Oxidised LDL internalisation by the LOX-1 scavenger receptor is dependent on a novel cytoplasmic motif and is regulated by dynamin-2

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Summary
The LOX-1 scavenger receptor recognises pro-atherogenic oxidised low-density lipoprotein (OxLDL) particles and is implicated in atherosclerotic plaque formation, but this mechanism is not well understood. Here we show evidence for a novel clathrin-independent and cytosolic-signal-dependent pathway that regulates LOX-1-mediated OxLDL internalisation. Cell surface labelling in the absence or presence of OxLDL ligand showed that LOX-1 is constitutively internalised from the plasma membrane and its half-life is not altered upon ligand binding and trafficking. We show that LOX-1-mediated OxLDL uptake is disrupted by overexpression of dominant-negative dynamin-2 but unaffected by CHC17 or μ2 (AP2) depletion. Site-directed mutagenesis revealed a conserved and novel cytoplasmic tripeptide motif (DDL) that regulates LOX-1-mediated endocytosis of OxLDL. Taken together, these findings indicate that LOX-1 is internalised by a clathrin-independent and dynamin-2-dependent pathway and is thus likely to mediate OxLDL trafficking in vascular tissues.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/13/2136/DC1

Key words: LOX-1, OxLDL, Endocytosis, Dynamin-2

Introduction
Oxidised low-density lipoprotein (OxLDL) is a key initiating factor in atherosclerosis that is recognised by vascular scavenger receptors and triggers conversion of macrophages into lipid-laden foam cells during plaque development, endothelial dysfunction and cellular apoptosis (Lusis, 2000). Lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1, also known as OLR1 and LOX1) is a class E scavenger receptor originally cloned as the major receptor for OxLDL on endothelial cells (Sawamura et al., 1997). LOX-1 is also expressed in macrophages, smooth muscle cells and platelets (Aoyama et al., 2000; Chen et al., 2001a; Yoshida et al., 1998). LOX-1 is a Type II membrane glycoprotein with an extracellular C-type lectin-like ligand-binding domain (Chen et al., 2001b; Ohki et al., 2005), which binds to a diverse range of ligands including OxLDL, phosphatidylserine, apoptotic bodies, bacteria and platelets (Kikutani et al., 2000; Murphy et al., 2006; Oka et al., 1998; Shimaoka et al., 2001).

Genetic and biochemical evidence indicates a role for LOX-1 in cardiovascular disease initiation and progression (Vohra et al., 2006; Dunn et al., 2008). LOX-1 levels are elevated within atherosclerotic plaques (Chen et al., 2000; Kataoka et al., 1999) and LOX-1 allelic polymorphisms can confer an increased risk of cardiovascular disease (Mango et al., 2003; Tatsuguchi et al., 2003). Furthermore, studies on LOX-1-null mice support the view that expression of the gene encoding LOX-1 accelerates the initiation and progression of atherosclerotic plaques (Mehta et al., 2007). LOX-1-mediated OxLDL binding in macrophages stimulates the formation of lipid-laden cells that resemble the foam cells found in atherosclerotic plaques (Smirnova et al., 2004).

However, the molecular and cellular biology underlying LOX-1-mediated OxLDL trafficking is not understood. The best understood mechanisms of receptor-ligand internalisation are clathrin-mediated endocytosis and caveolea- or lipid-raft-mediated uptake (Conner and Schmid, 2003; Mayor and Pagano, 2007) as candidates for LOX-1-mediated ligand uptake. However, the epidermal growth factor receptor (EGFR/ErbB1) can undergo regulated internalisation by both clathrin and lipid-raft-dependent routes. Clathrin-mediated endocytosis may result in EGFR recycling to the cell surface whereas lipid raft-mediated internalisation is thought to result in EGFR degradation in lysosomes (Sigismund et al., 2005). The route taken by EGFR is dependent on activation caused by binding of EGF ligand (Sigismund et al., 2005). Another receptor tyrosine kinase that is degraded in response to ligand binding is vascular endothelial growth factor receptor 2 (VEGFR2) (Ewan et al., 2006). Cellular responses to ligands that activate intracellular signalling require co-ordination with receptor-ligand trafficking, recycling or degradation in the endosome-lysosome system to ensure spatial and temporal regulation of physiological responses.

It is not known whether LOX-1 has a role as an OxLDL trafficking receptor. Here, we have utilised a biochemical and cellular approach to elucidate this mechanism: we have identified a constitutive internalisation pathway for LOX-1 that is regulated by a key GTPase in conjunction with recognition of a novel LOX-1 cytoplasmic motif. In our model, extracellular OxLDL undergoes...
constitutive internalisation via LOX-1 and delivery to the endosome-lysosome system.

**Results**

**OxLDL endocytosis is LOX-1 dependent**

HeLa cells were transfected with the full-length human LOX-1 cDNA containing an engineered FLAG-tag at the extreme C-terminus (LOX-1-FLAG) (Fig. 1A) and LOX-1 expression assessed by western blotting. HeLa cells transfected with the LOX-1-FLAG construct expressed a ~40 kDa polypeptide that was detected by anti-LOX-1 antibodies, but this species was absent in mock-transfected cells (Fig. 1B). This LOX-1 polypeptide corresponds to the molecular mass of human LOX-1 detected in vascular tissues and leukocytes (Xie et al., 2004; Yoshida et al., 1998). This LOX-1 polypeptide represents an N-glycosylated membrane protein, because the mature protein is sensitive to tunicamycin and PNGase F (see supplementary material Fig. S1). In addition, this LOX-1 molecule was detected on the surface of transfected cells (Fig. 1C) and corresponds to the steady-state localisation of endogenous or transfected LOX-1 mammalian orthologues from other studies (Chen and Sawamura, 2005; Sawamura et al., 1997).

