Oxidation of chylomicron remnant-like particles inhibits their uptake by THP-1 macrophages by apolipoprotein E-dependent processes

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Abstract

The influence of the oxidative state of chylomicron remnants (CMR) on the mechanisms of their uptake and induction of lipid accumulation by macrophages derived from the human monocyte cell line, THP-1, during foam cell formation was investigated using chylomicron-remnant-like particles (CRLPs) at 3 different levels of oxidation. The oxidative state of CRLPs was varied by exposure to CuSO4 (oxCRLPs) or incorporation of the antioxidant, probucol (pCRLPs) into the particles. oxCRLPs caused significantly less accumulation of triacylglycerol in the macrophages than CRLPs, and their rate of uptake was lower, while pCRLPs caused more lipid accumulation and were taken up faster. Uptake of all 3 types of particles was inhibited to a similar extent when entry via the low density lipoprotein (LDL) receptor related protein (80–90%), LDL receptor (−30–40%), CD36 (−40%) and phagocytosis (−35–40%) was blocked using lactoferrin, excess LDL, anti-CD36 and cytochalasin D, respectively, but blocking scavenger receptors-A or -B1 using poly inosinic acid or excess HDL had no effect. These findings show that oxidation of CRLPs lowers their rate of uptake and induction of lipid accumulation in macrophages. However, oxidation does not change the main pathways of internalisation of CRLPs into THP-1 macrophages, which occur mainly via the LRP with some contribution from the LDLr, while CD36 and phagocytosis have only a minor role, regardless of the oxidative state of the particles. Thus, the effects of CMR oxidation on foam cell formation contrast sharply with those of LDL oxidation and this may be important in the role of dietary oxidized lipids and antioxidants in modulating atherosclerosis.

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Keywords: Chylomicron remnants; Foam cells; Oxidized lipoproteins; Macrophages; Atherosclerosis

1. Introduction

Chylomicron remnants (CMR) carry lipids of dietary origin from the gut to the liver for processing [1] and there is now a large and growing body of evidence indicating that these lipoproteins are strongly atherogenic. They have been shown to be taken up into the artery wall as efficiently as LDL [2–5]; remnant-like particles containing apolipoprotein E (apoE) have been isolated from human aortic intima and atherosclerotic plaque [6,7]; and delayed clearance of CMR from the circulation correlates with lesion development [8,9]. Moreover, we and others have shown that CMR induce extensive lipid accumulation causing foam cell formation in human monocyte-derived macrophages (HMDM) [10] and in human and murine monocyte/macrophages cell lines [11,12].

Low density lipoprotein (LDL) plays a major role in atherogenesis and in foam cell generation, but oxidation of the lipoprotein particles, a process which can occur within the artery wall, is necessary before extensive lipid accumulation is induced [13]. In striking contrast, CMR do not require prior oxidation to cause macrophages to form foam cells [10–12]. However, our studies have demonstrated that incorporation of lipophilic antioxidants into the particles enhances, rather than inhibits, lipid uptake and accumulation in the cells [14,15], suggesting that the oxidative state of CMRs may play a role in their induction of foam cell formation, but in the opposite way to that of LDL. Oxidized CMR could occur either in the artery wall by the action of the cell-associated lipooxygenase and myeloperoxidase which are believed to oxidize LDL, or in the...
circulation, because dietary oxidised lipids, which are produced when fat is cooked at high temperatures, have been shown to be transported in these lipoproteins [16,17]. Clearly, therefore, it is important for the understanding of the atherogenicity of CMR to establish how their oxidation influences their uptake and induction of foam cell formation and the pathways by which CMR are internalised by the cells.

It has been demonstrated that CMR are taken up by the liver by apolipoprotein E (apoE)-dependent pathways mediated by the LDL receptor (LDLr) and the LDL receptor-like protein (LRP) [1]. The exact mechanisms by which CMR are taken up by macrophages, however, are not yet definitively established, and nothing is known about the effects of oxidation of the particles on the routes by which they are internalised. The LDL receptor (LDLr) appears to play a part [1], but as it is downregulated by the influx of cholesterol into cells, native LDL does not induce foam cell formation [16], and our studies [18] have suggested that the delivery of cholesterol to macrophages by CMR has a similar effect. Thus, other mechanisms are also likely to be involved, and evidence from experiments using antibodies to the LDLr and animals lacking the LDLr supports this view [10]. Candidates include the LRP [19], the apoB48 triacylglycerol-rich lipoprotein receptor (apoB48r) [20], an as yet unidentified 43 kDa protein described by Elsegoad et al. [21] and scavenger receptors such as scavenger receptor A (SR-A) and CD36 [1]. Phagocytosis has also been suggested as a possible mechanism [22]. Since the route of uptake of LDL by macrophages is profoundly changed after oxidation from the regulated LDLr to the unregulated scavenger receptors [16], it is important to establish whether the oxidative state also alters the mechanisms of uptake of CMR by the cells.

