Chylomicron-remnant-like particles inhibit receptor-mediated endothelium-dependent vasorelaxation in pig coronary arteries

Andrew B. GOULTER, Michael A. AVELLA, Jonathan ELLIOTT and Kathleen M. BOTHAM
Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College St, London NW1 0TU, U.K.

ABSTRACT

The influence of native and oxidized chylomicron-remnant-like particles (CMR-LPs) on endothelium-dependent relaxation in pig coronary arteries was studied. Artificial lipid particles of a size and lipid composition resembling chylomicron remnants and containing pig apolipoprotein E were used to investigate the effects of chylomicron remnants on the relaxation of isolated segments of pig coronary arteries in response to three endothelium dilators: 5-hydroxytryptamine (5-HT), bradykinin and the calcium ionophore A23187. CMR-LPs caused significant inhibition of the maximum relaxation response of the vessels to 5-HT, but not that to bradykinin or A23187 (P < 0.05). In contrast, CMR-LPs that had been oxidized by incubation with 10 μM CuSO₄ (oxidized CMR-LPs) were found to significantly reduce maximal relaxation to bradykinin by 13% (P < 0.05) and to reduce the sensitivity of the tissue to A23187 by 1.7-fold (P < 0.05). In experiments in which either the L-arginine/nitric oxide (NO) pathway or the endothelium-derived hyperpolarizing factor (EDHF) pathway was selectively inhibited, leaving the other intact, the inhibitory effect of oxidized CMR-LPs was observed only in vessels in which the L-arginine/NO-mediated pathway was operative. Furthermore, the oxidized particles had no inhibitory effect on the relaxation of the vessel segments to the non-endothelium-dependent agonists S-nitro-N-acetylpenicillamine, S'-((N-ethylcarboxamido)adenosine or pinicadil. These results demonstrate that CMR-LPs inhibit vascular relaxation in pig coronary arteries by an endothelium-dependent mechanism involving the L-arginine/NO pathway, but not the EDHF pathway, and provide evidence in support of a role for chylomicron remnants in the endothelial dysfunction associated with hypercholesterolaemia and atherogenesis.

INTRODUCTION

Endothelial dysfunction is an early event in the development of atherosclerosis, and is believed to play an important role in the progression of the disease [1]. Impaired endothelium-dependent vascular relaxation has been found to be associated with hypercholesterolaemia, and to precede the appearance of atherosclerotic lesions in arterial tissue [1]. This effect is due mainly to reduced bioavailability of nitric oxide (NO), and current evidence suggests that it is caused by raised plasma levels of atherogenic lipoproteins, such as low-density lipoprotein.
LDL) [2]. LDL has been shown to inhibit endothelium-dependent vasorelaxation by interfering with the production of NO via the l-arginine pathway [3] in a number of different vessel types from a variety of species [2]. However, most studies have concluded that oxidation of LDL, which is known to occur in the artery wall [4], is required before inhibition of endothelium-dependent vascular relaxation can be demonstrated [2].

The atherogenic nature of LDL has been established in a large number of studies over many years [5,6]. There is now considerable evidence, however, to suggest that lipoproteins of dietary origin also play an important part in atherosclerosis development [7]. Delayed clearance of chylomicron remnants, which carry dietary fat and cholesterol from the intestine to the liver for processing [8], has been found to be correlated with the progress of atherosclerotic lesions [9,10]. In addition, remnant particles [8], has been found to be correlated with the progress of cholesterol from the intestine to the liver for processing chylomicron remnants, which carry dietary fat and cholesterol from the intestine to the liver for processing [8]. Delayed clearance of lipoproteins of dietary origin also play an important part a large number of studies over many years [5,6]. There is vascular relaxation can be demonstrated [2]. However, most studies have concluded that oxidation of LDL, which is known to occur in the artery wall [4], is required before inhibition of endothelium-dependent vasorelaxation by interfering with the production of NO via the l-arginine pathway [3] in a number of different vessel types from a variety of species [2]. However, most studies have concluded that oxidation of LDL, which is known to occur in the artery wall [4], is required before inhibition of endothelium-dependent vasorelaxation can be demonstrated [2].

