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LDL receptors, caveolae and cholesterol in endothelial dysfunction: oxLDLs accomplices or victims?

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Oxidized LDLs (oxLDLs) and oxysterols play a key role in endothelial dysfunction and the development of atherosclerosis. The loss of vascular endothelium function negatively impacts vasomotion, cell growth, adhesiveness and barrier functions. While for some of these disturbances, a reasonable explanation can be provided from a mechanistic standpoint, for many others, the molecular mediators that are involved are unknown. Caveolae, specific plasma membrane domains, have recently emerged as targets and mediators of oxLDL-induced endothelial dysfunction. Caveolae and their associated protein caveolin-1 (Cav-1) are involved in oxLDLs/LDLs transcytosis, mainly through the scavenger receptor class B type 1 (SR-B1 or SCARB1). In contrast, oxLDLs endocytosis is mediated by the lectin-like oxidized LDL receptor 1 (LOX-1), whose activity depends on an intact caveolae system. In addition, LOX-1 regulates the expression of Cav-1 and vice versa. On the other hand, oxLDLs may affect cholesterol plasma membrane content/distribution thus influencing caveolae architecture, Cav-1 localization and the associated signalling. Overall, the evidence indicate that caveolae have both active and passive roles in oxLDL-induced endothelial cell dysfunction. First, as mediators of lipid uptake and transfer in the subendothelial space and, later, as targets of changes in composition/dynamics of plasma membrane lipids resulting from increased levels of circulating oxLDLs. Gaining a better understanding of how oxLDLs interact with endothelial cells and modulate caveolae-mediated signalling pathways, leading to endothelial dysfunction, is crucial to find new targets for intervention to tackle atherosclerosis and the related clinical entities.

KEYWORDS

endothelial dysfunction, LDL transcytosis, oxLDL, oxysterols and caveolae

1 | INTRODUCTION

The endothelium is a thin monolayer of cells that covers the luminal surface of the blood vessel wall, creating a barrier between blood and the surrounding tissues and playing an active role in vascular functioning and homeostasis. Physical and biochemical factors, that is glycocalyx, prostacyclin and NO, produced by endothelial cells are involved in the maintenance of vascular tone, antithrombotic activity

Abbreviations: ABCA1, ATP-binding cassette transporter-1; ALK1, activin receptor-like kinase 1; Cav-1, caveolin-1; eNOS, endothelial NO synthase; HMGB1, high-mobility group box 1; ICAM-1, intracellular adhesion molecule-1; LDLR, LDL receptor; LOX-1, lectin-like oxidized LDL receptor 1; MPO, myeloperoxidase; oxLDLs, oxidized LDLs; SR-B1/SCARB1, scavenger receptor class B type 1; TLR4, Toll-like receptor 4; VCAM-1, vascular cell adhesion molecule 1.

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and leukocyte trafficking. Endothelial cells also mediate blood-tissue exchange and participate in haemostasis and neovascularization, acting as a real organ.

Endothelial dysfunction, a complex event triggered by different agents, including cytokines and oxidized LDLs (oxLDLs) results in a pro-inflammatory and pro-coagulant phenotype of endothelial cells. The onset of this condition is considered as a crucial event in the pathogenesis of cardiovascular disease. The expression of cell surface adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule (E-selectin), has been proposed as a biomarker of endothelial cell activation. Also, the decreased synthesis of endothelium-derived NO, which acts as a vasodilator and antithrombotic agent, represents the earliest and one of the most important events contributing to endothelial dysfunction (Liao, 2013).

A reduction in NO availability may occur as a consequence of an accelerated degradation of NO under oxidative stress conditions, that is, NO is transformed into peroxynitrite in the presence of superoxide anions or by a decreased endothelial NO synthase (eNOS) protein expression and/or activity (Förstermann & Münzel, 2006). Direct binding of eNOS to the scaffolding domain of Cav-1, the most abundant protein associated with caveolae, is a recently described mechanism that inactivates eNOS (Chen et al., 2012). Caveolae are important mediators of endocytosis, transcytosis, lipid homeostasis and signal transduction in endothelial cells (Shvets, Ludwig, & Nichols, 2014). The endothelium is indeed permeable to small molecules with a diameter under 6 nm but is nearly impermeable to macromolecules. Thus the transport of lipoproteins, including oxLDLs, across the cell monolayer occurs via transcytosis (Zhang, Sessa, & Fernández-Hernando, 2018). Transcytosis of LDLs into the intima can be associated with their modification (e.g. oxidation) that favour endothelial cell dysfunction (Sun et al., 2010). On the other hand, it has been suggested that circulating oxLDLs and oxysterols may induce perturbations of membrane cholesterol, thus affecting the integrity and dynamics of cholesterol-rich domains such as those pertaining to caveolae (Levitan & Shentu, 2011).

In this review, we discuss the current knowledge on the potential interplay between the uptake and transcytosis of LDLs/oxLDLs and the alteration of caveolae architecture in relation to endothelial cell activation and dysfunction.

