Triacylglycerol-rich lipoprotein–gene interactions in endothelial cells

C.M. Williams1, V. Maitin and K.G. Jackson

Hugh Sinclair Unit of Human Nutrition, School of Food Biosciences, University of Reading, Reading RG6 6AP, U.K.

Abstract
Lipoproteins such as LDL (low-density lipoprotein) and oxidized LDL have potentially adverse effects on endothelial cells due to their ability to activate pro-inflammatory pathways regulated via the transcription factor NF-κB (nuclear factor κB). Triacylglycerol-rich lipoproteins (the chylomicrons, very-low-density lipoprotein and their respective remnant particles) have also been implicated in the induction of a pro-inflammatory phenotype and up-regulation of adhesion molecule expression. Although early studies supported the proposal that LPL (lipoprotein lipase)-mediated hydrolysis of TRLs (triglyceride-rich lipoproteins) at the endothelium could activate the NfκB pathway, more recent studies provide evidence of pro- and anti-inflammatory responses when cells are exposed to fatty acids or TRL particles. A large number of genes are up- and down-regulated when cells are exposed to TRL, with the net effect reflecting receptor- and non-receptor-mediated pathways that are activated or inhibited depending on fatty acid type, the lipid and apolipoprotein composition of the TRL and the presence or absence of LPL. Early concepts of TRL particles as essentially pro-inflammatory stimuli to the endothelium provide an overly simplistic view of their impact on the vascular compartment.

Introduction
The vascular endothelium functions as a homoeostatic organ, regulating vascular tone by the production of vasodilator and vasoconstrictor substances, and blood coagulation by the regulation of platelet aggregation and the production of a number of pro- and anti-coagulatory peptides. In addition, the endothelium regulates the adhesion of leucocytes to the vascular compartment by ensuring the presence of inflammatory adhesion molecules on the surface of the endothelial cell [1]. Endothelial activation, due to stimulation by inflammatory cytokines, bacterial activation or biomechanical stimuli, results in the expression of an inflammatory phenotype, characterized by increased cell-surface expression of endothelial-leucocyte adhesion molecules and chemo-attractants that allow migration and binding of leucocytes to the endothelial surface [2,3]. Although this represents a normal response to infection or injury, maladaptive changes in endothelial function, induced by chronic elevation in these stimuli, result in adverse consequences such as impaired antihaemostatic properties, hyperadhesiveness to blood leucocytes and an attenuated vasodilatory response to agonists that activate nitric oxide synthase [4]. It is now generally agreed that the early stages of diet-induced atherosclerosis are associated with a particular type of endothelial inflammatory response in which the monocyte plays a key role [5,6]. Although considerable information has been obtained on the cell–cell interactions, pathophysiological stimuli and intracellular signalling mechanisms that are involved in this process, the role of specific diet-related stimuli remains to be fully elucidated. This paper will focus on the cellular and molecular mechanisms by which circulating TRLs (triacylglycerol-rich lipoproteins), including CMs (chylomicrons), VLDL (very-low-density lipoprotein) and their remnant particles, are supposed to be involved in endothelial activation and the development of a pro-inflammatory endothelial phenotype.

Role of monocyte adhesion in atherogenesis
Although polymorphonuclear leucocyte adhesion to the endothelium of postcapillary venules is a manifestation of many acute states of inflammation, attraction and adherence of mononuclear leucocytes appears to be a unique feature of the early inflammatory events of atherosclerosis [5,6]. Infiltration of the endothelium with monocytes is typical of diet-induced atherosclerosis in the hypercholesterolaemic rabbit and apo (apolipoprotein) E-deficient mice models [7,8]. In the hypercholesterolaemic rabbit, the earliest indicator of endothelial activation, which precedes infiltration of the area with monocytes, is the increased expression of VCAM-1 (vascular cell adhesion molecule-1) on the endothelial surface [7]. VCAM-1 is a member of the immunoglobulin superfamily, which binds a heterodimeric integrin receptor, very late antigen 4, expressed on monocytes and lymphocytes, but not neutrophils. This targeted expression of adhesion

Abbreviations used:
apo, apolipoprotein; CM, chylomicron; COX2, cyclo-oxygenase 2; ICAM, intercellular cell-adhesion molecule; IL-6, interleukin 6; LOX1, lipoxygenase 1; LPL, lipoprotein lipase; M-CSF, macrophage colony stimulating factor; MCP-1, monocyte chemotactant protein-1; NFκB, nuclear factor κB; PPAR, peroxisome-proliferator-activated receptor; TNFα, tumour necrosis factor α; TRAIL, tracylglycerol-rich lipoprotein; (V)LDL, (very-)low-density lipoprotein; VCAM-1, vascular cell adhesion molecule-1.