The aim of this study is to investigate the effects of the oxidative state of CMR on their uptake by macrophages and on the accumulation of lipid within the cells, and to determine how oxidation affects the pathways involved in the internalisation of the particles. Chylomicron remnant-like particles (CRLPs) at three different levels of oxidation (CRLPs, oxidized CRLPs (oxCRLPs) and CRLPs containing the antioxidant probucol (pCRLPs)) and macrophages derived from the human monocyte cell line THP-1 were used as the experimental model, and the mechanisms of uptake were evaluated using specific inhibitors of the processes believed to be involved. The findings clearly demonstrate that oxidation of CRLPs reduces the rate of their uptake by THP-1 macrophages and decreases lipid accumulation in the cells, and further show that this is due to differential interaction with apoE dependent receptors.

2. Materials and methods

RPMI 1640 medium, fetal bovine serum (FBS), L-α-alanyl-L-glutamine (glutamax) penicillin/streptomycin and β-mercaptoethanol were obtained from Gibco (Paisley, UK). FBS was heat inactivated (56 °C, 30 min) before use. Trypan blue, fatty acid-free bovine serum albumin (BSA), phospholipids, cholesterol, cholesteryl oleate, phorbol 12-myristate 13-acetate (PMA), Oil red O, probucol, poly inosinic acid (poly I), lactoferrin and cytochalasin D were supplied by Sigma (Poole, UK). 1′,1′-dioctadecyl-3,3′,3′,3′-tetramethylindocarbocyanide perchlorate (DiI) was from Cambridge Bioscience (Cambridge, UK). The blocking antibody for CD36 was obtained from Immunodiagnostic Systems Ltd (Tyne and Wear, UK) and cholesterol oxidase from Merck Biosciences Ltd (Nottingham, UK). Dil-labelled acetylated LDL (acLDL) was purchased from Molecular Probes (Paisley, UK).

2.1. Preparation of lipoproteins

LDL and high density lipoprotein (HDL) were isolated from human plasma (National Blood Service, London UK) by ultracentrifugation. Plasma was layered under 0.9% NaCl (d=1.006 g/ml) centrifuged for 5 h at 100,000× g (4 °C), and the top fraction discarded. The density of the bottom layer was raised to 1.063 g/ml with KBr, layered under KBr (d=1.063 g/ml) and centrifuged for 16 h at 175,000× g (4 °C). LDL was collected from the top fraction by tube slicing. For the preparation of HDL, the bottom fraction was then adjusted to d=1.21 g/ml with KBr, layered under KBr (d=1.21 g/ml) and centrifuged at 175,000× g for 4 °C for 18 h and the top fraction containing HDL was collected by tube slicing. LDL and HDL were dialysed against saline for 48–72 h prior to used.

CRLPs were prepared by sonication (power setting 22–24 μm, 20 min, at 56 °C) of a lipid mixture containing 70% trilinolein, 2% cholesterol, 3% cholesteryl ester and 25% phospholipids in 0.9% NaCl (w/v) in Tris Buffer (20 mM, pH 7.4) followed by stepwise density gradient (2.5 ml d 1.065 g/ml, 2.5 ml d 1.020 g/ml, 3 ml d 1.006 g/ml) ultracentrifugation as described by Diard et al. [23] at 17,000× g for 20 min at 20 °C. The upper layer of grossly emulsified lipids was then removed and replaced with an equal volume of NaCl solution (d=1.020 g/ml) and tubes were centrifuged at 70,000× g for 1 h (20 °C). For apoE binding, lipid particles collected from the top layer were incubated with the dialysed d=1.063–1.21 g/ml fraction of human plasma (National Blood Transfusion Service, North London Centre, UK) prepared as described above at 37 °C with shaking for 4 h (1 volume of particles: 2 volumes plasma). The CRLPs containing apoE were then isolated by ultracentrifugation at d=1.006 g/ml (120,000× g for 12 h at 4 °C), harvested from the top layer, purified by a second centrifugation at the same density (202,000× g for 4 h at 4 °C) and stored at 4 °C under argon until required. All preparations were used within 1 week. For Dil-labelled CRLPs and pCRLPs, probucol (1 mg) and/or Dil were added to the lipid mixture prior to sonication. CRLPs were oxidized by incubation with CuSO4 (20 μM) with shaking for 5 h at 37 °C and the CuSO4 was then removed by dialysis (0.9% NaCl, 24 h, 4 °C). The oxidation process had no effects on the fluorescent properties of the Dil label.