2 | LDL OXIDATION, OXYSTEROL FORMATION, AND ENDOTHELIAL DYSFUNCTION

Cholesterol, an important component of membranes, is the most abundant lipid in eukaryotic cells. It is synthesized within cells in the endoplasmic reticulum, although this organelle contains only 0.5–5% of the total cell cholesterol (Iuliano, 2011; Lange, Ye, Rigney, & Steck, 1999) and is transported by LDLs which are leading contributors to atherosclerosis. The endothelium plays a pivotal role in regulating the exchanges of macromolecules between blood and peripheral tissues. Circulating LDLs are taken up by endothelial cells either by

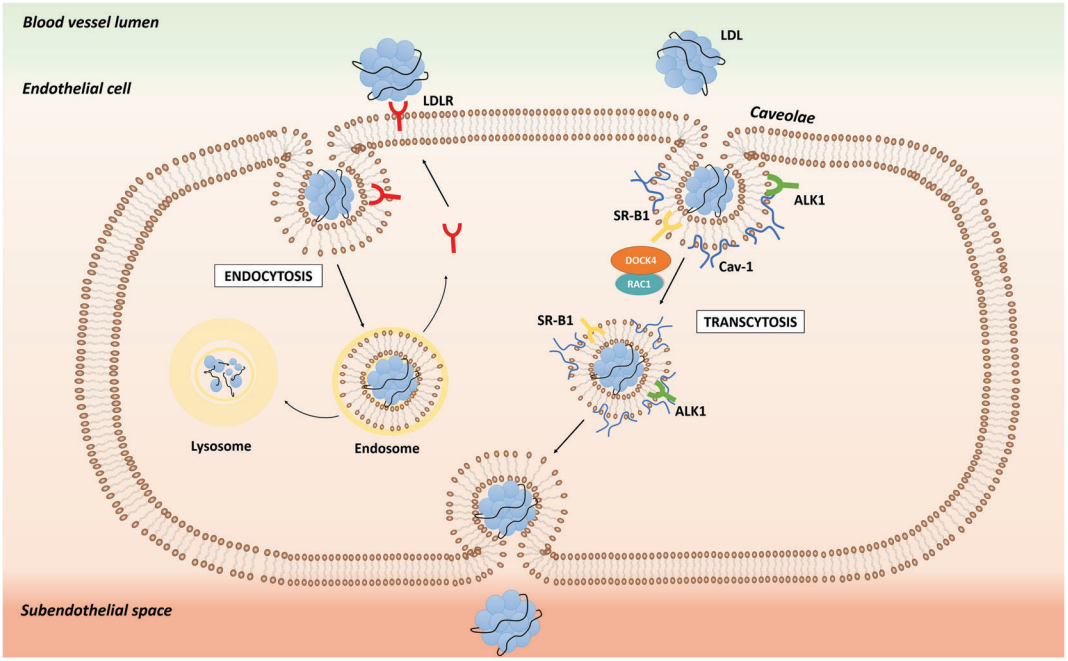

FIGURE 1 Endocytosis and transcytosis of circulating LDLs. The classical LDL receptor pathway mediates LDL uptake for degradation of LDLs in lysosomes (endocytosis). Upon entering the endosomes, LDLs are directed to the lysosomes, whereas the LDLR can be transported back to the cell surface for a new round of LDL binding and uptake. Binding of LDLs to SR-B1 and ALK1 receptors mediates LDL transcytosis. Internalization and transport are mediated by DOCK4 recruitment to SR-B1 receptor and RAC1 activation. Specific mechanisms involved in LDL-loaded vesicle movement and exocytosis are unknown. ALK1, activin receptor-like kinase 1; Cav-1, caveolin-1; LDLR, LDL receptor; SR-B1, scavenger receptor class B type 1; DOCK4, guanine nucleotide exchange factor dedicator of cytokinesis 4; RAC1, Rac family small GTPase 1

receptor-mediated endocytosis or transcytosis. Endocytic receptors bind and transport LDLs to the endolysosomal system where, upon degradation, their components are used for metabolic needs (Figure 1). The transcytotic receptors bind and transport LDLs across endothelial cells to underlying cells and tissues (Figure 1).

LDL transcytosis is stimulated by endothelial cell dysfunction and accumulation and retention of LDLs in the intima are an early step in atherogenesis.

LDL-dependent lipid laden cell formation, which governs initiation and progression of atherosclerotic lesions, is driven by LDL modification, consistently with the LDL oxidation/modification hypothesis of atherogenesis (Steinberg, 1997). Macrophages do not accumulate cholesterol via LDL-receptor uptake because this receptor is down-regulated by intracellular cholesterol content through sterol regulating element binding protein (SREBP). Instead, cholesterol accumulation and subsequent foam cell formation in macrophages occur via the scavenger receptor, which is up-regulated by oxLDL (Steimberg, 2002). In addition, oxLDLs escape interaction with proteoglycans, which retain LDLs in the extracellular matrix (Skålén et al., 2002), favouring their uptake by macrophage scavenger receptors (Öörni, Pentikäinen, Annila, & Kovanen, 1997).

An important question is where LDLs are oxidized in vivo considering that, under physiological conditions, oxidation is unlikely to occur in the circulating blood because LDL are protected by plasma antioxidants (Carmena et al., 1996). OxLDL production mainly occurs in areas of the vessel wall deprived of endothelial cell that have trapped phagocytes and in sub-endothelial matrix.

Oxidized LDLs are formed by diverse mechanisms based on free radicals produced by extra-cellular metal catalysts and enzymes and by activated cells (Kojima, Ino, Ishii, Nitta, & Yoshida, 2010). However, at present, the most important underlying physiological mechanism in vivo remains unclear. An important mechanism leading to the oxidation of LDLs occurs via **myeloperoxidase (MPO)** secreted by activated phagocytes. This enzyme generates oxLDLs by producing hypochlorous acid (HOCl) from H_2O_2 and chloride. Zhang et al. (2013) speculated that MPO could use **NADPH**-derived H_2O_2 in order to produce HOCl, thus promoting the oxidation of LDLs.