The present study was designed, therefore, to investigate the influence of native and oxidized chylomicron remnants on endothelium-dependent relaxation in pig coronary arteries. Because chylomicron remnants are formed from chylomicrons in vivo, and then cleared rapidly from the circulation [8], they cannot be isolated from the blood by the relatively simple procedures used for other lipoproteins, such as LDL. For this reason, artificial lipid particles resembling chylomicron remnants and containing pig apolipoprotein E (apoE) (chylomicron remnant-like particles, CMR-LPs) were used in the experiments reported here. ApoE was bound to the particles by incubation with the density > 1.006 g/ml fraction of pig serum, allowing it to transfer from high-density lipoprotein by the process that occurs in vivo [18].

**MATERIALS AND METHODS**

Pig hearts and blood were obtained from male or female animals (> 50 kg) after slaughter at a local abattoir (Cheale Meats, Brentwood, Essex, U.K.). After flushing the coronary arteries with ice-cold Krebs–Henseleit solution (KHS; 118 mM NaCl, 4.57 mM KCl, 1.27 mM CaCl₂, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄, 25 mM NaHCO₃, 5.55 mM glucose), the hearts were placed in KHS and transported to the laboratory on ice. Blood was collected as the pigs were bled out, and allowed to clot.

**Preparation and oxidation of CMR-LPs**

Lipid particles were prepared by a method based on that described by Diard et al. [19]. A lipid mixture (50 mg) comprising 70% (w/w) triolein, 25% (w/w) phospholipid, 3% (w/w) cholesterol, and 2% (w/w) cholesterol in 0.9% NaCl in Tricine buffer (20 mM, pH 7.4) was emulsified by sonication (20 min at 56 °C). The crude emulsion was then increased in density to 1.21 g/ml, layered under a stepwise gradient [20] and centrifuged at 17000 g (20 min, 20 °C). The top layer was discarded and replaced with an equal volume of 0.9% NaCl (1.006 g/ml), the tubes were centrifuged at 70000 g (60 min, 20 °C), and the lipid particles in the top layer were harvested.

For binding of pig apoE to the lipid particles, fresh pig blood was allowed to clot, centrifuged at 3000 g (35 min, 4 °C) and the serum was collected. After extensive dialysis against 0.9% NaCl, the serum was centrifuged (85000 g, 16 h, 4 °C). The top layer (1.5 ml), containing lipoproteins of density < 1.006 g/ml, was discarded and the bottom layer containing apoE was collected and frozen in aliquots at −20 °C until required. Lipid particles were then added to the apoE-containing serum fraction (1:2.5, v/v), and the mixture was incubated at 37 °C with shaking for 18 h, layered under 0.9% NaCl (1.006 g/ml) and centrifuged at 45000 g (18 h, 12 °C). CMR-LPs containing pig apoE were collected from the top 10–15 mm of the tubes. The preparations were stored under nitrogen and used within 1 week.

To oxidize the CMR-LPs, CuSO₄ (final concentration 10 μM) was added to CMR-LPs and the mixture was incubated at 37 °C with shaking for 16 h, then dialysed against 0.9% NaCl to remove the CuSO₄. The extent of oxidation achieved was determined by measuring the levels of thiobarbituric acid-reactive substances in the oxidized preparations before dialysis [21].

**Studies with pig vessels**

Rings 4–5 mm in length were prepared from the left descending coronary artery of each pig heart and prepared for isometric tension recording as previously described [15]. A resting tension of 2 g was applied and the vessel rings were allowed to equilibrate. When a stable tension was reached, the resting tension was gradually increased until a stable value of 4–5 g was attained. The vessels were then contracted by changing the
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Figure 1 Cumulative concentration–response relaxation curves to (A) 5-HT, (B) bradykinin (Bk) and (C) A23187 in pig coronary artery rings incubated with CMR-LPs (20 μM cholesterol) (●) or with an equal volume of saline (○). The ordinate scale represents relaxation expressed as a percentage of the contraction induced by U44069. Values represent means ± S.E.M. from five (bradykinin, A23187) or six (5-HT) experiments. Where no error bars are visible, they fall within the symbol.

The ordinate scale represents relaxation expressed as a percentage of the contraction induced by U44069. Values represent means ± S.E.M. from five (bradykinin, A23187) or six (5-HT) experiments. Where no error bars are visible, they fall within the symbol.