1To whom correspondence should be addressed (email c.m.williams@reading.ac.uk).
molecules explains the selective inhibition of monocyte, but not neutrophil, adhesion, by antibodies directed against VCAM-1 [9]. VCAM-1 is, therefore, key to the selectivity of monocyte recruitment in early atherogenesis. Ligand–receptor interactions between ICAM-1 (intercellular cell-adhesion molecule 1) and monocyte LFA-1 (lymphocyte function associated antigen-1) and CD11b/CD18, and between E-selectin and the monocytic sialylated Lewis X Factor, as well as specific chemoattractants, including MCP-1 (monocyte chemoattractant protein-1) and M-CSF (macrophage colony stimulating factor), are also believed to be involved in monocyte recruitment in early atherogenesis. Elevated circulating concentrations of the soluble forms of VCAM-1, ICAM-1, E- and P-selectins have been observed in subjects with atherosclerosis and those at increased risk due to diabetes and hyperlipidaemia [10–13]. The endothelium interacts continuously with plasma lipids and lipoproteins with considerable potential for adverse consequences in terms of endothelial activation, dysfunction and ultimately initiation of atherogenesis. It is, therefore, not surprising that the role of diet-related lipoprotein particles in endothelial activation and induction of adhesion molecule expression has received considerable attention in recent years.

**Stimulation of endothelial activation: transcriptional regulation of adhesion molecule expression**

Monocyte binding to cultured endothelial cells, and the expression of endothelial-leucocyte adhesion molecules on the surface of endothelial cells in *in vitro* have been extensively studied to elucidate the mechanisms underlying endothelial-monocyte interaction in diet-induced atherosclerosis. Apart from ICAM-1, which is constitutively expressed on the surface of endothelial cells in the rested state, the endothelial cell normally expresses negligible amounts of other adhesion molecules and chemoattractants. However, on activation, the gene expression of VCAM-1, E-selectin and soluble products such as MCP-1, M-CSF, IL-6 (interleukin 6) and IL-8 is up-regulated. This transcriptional regulation of adhesion molecule expression has received considerable attention in recent years.

**Atherogenic lipoproteins, endothelial activation and gene expression of proteins involved in monocyte adhesion**

Pro-atherogenic lipoproteins, particularly OxLDL (oxidized low-density lipoprotein), have been shown to cause up-regulation of adhesion molecule expression, especially VCAM-1 and E-selectin, and release of chemotactic factors such as MCP-1 and M-CSF. OxLDL induces NF-κB and activation protein 1 formation in endothelial cells and these effects can be inhibited by α-tocopherol [17,18]. However, in diseases such as diabetes where endothelial dysfunction is a marked pathophysiological feature, the dyslipidaemia is characterized by an elevation in TRL and small dense LDL particles, rather than LDL cholesterol. Furthermore, the metabolic degradation of TRL particles involving progressive hydrolysis of core triacylglycerol, and release of NEFAs (non-esterified fatty acids) and TRL remnants, occurs at the endothelium by the action of LPL (lipoprotein lipase), an endothelially bound enzyme. The possibility that postprandial processing of dietary-derived TRL through endothelially bound LPL provides a pro-inflammatory stimulus to the endothelium, is supported by *in vivo* studies that have shown impaired endothelial function in response to acute fat ingestion [19], although this is by no means a consistent finding [20]. Despite strong pathophysiological and metabolic justification for implicating TRLs in the initiation of endothelial activation, much less is known regarding the interaction of these particles and their products with the endothelium, compared with LDL and its oxidized products. From the relatively small number of studies that have been conducted, evidence is beginning to emerge suggesting that both pro- and anti-inflammatory signalling pathways are activated after stimulation with TRL, TRL remnants and NEFA, although the conditions that determine the net balance between the two are not well defined.