Oxidized LDLs are present not only within arterial walls, but they are also found into the circulation, particularly in patients with cardiovascular diseases (Ehara et al., 2001). Recent studies suggest that oxLDLs may be transferred between atherosclerotic lesions and the circulation (Itabe, Obama, & Kato, 2011). In addition, LDL transfer to the lymph (Michel, Nanjee, Olszewski, & Miller, 2015; Reichl, Postiglione, Myant, Pflung, & Press, 1975) suggests a re-circulating mechanism after their transit in the extracellular space, where LDL oxidation can take place (Cooke, Nazeem, Stepanovaa, Olszewskib, & Miller, 2004). Oxidized LDLs are particularly rich in oxysterols, 27-atom carbon compounds formed after enzymatic or non-enzymatic cholesterol oxidation in vivo (Iuliano, 2011). However, oxysterols can also be obtained through the diet. Methods of processing, preparation and storage expose the food to air, light or heat leading to the formation of oxysterols (Lordan, Mackrill, & O'Brien, 2009). Oxysterols are carried by lipoproteins, both as free and esterified forms. The incorporation of oxysterols into LDL particles makes LDLs more susceptible to oxidation (Staprans, Pan, Rapp, & Feingold, 2003; Vine, Mamo, Beilin, Mori, & Croft, 1998).

Oxysterols are involved in many physiological processes such as cholesterol metabolism, hormone and vitamin D synthesis, as well as transmembrane signalling as components of cholesterol enriched membrane microdomains, that is, lipid rafts and caveolae. On the other hand, the accumulation of oxysterols in tissues and organs has been associated with the progression of several diseases, including atherosclerosis, neurodegenerative diseases and cancer (Poli, Biasi, & Leonarduzzi, 2013; Voisin et al., 2017). A growing body of evidence suggests that oxysterols and oxLDLs play a key role in endothelial dysfunction by impairing the formation/production of NO, increasing the formation of reactive oxygen species (ROS), promoting the release of pro-inflammatory cytokines (Lubrano & Balzan, 2014; Maiolino et al., 2013) and inducing endothelial cell death (Luchetti et al., 2015; Luchetti et al., 2019). Oxidized LDLs and oxysterols were shown to markedly increase ROS intracellular levels by activating **NADPH oxidase** (NOX) isoenzymes. Vascular ROS are mainly produced by NOXs present in endothelial cells and smooth muscle cells as well as in infiltrating phagocytes. In endothelial cells induction of NOX by oxLDL has been reported by different authors (Heinloth, Heermeier, Raff, Wanner, & Galle, 2000; Rueckschloss, Galle, Holtz, Zerkowski, & Morawietz, 2001). Moreover, several in vitro studies demonstrated that oxysterols are able to induce the derangement of the mitochondrial membrane potential, thus amplifying ROS production, ultimately leading to mitochondrial dependent-apoptosis (Gargiulo, Gamba, Testa, Leonarduzzi, & Poli, 2016; Vurusaner et al., 2014).

3 | THE ROLE OF CAVEOLAE AND LDL RECEPTORS IN LDL TRANSCYTOSIS

The diameter of LDL particles is about 20–30 nm, that is much larger than that of gap-junctions (3–6 nm) between adjacent cells in continuous endothelium (Iuliano, Micheletta, & Violi, 2001). Hence, the only way for LDLs to cross the endothelium is through a process called caveolae-mediated transcytosis. This pathway operates via fluid phase or receptor-mediated ligand uptake (Fung, Fairn, & Lee, 2018). The classical LDL receptor (LDLR) pathway, which mediates the uptake of LDLs for internalization and subsequent degradation in the lysosomes, does not operate in transcytosis and, actually, does not explain the accumulation of LDLs in the subendothelium of systemic circulation (Dehouck et al., 1997). In addition, the LDLR-mediated pathway is down-regulated at high concentrations of LDLs, while LDLR-independent pathways are enhanced in conditions of hypercholesterolemia (Vasile, Simionescu, & Simionescu, 1983). Thus, transcytosis in endothelial cells is LDLR-independent and, importantly, requires the presence of caveolae (Figure 1).

Caveolae are specialized plasma membrane subdomains consisting of 50–100 nm invaginations of the apical plasma membrane that detach as vesicles to shuttle their cargo to the basolateral membrane where they fuse and release their contents (Figure 1).