Vessel rings, prepared as described above, were incubated in KHS with native or oxidized CMR-LPs (final cholesterol concentration of 20 μM) for 45 min, after which time they were contracted with U44069 (10–30 nM), and cumulative concentration–response relaxation curves were constructed to 5-hydroxytryptamine (5-HT; 1 nM–1 μM), bradykinin (1 nM–1 μM), A23187 (1 nM–1 μM), 3-nitroso-N-acetylpenicillamine (SNAP; 1 nM–0.1 μM), 5-(N-ethylycarboxamid)adenosine (NECA; 1 nM–1 μM) or pinacidil (0.1–30 μM). Two vessel rings from the same animal were tested in parallel, one being incubated with CMR-LPs and the second with an equal volume of 0.9% NaCl. One dose–response curve to one agonist was obtained with any given vessel segment.

To differentiate between the pathways mediated by NO and endothelium-derived hyperpolarizing factor (EDHF), which are both stimulated by bradykinin [22,23], vessel segments were treated as follows: (i) to block the EDHF pathway, leaving the NO pathway intact, segments were incubated with nifedipine (0.3 μM) for 30 min, followed by raising the KCl concentration of the bathing solution isotonically to 30 mM; (ii) to block the NO pathway, leaving the EDHF pathway intact, segments were incubated with Nω-nitro-L-arginine (L-NOARG; 300 μM) for 30 min [23]. The segments were then incubated with CMR-LPs, before contraction with U44069 and the construction of cumulative concentration–response relaxation curves to bradykinin as described above.

In all experiments, organ-bath bathing solutions contained 10 μM indomethacin to inhibit prostacyclin production.

Analytical methods

The total cholesterol and triacylglycerol contents of CMR-LPs were determined using kits from Boehringer Mannheim (Mannheim, Germany). For analysis of the apolipoprotein content of the particles, samples were delipidated [24] and separated by SDS/PAGE. For electron microscopy, the CMR-LPs were negatively stained, applied to grids and viewed using a Jeol 1200EX electron microscope.

Materials

Triolein, cholesterol, cholesteryl oleate, phospholipids and 1,1,3,3-tetraethoxypropane, and the drugs A23187 (sodium salt), bradykinin acetate, indomethacin, L-NOARG, SNAP, 9,11-dideoxy-9α,11α-epoxymethano-prostaglandin F2α (U44069), 5-HT creatinine sulphate, NECA, pinacidil and nifedipine, were all purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). All drug

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solutions were freshly prepared on the day of the experiment.

**Statistical analysis**

Data from organ bath experiments were expressed as the percentage decrease in tension of U44069-induced tone, and were plotted against the log of the concentration of agonist used. Cumulative concentration–response contraction curves for each vessel segment were fitted to a single-site logistic equation allowing for a variable slope using PRISM 3.0 (GraphPad Software Inc., San Diego, CA, U.S.A.):

\[
Y = \text{Bottom} + \frac{E_{\text{max}}}{1 + 10^{(\log EC_{50} - X) / \text{Hill slope}}}
\]

where \(Y\) is the response seen (decrease in tension) and \(X\) is the drug concentration. The maximum response (\(E_{\text{max}}\)), the \(EC_{50}\), and the Hill slope of the concentration–response curve were derived for each vessel segment and used to calculate the mean ± S.E.M. In each case, a paired Student’s \(t\) test was used to compare \(EC_{50}\) values between rings incubated with lipoprotein and control rings from vessels obtained from the same animal that were examined on the same experimental day.

**RESULTS**

**Characterization of CMR-LPs**

The triacylglycerol and total cholesterol content of CMR-LPs was 2.46 ± 0.47 and 0.40 ± 0.08 \(\mu\)mol/ml respectively \((n = 9)\), giving a triacylglycerol/total cholesterol ratio of 6.1:1 \((w/w)\), which is comparable with the value of 5.6:1 found for rat chylomicron remnants prepared in vivo by the method described by Lambert et al. [25]. This ratio was not changed after oxidation of the CMR-LPs. By electron microscopy the particles were observed to have a rounded appearance, similar to remnants prepared in vivo, and the mean diameter was estimated as 57.3 ± 2.0 nm. Analysis of the apolipoprotein content by SDS/PAGE showed that the CMR-LPs contained a protein with a molecular mass of approx. 34 kDa, which corresponds to apoE. This band was not present in the top fraction from serum incubated in the absence of lipid particles, indicating that the apoE was bound to the CMR-LPs, and that this binding occurred during the incubation period.