2.2. Culture of THP-1 cells

THP-1 monocytes were maintained in RPMI 1650 culture medium containing 10% (v/v) FBS, glutamax (2 mM), penicillin/streptomycin (100 U/ml/100 μg/ml) and β-mercaptoethanol (20 μM) (culture medium). The cells were differentiated into macrophages by incubation with PMA (200 ng/ml) for 72 h at 37 °C in 5% CO2-95% air. The medium containing PMA and any non-adherent cells were then removed and the macrophages were washed with PBS (3×1 ml) and incubated with CRLPs or DiI-labelled CRLPs (30 μg cholesterol/ml) in the presence or absence of specific inhibitors as indicated in the text. The inhibitors were added 1 h prior to the addition of the CRLPs. Cell viability as assessed by Trypan blue exclusion was >95% and was not affected by any of the CRLP types or conditions used. After the incubation, the macrophages were washed (culture medium 3×1 ml) and lipid accumulation was assessed by staining with Oil red O, or harvested for lipid analysis [24]. For studies with Dil-labelled CRLPs, the fluorescence associated with the cells was assessed by viewing with a Zeiss LMS 510 laser-scanning confocal microscope and quantified by absorbance volume analysis, or by fluorescence-activated cell analysis (FACS) using a BD FACScalibur flow cytometer (BD Biosciences, Oxford, UK). For FACS analysis, cells were harvested in PBS containing EDTA (5 mM), 0.1% lidocaine-HCl (1 mg/ml), incubated for 20 min at 37 °C and centrifuged at 10,000× g (2 min). The cell pellet was then resuspended and fixed in PBS containing 4% formalin.

2.3. Analytical methods

For mRNA analysis, total RNA was extracted from THP-1 macrophages before or after incubation with CRLPs (30 μg cholesterol/ml) using a kit from Sigma (Poole, UK) and first strand synthesis was carried out using a kit supplied by Promega (Southampton, UK) according to the manufacturer's instructions.
mRNA levels for the LDLr, LRP and the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) were determined by real time polymerase chain reaction using a SYBR green quantitative PCR kit (Sigma, Poole, UK) with an Opticon light cycler under the following conditions; denaturation at 94 °C for 2 min followed by 40 cycles of 94 °C for 15 s, 58 °C for 1 min, with a final extension at 72 °C for 1 min. The primers used and the product sizes are shown in Table 1. The Ct values were determined by automated threshold analysis using Opticon Monitor 2 software. Data were normalized with the values obtained for GAPDH and the fold change in mRNA expression in CRLP-treated as compared to untreated macrophages was determined by the method described by Pfaffl [25].

Lipids were extracted from cell samples by the addition of chloroform: methanol (2:1 v:v, 20 volumes) followed by 0.88% KCl (v:v) (40% total volume). The mixture was shaken and the chloroform layer containing the lipids was collected and dried under nitrogen. The triacylglycerol (TG) content of CRLPs and lipid extracts was determined using a fully enzymatic assay kit (Alpha Laboratories, Eastleigh, Hants, UK) in which glycerol released from triacylglycerol by a lipase is measured using glycerol phosphate oxidase and peroxidase. The total cholesterol (cholesterol+cholesteryl ester) content of CRLPs and lipid extracts was determined using cholesterol oxidase and peroxidase. The cholesterol ester concentrations were calculated by subtracting the values obtained for cholesterol from those for total cholesterol. The extent of oxidation of CRLPs and lipid extracts was determined using thiobarbituric acid-reacting substances (TBARS) in the preparations [27].

The apolipoprotein content of CRLPs was assessed by SDS-PAGE using the method described by Laemmli [28]. Visualisation of the bands was carried out using Coomassie blue. The protein bands corresponding to apoE were quantified by optical density volume analysis with an Opticon light cycler under the following conditions; denaturation at 94 °C for 2 min followed by 40 cycles of 94 °C for 15 s, 58 °C for 1 min, with a final extension at 72 °C for 1 min. The primers used and the product sizes are shown in Table 1. The Ct values were determined by automated threshold analysis using Opticon Monitor 2 software. Data were normalized with the values obtained for GAPDH and the fold change in mRNA expression in CRLP-treated as compared to untreated macrophages was determined by the method described by Pfaffl [25].