To assay for binding of OxLDL to LOX-1, fluorescent labelled DiI-OxLDL was incubated with mock-transfected or LOX-1-FLAG-expressing cells. DiI-OxLDL only bound to HeLa cells expressing LOX-1-FLAG (Fig. 1C). To further confirm that binding of OxLDL was LOX-1 dependent, LOX-1-FLAG-transfected cells were pre-incubated with JTX92, an anti-human LOX-1 antibody that blocks LOX-1 binding to ligand (Li et al., 2003). As expected, the JTX92 antibody blocked OxLDL binding to HeLa cells expressing LOX-1 (Fig. 1C). By contrast, LOX-1 transfected cells incubated with labelled OxLDL revealed accumulation of ligand in punctate perinuclear structures within 1 hour of incubation (Fig. 1D). The codistribution of labelled OxLDL and LOX-1 over time was quantified (Fig. 2). OxLDL colocalised extensively with LOX-1 during the first 15 minutes of internalisation but after 60 minutes, the colocalisation between OxLDL and LOX-1 had been reduced to 24% of that at the starting point (t=0). This indicated that the majority of LOX-1 and OxLDL dissociated from each other early in the endocytic pathway. We attempted to elucidate the effects of antibody labelling of cell surface LOX-1 and subsequent intracellular fate of LOX-1 but this was unsuccessful (data not shown).

**Ligand-independent endocytosis of LOX-1**

To determine whether LOX-1 internalisation is dependent on OxLDL binding, serum-starved LOX-1-FLAG-expressing cells were incubated with or without a saturating concentration of OxLDL (data not shown) at 4°C followed by cell surface labelling and biochemical analysis. Cell surface proteins were biotinylated

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**Fig. 1.** OxLDL binding to HeLa cells expressing LOX-1. (A) Domain structure of the human LOX-1-FLAG construct. CD, cytoplasmic domain; TMD, transmembrane domain. (B) Equal quantities of total cell lysate (30 μg) from mock-transfected and LOX-1-FLAG transfected cells were analysed by western blotting using sheep anti-LOX-1 antibodies or sheep antibodies that recognise a ubiquitous Golgi membrane glycoprotein and loading control (TGN46). (C) Mock and LOX-1-FLAG transfected HeLa cells were incubated with labelled DiI-OxLDL ligand (red) prior to cell fixation, processed and stained with a mouse anti-FLAG antibody. Ligand binding was also performed after pre-incubation with the JTX92 LOX-1 blocking antibody. Cell surface LOX-1 was detected by the mouse anti-FLAG and FITC-conjugated anti-mouse IgG antibodies (green). (D) HeLa cells transfected with LOX-1-FLAG were incubated with DiI-OxLDL (red) in DMEM prior to washing and chasing for different time periods before fixation. Nuclei were stained with DAPI (blue). Scale bar: 20 μm.
using sulfo-NHS-S-S-biotin before incubation at 37°C for 15 or 30 minutes to stimulate endocytosis. This was followed by treatment with the non-permeable reducing agent MESNA to cleave the biotin moiety from the remaining labelled cell surface proteins. Internalised and biotinylated proteins were isolated from detergent-solubilised cell lysates using neutravidin-agarose and analysed by western blotting using anti-LOX-1 antibodies (Fig. 3A). After 30 minutes, either 28% or 27% of cell surface LOX-1 is internalised in the presence or absence of OxLDL, respectively (Fig. 3A). Thus LOX-1 is constitutively internalised. The transferrin receptor TfR (Ajioka and Kaplan, 1986) was used as a control because it also shows constitutive internalisation in the absence of transferrin ligand (Harding et al., 1983) (Fig. 3A).

To test whether ligand recognition affected LOX-1 turnover and degradation, LOX-1-FLAG-transfected cells were incubated with (or without) a saturating concentration of OxLDL followed by cell surface biotinylation. After incubation at 37°C for different time periods, cells were lysed and analysed for biotinylated LOX-1. Total cellular biotinylated proteins were isolated after each incubation time period and probed by western blotting using anti-LOX-1 antibodies. The results clearly show that the half-life of LOX-1 is similar in either the presence or absence of OxLDL ligand (Fig. 3B). Six hours after cell surface biotinylation, 40% or 38% of biotinylated LOX-1 remained in either the presence or absence of OxLDL, respectively. These results, in conjunction with the finding that LOX-1 and OxLDL are separated into different pathways along the endocytic pathway (Fig. 2), indicate that LOX-1 is constitutively internalised at the cell surface and uncoupled from ligand in endosomes (supplementary material Fig. S2). A preliminary study on LOX-1 recycling in comparison to TfR was inconclusive (data not shown).

LOX-1 internalisation is regulated by dynamin-2

The dynamin-2 protein is a mechanoenzyme and a GTPase that regulates plasma membrane internalisation (Diatloff-Zito et al., 1995; Herskovits et al., 1993; van der Bliek et al., 1993). The K44A dominant-negative mutation locks the protein in a membrane-associated GTP-bound state, thus blocking plasma membrane scission, which depends on cycling through GTP and GDP-bound states. Overexpression of dominant-negative dynamin-2 inhibits transferrin internalisation by TfR (Damke et al., 1994) (Fig. 4A). Overexpression of wild-type dynamin-2 did not affect OxLDL internalisation and accumulation of labelled ligand in punctate perinuclear structures (Fig. 4B). However, overexpression of dominant-negative dynamin-2 blocked OxLDL internalisation causing ligand clustering at the plasma membrane (Fig. 4B).