After oxidation of the CMR-LPs with CuSO\(_4\), the levels of thiobarbituric acid–reactive substances present were approx. 5.4-fold higher than those found in the untreated particles (nmol of malondialdehyde/\(\mu\)mol of triacylglycerol: CuSO\(_4\)-treated, 1.84 ± 0.19; untreated, 0.34 ± 0.05; \(P < 0.05\)).

**Effects of CMR-LPs on the relaxation of pig coronary arteries to 5-HT, bradykinin and A23187**

The effects of CMR-LPs on the relaxation of pig coronary arteries in response to the endothelium-dependent vasodilators 5-HT, bradykinin and A23187 are shown in Figure 1, and the mean best-fit concentration–response curve parameters derived from the curves are given in Table 1. CMR-LPs inhibited the response of the vessels to 5-HT (Figure 1A), causing a large, significant reduction in the maximum percentage relaxation achieved (Table 1), indicating that the CMR-LPs had inhibited the extent to which these vessels could dilate in response to 5-HT. CMR-LPs had no significant effect on responses to bradykinin or A23187 (Figures 1B and 1C), although there was a trend for CMR-LPs to decrease the sensitivity to A23187. However, the maximum relaxation observed with 5-HT in the absence of CMR-LPs was <10% of the U44069-induced tone, a very low value compared with the relaxations of 90–100% found with bradykinin and A23187.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>(EC_{50}) (M)</th>
<th>Maximum relaxation (%)</th>
<th>Hill slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>(4.32 ± 2.13) \times 10^{-8}</td>
<td>7.88 ± 1.17†</td>
<td>0.90 ± 0.27</td>
</tr>
<tr>
<td>5-HT + CMR-LPs</td>
<td>(1.75 ± 0.37) \times 10^{-8}</td>
<td>2.42 ± 0.61†</td>
<td>0.88 ± 0.20</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>(1.55 ± 0.51) \times 10^{-8}</td>
<td>108.6 ± 4.08</td>
<td>2.17 ± 0.46</td>
</tr>
<tr>
<td>Bradykinin + CMR-LPs</td>
<td>(1.51 ± 0.27) \times 10^{-8}</td>
<td>110.2 ± 7.09</td>
<td>2.22 ± 0.22</td>
</tr>
<tr>
<td>A23187</td>
<td>(7.28 ± 1.31) \times 10^{-8}</td>
<td>96.0 ± 1.65</td>
<td>3.01 ± 0.29</td>
</tr>
<tr>
<td>A23187 + CMR-LPs</td>
<td>(14.00 ± 0.46) \times 10^{-8}</td>
<td>94.1 ± 1.64</td>
<td>2.95 ± 0.21</td>
</tr>
</tbody>
</table>

* \(P < 0.05\) compared with corresponding control value (Student’s paired \(t\) test).
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Figure 2 Cumulative concentration–response relaxation curves to (A) bradykinin (Bk) and (B) A23187 in pig coronary artery rings incubated with oxidized CMR-LPs (20 μM cholesterol) (◼) or with an equal volume of saline (⊙). The ordinate scale represents relaxation expressed as a percentage of the contraction induced by U44069. Values represent means ± S.E.M. from seven experiments. Where no error bars are visible, they fall within the symbol.

Effects of oxidized CMR-LPs on the relaxation of pig coronary arteries to bradykinin and A23187

Figure 2 shows the effects of oxidized CMR-LPs on the relaxation of pig coronary arteries in response to bradykinin and A23187, and the mean best-fit concentration–response curve parameters derived from the curves are presented in Table 2. In view of the limited response of the vessels to 5-HT, and the almost complete inhibition of this response by native CMR-LPs (Figure 1A), the effects of oxidized CMR-LPs on responses to this agonist were not studied.