The lipid composition of CRLPs, oxCRLPs and pCRLPs is shown in Table 2. The concentration of TG and TC in CRLPs and pCRLPs was similar, but that of oxCRLPs was a little lower, reflecting the dilution of the preparations during the oxidation procedure. The mean TG:TC ratio, however, was between 5 and 6 for all 3 CRLP types. Previous work in our laboratory has shown that the phospholipid content of CRLPs, oxCRLPs and pCRLPs prepared by the methods used here are not significantly different [15,29]. Analysis of the TBARS content of the particles showed that there were significant differences in their oxidative states, with values being higher in oxCRLPs as compared to CRLPs, while those for pCRLPs were lower (one way ANOVA, Bonferroni’s test post hoc) (Table 2).

The apoE content of CRLPs, oxCRLPs and pCRLPs was evaluated by SDS PAGE electrophoresis. The results showed that the particles contained apoE, but no apoE was detected in the top fraction of the d 1.063–1.21 g/ml fraction of human plasma incubated and centrifuged in the absence of lipid particles (Fig. 1A), indicating that the apoprotein was bound to the CRLPs during the incubation. No apoCs or other apolipoproteins were detectable. There were no significant differences in the apoE

### Table 1

<table>
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<tr>
<th>Parameter</th>
<th>CRLPs</th>
<th>oxCRLPs</th>
<th>pCRLPs</th>
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<td>TG (μmol/ml)</td>
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<td>0.70±0.11(23)</td>
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<td>CE (μmol/ml)</td>
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<td>0.48±0.29(23)</td>
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<tr>
<td>UC (μmol/ml)</td>
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<td>0.12±0.07(23)</td>
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<td>TG:TC</td>
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<td>4.12±0.20(19)</td>
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<td>TBARS (nmol MDA/μmol TG)</td>
<td>5.93±0.36(23)</td>
<td>7.52±3.03(23)**</td>
<td>0.38±0.26(19)*</td>
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CRLPs were prepared as described in Materials and methods and their triacylglycerol (TG), cholesteryl ester (CE), unesterified cholesterol (UC), total cholesterol (TC) and TBARS content was determined. Data shown are the mean±SEM (number of preparations). *P<0.05, **P<0.01 vs. CRLPs (one-way ANOVA, Bonferonni’s test post hoc).

### 3. Results

#### 3.1. Characteristics of CRLPs

The lipid composition of CRLPs, oxCRLPs and pCRLPs is shown in Table 2. The concentration of TG and TC in CRLPs and pCRLPs was similar, but that of oxCRLPs was a little lower, reflecting the dilution of the preparations during the oxidation procedure. The mean TG:TC ratio, however, was between 5 and 6 for all 3 CRLP types. Previous work in our laboratory has shown that the phospholipid content of CRLPs, oxCRLPs and pCRLPs prepared by the methods used here are not significantly different [15,29]. Analysis of the TBARS content of the particles showed that there were significant differences in their oxidative states, with values being higher in oxCRLPs as compared to CRLPs, while those for pCRLPs were lower (one way ANOVA, Bonferroni’s test post hoc) (Table 2).

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### Table 2

<table>
<thead>
<tr>
<th>Lipids and TBARS content of CRLPs</th>
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<tr>
<td>Parameter</td>
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<tr>
<td>TG (μmol/ml)</td>
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<tr>
<td>CE (μmol/ml)</td>
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<td>UC (μmol/ml)</td>
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<tr>
<td>TG:TC</td>
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<td>TBARS (nmol MDA/μmol TG)</td>
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content of the CRLPs, oxCRLPs or pCRLPs as assessed by optical density volume analysis (Fig. 1B).

3.2. Effect of the oxidative state of CRLPs on the induction of lipid accumulation in macrophages

Incubation of THP-1 macrophages with CRLPs or oxCRLPs for 5, 24 or 48 h caused a marked increase in the total lipid found in the cells (Fig. 2A) at all time points, but lipid accumulation was greater with CRLPs as compared to oxCRLPs ($P<0.001$). This effect was mainly due to greater accumulation of TG in the presence of CRLPs ($P<0.001$) (Fig. 2B), as the trend for an increase in TC in CRLP- as compared to oxCRLP-treated macrophages did not reach significance (Fig. 2C).