Overexpression of dominant-negative dynamin-2 also caused LOX-1 clustering at the plasma membrane in contrast to wild-type dynamin-2 overexpression, which had no significant effects (Fig. 4C). A 0.5 μm confocal section through a transfected cell coexpressing LOX-1 and dominant-negative dynamin-2 in transfected cells revealed substantial colocalisation with OxLDL ligand at the plasma membrane positioned over the cell apex (Fig. 4C). By contrast, a similar analysis of transfected cells overexpressing both LOX-1 and wild-type dynamin-2 proteins showed LOX-1 accumulation at the plasma membrane, whereas OxLDL accumulated in punctate perinuclear structures showing significant divergence in post-plasma-membrane trafficking (Fig. 4C). This suggests that prolonged inhibition of endocytosis due to expression of dominant-negative dynamin-2 caused LOX-1 to
become clustered at the plasma membrane. To verify that the clustered LOX-1 was indeed arrested at the cell surface, extracellular N-linked carbohydrates were labelled with a fluorescent-labelled lectin, concanavalin A (Con A) (Bhattacharyya et al., 1991) (Fig. 4D). The LOX-1 glycoprotein showed substantial colocalisation with Con-A-labelled N-linked carbohydrates supporting the accumulation of glycosylated species at the plasma membrane including the mature N-glycosylated LOX-1 (Fig. 4D, arrow). Quantification showed that >90% of cells expressing wild-type dynamin-2 protein showed OxLDL accumulation in punctate perinuclear structures whereas <5% of cells expressing dominant-negative dynamin-2 displayed perinuclear OxLDL accumulation (Fig. 4E). Thus a functional dynamin-2 GTPase activity is required for LOX-1-dependent accumulation of OxLDL within the cell.

Hypertonic treatment blocks LOX-1-mediated OxLDL internalisation

To test for requirement for clathrin and/or cytosolic factors in LOX-1-mediated OxLDL internalisation, LOX-1-transfected cells were subjected to potassium depletion or hypertonic treatment. These conditions disrupt clathrin-coated pit assembly and endocytosis (Heuser and Anderson, 1989; Larkin et al., 1983). Both potassium depletion or hypertonic treatment inhibited TfR-regulated transferrin endocytosis (Fig. 5A). Potassium depletion caused a 55% reduction in endocytosis of OxLDL (Fig. 5D). Similarly, hypertonic treatment of cells caused a 62% reduction in OxLDL endocytosis (Fig. 5D). These inhibitory effects on OxLDL endocytosis caused by either potassium depletion or hypertonic treatment were reversible, thus showing that such conditions were not causing cytotoxic effects that irreversibly blocked clathrin-mediated uptake (Fig. 5C). To test further whether potassium depletion and hypertonic treatment were specific for clathrin-mediated uptake, a labelled ganglioside GM1 was used. GM1 can be internalised by both clathrin and caveolae or raft-based pathways (Singh et al., 2003). Neither potassium depletion nor hypertonic treatment prevented the internalisation of fluorescent GM1 (data not shown), thus indicating that caveolae or lipid-raft-linked internalisation pathways are relatively unaffected under such conditions.