In the presence of oxidized CMR-LPs, the relaxation of the vessel rings to bradykinin (Figure 2A) and A23187 (Figure 2B) was significantly inhibited. In both cases, the concentration–response curves were shifted over to the right in the presence of the oxidized CMR-LPs, showing that the tissues became less able to relax in response to both agonists. The maximum relaxation achieved in response to bradykinin was significantly reduced, indicating that the CMR-LPs had inhibited the extent to which these vessels could dilate in response to bradykinin. This decrease in the maximum response to bradykinin meant that the change in $EC_{50}$, although numerically larger in the presence of oxidized CMR-LPs, did not reach statistical significance. In the case of A23187, the small decrease in the maximum response seen in the presence of oxidized CMR-LPs failed to reach statistical significance, but the $EC_{50}$ value found for A23187 was significantly increased (Table 2), indicating reduced sensitivity of the arteries to this agonist.

In order to investigate whether the oxidized CMR-LPs inhibit vascular relaxation selectively via the NO- or the EDHF-mediated pathway, both of which are known to be stimulated by bradykinin and A23187 [23,26], further experiments were carried out on bradykinin-induced relaxation using vessel rings pretreated with L-NOARG or with nifedipine followed by a raised KCl concentration, to block the L-arginine/NO and EDHF pathways respectively. Initial experiments showed that the maximum relaxation response achieved in the absence of CMR-LPs following preincubation of the vessel rings with nifedipine/KCl was not significantly changed (control, 83.9 ± 6.1%; nifedipine/KCl-treated, 78.3 ± 12.3%; $n = 5$), whereas that achieved after pretreatment with L-NOARG (48.6 ± 2.7%; $P < 0.05$, $n! = 5$) was significantly decreased. Vessels rings pretreated with L-NOARG and nifedipine/KCl to block both pathways showed little relaxation (maximum response 8.9 ± 5.9%; $n = 5$).

The results of the experiments with oxidized CMR-LPs are shown in Figure 3, and the mean best-fit

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$EC_{50}$ (M)</th>
<th>Maximum relaxation (%)</th>
<th>Hill slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradykinin Control</td>
<td>(1.57 ± 0.34) × 10^{-4}</td>
<td>91.3 ± 4.15</td>
<td>2.10 ± 0.20</td>
</tr>
<tr>
<td>Bradykinin + Oxidized CMR-LPs</td>
<td>(2.50 ± 0.37) × 10^{-4}</td>
<td>79.4 ± 6.69*</td>
<td>2.29 ± 0.25</td>
</tr>
<tr>
<td>A23187 Control</td>
<td>(5.95 ± 1.56) × 10^{-4}</td>
<td>121.0 ± 2.51</td>
<td>2.86 ± 0.41</td>
</tr>
<tr>
<td>A23187 + Oxidized CMR-LPs</td>
<td>(10.3 ± 2.50) × 10^{-4}</td>
<td>114.7 ± 5.83</td>
<td>2.95 ± 0.21</td>
</tr>
</tbody>
</table>
Pig coronary artery rings were incubated with (A) L-NOARG (300 μM) for 30 min or (B) nifedipine for 30 min, followed by raising the KCl concentration of the bathing solution isotonically to 30 mM, and then with oxidized CMR-LPs (20 μM cholesterol) (●) or with an equal volume of saline (○) for 45 min before being contracted with U44069. The ordinate scale represents relaxation expressed as a percentage of the contraction induced by U44069. Values represent means ± S.E.M. from five experiments. Where no error bars are visible, they fall within the symbol.

**Figure 3 Cumulative concentration–response relaxation curves to bradykinin (BK)**

Pig coronary artery rings were incubated with (A) L-NOARG (300 μM) for 30 min or (B) nifedipine for 30 min, followed by raising the KCl concentration of the bathing solution isotonically to 30 mM, and then with oxidized CMR-LPs (20 μM cholesterol) (●) or with an equal volume of saline (○) for 45 min before being contracted with U44069. The ordinate scale represents relaxation expressed as a percentage of the contraction induced by U44069. Values represent means ± S.E.M. from five experiments. Where no error bars are visible, they fall within the symbol.

concentration–response curve parameters derived from the curves are given in Table 3. Oxidized CMR-LPs had no significant effect on vessel rings pretreated with L-NOARG (EDHF pathway only intact). In those vessel segments exposed to nifedipine/KCl (l-arginine/NO pathway only intact), the same pattern of the effect of oxidized CMR-LPs was seen as had been noted for vessels with both pathways intact. Thus the concentration–response curves were shifted to the right, indicating that the tissue was less able to respond to bradykinin in the presence of the lipoprotein particles. In this case, the presence of the CMR-LPs resulted in both a decrease in the maximum percentage relaxation and an increase in the EC<sub>50</sub> value, indicating that the particles had reduced both the extent of relaxation possible to bradykinin and the sensitivity of the tissue to this agonist.