3.3. Effect of the oxidative state of CRLPs on their uptake by macrophages

The uptake of Dil-labeled CRLPs, oxCRLPs and pCRLPs by THP-1 macrophages was assessed by confocal microscopy and FACS analysis (Fig. 3). Examination of cells incubated with Dil-labelled particles for periods up to 24 h with the confocal microscope showed that the fluorescence associated with the cells increased with time in all cases, but that there was clearly more in pCRLP- and less in oxCRLP-treated as compared to CRLP-treated cells (Fig. 3A). Quantification of the cell-associated fluorescence and analysis by ANOVA repeated measures showed that the rate of uptake of CRLPs over 24 h was significantly higher than that of oxCRLPs ($P<0.01$) and significantly lower than that of pCRLPs ($P<0.01$) (Fig. 3B), and a similar result was obtained using FACS ($P<0.05$, CRLPs vs. oxCRLPs or pCRLPs (Fig. 3C).

3.4. Role of apoE in the uptake of CRLPs of different oxidative states

To investigate whether apoE is necessary for uptake of CRLPs, THP-1 macrophages were incubated with Dil-labelled CRLPs or CRLPs without apoE for 2 h and the fluorescence associated with the cells was determined by FACS analysis. In the absence of apoE, the uptake of CRLPs was reduced by about 90% (fluorescence values: CRLPs, 98.6±7.1; CRLPs without apoE, 8.4±0.9, $n=3$).

The potential role of apoE dependent receptors in the uptake of CRLPs by the cells was studied using excess LDL and the LRP ligand, lactoferrin, to block entry via the LDLr and LRP, respectively. The effects of lactoferrin (2 mg/ml) on lipid accumulation in macrophages exposed to CRLPs or oxCRLPs as assessed by Oil red O staining are shown in Fig. 4. Lactoferrin had no effect on the lipid content of cells incubated in the absence of CRLPs, but reduced that in macrophages treated with either CRLPs or oxCRLPs ($P<0.05$, both cases).

When the effects of excess LDL (200 μg cholesterol/ml) and lactoferrin (2 mg/ml), on the fluorescence associated with the macrophages after incubation for 1, 4, 16 and 24 h with CRLPs, oxCRLPs or pCRLPs were assessed by confocal microscopy (Fig. 5A–C), no significant inhibition was observed in the presence of excess LDL, but lactoferrin caused a decrease of >90% in experiments with CRLPs (Fig. 5A) and oxCRLPs (Fig. 5B) and >80% with pCRLPs (Fig. 5C) ($P<0.0001$, all cases). Addition of LDL and lactoferrin together completely abolished detectable uptake of all three types of particles (Fig. 5A–C). FACS analysis of macrophages treated with CRLPs, oxCRLPs or pCRLPs in the presence or absence of lactoferrin (2 mg/ml), LDL (300 μg cholesterol/ml) or LDL+lactoferrin for 2 h also showed that lactoferrin caused a marked decrease of about 75% in the uptake of all three types of CRLPs ($P<0.01$), and in this case

![Fig. 2. THP-1 macrophages were incubated in the absence (control) or presence of CRLPs or oxCRLPs (30 μg cholesterol/ml) for the times indicated and the lipid content of the cells was determined. (A) Total lipid (TG+TC); (B) triacylglycerol (TG); (C) total cholesterol (TC). Data shown are the mean from 4 separate experiments. Error bars show the SEM. Total lipid and TG, CRLPs vs. oxCRLPs, $P<0.001$ (ANOVA repeated measures).](image-url)
significant inhibition (−30–40%) was observed in the presence of LDL (P<0.05) (Fig. 5D). Moreover, in a further separate experiment in which the excess of LDL added was increased by lowering the concentration of CRLPs to 10 μg cholesterol/ml and raising that of LDL to 500 μg cholesterol/ml, the uptake of both CRLPs and oxCRLPs was also significantly inhibited (fluorescence values (n=3): CRLPs, 94.8±6.2; CRLPs + LDL, 58.7±5.8 (P<0.05); oxCRLPs

Fig. 3. Dil-labelled CRLPs, oxCRLPs or pCRLPs (30 μg cholesterol/ml) were incubated with THP-1 macrophages for times up to 24 h and cell-associated fluorescence was evaluated by confocal microscopy or FACS. (A) Confocal microscopy images after 16 h incubation; (B) uptake of fluorescence by the cells over 24 h as assessed by confocal microscopy; (C) uptake of fluorescence by the cells over 6 h as assessed by FACS. Each point (B and C) is the mean from 3 experiments. Error bars show the SEM. Significance limits: B (confocal microscopy), CRLPs vs. oxCRLPs or pCRLPs, P<0.01; C (FACS), CRLPs vs. oxCRLPs or pCRLPs, P<0.05 (ANOVA repeated measures).

