LOX-1 scavenger receptor mediates calcium-dependent recognition of phosphatidylserine and apoptotic cells

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The LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1) scavenger receptor regulates vascular responses to oxidized-low-density-lipoprotein particles implicated in atherosclerotic plaque formation. LOX-1 is closely related to C-type lectins, but the mechanism of ligand recognition is not known. Here we show that human LOX-1 recognizes a key cellular phospholipid, PS (phosphatidylserine), in a Ca^{2+} -dependent manner, both *in vitro* and in cultured cells. A recombinant, folded and glycosylated LOX-1 molecule binds PS, but not other phospholipids. LOX-1 recognition of PS was maximal in the presence of millimolar Ca^{2+} levels. Mg^{2+} was unable to substitute for Ca^{2+} in LOX-1

binding to PS, indicating a Ca^{2+} -specific requirement for bivalent cations. LOX-1-mediated recognition of PS-containing apoptotic bodies was dependent on Ca^{2+} and was decreased to background levels by bivalent-cation chelation, LOX-1-blocking antibodies or PS-containing liposomes. The LOX-1 membrane protein is thus a Ca^{2+} -dependent phospholipid receptor, revealing novel recognition of phospholipids by mammalian lectins.

Key words: Ca^{2+} , lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), liposomes, phosphatidylserine, protein-lipid overlay, recombinant protein.

INTRODUCTION

LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1) is a mammalian scavenger receptor cloned as a receptor for OxLDL (oxidized low-density lipoprotein) [1]. LOX-1 expression can be up-regulated by pro-inflammatory stimuli including $\text{TNF}\alpha$ (tumour necrosis factor- α), PMA and IL-1 β (interleukin 1 β) [2,3] and is detected on endothelial cells, macrophages, smooth-muscle cells and platelets [4–6]. LOX-1 is implicated in the pathogenesis of atherosclerotic lesions, as OxLDL binding can trigger elevation in the levels of reactive oxygen species, monocyte chemoattractant protein-1 and matrix metalloproteinases [7–9]. LOX-1 can be detected in atherosclerotic lesions, and allelic variants are linked to the incidence of cardiovascular disease [10,11]. Furthermore, LOX-1 binds to a diverse variety of ligands, including OxLDL, apoptotic cells, activated platelets and bacteria [12–14].

LOX-1 is a structurally distinct member of the scavenger-receptor family [15] with an extracellular C-type lectin-like domain highly homologous with that of NK (natural killer)-cell receptors [16], all part of a single gene cluster on human chromosome 12 [17]. C-type lectins are classically defined as proteins that bind carbohydrate moieties in a Ca^{2+} -dependent manner [18], but NK cell receptors mediate Ca^{2+} -independent, lectin-dependent protein–protein recognition of MHC-Class I-related molecules [19,20]. The crystal structure of the LOX-1 C-type lectin-like domain reveals a lectin fold and a propensity to form homodimers [16,21].

hLOX-1 (human LOX-1) is a type II membrane protein (Figure 1A) that binds OxLDL via positively charged and neutral hydrophilic residues in the extracellular lectin-like domain [22–24], but its Ca^{2+} -dependent properties are unknown. LOX-1

has three disulphide bonds in the lectin-like domain, and the bovine orthologue can be cleaved by cellular proteases to generate a soluble extracellular LOX-1 fragment [25]. We have expressed a truncated soluble recombinant LOX-1 extracellular domain lacking the cytoplasmic and transmembrane domains and investigated its ability to bind membrane ligands. Full-length LOX-1 protein was expressed in transfected human cells to further evaluate the ability of the native protein to discriminate between ligands. We show that human LOX-1 mediates Ca^{2+} -dependent recognition of PS (phosphatidylserine) and apoptotic bodies.

EXPERIMENTAL

Reagents

All chemicals were obtained from Sigma unless otherwise stated. Mammalian- and insect-cell culture media and supplements were from Invitrogen.

Plasmids

Bacterial and baculovirus expression involved using PCR to amplify a DNA sequence encoding residues 68–273 of the extracellular domain of hLOX-1 using a complete human expressed-sequence-tag cDNA (GenBank[®] accession number BG547497; Geneservice). The PCR product was digested and cloned into either the pET-15b bacterial expression vector (Novagen) or a modified pTriEX1.1 insect-cell expression vector (Novagen) containing a signal sequence from the baculovirus major envelope glycoprotein gp67 (kindly provided by Dr Kevin Dalton and Professor Ian Jones, Microbiology Division, School of

Abbreviations used: ApoB-100, apolipoprotein B-100; bv-LOX-1, baculovirus/insect cell-expressed lectin-like oxidized low-density lipoprotein receptor-1; ec-LOX-1, bacterially expressed LOX-1; GST, glutathione S-transferase; His₆, hexahistidine; hLOX-1, human LOX-1; HRP, horseradish peroxidase; IL-1 β , interleukin-1 β ; IPTG, isopropyl β -D-thiogalactoside; MOI, multiplicity of infection; Ni-NTA, Ni²⁺-nitrilotriacetate; NK, natural killer; OxLDL, oxidized low-density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PNGase F, N-glycosidase F; PS, phosphatidylserine; sTGN46, soluble His₆-tagged trans-Golgi-network protein 46; TBS, Tris-buffered saline; $\text{TNF}\alpha$, tumour necrosis factor- α .