**Table 3 Concentration–response relaxation curve parameters of pig coronary arteries to bradykinin in the presence or absence of oxidized CMR-LPs after selective blocking of the NO or EDHF pathways**

Pig coronary vessels with either the NO pathway intact (nifedipine + KCl) or the EDHF pathway intact (L-NOARG) were incubated in the presence or absence (control) of oxidized CMR-LPs (20 μM cholesterol) and preconstricted with U44069. Cumulative concentration–response curves to bradykinin were then obtained. Maximum relaxation is expressed as a percentage of U44069-induced tone. Values represent means ± S.E.M. from five experiments; *P < 0.05 compared with corresponding control value (Student’s paired t test).

<table>
<thead>
<tr>
<th>Condition</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (M)</th>
<th>Maximum relaxation (%)</th>
<th>Hill slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NOARG Control</td>
<td>(5.36 ± 2.71) × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>68.1 ± 5.39</td>
<td>2.00 ± 0.22</td>
</tr>
<tr>
<td>L-NOARG + CMR-LPs</td>
<td>(3.53 ± 1.50) × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>62.7 ± 9.42</td>
<td>2.01 ± 0.21</td>
</tr>
<tr>
<td>Nifedipine + KCl Control</td>
<td>(0.89 ± 0.12) × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>104.2 ± 7.38</td>
<td>1.84 ± 0.39</td>
</tr>
<tr>
<td>Nifedipine + KCl + CMR-LPs</td>
<td>(2.12 ± 0.60) × 10&lt;sup&gt;-8&lt;/sup&gt;*</td>
<td>91.3 ± 5.66*</td>
<td>1.82 ± 0.23</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Since chylomicrons are converted rapidly into chylomicron remnants in vivo, it is inherently difficult to obtain preparations of chylomicron remnants from animals that are suitable for in vitro studies, necessitating the use of artificial CMR-LPs in the present study. However, the metabolism of lipid emulsions of a size and composition similar to those of physiological chylomicrons is almost identical [27–29], and particles resembling chylomicron remnants containing apoE have been found to have effects that mimic those of physio-
Figure 4 Cumulative concentration–response relaxation curves to (A) SNAP, (B) NECA and (C) pinacidil in pig coronary artery rings incubated with oxidized CMR-LPs (20 μM cholesterol) (■) or with an equal volume of saline (○). The ordinate scale represents relaxation expressed as a percentage of the contraction induced by U44069. Values represent means ± S.E.M. from five (pinacidil), six (SNAP) or eight (NECA) experiments. Where no error bars are visible, they fall within the symbol.

Logical remnants in isolated hepatocytes [19,30,31]. The CMR-LPs used here had a triacylglycerol/cholesterol ratio and a mean diameter similar to those reported for physiological remnants [8,18,32]. Furthermore, they were shown to contain apoE and no other lipoproteins derived from pig serum. Thus, although not ideal, these particles are the best model available for the study of the effects of chylomicron remnants on endothelium-dependent vascular relaxation in pig coronary arteries. We examined large-diameter coronary arteries under static conditions, whereas in vivo these vessels would be subjected to shear forces exerted by pulsatile flow. These vessels are conductance rather than resistance vessels, but they are of direct relevance to atherosclerosis. Studying their relaxant responses to endothelium-dependent vasodilator agonists represents a sensitive method of assessing endothelial cell function, although other functions, such as their anti-adhesive properties, may be more pertinent to the development of atheroma in conductance vessels.

The three major mediators involved in endothelium-dependent vasorelaxation in pig coronary arteries are NO, EDHF and prostacyclin [23]. In our experiments, prostacyclin production was inhibited by the presence of indomethacin in all bathing solutions.