Fig. 4. THP-1 macrophages were incubated without (control) or with CRLPs or oxCRLPs (30 μg cholesterol/ml) in the presence of lactoferrin (2 mg/ml) for 5, 24 or 48 h. The cells were then stained with Oil red O and the staining density was measured. Data shown are the mean from 4 experiments. Error bars show the SEM. CRLPs vs. CRLPs+lactoferrin, oxCRLPs vs. oxCRLPs+lactoferrin, P<0.05 (ANOVA repeated measures).
56.7 ± 1.5; oxCRLPs + LDL, 29.2 ± 7.3 (P < 0.05)). As observed in the experiments using confocal microscopy, adding both LDL and lactoferrin to the incubations caused a further decrease to similar minimal levels in the uptake of all three types of CRLPs.

Assessment of the fluorescence associated with THP-1 macrophages after incubation with DiI-labelled acetylated LDL (acLDL) (10 μg cholesterol/ml) for 2 h showed that acLDL uptake, in contrast to that of CRLPs, was not significantly inhibited by excess LDL (500 μg cholesterol/ml) or lactoferrin (2 mg/ml) (Fig. 6).

3.5. Role of scavenger receptors and phagocytosis in the uptake of CRLPs of different oxidative states

The effects of blocking the class A scavenger receptor SR-A, the class B receptors CD36 and SR-B1 or phagocytosis on the uptake of CRLPs by THP-1 macrophages was investigated using poly-I, a known ligand for SR-A, a blocking antibody to CD36, excess HDL, which binds to SR-B1, and cytochalasin D, which blocks the polymerization of actin microfilaments [30]. Evaluation of the fluorescence associated with the cells after incubation with DiI-labelled CRLPs, oxCRLPs or pCRLPs for 1, 4, 16 or 24 h by confocal microscopy (Fig. 7A–C) show no significant change in the presence of poly-I (5 μg/ml) or excess HDL (300 μg/ml). Significant decreases, however, were observed in the presence of anti-CD36 (1 μg/ml) (38% after 24 h (P < 0.05) and cytochalasin D (10 μg/ml) (27% after 24 h) (P < 0.01) in experiments with CRLPs (Fig. 7A), although no change in the uptake of oxCRLPs or pCRLPs was detected with these treatments (Fig. 7B, C).

FACS determinations after incubation of macrophages with CRLPs, oxCRLPs or pCRLPs for 2 h confirmed that poly-I and HDL did not inhibit the uptake of the particles (Fig. 7D).
In contrast, however, the uptake of DiI-labelled acLDL was decreased by about 90% in the presence of poly-I (50 μg/ml) (Fig. 6). As found in the experiments using confocal microscopy, anti-CD36 and cytochalasin D significantly inhibited the uptake of CRLPs, but in this case effects of a similar magnitude were also seen with oxCRLPs and pCRLPs (After 24 h: anti-CD36; CRLPs, −38%, oxCRLPs −40%, pCRLPs, −39%; cytochalasin D; CRLPs −39%, oxCRLPs −35%, pCRLPs −38%), although the changes with oxCRLPs did not reach significance (Fig. 7D).

3.6. Expression of mRNA for the LDLr and LRP in THP-1 macrophages

In THP-1 macrophages exposed to CRLPs (30 μg cholesterol/ml) for 24 h, LRP mRNA levels were increased in CRLP-treated THP-1 cells (fold change, 2.7 ± 1.01, n = 3), while LDLr mRNA abundance was decreased (0.27 ± 0.11(range), n = 2).

4. Discussion

Since homogeneous CMR cannot be obtained easily from human blood without contamination with other lipoproteins of a similar density such as chylomicrons and very low density lipoprotein (VLDL), model CRLPs were used in this study. These particles are similar in size, density and lipid composition to physiological remnants [23], and also contained human apoE, thus they differ from physiological CMR only in lacking apoB48. Importantly, extensive previous studies in both humans and experimental animals have demonstrated that chylomicron- and chylomicron remnant-like particles without apoB48 are cleared from the blood and metabolised in a similar way to the corresponding physiological lipoproteins [31–34]. In addition, CRLPs lacking apoB48, but containing apoE from the appropriate species, have been found to have effects which mimic those of physiological remnants in rat hepatocytes and pig endothelial cells [23,35], and our earlier work has shown that CRLPs cause extensive lipid accumulation in THP-1 macrophages and human monocyte derived macrophages (HMDM) which is comparable to that found in experiments with physiological CMR from rats and the murine macrophage cell line J774 [24,36]. For the current investigation, the use of CRLPs facilitated the manipulation of the oxidative state of the particles by inclusion of the lipophilic antioxidant probucol or by exposure to oxidising conditions similar to those used extensively in studies with oxLDL [16]. In previous work, we have shown that incorporation of probucol into CRLPs increases their uptake by THP-1 macrophages [15], and that this is due to protection of the particles from oxidation, since lycopene, a chemically unrelated antioxidant, has a similar effect [14]. For the present study, therefore, we used pCRLPs in addition to CRLPs and oxCRLPs, so that 3 different oxidative states were tested. Assessment of TBARS in the particles used