LOX-1-mediated internalisation by a clathrin-independent pathway

To further characterise internalisation of LOX-1-OxLDL, we investigated the colocalisation of internalised OxLDL with clathrin and caveolin-1, which represent two major dynamin-2-regulated pathways for plasma membrane uptake. Microscopy was carried out on transfected HeLa cells expressing tagged LOX-1, which were labelled on ice with OxLDL and then warmed to 37°C for a short period (10 minutes) to allow internalisation. Analysis of labelled OxLDL or LOX-1 with clathrin light chain or caveolin-1 indicated increased codistribution with clathrin but not caveolin-1 (supplementary material Fig. S3). This was also similar to results observed for TfR (supplementary material Fig. S3). To further analyse LOX-1 endocytosis dependence on clathrin and cytosolic factors, we carried out RNAi to deplete clathrin heavy chain (CHC17) or the μ2 subunit (of the AP2 adaptor complex) followed by endocytosis of labelled transferrin and OxLDL in LOX-1-transfected HeLa cells (Fig. 6). Depletion of either CHC17 or μ2 blocked transferrin uptake (via TfR) and showed plasma membrane transferrin accumulation (Fig. 6A, arrows in left panels). However, analysis of the same cells overexpressing LOX-1 showed that OxLDL internalisation was not blocked (Fig. 6A). Western blotting revealed >90% depletion of CHC17 or μ2 using RNAi (Fig. 6B). Quantification of internalised labelled transferrin and OxLDL in controls, CHC17 or μ2-depleted cells showed that whereas OxLDL uptake was relatively unaffected, transferrin uptake was reduced by ~40% upon CHC17 depletion or ~50% upon μ2 depletion (Fig. 6C). This suggests that CHC17 and μ2 are not required for LOX-1-mediated OxLDL uptake. Thus LOX-1 and OxLDL endocytosis must occur via a clathrin-independent pathway.
Fig. 4. Dynamin-2 regulates LOX-1-mediated OxLDL internalisation. HeLa cells cotransfected with tagged LOX-1 and Myc-tagged wild-type (WT) dynamin-2 or Myc-tagged K44A dominant-negative dynamin-2 were incubated with (A) rhodamine-conjugated transferrin (red) for 15 minutes or (B) pulsed with OxLDL (red) for 5 minutes followed by chase at 37°C (for 55 minutes) before fixation and labelling with mouse anti-Myc and FITC-conjugated anti-mouse IgG antibodies (green). Panels in A represent whole cell projections and nuclei are stained with DAPI (blue). Images in B represent 0.5 μm optical sections through the cell nucleus (middle) or at the cell apex (top). Asterisks indicate cell nuclei. (C) HeLa cells cotransfected with tagged LOX-1 and Myc-tagged WT dynamin-2 or Myc-tagged dominant-negative dynamin-2 were pulsed with labelled OxLDL (red, i) followed by fixation and incubation with sheep anti-LOX-1 and Cy5-conjugated anti-sheep IgG antibodies (displayed as green, ii) and mouse anti-Myc and FITC-conjugated anti-mouse IgG antibodies (not shown). (iii) Merged images with colocalisation of labelled OxLDL and LOX-1 shown as yellow. (iv) Z-axis image view of cells shown in panel iii. Images represent projected stacks of whole cells. (D) Labelling of cell surface glycoproteins and LOX-1 on HeLa cells transfected with LOX-1 and dominant-negative dynamin-2 using FITC-conjugated Con A (Con A, green, i) followed by sheep anti-LOX-1 and Alexa Fluor 594-conjugated anti-sheep IgG antibodies (red, ii) and mouse anti-Myc and Alexa Fluor 633-conjugated anti-mouse IgG antibodies (not shown). Con A labelling was performed on ice prior to fixation and processing for microscopy. (iii) Merged image with colocalisation appearing yellow. (iv) Z-axis image view of cells shown in panel iii. Images represent projected stacks of whole cells. Scale bars: 10 μm. (E) The percentage of WT or DN dynamin-2 transfected cells with internalised labelled OxLDL was evaluated. Data represent the mean ± s.e.m. (n=3 experiments). Comparison to control to calculate P values, *P<0.001.
Fig. 5. LOX-1-dependent OxLDL endocytosis is inhibited by potassium depletion. (A) HeLa cells were incubated with rhodamine-conjugated transferrin (red) for 15 minutes under control conditions or in potassium-free or hypertonic buffers as described in Materials and Methods. The plasma membrane was labelled with FITC-conjugated Con A (green) immediately before fixation. (B) LOX-1 expressing HeLa cells were pulsed with labelled OxLDL (red) for 5 minutes and chased at 37°C (for 55 minutes) in control, potassium-free or hypertonic buffers before labelling the plasma membrane with FITC-conjugated Con A (green) and fixation. (C) Addition of exogenous 10 mM KCl or addition of normal media for 1 hour reverses the inhibitory effects (washout) on labelled OxLDL uptake. Scale bars: 10 μm. Asterisks indicate cell nuclei. (D) The amount of internalised OxLDL under these conditions was again calculated as described in Materials and Methods (n=3 separate experiments in which five cells were quantified, values are mean ± s.e.m.). Comparison with control to calculate P values, *P<0.05.
A novel cytoplasmic acidic motif within LOX-1 regulates internalisation

Short cytoplasmic motifs regulate receptor-mediated endocytosis in eukaryotes: these include YxxΦ, NPxY and di-leucine-based motifs (Bonifacino and Traub, 2003). Multiple sequence alignment of the LOX-1 cytoplasmic domain from mammalian species (Fig. 7A) reveals a lack of conserved sequences that bear homology to previously characterised endocytic signals. However, the existence of highly conserved acidic residues was noticed including a completely conserved di-aspartate motif (residues 4 and 5) and another completely conserved aspartic acid residue (residue 16). In addition, a partially conserved GL motif (residues 26-27) may be a potential di-leucine-based motif (Fig. 7A). In human LOX-1, the non-conserved aromatic residues such as a phenylalanine (residue 3) and a tyrosine (residue 31) could be part of non-conventional endocytosis motifs. Site-directed mutagenesis was used to substitute each residue for alanine within tagged LOX-1 (Table 1).

Fig. 6. Clathrin and AP2-independent uptake of OxLDL by LOX-1. Following RNAi, LOX-1 was expressed in transfected HeLa cells and labelled transferrin and OxLDL uptake was monitored (see Materials and Methods) by microscopy (A) and quantified (C). (A) HeLa cells subjected to RNAi through mock treatment (mock), a control scrambled siRNA duplex (scrambled), a siRNA duplex specific for the clathrin heavy chain (CHC17) or a siRNA duplex specific for the μ2 subunit of the AP2 adaptor complex (μ2) on cells expressing LOX-1-FLAG. After 12 hours, cells were incubated with Alexa Fluor 488-transferrin and Dil-OxLDL for 15 minutes followed by 30 minute chase and then fixation and confocal laser-scanning microscopy. Arrows (left hand panels) indicate plasma membrane transferrin accumulation in cells (*) where clathrin or AP2-mediated uptake is inhibited. In right panels, transverse z-axis sections are also shown to visualise intracellular staining; small arrows denote endosomes containing labelled OxLDL. Scale bars: 10 μm. (B) Western blotting to demonstrate depletion of endogenous protein levels after RNAi treatment using CHC17 siRNA (lane 1), μ2 siRNA (lane 2), scrambled siRNA (lane 3) and mock-transfected cells (lane 4). (C) Quantification of uptake of labelled transferrin and OxLDL ligands in LOX-1-transfected HeLa cells (n=30, error bars indicate s.e.m.) was carried out as described in Materials and Methods.
Endocytosis of OxLDL via LOX-1

The LOX-1 mutants were assessed for their ability to internalise labelleled OxLDL compared with wild-type LOX-1. Transfection and expression of each LOX-1 mutant protein was verified by western blot analysis and expression of an endogenous membrane protein (TGN46): the different mutations did not affect LOX-1 expression, stability or turnover (data not shown). All wild-type and LOX-1 mutants were localised to the plasma membrane (Fig. 7B; supplementary material Fig. S4B). The LOX-1 mutants were assessed for their ability to internalise labelled OxLDL compared with wild-type LOX-1 (Fig. 7B). Transfected HeLa cells were pulsed with labelled OxLDL and cells with internalised OxLDL were quantified (Fig. 7C). Alanine substitution of LOX-1 residues that caused the most significant effects on OxLDL uptake were D4 (40% inhibition), D5 (92% inhibition) and L6 (43% inhibition) (Fig. 7C). By comparison, other mutations around this site had little effect on LOX-1-mediated OxLDL uptake.