**Fatty acids, TRL and TRL remnant and adhesion molecule expression**

Early evidence supported a pro-oxidant, pro-inflammatory paradigm for TRLs and NEFA, similar to that shown for OxLDL and consistent with activation of NF-κB and up-regulation of adhesion molecule expression. *In vitro* studies of endothelial cells showed that LPL-derived remnants of TRLs isolated from hypertriglyceridaemic subjects, as well as incubation with specific unsaturated fatty acids, have the ability to disrupt endothelial integrity [21,22]. CMs oxidized with copper sulphate *in vitro* have been shown to cause greater induction of E-selectin in endothelial cells, when obtained after ingestion of sunflower compared with olive oil [23] and oxidized CMs have been shown to cause greater stimulation of monocyte adhesion to the endothelium [24]. *In vitro* studies using individual fatty acids showed unsaturated fatty acids, generally, to be more pro-inflammatory than saturated fatty acids. Studies showed linoleic acid to have greater capacity to induce oxidative and inflammatory stress than other fatty acids, including those with greater numbers...
of double bonds, such as α-linolenic acid. Addition of linoleic acid to endothelial cells in relatively short-term incubations (6 h) induced cellular oxidation, reduced intracellular glutathione levels and promoted NF-κB activation and transcriptional activity, whereas addition of vitamin E attenuated these effects [25]. However, later studies by the same authors suggested that the pro-inflammatory effects of linoleic acid may be time-dependent. Preincubation of cells with linoleic acid was shown to attenuate NF-κB binding to DNA and reduce the expression of IL-8 and ICAM-1 in response to TNFα. These effects were suggested to be due to the ability of linoleic acid to enhance the recovery of IκB to non-stimulated levels and reduce cell oxidative stress compared with TNFα treatment alone, although the effects on IκB were found to be highly variable over time [26]. A series of dose-response studies in which cells were preincubated with saturated, monounsaturated and $n - 6$ and $n - 3$ polyunsaturated fatty acids, also support inhibitory effects of unsaturated fatty acids on IL-1 or TNFα-mediated VCAM-1 expression in endothelial cells [27,28]. Inhibitory effects of docosahexaenoic acid on VCAM-1 expression were highly correlated with the time course of incorporation of this fatty acid into membrane phospholipids [28]. The signalling pathway (s) mediating inhibitory effects of unsaturated fatty acids on VCAM-1 have not been elucidated, although addition of indomethacin did not block the effects of docosahexaenoic acid, suggesting that attenuation of an eicosanoid-dependent pathway was not involved.

The TRL particles themselves have also been shown to induce pro- and anti-inflammatory responses in endothelial cells in vitro. CM preparations obtained from human plasma 4 h after a standard fat-containing meal were shown to induce a 3-fold increase in E-selectin and 10-fold increase in VCAM-1 expression in endothelial cells [29]. In contrast with earlier studies that showed pro-inflammatory effects of VLDL to be dependent on the presence of LPL [30], this study showed up-regulation of the adhesion molecule expression to be markedly attenuated when LPL was added to the CM preparations. That TRL does not require the presence of LPL to promote adhesion molecule expression, supports the possibility that TRL particles are themselves pro-inflammatory, independent of their ability to release fatty acids. The ability of TRL to promote endothelial adhesion molecule expression also does not appear to depend on the oxidation of the particle. In contrast with the pro-oxidant paradigm, Dichtl et al. [31] showed VLDL, but not OxVLDL, to be a potent activator of NF-κB in EAhY926 and HUVEC cells and to be more potent than OxLDL. In the same study, intravenous injection of human VLDL into rats resulted in arterial activation of NF-κB as shown by aortic immunostaining for the active NF-κB RelA (p65) subunit, and this activation was not blocked by pretreatment of the animals for 8 weeks with the antioxidant probucol.

**TRL particle heterogeneity**

Recent evidence suggests that the nature of the TRL particles used and the presence or absence of LPL in the incubation are important conditions that can determine the pro- or anti-inflammatory response to TRL. The TRL preparations used in most studies comprise a highly heterogeneous mixture of TRLs consisting of CMs, VLDL and remnant particles of varying size and density that have different fatty acid, lipid and apolipoprotein compositions. TRLs that enter the circulation from the gut (CMs) or liver (VLDL) contain apo B but acquire apos CII, CIII and E by exchange with other lipoproteins. Because of their roles as co-factors for LPL (apos CII and CIII) and receptor-mediated uptake of TRL and TRL remnants (apo E), the apo content of TRLs could be important determinants of their interactions with endothelial cells. We have recently shown that TRLs formed after ingestion of saturated fats have a significantly higher content of apo CIII and apo E than TRLs formed after ingestion of unsaturated fats [32]. On the basis of this finding and using sequential gradient ultracentrifugation, we prepared three TRL fractions (CMs, $S_i > 400$; VLDL1, $S_i 60–400$ and VLDL2, $S_i 20–60$) from plasma obtained after meals enriched with either saturated, monounsaturated or polyunsaturated fatty acids. We studied the ability of the three fractions obtained under different meal conditions, to promote expression of 13 genes implicated in endothelial activation. All three TRL fractions were shown to cause up-regulation of the expression of E-selectin, VCAM-1 and the LOX1 (lipooxygenase 1) genes and down-regulation of the LDL-r and the COX2 (cyclo-oxygenase 2) genes (Figure 1). However, the impact on gene expression varied according to the TRL fraction used and the nature of the meal fed before collection of the TRL sample. In particular, after stimulation of cells with the VLDL2 particle (usually referred to as the remnant fraction), a consistent pattern of gene expression was observed, where pro-inflammatory genes were less up-regulated (E-selectin, VCAM-1, LOX1) or more down-regulated (LDL-r, COX2) as the TRL fatty acid composition became progressively enriched with unsaturated fatty acids. A consistent fatty acid-specific effect on the expression of pro-inflammatory genes was not observed with the VLDL1 fraction.

