl ester synthesis in THP-1 cells

THP-1 macrophages were incubated in the presence of \(^{14}\text{C}\)oleate and native or oxidized CRLPs (5–40 μg of cholesterol/ml) containing trilinolein or triolein labelled in triacylglycerol with \(^{3}\text{H}\)oleate, and the incorporation of \(^{3}\text{H}\) and \(^{14}\text{C}\) into cellular triacylglycerol and cholesteryl ester was determined. Data are expressed as the percentage contribution of the \(^{3}\text{H}\) label to the substrate pool for cholesteryl ester and triacylglycerol synthesis, and are means ± S.E.M. from three separate experiments.

<table>
<thead>
<tr>
<th>CRLP type</th>
<th>Concentration (μg/ml)</th>
<th>Cholesteryl ester</th>
<th>Triacylglycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Native CRLPs</td>
<td>Oxidized CRLPs</td>
</tr>
<tr>
<td>Trilinolein</td>
<td>5</td>
<td>16.5 ± 2.4</td>
<td>17.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>21.9 ± 3.3</td>
<td>23.6 ± 4.6</td>
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<tr>
<td></td>
<td>40</td>
<td>24.3 ± 3.1</td>
<td>27.8 ± 4.4</td>
</tr>
<tr>
<td>Triolein</td>
<td>5</td>
<td>14.5 ± 2.2</td>
<td>16.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>27.3 ± 1.4</td>
<td>27.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>30.1 ± 3.5</td>
<td>32.8 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.1 ± 3.7</td>
<td>16.3 ± 4.3</td>
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<td></td>
<td></td>
<td>29.5 ± 1.6</td>
<td>29.7 ± 4.2</td>
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<td></td>
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<td>31.0 ± 4.0</td>
<td>32.9 ± 4.8</td>
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<td></td>
<td></td>
<td>17.5 ± 21.7</td>
<td>17.9 ± 1.3</td>
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<td></td>
<td></td>
<td>32.5 ± 2.3</td>
<td>31.6 ± 2.3</td>
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<tr>
<td></td>
<td></td>
<td>35.2 ± 5.4</td>
<td>35.9 ± 2.7</td>
</tr>
</tbody>
</table>

Cholesteryl ester formed was derived from \(^{3}\text{H}\)oleate at a concentration of 40 μg of CRLP cholesterol/ml, although this was not sufficient to account for the total decrease (40%) in cholesterol esterification seen (Figure 1A). Furthermore, there were no significant differences in the relative incorporation of \(^{3}\text{H}\) into cholesteryl ester in cells treated with native or oxidized particles, or triolein or trilinolein CRLPs (Table 3). The contributions of \(^{14}\text{C}\)oleate and \(^{3}\text{H}\)oleate to triacylglycerol synthesis in these experiments showed a similar pattern (Table 3).

After oestrogen treatment, cholesterol esterification was decreased to a significantly greater extent than in control cells (maximum approx. 65%) (Table 2). As a consequence there was no significant difference in the absolute values (control, 32.0 ± 8.2 pmol of \(^{3}\text{H}\)oleate incorporated·h\(^{-1}\)·mg\(^{-1}\) cell protein; oestrogen-treated, 34.3 ± 5.3 pmol·h\(^{-1}\)·mg\(^{-1}\)) found at the highest concentration of CRLP cholesterol used. The increase in triacylglycerol synthesis caused by oestrogen (Table 1) was also abolished by trilinolein CRLPs (values at 40 μg of cholesterol/ml: control, 6.4 ± 1.0 pmol·h\(^{-1}\)·mg\(^{-1}\) cell protein; oestrogen-treated, 6.1 ± 1.7 pmol·h\(^{-1}\)·mg\(^{-1}\)) (Figure 1B). Triolein CRLPs had effects similar to those of the trilinolein particles on the incorporation of \(^{3}\text{H}\)oleate into the two lipid classes (Figure 2; Table 2). When the mean values obtained with trilinolein and triolein CRLPs were corrected for the dilution of the radiolabel in the substrate pool using the data shown in Table 3, the pattern of the changes observed in cholesteryl ester and triacylglycerol synthesis in control and oestrogen-treated cells remained similar (Figures 1 and 2).
Effects of native triolein CRLPs on lipid metabolism in THP-1 macrophages

Control (●) or oestrogen-treated (▲) THP-1 macrophages were incubated in the presence or absence of CRLPs containing triolein for 4 h, and the incorporation of [3H]oleate into (A) cholesteryl ester and (B) triacylglycerol was determined. Each point represents the mean from three separate experiments; error bars indicate S.E.M. Mean values corrected for the contribution of CRLP triacylglycerol to the fatty acid substrate pool for triacylglycerol and cholesteryl ester synthesis are also shown: ○, control cells; ▲, oestrogen-treated cells.