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Figure 1 Expression of recombinant LOX-1 in bacteria, insect cells and mammalian cells

(A) Schematic representation of the domain structure of hLOX-1 (N, N-terminus; TMD, transmembrane domain; C, C-terminus). (B) hLOX-1 (residues 68–273) was expressed as His₆-tagged proteins in *E. coli* and Sf9 insect cells (see the Materials and methods section) and purified to produce recombinant ec-LOX-1 and bv-LOX-1 respectively. (C) Western blotting using recombinant ec-LOX-1 and recognition by rabbit anti-LOX-1 and sheep anti-LOX-1 antibodies. (D) Expression of LOX-1-FLAG construct in transfected HeLa cells. Cells were fixed and LOX-1-FLAG detected using sheep anti-LOX-1, mouse anti-FLAG and AlexaFluor-labelled secondary antibodies. Cell images represent a deconvolved, projected stack of optical sections. The scale bar represents 10 μ m.

Animal and Microbial Sciences, University of Reading, Reading, Berks., U.K.). Both constructs now contained an N-terminal His₆ (hexahistidine) tag fused to the LOX-1 sequence. For mammalian-cell expression, residues 1–273 of hLOX-1 were amplified using PCR and cloned into a mammalian expression vector pCDNA3.1+ (Invitrogen) in conjunction with a FLAG peptide at the hLOX-1 C-terminus. Further details are given in the supplementary material (<http://www.BiochemJ.org/bj/393/bj3930107add.htm>).

Bacterial expression and protein purification

The pET-15b/LOX-1 plasmid was transformed into *E. coli* Rosetta (DE3)pLysS (Novagen). Exponential-phase cultures were induced with 0.1 mM IPTG (isopropyl β -D-thiogalactoside) at 37°C for 6 h, pelleted by centrifugation at 4000 *g* for 30 min, and lysed in 10 mM Tris/HCl (pH 7.8)/1 mg/ml lysozyme/protease-inhibitor cocktail (Roche Diagnostics). After incubation for 30 min at 4°C, the bacterial lysate was briefly sonicated and

centrifuged at 10 000 *g* for 15 min. An inclusion-body pellet was solubilized in 10 mM Tris (pH 8.0)/6 M guanidinium chloride/100 mM NaH₂PO₄ and sonicated briefly before mixing at 4°C for 30 min. After centrifugation at 100 000 *g* for 30 min, solubilized His₆-tagged LOX-1 (ec-LOX-1) was purified from the supernatant using Ni-NTA (Ni²⁺-nitrilotriacetate)-agarose resin (Qiagen). sTGN46 (soluble His₆-tagged *trans*-Golgi-network protein 46) was produced exactly as described previously by Prescott et al. [26]. GST (glutathione S-transferase)-tagged annexin VI was produced as described by Davis et al. [27] and the GST moiety removed using thrombin protease digestion and adsorption on glutathione-agarose resin.

Insect cell expression and culture

Sf9 [*Spodoptera frugiperda* (fall armyworm)] insect cells (1×10^6) grown as a monolayer culture in six-well plates were co-transfected with 0.5 μ g of pTriEx-LOX-1 and 0.15 μ g of the linearized bacmid BAC10:KO₁₆₂₉ [28] (kindly provided by

Dr Kevin Dalton and Professor Ian Jones) using 8 μ g of Lipofectin® (Invitrogen) in serum-free TC-100 medium at 28 °C. After 16–20 h, the DNA/Lipofectin® mix was removed and replaced with complete TC-100 medium [100 units of penicillin/ml, 100 μ g of streptomycin/ml and 10 % (v/v) fetal bovine serum]. Supernatants containing recombinant baculovirus were harvested after 2–5 days incubation at 28 °C. High-titre viral stocks (>10⁹ plaque-forming units/ml) were produced by two sequential passages in 2 × 10⁶ Sf9 cells/ml infected at an MOI (multiplicity of infection) of 0.1. Recombinant virus amplification and protein production were carried out using suspension cultures of Sf9 cells grown in shaker flasks containing Sf-900 II serum-free medium, 100 units of penicillin/ml and 100 μ g of streptomycin/ml. Large-scale cultures of Sf9 cells (1 litre) grown to a density of 2 × 10⁶ cells/ml in serum-free medium were infected with high-titre recombinant virus at an MOI of 10 for 4 days. Cells were pelleted by low-speed centrifugation and the supernatant containing recombinant His₆-LOX-1 was incubated with 2 ml of Ni-NTA resin overnight at 4 °C. The Ni-NTA resin was washed sequentially with 50 mM NaH₂PO₄, pH 8, 300 mM NaCl, 0.05 % (v/v) Tween-20 and PBS, pH 7.4, before elution of bv-LOX-1 (baculovirus/insect-cell-expressed LOX-1) in PBS containing 250 mM imidazole. Bv-LOX-1-containing fractions were pooled and dialysed against buffer containing 20 mM sodium acetate pH 5.0, and 100 mM NaCl.