Caveolae are present in most cell types but are particularly abundant in endothelial cells, adipocytes, fibroblasts and smooth muscle cells (Chidlow & Sessa, 2010). Like lipid rafts, caveolae are rich in cholesterol, glycosphingolipids and lipid-anchored proteins. Unlike lipid rafts, caveolae are coated with the protein caveolin, a cholesterol binding protein (Sharma, Yu, & Bernatchez, 2010). To date, three caveolin isoforms (Cav-1, -2 and -3), which are expressed at different densities depending on the cell type, have been identified. Cav-1 and Cav-2 are the most expressed in endothelial cells, where caveolae cover up to 40% of the luminal surface of the vascular endothelium. In addition, equipped with a complete set of effector proteins, from extracellular receptors to intracellular transducers, caveolae are involved in signal transduction. Caveolae serve to compartmentalize, modulate and integrate signalling events at the cell surface. Lipid modification (myristoylation and palmitoylation) of proteins appears to help regulating the movement of molecules into and out of caveolae (Galbiati, Razani, & Lisanti, 2001). Specifically, Cav-1 appears to act as a scaffolding protein able to recruit and modulate the activity of caveolae-localized signalling molecules (Patel, Murray, & Insel, 2008). However, despite the wealth of literature supporting the occurrence of this mechanism, structural and bioinformatic analyses do not support such direct physical interactions (Collins, Davis, Hancock, & Parton, 2012), implying that other mechanisms may be involved. Caveolae microdomains are particularly enriched in GPCRs and G proteins. G proteins are likely to directly bind Cav-1 in their **GDP**-bound inactive state. The binding effectively suppresses GTPase activity by inhibiting GDP/**GTP** exchange (Nunez-Wehinger et al., 2014). Small GTP-binding proteins of the **Ras** superfamily also reside in caveolae (Song et al., 1996). Other signalling molecules, which are recruited in their inactive state are certain nonreceptor tyrosine kinases (e.g. **c-Src**, **Fyn** and **Lyn**) (Li, Couet, & Lisanti, 1996) and eNOS, which are held by caveolin in the off state (Busija, Patel, & Insel, 2017). Cav-1 has been shown to regulate downstream effectors of different receptor tyrosine kinases (**RTKs**). It has been reported, for example, that Cav-1 inhibits vascular endothelial growth factor (VEGF) receptor 2 (**VEGFR-2**) signalling through the formation of a molecular complex that rapidly dissociates upon stimulation by **VEGF**, enabling Cav-1 to serve as a substrate for Src kinases (Labrecque et al., 2003). Multiple downstream effectors of RTK such as p42/44 **MAPK** localize to caveolae and are negatively regulated by Cav-1. In addition, several mediators of Ca^{2+} signalling have been found to be associated with caveolae and Cav-1 deficiency in endothelial cells has been shown to impair plasma membrane Ca^{2+} entry (Fujimoto, 1993). All these pathways have important implications for diverse processes in endothelial cells including the response to shear/mechanical stress, cellular proliferation/migration, regulation of vascular permeability/tone and angiogenesis.

Recent studies have reported that Cav-1 deletion suppresses atherosclerosis by attenuating LDL transcytosis. In particular, LDL accumulation in atherosclerosis-prone areas was significantly reduced in Cav-1 deficient mice (Ramírez et al., 2019). In the endothelial cell luminal plasma membrane, the number of caveolae and the protein level of Cav-1 could be locally affected by haemodynamic and mechanical stress, thus favouring LDL infiltration in atherosclerosis-prone regions (Boyd et al., 2003; Frank & Lisanti, 2006).

Both SR-B1 and activin receptor-like kinase 1 (**ALK1**) receptors, which are localized within caveolae, are reportedly involved in LDL loading and subsequent trafficking across endothelial cells. The signalling cascade downstream these receptors which is involved in LDL trafficking and exocytosis remains to be elucidated. Armstrong et al. (2015) provided evidence that SR-B1 plays a role in LDL transcytosis by showing that the infiltration of LDL in the subendothelial space is inhibited in SR-B1 deficient mice. More recently, Huang et al. (2019) reported that SR-B1 directly binds LDL and recruits the guanine nucleotide exchange factor dedicator of cytokinesis 4 (DOCK4) through its cytoplasmic domain. DOCK4 functions as a guanine nucleotide exchange factor (**GEF**) and participates in regulating the actin cytoskeleton (Gadea & Blangy, 2014). DOCK4 serves as GEF for the RHO GTPase **Rac1** that, in turn, is required to sustain SR-B1-mediated LDL internalization and transport (Huang et al., 2019) (Figure 1). Mechanisms involved in LDL-loaded caveolae movement and exocytosis at the basolateral site are poorly understood. Caveolae-mediated endocytosis usually implies activation of Src-family tyrosine kinases which control interactions of actin with Cav-1, thereby regulating caveolae detachment and trafficking (Sverdlov, Shajahan, & Minshall, 2007). Conversely, fusion of the vesicles with the basal membrane of endothelial cells may require soluble **N-ethylmaleimide** sensitive factor attachment protein receptor (SNARE) proteins (Jahn & Scheller, 2006). Interestingly, a higher SR-B1 expression level has been reported before lesion formation in atherosclerosis-prone regions of mouse aorta and in human atherosclerotic arteries compared to normal arteries. This observation supports the notion that atherosclerosis is favoured by increased LDL transcytosis in altered areas of the endothelial barrier rather than by paracellular leaks. A second receptor involved in LDL transcytosis has been identified in ALK1 that functions as a low affinity receptor for LDLs in endothelial cells (Kraehling et al., 2016) (Figure 1). ALK1 is an endothelial cell-restricted TGF β-type receptor that mediates LDL transcytosis independently of its kinase activity. ALK1 is localized in endothelial caveolae, where it functionally interacts with Cav-1 and co-localization of the two proteins is drastically reduced under conditions of plasma membrane cholesterol depletion (Santibanez et al., 2008).

Gerbod-Giannone et al. (2019) recently demonstrated that LDL endocytosis is reduced in Cav-1 or in **CD36**-deficient endothelial cell, suggesting that **CD36** may be involved in the transcytosis of native LDLs across the endothelium as well. However, Huang et al. (2019) point to the role of the CD36 receptor in LDL uptake but not in transcytosis, while identifying ALK1 and SR-B1 as the only receptors involved in transcytosis.