Effects of oxidized CRLPs on lipid synthesis in control and oestrogen-treated THP-1 macrophages

Figures 3 and 4 show the effects of oxidized trilinolein and triolein CRLPs respectively on lipid synthesis in control and oestrogen-treated THP-1 macrophages. In untreated cells, in contrast with the decrease observed with native trilinolein CRLPs (Figure 1A), cholesterol ester synthesis was unaffected by the particles after oxidation (Figure 3A). Thus the percentage changes in the presence of 40 μg/ml cholesterol as compared with 0 μg/ml CRLP were +18% and −40% with oxidized and native CRLPs respectively, and this difference was statistically significant (Table 2). After oestrogen treatment, however, the oxidized particles caused a decrease in cholesterol esterification that was not significantly different from that seen with native CRLPs (Figures 1A and 3A; Table 2). The effects of oxidized CRLPs on triacylglycerol formation in the treated and untreated cells differed from those of native particles, in that a decrease of 52–60% (rather than no change) was observed (Figures 1B and 3B; Table 2).

Unlike oxidized trilinolein CRLPs, the oxidized triolein particles had effects on cholesterol esterification similar to those observed with native triolein CRLPs, causing a decrease compared with untreated macrophages (Figure 4A; Table 2), and stronger inhibition in oestrogen-treated cells, so that the values found with 40 μg/ml CRLP cholesterol were similar in the two cell types (untreated, 30.9±6.2 pmol of [3H]oleate incorporated h⁻¹ mg⁻¹ cell protein; oestrogen-treated, 31.8±11.9 pmol h⁻¹ mg⁻¹). Triacylglycerol synthesis, on the other hand, was decreased by oxidized triolein CRLPs, both in untreated cells and in those exposed to oestrogen (Figure 4B; Table 2).

As found with native CRLPs, after correction of the mean values from experiments with both types of CRLPs, the pattern of changes in cholesteryl ester and triacylglycerol formation in control and oestrogen-treated cells was not changed (Figures 3 and 4).
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Figure 4  Effects of oxidized triolein CRLPs on lipid metabolism in THP-1 macrophages

Control (●) or oestrogen-treated (▲) THP-1 macrophages were incubated in the presence or absence of oxidized CRLPs containing triolein for 4 h, and the incorporation of [3H]oleate into (A) cholesteryl ester and (B) triacylglycerol was determined. Each point represents the mean from three separate experiments; error bars indicate S.E.M. Mean values corrected for the contribution of triacylglycerol from oxidized CRLPs to the fatty acid substrate pool for triacylglycerol and cholesteryl ester synthesis are also shown: ○, control cells; ▲, oestrogen-treated cells.

DISCUSSION

The CRLPs used in the present study had a triacylglycerol/cholesterol ratio of 5.4:1, and we have shown previously that particles prepared by the methods used have a mean diameter of approx. 57 nm [34], which is comparable with that found for chylomicron remnants from in vivo sources [7]. Thus, in terms of their size, density and lipid composition, the CRLPs resemble native chylomicron remnants, and in addition they contain human apoE. Furthermore, similar particles containing apoE from the relevant species have been found to have effects which mimic those of physiological remnants in rat hepatocytes and in pig endothelial cells [27,34,37,38]. We conclude, therefore, that the CRLPs are suitable for the study of the influence of chylomicron remnants on lipid metabolism in THP-1 macrophages in the absence of other lipoproteins such as chylomicrons and VLDL, which may be present in remnants obtained from in vivo sources.