The presence of LPL in the incubation media has been shown recently to shift the response to TRL towards an anti-inflammatory response that is PPAR (peroxisome-proliferator-activated receptor)-dependent [33]. Endothelial cells harvested from wild-type mice showed a modest up-regulation of VCAM-1 in response to VLDL, but down-regulation in response to VLDL + LPL. Down-regulation mimicked that seen on addition of a PPAR agonist, and PPAR knockout animals responded neither to agonist nor to VLDL + LPL. We propose that endothelial LPL directs the hydrolysis of TRL to an anti-inflammatory PPAR-dependent pathway. Further evidence of multiple pathways involved in TRL interaction with the endothelium is provided by a study that used a human 8K gene array to evaluate gene expression profiling in EAhY926 cells exposed to VLDL or OxVLDL [34]. The results showed up-regulation in a total of 115 and 206 genes for VLDL and OxVLDL respectively, and down-regulation in 81 and 106 genes for VLDL and
Lipoproteins were isolated from pooled plasma collected 3–8 h after meals enriched in saturated fatty acids (V1S, V2S), monounsaturated fatty acids (V1M, V2M) and polyunsaturated fatty acids (V1P, V2P). EAhy926 cells were treated with isolated VLDL1 and VLDL2 (5 µg/ml apoB) for 6 h, in the presence of 10 ng/ml TNFα. Gene expression was determined by real-time quantitative PCR and normalized to β-actin. The results are presented as fold change in gene expression with respect to the control (+10 ng/ml TNFα, no added lipoprotein). The data represent means ±S.E.M. from six experiments (⁎P < 0.05 compared with saturated VLDL1 or VLDL2, Mann–Whitney U-test).

OxVLDL respectively. For VLDL, many of the up-regulated genes were receptors, signalling kinases and transcription factors, suggesting marked activation of the endothelium in response to physiological concentrations of VLDL. Activation of ERK (extracellular-signal-regulated kinase) 1/2 and inhibition of IκB by VLDL, and activation of p38 MAPK (mitogen-activated protein kinase) by OxVLDL were demonstrated using Western-blot analysis for phosphorylated forms of the signalling proteins. Activation of ERK1/2 strongly suggests receptor-mediated interaction of TRLs with the endothelium, supporting previous evidence that an apo E-dependent mechanism is involved in CM remnant activation of endothelial cells [35].

It is clear that early concepts of TRL particles as essentially pro-inflammatory stimuli to the endothelium, provides an overly simplistic view of their impact on the vascular compartment. Given their close metabolic interaction with the endothelial surface, and the fluctuation in their concentrations throughout the day in response to lipid ingestion, it is probable that highly regulated mechanisms have evolved to ensure that a balance between pro- and anti-inflammatory responses is maintained. Although recent evidence suggests that part of the balance may reflect the relative activation of NF-κB and PPAR-dependent processes, it is clear that others may also be involved. A key question that remains to be determined relates to the compositional characteristics of TRL particles that determine relative activation of pro- and anti-inflammatory pathways. Previous hypotheses, driven by the pro-oxidant-NF-κB paradigm, have focused on the degree of unsaturation of the fatty acid moieties of TRL. It is clear that other compositional characteristics, which may themselves be dependent on the quality and quantity of ingested lipid, will also determine variability in inflammatory response to TRL exposure. Of particular relevance are the apo CII, CIII and E contents, which determine activation/inhibition of LPL and receptor-mediated uptake respectively. Recognition that variation in circulating levels of apos is observed in response to fat ingestion [31], and in certain pathophysiological states [36,37], provides a potentially new avenue of investigation for understanding the normal physiological interaction of these particles with the endothelium and disturbances that lead to endothelial dysfunction.

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References