While there is a considerable body of evidence supporting the role of Cav-1, conflicting results are reported in the literature concerning the type of receptors involved in LDL transcytosis. Since LDL uptake and transcytosis are important contributors to

atherosclerotic lesion development and receptors represent important pharmacological targets, these discrepancies underscore the need to further investigate in this area.

4 | THE INTERPLAY BETWEEN oxLDLs AND CAVEOLAE/CAVEOLIN AND ITS IMPACT ON ENDOTHELIAL DYSFUNCTION

Uptake and transcytosis of circulating oxLDLs, together with oxidation of LDLs in the subendothelium, play an important role in the development of atherosclerosis. Transcytosis of oxLDLs via caveolae was suggested by Sun et al. (2010), who showed that two caveolae specific inhibitors, filipin and nocodazole, decrease the uptake of oxLDLs by human umbilical vein endothelial cells and inhibit their efflux. LOX-1 is the major receptor for binding, internalization and degradation of oxLDLs in endothelial cells. LOX-1 is naturally present in caveolae-enriched lipid rafts (Matarazzo et al., 2012; Pirillo, Norata, & Catapano, 2013) and its expression is up-regulated by oxLDLs (Li & Mehta, 2000; Sawamura et al., 1997) (Figure 2). Interestingly, Cav-1 expression is also up-regulated by oxLDLs, while Cav-1 silencing results in decreased LOX-1 expression upon oxLDL administration, suggesting that caveolin participates in LOX-1 regulation. It is known that the binding of oxLDLs to LOX-1 stimulates the development of atherosclerosis through different mechanisms involving:- (i) the activation of MAPK proteins, which causes increased expression of adhesion molecules and chemo-attractants; (ii) the stimulation of NADPH oxidase activity, leading to ROS production, oxidative stress and consequent reduction of NO levels and (iii) the activation of the NF-κB signalling pathway, resulting in cytokine and adhesion molecule production as well as increased expression of LOX-1 itself, thus creating a vicious cycle of proinflammatory signalling (Kattoor, Goel, & Mehta, 2019) (Figure 2).

More recently, the role of LOX-1 in oxLDL uptake has been questioned by LOX-1 knockdown experiments in human aortic endothelial cells. Whereas LOX-1 knockdown did not influence oxLDL internalization, the silencing of LDL and CD36 receptors attenuated oxLDL uptake (Huang et al., 2019). By contrast, a decrease in oxLDL transfer was observed when the expression of SR-B1 was down-regulated, thus suggesting that transcytosis of