Importantly, cells expressing LOX-1 containing either the D4A, D5A or L6A mutations were not only unable to internalise OxLDL but also displayed substantial colocalisation of OxLDL ligand with clustered LOX-1 at the cell surface (Fig. 7B, arrows). This morphology was qualitatively similar to that previously observed in cells expressing dominant-negative dynamin-2 and LOX-1 (Fig. 4C). Again, the plasma membrane clusters of LOX-1-D4A, -D5A or -L6A mutants showed substantial colocalisation with labelled Con A used to detect N-glycosylated plasma membrane proteins (Fig. 7B). The LOX-1-DD(4,5)AA double mutant displayed an intermediate effect with ~50% inhibition in OxLDL uptake; however, the LOX-1-D16A, GL(25,26)AA and Y31A mutants all exhibited no significant effects on OxLDL uptake (supplementary material Fig. S4). Thus, a partially conserved acidic tripeptide motif (DDL) mediates constitutive internalisation of the LOX-1 membrane protein.

Discussion

Although the pro-atherogenic nature of OxLDL is well established, relatively little is known about scavenger receptor-mediated lipid particle internalisation, trafficking and processing during atherosclerotic plaque formation and progression (Bobryshev, 2006). In this study on the human LOX-1 scavenger receptor, we have delineated a clathrin-independent but dynamin-2-dependent pathway that regulates the endocytosis and accumulation of OxLDL. First, OxLDL internalisation via LOX-1 was inhibited by alanine substitution of three conserved acidic residues in the LOX-1 cytoplasmic domain (DDL). These results are consistent with previous studies showing that D- and E-rich endosomal domains mediate clathrin- and dynamin-dependent endocytosis in other proteins (Schmid and Simons, 1997; Anderson et al., 1999). The partial conservation of these motifs among species (Table 1) suggests that DDL-mediated internalisation is evolutionarily conserved.

Table 1. Mutational analysis of the LOX-1 cytoplasmic domain*

| Point mutation | LOX-1 cytoplasmic domain sequence |
|----------------|----------------------------------|
| Wild-type      | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| T2A           | MTFAKLQKTVQDPEKSKNGKAKGLQFLSP   |
| F3A           | MTAFKLQKTVQDPEKSKNGKAKGLQFLSP   |
| D4A           | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| D5A           | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| L6A           | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| K7A           | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| F8A           | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| Q9A           | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| DD(4,5)AA     | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| D16A          | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| GL(26,27)AA   | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |
| Y31A          | MTFDDLQKTVQDPEKSKNGKAKGLQFLSP   |

*Alanine-scanning mutagenesis of specific residues within the LOX-1 cytoplasmic domain is indicated by bold underlined lettering.

![Fig. 7. The LOX-1 cytoplasmic domain contains a novel aspartate-based endocytic motif. (A) Multiple sequence alignment of mammalian LOX-1 cytoplasmic domains. Completely conserved amino acid residues are indicated by an asterisk, conservative amino acid substitutions are indicated by a colon and predominantly conserved amino acid substitutions are indicated by a full stop. LOX-1 wild-type and mutant proteins were expressed and OxLDL uptake monitored (see Materials and Methods) by microscopy (A) and quantified (B). (A) Fixed cells were labelled with mouse anti-FLAG and FITC-conjugated anti-mouse IgG antibodies. Arrows indicate areas of clustered LOX-1 and OxLDL ligand at the plasma membrane. (C) The uptake of labelled OxLDL in LOX-1-transfected HeLa cells were quantified (see Materials and Methods). The percentage of transfected cells with internalised OxLDL was counted (n=3 separate experiments, 50 cells from each experiment, mean ± s.e.m.). Comparison with control LOX-1 WT was used to calculate P values, *P<0.01.](image-url)
by the expression of a dynamin-2 mutant that blocks receptor-ligand uptake. Second, hypertonic treatment completely blocked uptake of OxLDL by LOX-1. Furthermore, site-directed mutagenesis of the LOX-1 cytoplasmic domain identified a novel partially conserved tripeptide motif (DDL) proximal to the N-terminus (+4 to +6) that regulates efficient receptor-mediated endocytosis. The di-aspartate sequence is completely conserved in LOX-1 mammalian orthologues (Fig. 8), indicating the functional significance of this motif. Finally, the receptor undergoes constitutive internalisation and the binding of OxLDL ligand does not significantly alter receptor trafficking or degradation.

Our results also show that LOX-1 undergoes constitutive endocytosis and neither its rate of internalisation nor its half-life is significantly altered upon binding OxLDL; the effects of antibody crosslinking on receptor trafficking were not addressed in this study. Intracellular LOX-1 staining diverges from internalised OxLDL ligand after 30 minutes of trafficking along the endocytic pathway. The LOX-1-OxLDL complex probably dissociates within the acidic environment of the endosome, a feature common for other lipoprotein particle receptors such as LDL-R (Davis et al., 1987). One possibility is that LOX-1 is recycled, but our current assay is currently not sensitive enough to detect this aspect of trafficking. Alternatively, LOX-1 could be stationed in a different endosomal compartment after uncoupling from bound OxLDL; in this case, LOX-1 may have a relatively long half-life and thus might be protected from degradation by lysosomal proteases.