Endothelium-dependent relaxation in response to 5-HT in pig coronary arteries is believed to be mediated predominantly by NO [34], with little or no contribution from EDHF, while bradykinin and A23187 are known to activate both pathways [23,26]. In the present study, CMR-LPs caused significant inhibition of the relaxation of pig coronary arteries to 5-HT (Figure 1A, Table 1). However, the maximum relaxation to 5-HT that could be demonstrated in the absence of the lipid particles was less than 10% of the U44069-induced tone. Despite being smaller in magnitude than those reported in other studies (40–60%) [35,36], the relaxations were dose-dependent, reproducible, and abolished by removal of the endothelium (results not shown). Our results, therefore, are consistent with previous work showing that coronary arteries from hypercholesterolaemic pigs are less responsive to 5-HT [37,38], and that oxidized LDL decreases the endothelium-dependent relaxation of epicardial pig coronary arteries to 5-HT [39].

Inhibition of the maximum relaxation of pig coronary arteries in response to bradykinin was demonstrated to occur in the presence of oxidized, but not native, CMR-LPs (Figures 1B and 2A, Table 1 and 2). We did not investigate the effects of oxidized CMR-LPs on responses to 5-HT, because native CMR-LPs almost completely inhibited the responses to this agonist. Examination of the effects of oxidized CMR-LPs on 5-HT-induced relaxations, therefore, would have told us little about the relative efficacy of oxidized compared with native particles. The endothelial cell receptors involved in bradykinin-induced vascular relaxation are thought to be G_q-protein-linked, whereas responses to 5-HT are mediated through G_t-protein-linked receptors [40]. Current evidence suggests that G_q-protein-linked receptors are less susceptible than G_t-protein-linked pathways to inhibition by oxidized LDL [2,39]. Oxidized LDL and its component lysophosphatidic acid have been shown to inhibit relaxation of pig coronary arteries to 5-HT, but not bradykinin [39,41]. Our findings that the effects of CMR-LPs on bradykinin-induced, but not 5-HT-induced, relaxation require prior oxidation of the particles suggest that there is a similar difference in the sensitivity of G_t- and G_q-protein-linked receptors to
chyramicron remnants in pig coronary arteries. However, as bradykinin stimulates both the NO and EDHF pathways, the inhibition of bradykinin-induced relaxation by CMR-LPs by one of these pathways may be compensated for by up-regulation of the other, and this may be an alternative explanation for the difference in the effects of native particles on 5-HT- and bradykinin-induced responses. Finally, the lower efficacy of 5-HT as a vasorelaxant, particularly in the present study, may partially explain the highly sensitive nature of the responses to non-competitive inhibition by CMR-LPs.

The calcium ionophore A23187 activates endothelial cells by allowing transfer of calcium across the plasma membrane, leading to enhanced activity of endothelial NO synthase [42] and hyperpolarization by activation of potassium channels [43]. Thus both the NO and the EDHF pathways are stimulated. Our experiments indicate that oxidized CMR-LPs significantly reduce the sensitivity of pig coronary arteries to A23187, as indicated by an increased EC_{50} value, without changing the maximum response (Figures 1C and 2B, Table 1 and 2). These results suggest that the pig coronary artery has a greater reserve capacity for A23187 to stimulate the NO pathway when compared with bradykinin, where a reduction in the maximum response was observed in the presence of oxidized CMR-LPs. Furthermore, the results of the present study contrast with our previous findings using rat aorta, where neither native [12] nor oxidized (D. J. Grieve, M. A. Avella, K. M. Botham and J. Elliott, unpublished work) chylomicron remnants had a significant effect on relaxations to A23187. Doi et al. [16], however, found that both native and chemically modified remnants from human postprandial plasma inhibited A23187-induced relaxation in rabbit aorta.

As both bradykinin and A23187 activate both the L-arginine/NO pathway and the EDHF pathway, inhibition of either pathway could explain our results. However, further investigations on responses to bradykinin, using vessels in which either the NO or the EDHF pathway was blocked leave the other intact [26], clearly demonstrated that the oxidized particles inhibit bradykinin-induced vascular relaxation mediated by the L-arginine/NO pathway, but not that mediated by the EDHF pathway (Figure 3, Table 3). If a common mechanism of action accounts for the inhibitory effects of oxidized CMR-LPs on bradykinin- and A23187-induced activation of the NO pathway, this would support a site of action downstream of the second messenger system, possibly involving factors regulating endothelial NO synthase activity, such as the membrane protein caveolin, as has been proposed for LDL [44]. Further work is necessary to establish the mechanism of the inhibitory effect of oxidized CMR-LPs on the bradykinin- and A23187-mediated activation of the NO pathway in the pig coronary artery.