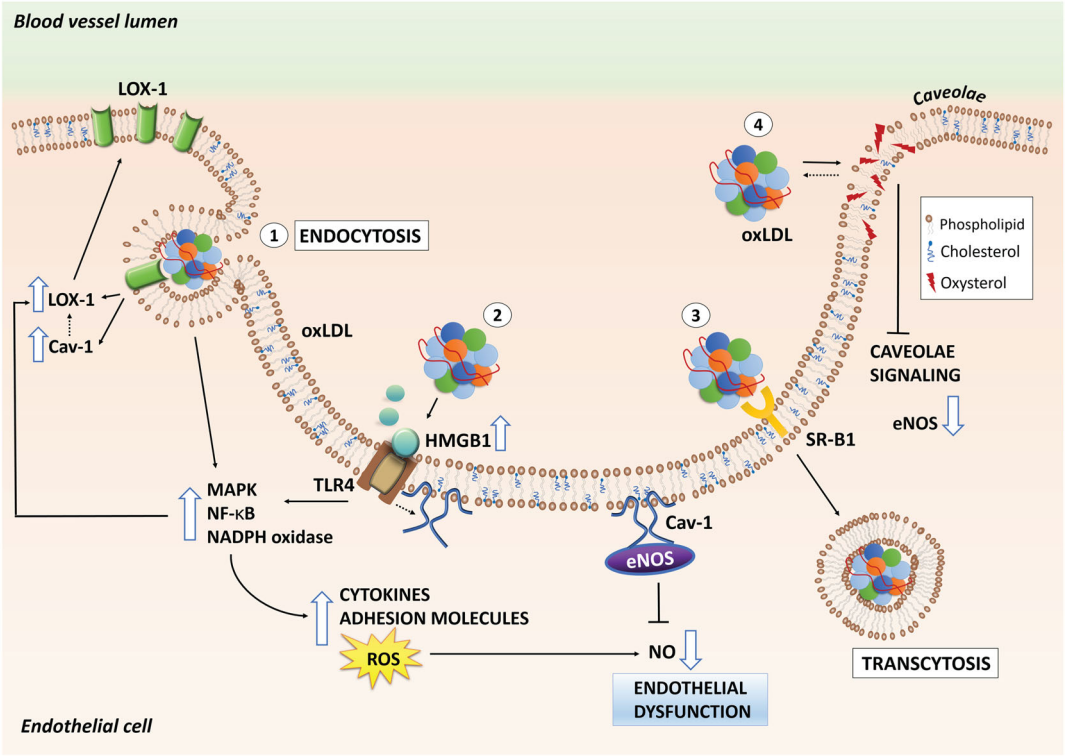

FIGURE 2 Overview of the main interactions occurring between oxLDLs and caveolae and their impact on intracellular signalling in endothelial cells (ECs). ① oxLDLs can be taken up by the EC through LOX-1 receptors to be degraded within lysosomes; oxLDLs up-regulate both LOX-1 and Cav-1 expression, the latter being involved in LOX-1 up-regulation; oxLDLs by binding LOX-1 activate intracellular pro-inflammatory signalling cascades leading to LOX-1 expression and NO overproduction; Cav-1, by binding eNOS reduces NO levels; ② oxLDLs induce HMGB1 extracellular release which in turn activates TLR4-mediated pro-inflammatory signalling; HMGB1 increases Cav-1 and TLR4 expression, the latter being involved in Cav-1 induction; ③ ox-LDLs bind SR-B1 receptors and mediate oxLDL transcytosis; ④ oxLDLs affect membrane cholesterol content by exchanging oxysterols with cholesterol and by inducing cholesterol depletion/redistribution, thus affecting caveolae signalling, including the eNOS/NO pathway. eNOS, endothelial NO synthase; HMGB1, high-mobility group box 1; LOX-1, lectin-like oxidized LDL receptor 1; oxLDLs, oxidized LDLs; SR-B1, scavenger receptor class B type 1; TLR4, toll-like receptor 4; Cav-1, caveolin-1; SR-B1, scavenger receptor class B type 1

oxLDLs in endothelial cells occurs via the SR-B1 receptor (Huang et al., 2019) (Figure 2).

Caveolae are rich in free cholesterol and changes in the content of this lipid can affect the morphology and signalling mediated by caveolae. In this regard, Smart, Ying, Conrad, and Anderson (1994) were the first to show that cholesterol oxidation results in the translocation of caveolin from plasma membrane to Golgi with a modest reduction in the number of caveolae. Subsequently, oxLDLs were shown to deplete caveolar cholesterol and induce the transfer of Cav-1 and eNOS to intracellular compartments, thus enabling NO production (Blair, Shaul, Yuhanna, Conrad, & Smart, 1999) (Figure 2).

The mechanism through which oxLDLs may deplete cholesterol is unknown. It has been hypothesized that oxLDLs may act as a cholesterol acceptor to remove cholesterol from cellular membranes rather than loading cells with cholesterol. A higher efflux of cholesterol induced by oxLDLs has also been proposed, in a mechanism that involves the binding of oxLDLs to CD36 receptors. Finally, a redistribution of cholesterol between membrane-rich and cholesterol-poor domains has been proposed (Shentu et al., 2010). Even though the effects of oxLDLs on membrane cholesterol remain elusive and controversial, the effects of oxLDLs on endothelial cell function impairment are very similar to those observed after experimental-induced cholesterol depletion, suggesting a common mechanism of action (Levitan & Shentu, 2011).

In human umbilical vein endothelial cells, Zhu et al. (2005) demonstrated that oxLDLs can inhibit the transcription of ATP-binding cassette transporter-1 (**ABCA1**), which mediates the active efflux of cholesterol and/or phospholipids. The regulation of ABCA1 by oxLDLs occurs at the transcriptional level through the inhibition of endogenous liver X receptor (**LXR**) ligand production. The role of caveolin in cholesterol homeostasis is less clear. Overexpression of Cav-1 has been shown to up-regulate ABCA1 expression and enhance cholesterol efflux to extracellular effectors (Lin, Ma, Hsu, Lo, & Yang, 2007). Conversely, Cav-1 knockdown has been associated with reduced free cholesterol and increased esterified cholesterol, against minimal effects on cellular cholesterol efflux (Frank et al., 2006). Whether oxLDLs might affect cholesterol homeostasis by directly interfering with Cav-1 levels is not known. Cav-1 seems to be regulated by cellular cholesterol levels (Bist, Fielding, & Fielding, 1997) and caveolin mRNA levels have been found to be up-regulated by free cholesterol but down-regulated by oxysterols in fibroblast monolayers (Fielding, Bist, & Fielding, 1997).

Finally, an exchange in free cholesterol between plasma LDL particles and the luminal surface of endothelial cells is thought to occur (Stender, 1982). In this context, oxLDLs have been shown to induce an increase in endothelial stiffness by direct incorporation of oxysterols into the endothelial plasma membrane (Figure 2) (Shentu et al., 2012). It has been hypothesized that this event could result in the disruption of the structure of lipid-ordered domains, including caveolae. Moreover, there is evidence that oxysterols interact with Cav-1 (Sleer, Brown, & Stanley, 2001). Possibly by interacting with caveolin, 7-ketocholesterol has been shown to increase the activity of src kinases (Myers & Stanley, 1999). Whether this effect influences endothelial cell dysfunction and/or LDL transcytosis has not been elucidated. Thus, although the molecular mechanisms through which oxLDLs lead to endothelial dysfunction need to be fully elucidated, a growing body of evidence points to the important role of direct/indirect disruption of cholesterol homeostasis, which in turn may affect caveolae function and modulate signalling pathways involved in atherosclerosis.

5 | oxLDLs INFLUENCE CAVEOLAE/Cav-1 SIGNALLING

In endothelial cells, caveolae sense and transduce haemodynamic changes into biochemical signals to regulate vascular function. Caveolae compartmentalize signalling proteins in the plasma membrane through direct/indirect interactions with Cav-1 allowing to the fine-tuning the magnitude of signalling cascades.