The dynamin-2 GTPase regulates multiple plasma membrane internalisation events including clathrin-dependent, caveolae-dependent and clathrin/caveolin-independent steps (Conner and Schmid, 2003; Mayor and Pagano, 2007). Depletion of either the clathrin heavy chain (CHC17) or μ2 subunit of AP2 adaptor complex blocked transferrin uptake, but OxLDL internalisation via LOX-1 was not affected. In this model for OxLDL uptake via LOX-1, a clathrin-independent pathway is utilised to effect delivery of the receptor-ligand complex to the endosomal network (Fig. 8).

How does the LOX-1 cytoplasmic motif (DDL) compare with other trafficking motifs for plasma membrane endocytosis? Importantly, cytoplasmic signals for clathrin-independent uptake are not well defined (Mayor and Pagano, 2007); our study is one of the first to define a tripeptide cytoplasmic motif that regulates receptor-ligand uptake by such a route. In contrast to the well-characterised YxxΦ and di-leucine motifs that interact with the AP2 adaptor complex to specify inclusion into clathrin-coated vesicles, the NPxY motif found in LDL-R interacts with other clathrin-associated adaptor proteins such as ARH and Dab2 (Bonifacino and Traub, 2003). It has also been noted that plasma membrane LDL-R and EGFR appear to undergo endocytosis via different clathrin-dependent pathways (Motley et al., 2003). The DDL motif does not resemble such motifs and the RNAi depletion of clathrin heavy chain or μ2 (AP2) point to another cytosolic factor or ‘coat complex’ that mediates LOX-1 recognition and endocytosis. Intriguingly, hypertonic treatment is well known to perturb clathrin and AP2-mediated endocytosis; this treatment also completely blocks LOX-1-mediated ligand uptake. These findings suggest that LOX-1-mediated endocytosis is dependent on recognition of this unique tripeptide motif by cytosolic factors that are clathrin-independent but still susceptible to perturbation by hypertonic conditions. In support of this, it has been noted that the clathrin-independent endocytosis of certain G-protein-coupled receptors (Cinar and Barnes, 2001; Idkowiak-Baldys et al., 2006) and the Menkes’ disease copper ATPase (ATP7A) (Lane et al., 2004) are also blocked by hypertonic treatment.

The acidic motif (DDL) is required for LOX-1-mediated ligand uptake (Fig. 7). Clusters of acidic residues can influence the trafficking of proteins, such as the human cytomegalovirus glycoprotein B (Tugizov et al., 1999) and the pseudorabies virus Us9 envelope protein (Brideau et al., 1999). Recently, glutamate-based motifs in the cytoplasmic domain of the potassium channel Kir3.4 subunit have been shown to mediate the endocytosis and/or recycling of membrane proteins via a clathrin-independent Arf6-dependent pathway (Gong et al., 2007). Acidic sequences are also responsible for basolateral sorting of the LDL-R and for targeting the dendritic cell receptor DEC-205 (CD205) to late endosomes (Matter et al., 1994; Mahnke et al., 2000).

LOX-1 belongs to one of several diverse classes of eukaryote scavenger receptors that bind OxLDL, apoptotic bodies and phospholipid bodies (Murphy et al., 2005). LOX-1 is a scavenger receptor whose levels are elevated in vascular tissues under pro-inflammatory conditions; this molecule could play a major role in contributing to lipid accumulation during atherosclerotic plaque formation (Smirnova et al., 2004). Our results provide a model (Fig. 8) in which LOX-1 undergoes constitutive endocytosis by a clathrin-independent pathway and delivery to endosomes. In this way, LOX-1 would act as a true ‘scavenging’ receptor, allowing the continuous uptake of OxLDL into the cell.

Fig. 8. Model for trafficking of the LOX-1-OxLDL complex. The findings indicate that LOX-1 constitutively cycles between the plasma membrane (PM) and endosomes (E) in the absence of ligand. LOX-1 and LOX-1-OxLDL complexes are internalised via a dynamin-2 and clathrin-independent mechanism, which may involve the recruitment of cytosolic factors. The majority of LOX-1 dissociates from OxLDL early in the endocytic pathway and may recycle to the plasma membrane, whereas the OxLDL traffics to later endocytic compartments (L). It is likely that some LOX-1 does not dissociate from OxLDL and traffics to later endocytic compartments with subsequent degradation in a late endocytic or lysosomal compartment. Thus, LOX-1 is able to mediate the continuous uptake of OxLDL into the cell.
l-ligand binding

Cells were plated on sterile glass coverslips and transfected as described previously (Murphy et al., 2006) with the indicated plasmids. Cells were pulsed with 10 μg/ml Dil-OxLDL in complete medium for 5 minutes at 37°C and then washed three times with PBS. Cells were fixed and processed for immunofluorescence microscopy or chased for the indicated time periods at 37°C before fixation and processing. To block OxLDL binding, cells were incubated with 10 μg/mL LOX-1 blocking antibody JTX02 (Li et al., 2003) in complete medium for 30 minutes at 37°C and washed three times before addition of labelled OxLDL. To monitor transferrin internalisation by microscopy, 50 μg/ml tetramethylrhodamine-conjugated transferrin (Invitrogen) was incubated with cells in the indicated buffers for 15 minutes at 37°C prior to washing, fixation and processing. For control uptake, 1 μM BODIPY FL C2-ganglioside GM1 (Invitrogen) was incubated with cells for 30 minutes at 37°C in the indicated buffers prior to washing, fixation and processing.