![Fig. 7. DiI-labelled CRLPs, oxCRLPs or pCRLPs (30 μg cholesterol/ml) were incubated with THP-1 macrophages in the presence/absence of poly I, excess HDL, anti-CD36 or cytochalasin D and cell-associated fluorescence was evaluated by confocal microscopy after 1, 4, 16 or 24 h (A, CRLPs; B, oxCRLPs; C, pCRLPs) or by FACS after 2 h (D). Data are the mean from 3 experiments and error bars show the SEM. Confocal microscopy, CRLPs vs. CRLPs+anti CD36 or+cytochalasin D, P<0.05 (ANOVA repeated measures). FACS, *P<0.05 vs. corresponding no additions (one way ANOVA).]
showed that the oxidative states of CRLPs, oxCRLPs or pCRLPs were significantly different, and there were no significant differences in their apoE content, thus they provided a convenient and suitable model for the study.

Earlier work in our laboratory and others has demonstrated that CMR cause the extensive lipid accumulation associated with foam cell formation in alveolar macrophages and murine macrophage cell lines [24,36,37]. In experiments with THP-1 macrophages and HMDM, we found that CRLPs induced a greater increase in the intracellular total lipid content than oxLDL (at the equivalent cholesterol level), and that, as might be expected, this was mainly due to greater accumulation of TG, while TC levels were raised to a comparable extent with both types of lipoprotein [24]. In the current study we compared the effects of oxidized and non-oxidized particles and, although both caused rises in cellular lipid content compared to untreated cells, the increase was clearly smaller with oxCRLPs as compared to CRLPs, mainly because of decreased accumulation of TG (Fig. 2). These results suggest that the uptake of CRLPs by the cells may be inhibited by oxidation, and this was confirmed in confocal microscopy and FACS studies (Fig. 3), which showed that the rate of uptake of CRLPs was decreased by oxidation and increased by incorporation of probucol into the particles to protect them from oxidation (Table 2). These results clearly demonstrate that oxidation of CMR inhibits their uptake by macrophages and attenuates foam cell formation. This is an important new finding, since this effect is strikingly different from that found with LDL, where oxidation is required to induce macrophages to form foam cells.

CMR are known to be cleared from the circulation by the liver mainly by apoE-dependent pathways involving the LDLr and the LRP, and the present study shows that apoE also plays an important role in the entry of CRLPs into THP-1 macrophages, since a markedly reduced rate of uptake was observed when apoE was not present in the particles. These findings are in agreement with earlier work which has suggested that both the LDLr and the LRP are involved in the uptake of CMR by macrophages [10,19,38]. Our results indicate that both receptors are able to mediate the uptake of CRLPs regardless of their oxidative state, although lactoferrin, a ligand for the LRP, caused a marked reduction in lipid accumulation and uptake after exposure of THP-1 macrophages to all three types of CRLPs (Figs. 4, 5), while the maximum effect of excess LDL on uptake observed was more modest in all cases (Fig. 5). This lesser role of the LDLr may be due to its down-regulation on the influx of lipoprotein into cells. We have shown previously that the expression of mRNA for the LDLr in THP-1 macrophages is decreased by CRLPs while the expression of LRP mRNA is increased [18], and the present study, which shows a decrease of about 73% in LDLr mRNA and a 2.7-fold rise in LRP mRNA after exposure of the macrophages to CRLPs, is in agreement with these findings.