It is known that oxidation of LDL greatly enhances its atherogenic properties [5], and dietary fatty acid composition has been shown to influence the rate and extent of this process [39]. Dietary mono-unsaturated fatty acids, such as oleic acid, have a protective effect [40,41], while polyunsaturated fatty acids, such as linoleic acid, have been found to increase LDL oxidation [42,43]. As the function of chylomicron remnants is to carry lipid of dietary origin to the liver for processing [7], the fatty acid composition of these particles is particularly responsive to that of the diet [28], and this may be important for the effects of chylomicron remnants in promoting atherosclerosis. For this reason, the effects of CRLPs containing oleic acid and linoleic acid (in the triacylglycerols triolein and trilinolein respectively) on cholesteryl ester and triacylglycerol synthesis in THP-1 macrophages were compared in the present study.

As cholesteryl ester and triacylglycerol synthesis was determined by measuring the incorporation of [3H]oleate into the two lipid classes, it is possible that the uptake of triacylglycerol from the CRLPs may affect the results by contributing fatty acid to the intracellular substrate pools. Double-label experiments using [14C]oleate and CRLPs labelled with [3H]oleate in triacylglycerol showed that fatty acid from CRLPs contributed approx. 25–35% of the total cholesteryl ester and triacylglycerol synthesized by the cells in the presence of the highest concentration of CRLP cholesterol used (40 μg/ml) (Table 3). Importantly, however, these proportions did not change significantly when CRLPs containing trilinolein or triolein were used, or after oxidation of the particles. Thus our results will underestimate the synthesis of cholesteryl ester and triacylglycerol in the presence of CRLPs by 25–35%. Correction of the values obtained for this factor, however, showed that the general pattern of changes observed was not affected (Figures 1–4). The results of the double-label experiments, therefore, show that any differences observed between native and oxidized CRLPs or between trilinolein and triolein CRLPs are not due to differential dilution of the fatty acid substrate pools.

Our findings indicate that, without prior exposure to oxidizing conditions, triolein and trilinolein CRLPs had similar effects on lipid synthesis in THP-1 macrophages, decreasing the incorporation of [3H]oleate into cholesteryl ester, but having little effect on incorporation into triacylglycerol (Figures 1 and 2). The contribution of fatty acid from CRLPs to the fatty acid substrate pool for cholesteryl ester synthesis (maximally approx. 24–28%) accounts for some, but not all, of the decrease (40–48%) in the formation of cholesterol [3H]oleate. A recent report suggested that chylomicron remnants cause cytotoxicity in human monocyte-derived macrophages [15]; however, a level of 150 μg of remnant cholesterol/ml was required to produce a significant decrease in cell viability at time points of less than 10 h. Moreover, we did not
detect any differences in the morphology of cells treated with CRLPs as compared with that of control cells. In addition, in another study we have found that necrosis of THP-1 macrophages, assessed by the staining of DNA with propidium iodide in a flow cytometry assay, was also not significantly affected by incubation with CRLPs for 5 h at a concentration of 30 \( \mu \text{g of cholesterol/ml} \) (K. Batt and K. M. Botham, unpublished work). The changes in cholesteryl ester formation observed in our experiments, therefore, are unlikely to be due to remnant toxicity. Thus our results indicate that cholesterol esterification in THP-1 cells tends to be lowered rather than raised in response to CRLPs. These findings are in agreement with those of Ellsworth et al. \([44]\), who found that chylomicron remnants decreased the incorporation of radiolabelled olate into cholesteryl ester in the murine macrophage cell line J774. On the other hand, using a similar assay, Yu and Mamo \([17]\) reported that chylomicron remnants increased cholesteryl esterification in primary rabbit macrophages. The effects of dilution of the substrate pool by fatty acid from triacylglycerol from the remnant particles, however, was not quantified in either of these studies. Although the decrease in cholesteryl esterification on incubation of macrophages with CRLPs observed in our experiments was relatively modest, it contrasts clearly with the effects of CRLPs observed in our experiments was relatively modest. In our experiments, there was a clear increase in cholesteryl ester synthesis found on incubation of human monocyte-derived macrophages with acetylated LDL \([23]\). The effects of the hormone on lipid synthesis in macrophages in the absence of exogenous lipoprotein, however, have not been reported previously, as far as we are aware. In our experiments, there was a clear increase in cholesteryl esterification, and a smaller, but significant, increase in triacylglycerol synthesis when THP-1 macrophages were treated with oestrogen (Table 1). These findings are consistent with two earlier reports of increased rates of cholesterol esterification induced by oestrogen in human blood peripheral mononuclear cells \([48]\) and human aortic smooth muscle cells \([49]\). Our further experiments also demonstrated that, on incubation of the cells with either triolein or trilinolein CRLPs, the differences in cholesterol esterification and triacylglycerol formation between oestrogen-treated and untreated THP-1 macrophages were abolished (Figures 1 and 2; Table 2). After exposure of the cells to oestrogen, therefore, the down-regulatory effect of CRLPs on cholesterol esterification is enhanced.