Immunofluorescence microscopy

Immunofluorescence was performed as exactly described previously (Towler et al., 2000). Cells were fixed in 3% (v/v) paraformaldehyde in PBS and quenched with 50 mM ammonium chloride in PBS. Where stated, cells were permeabilised with 0.1% (v/v) Triton X-100 in PBS for 4 minutes. For experiments involving Dil-labelled OxLDL, cells were permeabilised with 0.3% (v/v) Tween-20 in PBS for 4 minutes (Lukas et al., 1998). All subsequent incubations were carried out in 0.2% (v/v) fish skin gelatin in PBS. Coverslips were mounted in Fluoromount G (SouthernBiotech, Birmingham, AL). To label N-linked carbohydrates attached to cell surface glycoproteins, samples were incubated with 10 μg/ml FITC-conjugated Concanavalin A (Sigma) in PBS containing 1 mM CaCl₂ for 5 minutes at 4°C immediately before fixation.

Microscopy and quantification

High-resolution images were collected using the DeltaVision Optical Restoration Microscopy System (Applied Precision, Issaquah, WA) with an Olympus IX-70 epifluorescence microscope and 60x objective. 0.2-μm-thick optical sections were collected and datasets deconvolved using the SoftWoRx deconvolution algorithm. Quantification of colocalisation was determined using the IMARIS software suite (Bitplane AG, Zurich, Switzerland) on selected cell regions as described previously (Herbert et al., 2005). Background was eliminated by excluding pixel values lower than 10% of the maximum pixel intensity. Colocalised pixels were expressed as a percentage of the total pixels selected.

For quantification of accumulated OxLDL uptake, samples were analysed using a Zeiss LSM 510 Meta confocal microscope attached to an Axiovert 200M inverted microscope using AIDA (Advanced Image Data Analyzer) 2.11 software (FujiFilm, Japan). The region of interest (ROI) within each fluorescence channel image was measured using AIDA (Bitplane AG, Zurich, Switzerland) on selected cell regions as described previously (Towler et al., 2000). Background pixel intensities were subtracted from all readings.

For quantification of accumulated labelled transferrin and OxLDL in RNAi-treated cells and control samples, an intensity threshold was previously described for immunofluorescence microscopy. Images were captured in sequential scanning mode using a Zeiss LSM-510 Meta confocal microscope and a Zeiss Plan Apochromat 63× oil immersion lens (NA 1.4). The pinhole was set such that each optical section was 1 μm thick. DIC contrast enhanced transmitted light images were captured simultaneously. All the images were acquired at a 1024×1024 pixel resolution with 4096 grey levels per pixel. Each channel image was then exported as a 12-bit black and white TIFF into Metamorph 5.0 for quantification (Molecular Devices, Sunnyvale, CA). The region of interest (ROI) within each cell was traced using the MetaMorph Trace Region tool. The ROI outline was transferred (copied) into each fluorescence channel image to quantify the fluorescence intensity within the same region of the cell. The mean pixel intensity was calculated for each channel in the ROI. To eliminate any background fluorescence the pixel intensity was only measured in the brighter cellular regions with pixel intensity threshold (20% of the total gray level scale) was measured, where pure black pixel=0 and pure white=4095. At least 30 cells were analysed per treatment. Raw data was exported into Excel, transferred and presented using GraphPad Prism 5.0. The mock-transfected cells were used as the baseline control and normalised as 100% transferrin or OxLDL uptake per cell profile.

Cell surface biotinylation assay

These assays are based on a previously described procedure (Marijanovic et al., 2006). Cells were seeded in six-well dishes and transfected with LOX-1-FLAG as described. After 48 hours, cells were starved in serum-free medium for 2 hours, chilled on ice and incubated with or without 10 μg/ml OxLDL in serum-free medium for 1 hour at 4°C. Cells were then washed three times with PBS+ (PBS containing 0.7 mM CaCl₂, 0.5 mM MgCl₂) and incubated with 0.3 mg/ml EZ-Link-Sulfo-NHS-S-S-biotin (Pierce, Rockford, IL) in PBS+ for 30 minutes at 4°C to biotinylate cell surface proteins. Cells were then washed three times with TBS to remove and quench unbound

Materials and Methods

Reagents

Well-characterised affinity-purified sheep anti-LOX-1 and sheep anti-TGNa46 antibodies have been previously described (Murphy et al., 2006; Towler et al., 2000). Mouse monoclonal anti-LOX-1 blocking antibody (JTX02) was a kind gift from T. Sawamura (National Cardiovascular Center Research Institute, Osaka, Japan). Rabbit anti-clathrin light chain polyclonal and mouse anti-CHC17 (X22) monoclonal antibodies were kindly provided by F. Brodsky (University of California, San Francisco, CA). Anti-Myc monoclonal antibody was obtained from the mouse 9E10 hybridoma cell line from the European Cell and Animal Cultures Collection (ECACC, Porton Down, UK). Mouse M2 anti-FLAG monoclonal antibody was from Sigma (Poole, UK). Mouse anti-TIR monoclonal antibody (clone H68.4) was from Invitrogen. Rabbit anti-caveolin-1 was from BD Transduction Laboratories (Oxford, UK). Mouse monoclonal anti-LOX-1 blocking antibody (clone H68.4) was from Jackson ImmunoResearch Laboratories (West Grove, PA); secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 were from Invitrogen, (Amsterdam, The Netherlands). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

Preparation and labelling of oxidised LDL

Human LDL particles were isolated from plasma, chemically modified to make OxLDL and analysed using established procedures as described previously (Vohra et al., 2007). OxLDL particles were labelled with a fluorescent dye, 1,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen), also as described previously (Vohra et al., 2007).