The differences in rates of uptake of oxCRLPs, CRLPs and pCRLPs were retained in the presence of inhibitors of both the LDLr and the LRP, although when both the LDLr and the LRP were blocked, uptake of oxCRLPs, CRLPs and pCRLPs was reduced to a similar low level (Fig. 5), suggesting that the difference in their rate of uptake is apoE-dependent. Thus, an important novel finding of this study is that oxidation of CRLPs, unlike LDL, does not change their major routes of uptake by macrophages, with apoE-dependent receptors being of major importance and the LRP playing the predominant role, regardless of the oxidative state of the particles. ApoE does not bind to the LDLr family in its lipid-free state, as interaction with lipid is necessary to induce a conformational change which promotes high affinity for the receptors [39]. In addition, apoE has been shown to adopt different conformations when complexed to different lipids. Thus, changes in the lipid composition of lipoproteins such as an increased content of oxidized lipids or the presence of lipophilic compounds such as probucol may alter the conformation of the protein on their surface, and not all apoE molecules on a particular remnant particle may be able to act as ligands [39,40]. The different rates of uptake of CRLPs oxCRLPs and pCRLPs by macrophages demonstrated here, therefore, could be explained by effects on interaction with the LDLr and the LRP caused by differences both in the conformation of apoE and in the number of apoE molecules able to bind to the receptors, even though the total amount of apoE associated with the particles is not changed. Although we cannot rule out the possibility that the faster internalization of pCRLPs is due a specific effect of probucol on the surface structure of the particles, the finding that CRLPs are taken up more slowly after oxidation and more rapidly when the antioxidant is present suggests the changes are more likely to be related to the amounts of oxidized lipid in the particles.

The expression of scavenger receptors such as SR-A and the class B receptors CD36 and SR-B1 is a characteristic feature of macrophages, and SR-A and CD36 are known to play a part in the induction of foam cell formation by oxidized or chemically modified LDL [41]. It is possible, therefore, that this type of receptor may also be involved the uptake of oxCMR by macrophages. In the present study, poly I, a ligand for SR-A, reduced the uptake of acLDL by THP-1 cells by about 90% as expected, but had no significant effect on the uptake of CRLPs, irrespective of their oxidative state. In addition, excess HDL, which binds to SR-B1, did not prevent the entry of CRLPs, oxCRLPs or pCRLPs into the cells. In contrast, in the presence of anti-CD36, the uptake of all three CRLP types was inhibited by about 35–40% so that the differences in their rates of uptake were retained (Fig. 7). Thus, SR-A and SR-B1 do not appear to play a significant part in the uptake of oxCRLPs by macrophages, although CD36 may have a role which is unaffected by the oxidative state of the particles.

Mamo et al. [22] have reported previously that the phagocytosis may be important in the uptake of CMR by rabbit alveolar macrophages, although in a later electron microscopy study with HMDM they found no evidence for entry via this route [42]. In our experiments with THP-1 macrophages, however, cytochalasin D inhibited the uptake of all CRLPs tested by 35–40%, suggesting that some phagocytosis of CRLPs of all oxidative states does occur in these cells (Fig. 7).

It has been suggested that the apoB48r may be involved in the uptake of chylomicron remnants by macrophages [1,20], and Kawakami et al. [43] have reported that it is responsible for
the induction of macrophage foam cell formation by remnant lipoproteins from hyperlipidemic patients. Since apoB48 is an integral protein incorporated during the assembly of chylomicrons in intestinal cells, it is not possible to bind it to model CRLPs in a physiological way. The particles used in the present work, therefore, do not contain apoB48 and we were unable to study involvement of this receptor in the uptake of CMR by macrophages. However, antibodies to apoB48 have been found not to inhibit the uptake of chylomicron remnants by rat macrophages [44], and Elsegood et al. [21] who were unable to detect binding of chylomicron remnants to a protein with a molecular weight corresponding to the apoB48 receptor in THP-1 macrophages have suggested that it may be specific for VLDL remnants rather than chylomicron remnants.

The results of this study demonstrate that oxidative modification of CMR as compared to LDL has profoundly different effects on the uptake of the particles and the subsequent induction of lipid accumulation in macrophages. Instead of markedly enhancing foam cell formation, oxidation of CMR slows their uptake by macrophages and reduces the amount of lipid subsequently accumulated in the cells. This difference may be due to the different receptor mechanisms involved, since oxidation of LDL shifts the main route of uptake from the regulated LDLr to the unregulated scavenger receptors, while our experiments suggest that CMR are taken up mainly by the LRP with some contribution from the LDLr, with CD36 and phagocytosis playing only minor roles, irrespective of their oxidative state. These findings provide important new information about the way in which oxidation of CMR influences their induction of foam cell formation and the mechanisms involved, and has important implications for the role of dietary factors such as oxidized lipids and antioxidants which are transported in CMR in the promotion of atherosclerosis.

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