Within caveolae, Cav-1 functions as a scaffold for several proteins such as eNOS (Shaul, 2003; Williams & Lisanti, 2004) and NADPH oxidase (Chen et al., 2014; Patel & Insel, 2009) two enzymes that have a pivotal role in endothelial dysfunction.

eNOS are a family of enzymes that produce NO using L-arginine as substrate. Three NOS isoforms have been identified:- neuronal NOS (**nNOS**, NOS1), inducible NOS (**iNOS**, NOS2) and eNOS (NOS3), all of which differ slightly in terms of their function and structure. eNOS is constitutively expressed in endothelial cells and the produced NO regulates vascular tone and inhibits platelet aggregation and neutrophil-endothelium interaction (Brunner et al., 2003). eNOS exerts a slow basal activity of NO generation, which in endothelial cells is enhanced by agonists such as **ACh**, **bradykinin** and **histamine**. These agonists increase intracellular calcium, whereas shear stress and hormones increase eNOS activity independently of changes in intracellular calcium (Chen et al., 2018).

eNOS is abundantly represented in endothelial cells and is located in the plasma membrane in close association with Cav-1, Golgi apparatus, cytosol, cytoskeleton and even in the nucleus. However, eNOS is mainly active in the plasma membrane (Fulton et al., 2002). Co-localization and co-immunoprecipitation experiments have shown that the binding of eNOS to Cav-1 inhibits enzyme activity, resulting in reduced NO production (Bucci et al., 2000).

In this context, oxLDLs cause selective depletion of cholesterol within the caveolae resulting in eNOS intracellular redistribution and an attenuated capacity to activate eNOS enzyme (Shaul, 2003) (Figure 2). In addition, oxLDLs promote the expression of several pro-inflammatory mediators, including iNOS, presumably via the MAPKs/NF-κB pathway. This leads to an imbalance between eNOS and iNOS activity with the production of high amounts of NO, which acts as a free radical with bactericidal and inflammatory function (Figure 2) (Gliozzi et al., 2019). The production of high NO concentrations by iNOS causes the generation of high levels of peroxynitrite which has been correlated with apoptosis in endothelial cells (Salvemini, Kim, & Mollace, 2013). Gliozzi et al. suggested that this inflammatory

condition promotes the nuclear translocation of NF-κB switching the signalling from the pro-survival to the pro-apoptotic (Gliozzi et al., 2019; Mollace et al., 2015). In agreement with these findings, we reported that endothelial cell exposed to high concentrations of secosterol B, a product of cholesterol oxidation, undergo apoptosis via a pathway that involves early phosphorylation of the alpha subunit of **eukaryotic translation initiation factor 2 (eIF2α)** and NF-κB activation (Luchetti et al., 2019). Potje, Grando, Chignalia, Antoniali, and Bendhack (2019) recently reported that cholesterol depletion reduced the number of caveolae and promotes eNOS uncoupling which results in free radical production at the expense of NO generation. NO is a competitive inhibitor of oxygen in the cytochrome oxidase present in mitochondrial complex IV leading to O_2 formation. The authors demonstrated that neither siRNA-mediated eNOS knockdown nor pharmacological inhibition of eNOS are able to block the effect of Cav-1 knockdown on increased ROS production suggesting a direct effect of Cav-1 on mitochondrial oxidative metabolism (Shiroto et al., 2014). In addition, Cav-1 deficiency impairs mitochondria function by promoting an increased influx and accumulation of free cholesterol in mitochondrial membranes. This phenomenon affects the efficiency of the respiratory chain and the intrinsic antioxidant defence leading to accumulation of ROS resulting in cell death (Bosch et al., 2011).

Interestingly, in cells exposed to LPS, Cav-1 is phosphorylated at Tyr (14) promoting Cav-1 and Toll-like receptor 4 (**TLR4**) interaction and, thereby, TLR4 activation of MyD88, leading to NF-κB activation and the generation of proinflammatory cytokines (Jiao et al., 2013). Notably, the effects observed in **LPS**-treated cells are mimicked by high-mobility group box 1 (HMGB1), a protein known to accumulate in atherosclerotic lesions and to mediate vascular inflammation. TLR4 activation by HMGB1 in human aortic endothelial cells has been demonstrated by Yang, Han, Kim, Lee, and Park (2016), as shown by the expression of its downstream partner MyD88. Treatment with recombinant HMGB1 was found to increase **ERK** phosphorylation and nuclear translocation of NF-κB. Thus, HMGB1-induced activation of TLRs initiates pro-inflammatory signalling pathways and mediates the release of cytokines and chemokines, thus contributing to vascular inflammation and endothelial dysfunction (Jiao et al., 2013). While there is evidence that oxLDLs can promote cytoplasmic relocation and extracellular release of HMGB1 by endothelial cells (Yu et al., 2012; Zhou, Zhu, Hu, & Shu, 2016) (Figure 2), the role of caveolin in HMGB1-induced TLR4 activation is not clear. HMGB1 increases endothelial cell Cav-1 and TLR4 protein expression, suggesting that TLR4 and Cav-1 may act together. These proteins colocalize in human umbilical vein endothelial cells and knockdown of TLR4 abrogates Cav-1 induction (Jiang et al., 2014). More recently, Lin et al. (2018) provided evidence that oxLDLs promote phosphorylation of Cav-1 in human umbilical vein endothelial cells and increase oxLDL uptake. Intracellular accumulation of oxLDLs induces NF-κB activation and HMGB1 translocation from the nucleus to the cytoplasm resulting in cell apoptosis. NF-κB activation also facilitates Cav-1 phosphorylation and HMGB1 expression. Considering that HMGB1 enhances oxLDL uptake through induction of LOX-1 (Lee et al., 2012), it is plausible that a tight crosstalk between HMGB1, TLR4, NF-κB, LOX-1 and caveolin may occur in response to oxLDLs.