The increase in cholesterol esterification found in oestrogen-treated THP-1 macrophages in our experiments seems to conflict with previous work indicating that the hormone reduces foam cell formation \([19,21–23,47]\). It has been reported recently, however, that pharmacological inhibition of cholesterol esterification in macrophages increases, rather than decreases, atherosclerotic plaque formation in experimental animals \([50]\). Cholesteryl ester accumulation in cells depends on the rates of cholesteryl ester hydrolysis and cholesterol efflux, in addition to the rate of cholesteryl ester synthesis. In a recent study with human monocyte-derived macrophages, we found that oestrogen treatment increased the activities of the enzymes regulating both cholesterol esterification and hydrolysis, as well as the rate of efflux of cholesterol from the cells in the presence of high-density lipoprotein (M. Napolitano and E. Bravo, unpublished work). In addition, Tomita et al. \([47]\) have also reported activation of macrophage neutral cholesteryl ester hydrolase by oestrogen. These findings suggest that the up-regulation of cholesterol esterification by oestrogen may be associated with increased flux of cholesterol through the cholesteryl ester cycle, and its increased removal from the cells. In this context, therefore, the effects of CRLPs in decreasing cholesteryl ester synthesis to the level found in control cells may oppose the protective effect of oestrogen.

As would be expected from the known chemical properties of mono-unsaturated and \(n-6\) polyunsaturated fatty acids \([39]\), exposure of triolein and trilinolein CRLPs to identical oxidizing conditions caused the particles to become oxidized to different extents, so that the level of TBARS was increased by 2.2- and 6.3-fold respectively. Furthermore, the two types of oxidized CRLPs had differential effects on cholesterol esterification in THP-1 macrophages: the inhibitory effect of the trilinolein particles on cholesterol esterification was abolished, while that of the triolein particles was retained (Figures 3A and 4A; Table 2). Thus the rate of cholesteryl ester formation was higher in macrophages exposed to oxidized CRLPs containing linoleic acid as compared with that in macrophages exposed to native particles or to oxidized particles containing oleic acid, suggesting that the loss of the regulatory effect of CRLPs on cholesterol esterification is related to the greater degree of oxidation of the particles. Cells treated with oxidized trilinolein CRLPs had a similar morphology to control cells under light microscopy. In addition, in other work using a flow cytometry assay after staining of DNA with propidium iodide, we found that, as with native CRLPs, a concentration of 30 \(\mu\text{g of oxidized CRLP}}
cholesterol/ml did not increase necrosis of the cells compared with that in controls after 5 h of incubation (K. Batt and K. M. Botham, unpublished work). The mechanism of uptake of chylomicron remnants by macrophages is not clear, but phagocytosis [51], the LDL receptor [52], the LDL-receptor-related protein (LRP) [53] and a novel receptor-recognizing apoB48 [54] have all been implicated, although scavenger receptors do not appear to be involved [51]. The CRLPs used in the present experiments did not contain apoB48. However, we have found that both native and oxidized trilinolein CRLPs cause extensive lipid accumulation in THP-1 macrophages after 48 h of incubation (K. Batt and K. M. Botham, unpublished work), suggesting that the lack of apoB48 does not prevent lipids from the particles entering the cells, and that oxidation of the CRLPs does not inhibit their uptake. In addition, triacylglycerol synthesis was decreased by oxidized CRLPs in the experiments reported here. Thus the difference in the rate of cholesterol esterification in macrophages exposed to native and oxidized trilinolein CRLPs is unlikely to be caused either by differences in their cytotoxicity or by lack of uptake of the oxidized particles.