Gene manipulation, transfection and RNAi

Full length human LOX-1 was cloned into pcDNA3.1+ (Invitrogen) in conjunction with a FLAG tag at the C-terminus of LOX-1 as described previously (Murphy et al., 2006). Alamine substitutions were introduced into the LOX-1 sequence by site-directed mutagenesis using Quikchange (Stratagene). All mutations were confirmed by DNA sequencing. A mammalian expression plasmid (pCMV5) containing either rat wild-type dynamin-2 or dominant-negative (K44A) dynamin-2 DNA fused to a Myc tag at the N-terminus was a kind gift of H. McMahon (MRC Laboratory of Molecular Biology, Cambridge, UK). HeLa cells were transfected with DNA plasmids using a calcium phosphate method (Towler et al., 2000) and assayed 48 hours post transfection.

Synthetic oligonucleotide duplexes (Ambion) corresponding to the mRNAs of clathrin heavy chain (GGGGUCGAGAUAUCAAU) or µ2 (GGGUGUUAGACCGAAGCU) were used in conjunction with Oligofectamine as specified by the manufacturer (Invitrogen, Amsterdam, The Netherlands). Subconfluent HeLa cells were incubated with preformed lipid-siRNA complexes (100 nM siRNA) for 6 hours, after which fresh medium was added. After 36 hours, cells were transfected onto coverslips and 60 hours after the initial siRNA transfection, cells were subsequently transfected with LOX-1-FLAG. Following LOX-1 transfection after 12 hours, cells were processed for internalisation assays by incubation with Alexa Fluor 488-transferrin (Invitrogen) and Dil-OxLDL for 15 minutes at 37°C, followed by chase at 37°C for 30 minutes with fresh media. Cells were fixed and processed as previously described (Towler et al., 2000) using confocal microscopy (see later).

Western blotting

Cellular proteins were analysed by western blotting using enhanced chemiluminescence (Murphy et al., 2006). Primary antibodies used were sheep anti-LOX-1 (0.5 μg/ml), sheep anti-TGN46 (0.1 μg/ml) or tissue culture supernatant of mouse anti-transferrin receptor (1:5000). Levels of protein expression were evaluated from multiple western blotting datasets (n=3) using AIDA (Advanced Image Data Analyzer) 2.11 software (Fujifilm, Japan). For the RNAs experiments, cell lysates (10 μg total protein per lane) were run on 3-8% Tris-acetate SDS-PAGE gels (Invitrogen). Mouse monoclonal antibodies to clathrin heavy chain and the µ2 subunit of AP2 (BD Biosciences, Oxford, UK) or actin (Sigma, Poole, UK) were used for western blot detection.
bion. To assess LOX-1 degradation, biotinylated cells were incubated at 37°C in serum-free media for the indicated times and then lysed in 1% (v/v) NP-40, 50 μM Tris-HCl pH 7.5, 150 mM NaCl. To assess LOX-1 internalization, biotinylated cells were incubated at 37°C in serum-free medium for the indicated times, then re-chilled on ice and washed three times with PBS. Cell surface biotin labelling was removed by three 10-minute incubations with ice-cold reducing buffer (100 mM MESNA, 50 mM Tris-HCl pH 8.6, 100 mM NaCl, 1 mM EDTA, 0.2% (w/v) BSA). Cells were washed and excess reducing agent quenched by incubation with 120 mM iodoacetamide in PBS for 10 minutes at 4°C before cell lysis. Biotinylated proteins were recovered by incubation with 30 μl neutravidin-agarose (Pierce) overnight at 4°C. The isolated biotinylated proteins were washed three times with lysis buffer, boiled in SDS-PAGE sample buffer and probed for LOX-1 using western blotting.

Hypertonic treatment and potassium depletion
For hypertonic treatment (Heuser and Anderson, 1989), cells were incubated with complete medium containing 0.45 M sucrose for 15 minutes at 37°C; this was followed by a 5 minute pulse with 10 μg/ml Dil-labelled OxLDL followed by a 55 minute chase, in complete media with 0.45 M sucrose. Control incubations were carried out in complete medium. Cells were then fixed and processed for immunofluorescence microscopy. For potassium depletion (Hansen et al., 1993), cells were washed with K+-free buffer (20 mM HEPES pH 7.4, 140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM D-glucose), incubated with hypotonic buffer (K+-free buffer diluted 1:1 with water) for 5 minutes at 37°C, washed three times with K+-free buffer and incubated with K+-free buffer for 30 minutes at 37°C. Cells were then pulsed with 10 μg/ml Dil-labelled OxLDL for 5 minutes followed by a 5 minute chase, both in K+-free buffer and at 37°C. Control cells were assayed in the same buffers containing 10 mM KCl. Cells were then fixed and processed for immunofluorescence microscopy.

Quantification of OxLDL uptake
Internalisation of Dil-labelled OxLDL in potassium-depleted or hypertonic treated cells was quantified using ImageJ as described above. In all other cases, quantification in internalisation experiments was assessed using data from three separate experiments (n=150 cells in total). Plasma membrane and intracellular punctate staining patterns were compared. Cells pulsed and chased with labelled ligand displayed intracellular punctate structures with little, if any, plasma membrane staining; the converse was true where labelled ligand was bound but not internalised.

This work was supported by a British Heart Foundation project grant (S.P., J.H.W. and S.H.V.), a BBSRC DTA PhD studentship (J.E.M.), a Wellcome Trust Senior Fellowship (T.S.P., J.H.W. and S.H.V.), ahall, 61-72.

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