Only a few studies on the impact of oxLDL/caveolae interaction on caveolae/Cav-1-mediated signalling are reported in the literature. Therefore, more research focusing on caveolae/Cav-1 and oxLDL signal transduction is urgent in order to better understand the mechanism of atheroma formation and find new targets of intervention.

6 | CAVEOLAE AND Cav-1 AS DRUGGABLE TARGETS IN oxLDLs-INDUCED ENDOTHELIAL DYSFUNCTION

The evidence that caveolae and Cav-1 are involved both in oxLDL endocytosis and oxLDL/LDL transcytosis makes them important targets for therapeutic interventions in atherosclerosis. The proof of concept comes in Cav-1 knockout studies and it is further supported by studies on the molecular mechanisms of drugs already in clinical use. Statins (3-hydroxy-3-methylglutaryl CoA reductase inhibitors) are the first-line choice for lowering total and LDL cholesterol levels in the atherosclerotic patients. Statins disrupt cholesterol-rich membrane microdomains leading to LOX-1 receptor disorganization at the plasma membrane and impairing oxLDL binding and internalization. Moreover, statins reduce transcytosis, probably by decreasing the number of caveolae in the cell membrane. By decreasing plasma membrane caveolin levels, they also stimulate endothelial NO production (Feron, 2005). However, statins exert effects which are independent of their plasma cholesterol lowering properties. In particular, statins inhibit the synthesis of farnesylpyrophosphate and geranylgeranylpyrophosphate, which cause isoprenylation and thus membrane attachment of small GTPases, including those which are involved in Cav-1 signal transduction and transcytosis (Sessa, 2001).

Among direct caveolin-modulating strategies, a particularly effective intervention involves the use of cell permeable peptides encompassing the Cav-1 scaffold domain. In vitro and in vivo studies have demonstrated that these peptides allow caveolin to uncouple from the signalling machinery in the inner plasma membrane (Navarro, Borroto-Escuela, Fuxe, & Franco, 2014; Sellers, Trane, & Bernatchez, 2012). On the other hand, loss of Cav-1 has been shown to cause ERK1/2 hyperactivation resulting in ventricular hypertrophy (Zhao et al., 2002). In addition, Cav-1 is implicated in cancer, both as a promoter and a suppressor of this disease. Thus, due to the multiple implications of caveolae and caveolins in several cellular processes and cell types, caveolin targeted therapeutics require further development to ensure specificity before clinical applicability can be attained. To this end, it will be important to develop highly specific targets within Cav-1 signalling.

7 | CONCLUSIONS

It is widely accepted that oxLDLs play a pivotal role in endothelial dysfunction and atheroma formation; the mechanisms involved are

less clear. Recent research suggests that oxLDLs may affect caveolae architecture and function in a complex interplay which leads to endothelial dysfunction. Caveolae are widely expressed on endothelial cells where they act as gatekeepers for LDL infiltration into the intima. Subendothelial accumulation and subsequent oxidation of LDL represent key events in atherogenesis. LDL transcytosis in endothelial cells occurs via caveolae and SR-B1 and ALK1 are the main receptors that are involved. On the other hand, oxLDLs are also present in circulation from where they can be taken up at the endothelial cell interface by LOX-1 receptors, inducing the activation of many signalling pathways and leading to the establishment of a pro-inflammatory and pro-coagulant state in endothelial cells. Although SR-B1 has recently been shown to be involved in oxLDL transcytosis, excluding a role for LOX-1, crosstalk between caveolae and LOX-1 seems to exist. Firstly, LOX-1 activity depends on an intact caveolae system. Secondly, its activity regulates the expression of the caveolar protein Cav-1 and vice versa. On the other hand, caveolae are important for signal transduction as they can concentrate and/or segregate not only receptors but also signalling intermediates. Their function is strictly correlated to their rich cholesterol composition, which allows a certain degree of plasticity. Oxidized LDLs and oxysterols have been reported to affect the content and/or the distribution of cholesterol in caveolae. This event is thought to result in aberrations in signalling cascades, which are important in the development of atherosclerosis, but such aberrations have yet to be established. The ability of oxLDLs to act as cholesterol "loaders" as well as "depletors"/plasma membrane "disruptors" highlights the dichotomy concerning our understanding of how oxLDLs impair endothelial function. To reconcile these apparently opposing perspectives, it could be speculated that in the early phases of the atherogenic process, low levels of circulating oxLDLs could promote cholesterol loading. By contrast, in advanced phases, high levels of circulating oxLDLs, released from atherosclerotic plaques, may affect caveolae lipid composition and/or functions. Thus, even though a large body of evidence points to an interplay between caveolae and oxLDLs, as one of the mechanisms underlying oxLDL-induced endothelial cell dysfunction, some aspects of this interaction remain contradictory and the information fragmentary.

Future research is expected to shed light on the connections between these players, focusing on the crosstalk between LDL receptor- and caveolae-mediated signalling and how this may be affected by changes in membrane cholesterol. This is a relevant area of research because of the therapeutically implications of targeting these pathways.

7.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019a; Alexander et al., 2019b; Alexander et al., 2019c).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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