Oxidative modification of lipoproteins is known to alter the apolipoprotein as well as the lipid content of lipoproteins [5,55]. It is possible, therefore, that the behavior of oxidized CRLPs might be altered if apoB48 were present. In another recent study, however, we investigated the effects of native and oxidized chylomicron remnants derived from corn oil (rich in linoleic acid) prepared in rats in vivo (i.e. containing apoB48) on cholesteryl ester synthesis in a mouse liver cell line [56], and the results were similar to those found here with trilinolein CRLPs and THP-1 cells, in that oxidation of remnants abolished the decrease observed with native particles. Furthermore, we have also demonstrated that oxidized rat chylomicron remnants inhibit vasorelaxation by interfering with NO production [10,57], and that equivalent effects are brought about in pig coronary arteries by oxidized CRLPs containing pig apoE [34,58].

Oxidized CRLPs, therefore, appear in many respects to behave in a similar way to chylomicron remnants containing apoB48.

As oestrogen is known to modulate the effects of oxidized or chemically modified LDL on macrophage lipid metabolism [19,22,23], we investigated the question of whether it also alters the action of oxidized CRLPs. The effects of oxidized CRLPs on triacylglycerol formation were unaffected by prior exposure of the cells to oestrogen (Figures 3 and 4). However, oxidized CRLPs containing trilinolein caused a decrease in cholesterol esterification of approx. 50% in oestrogen-treated cells, in contrast with their lack of effect in untreated macrophages (Figure 3A; Table 2). These results indicate that oestrogen modifies the influence of oxidized CRLPs on cholesteryl ester synthesis, restoring their ability to down-regulate the process. It is possible that this effect may be related to the antioxidant activity of oestrogen, which is believed to be one of the mechanisms responsible for its anti-atherogenicity [59].

In summary, the results reported here demonstrate that CRLPs influence lipid metabolism and, in particular, lower the rate of cholesterol esterification in THP-1 macrophages. Moreover, the effects are modified by oxidation of the particles and the type of fatty acid that they contain, as well as by exposure of the cells to oestrogen. In the presence of CRLPs, cholesterol esterification is reduced to a greater extent in oestrogen-treated than in control cells. Furthermore, after exposure to identical oxidizing conditions, CRLPs containing the \( n - 6 \) polyunsaturated fatty acid, linoleic acid, lose their ability to down-regulate cholesterol esterification in cells not exposed to oestrogen, while those containing the mono-unsaturated fatty acid, oleic acid, do not; this may be due to the greater susceptibility to oxidation of polyunsaturated fatty acids. After oestrogen treatment, however, oxidized CRLPs containing linoleic acid regain their ability to inhibit cholesteryl ester formation.

Our findings using CRLPs suggest that chylomicron remnants have significant effects on lipid metabolism in macrophages which interact with those of oestrogen, and may be altered by oxidation of the particles. The oxidation of LDL in vivo is believed to occur mainly after its uptake by the artery wall [5]. As chylomicron remnants are also taken up by the artery [6–11], they are likely to be exposed to the same oxidative processes as LDL. Despite this, little attention has been paid to the role of oxidized chylomicron remnants in atherogenesis, and further investigation is required to establish its importance.

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**REFERENCES**

8 Mamo, J. C., Proctor, S. C. and Smith, D. (1998) Retention of chylomicron remnants by arterial tissue; importance of an efficient clearance mechanism from plasma. Atherosclerosis 141 (Suppl. 1), S63–S69
11 Mamo, J. C. L. and Wheeler, J. R. (1994) Chylomicrons or their remnants penetrate rabbit thoracic aorta as efficiently as do smaller macromolecules, including low-density lipoprotein, high-density lipoprotein and albumin. Coron. Artery Dis. 5, 695–705

Ellsworth, J. L., Cooper, A. D. and Kraemer, F. B. (1986) Evidence that chylomicron remnants and VLDL are transported by the same receptor pathway in J774 murine macrophage-derived cells. J. Lipid Res. 27, 1062–1072


Fujio, Y., Cooper, A. D. and Fong, L. G. (1998) Multiple processes are involved in the uptake of chylomicron remnants by mouse peritoneal macrophages. J. Lipid Res. 39, 2339–2349


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