In experiments with SNAP (an NO donor), NECA (an A\_2 adenosine receptor agonist) [45] or pinacidil (an ATP-sensitive potassium channel activator) [46], no significant inhibitory effects of oxidized CMR-LPs were found (Figure 4, Table 4). Indeed, there was a small, but significant, potentiation of SNAP-induced responses. One possible explanation for this finding is that oxidized CMR-LPs inhibit basal NO production, making the vessels apparently more receptive to NO donors such as SNAP [47]. The results obtained with SNAP and pinacidil rule out the possibility that oxidized CMR-LPs have a direct inhibitory effect on NO-mediated relaxation at the level of the smooth muscle cell, and also show that the particles do not affect the ability of preconstricted pig coronary arteries to respond to hyperpolarizing stimuli.

In conclusion, the results presented here demonstrate that CMR-LPs inhibit endothelium-dependent vascular relaxation in pig coronary arteries by a selective effect on the L-arginine/NO pathway. Previous studies have shown that, in the first 6 h after a fat meal, cholesterol concentrations in the \(< 1.019 \text{ g/ml plasma fraction (containing chylomicrons, chylomicron remnants and very-low-density lipoprotein) in normolipidaemic men increase by about 350 } \mu \text{M} [48]. Since a good proportion

### Table 4 Concentration–response relaxation curve parameters of pig coronary arteries to SNAP, NECA and pinacidil in the presence or absence of oxidized CMR-LPs

<table>
<thead>
<tr>
<th>Agonist</th>
<th>EC_{50} (M)</th>
<th>Maximum relaxation (%)</th>
<th>Hill slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAP Control</td>
<td>(4.96 ± 2.00) x 10^{-4}</td>
<td>91.5 ± 3.79</td>
<td>1.27 ± 0.08</td>
</tr>
<tr>
<td>SNAP + Oxidized CMR-LPs</td>
<td>(4.18 ± 2.41) x 10^{-4}</td>
<td>99.7 ± 2.90*</td>
<td>1.59 ± 0.10</td>
</tr>
<tr>
<td>NECA Control</td>
<td>(0.26 ± 0.05) x 10^{-4}</td>
<td>108.5 ± 3.82</td>
<td>1.49 ± 0.08</td>
</tr>
<tr>
<td>NECA + Oxidized CMR-LPs</td>
<td>(0.33 ± 0.05) x 10^{-4}</td>
<td>113.3 ± 5.65</td>
<td>1.38 ± 0.08</td>
</tr>
<tr>
<td>Pinacidil Control</td>
<td>(49.0 ± 2.80) x 10^{-4}</td>
<td>106.7 ± 2.52</td>
<td>2.29 ± 0.23</td>
</tr>
<tr>
<td>Pinacidil + Oxidized CMR-LPs</td>
<td>(50.8 ± 4.00) x 10^{-4}</td>
<td>110.7 ± 3.30</td>
<td>2.29 ± 0.28</td>
</tr>
</tbody>
</table>

Cumulative concentration–response curves to SNAP, NECA and pinacidil were obtained in vessels preconstricted with U44069. Maximum relaxation is expressed as a percentage of U44069-induced tone. Values represent means ± S.E.M. from five (Pinacidil), six (SNAP) or eight (NECA) experiments; *P < 0.05 compared with corresponding control value (Student’s paired t test).
of this rise will be due to cholesterol in chylomicron remnants, it is clear that the concentration of CMR-LP cholesterol (20 μM) used in the present study is well within the physiological range. Our findings, therefore, together with those from our earlier studies with the rat model,[12,15], provide good evidence to support the view that chylomicron remnants play a part in the endothelial dysfunction that is known to be associated with hypercholesterolaemia and the development of atherosclerosis in humans. This suggests that dietary lipids influence atherogenesis through direct effects on the endothelium during their transport from the gut to the liver, in addition to their well documented effects on plasma LDL levels [49], and highlights the need for further studies in order to elucidate the mechanism behind these effects at the cellular level.

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REFERENCES

13 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
33 Reference deleted

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