LOX-1 antibody production

Sheep and rabbits were immunized with ec-LOX-1 (bacterially expressed LOX-1) or a keyhole-limpet-haemocyanin-conjugated peptide corresponding to an extracellular hLOX-1 sequence (107–120) respectively [see the supplementary material (<http://www.BiochemJ.org/bj/393/bj3930107add.htm>) for further details]. Polyclonal antibodies were affinity-purified using antigen-immobilized columns.

Western blotting

Protein samples were fractionated by SDS/PAGE, transferred to nitrocellulose membranes and non-specific binding sites were blocked using 5 % (w/v) skimmed milk in PBS. Sheep anti-LOX-1 (1:1000) or rabbit anti-LOX-1 (1:1000) primary antibodies were incubated with blots for 16 h, extensively washed with PBS, followed by incubation with the appropriate anti-species HRP (horseradish peroxidase) conjugate secondary antibody (GE Diagnostics; used at 1:3000) for 2 h. Membranes were washed extensively again and bound antibodies were visualized using West Pico enhanced chemiluminescence (Perbio Science).

Far-UV CD analysis

Bv-LOX-1 was dialysed into 20 mM sodium acetate buffer, pH 5.0, at a final protein concentration of 11.3 μ M, and CD measurements were recorded on a Jasco J175 spectropolarimeter over the range 200–250 nm in a 1 mm path-length cell. Spectra were obtained over a range of temperatures and blank buffer subtraction was used for averaging and baseline correction. Measurements were converted into mean residue molar ellipticity [θ]_{MRW}.

PNGase F (N-glycosidase F) digestion and analysis

Aliquots (10 μ g) of bv-LOX-1 were deglycosylated using PNGase F (New England Biolabs) by incubation for either 2 or 16 h at 37 °C (see the supplementary material at <http://www.BiochemJ.org/bj/393/bj3930107add.htm> for further details). Con-

trols and digested samples were subjected to SDS/PAGE and stained with Coomassie Blue.

Protein lipid overlay assay

The protein lipid overlay assay was performed essentially as described [29] in buffer containing 2 mM Ca²⁺, Mg²⁺ or EDTA supplemented with either 100 nM bv-LOX-1, sTGN46 or annexin VI. Bound protein–phospholipid complexes were detected by incubation with sheep anti-LOX-1 (1:1000), rabbit anti-(annexin VI) (1:1000) or sheep anti-TGN46 (1:1000), followed by appropriate HRP-conjugated secondary antibodies and West Pico enhanced chemiluminescence. A Fuji Intelligent Dark Box II image reader using Fuji Las-1000 Pro software was used to capture images. Dot intensities were determined densitometrically by using Fuji Aida (Advanced Image Data Analyzer) 2.11 software. For further details, see the supplementary material at <http://www.BiochemJ.org/bj/393/bj3930107add.htm>.

Apoptotic-body binding assay

HeLa and HEK-293T cells were maintained in complete DMEM [with 10 % (v/v) fetal bovine serum, 100 units penicillin/ml, 100 μ g streptomycin/ml, 2 mM L-glutamine and 2 mM non-essential amino acids; Invitrogen]. HeLa cells were transiently transfected using the calcium phosphate method [30]. HeLa cells grown on coverslips were transfected with pCDNA3.1/LOX-1-FLAG and incubated with apoptotic HEK-293T cells that had been treated with 1 μ M staurosporine for 1 h (apoptotic bodies) in DMEM (Dulbecco's modified Eagle's medium) containing 2.5 mM Ca²⁺ at 37 °C for 30 min. To block LOX-1-mediated recognition, HeLa cells were pre-incubated with 10 μ g/ml of LOX-1 blocking antibody JTX92 [9] or 10 μ g/ml sheep anti-LOX-1 in DMEM for 30 min at 37 °C and washed three times with DMEM before the addition of apoptotic bodies. Synthetic liposome competition assays contained transfected HeLa cells pre-incubated with 100 μ M PS or PC (phosphatidylcholine) liposomes (see the supplementary material at <http://www.BiochemJ.org/bj/393/bj3930107add.htm>) in TBS containing 2.5 mM Ca²⁺ for 30 min at 37 °C before addition of apoptotic bodies (also containing 100 μ M PS or PC liposomes). Cells bound to apoptotic bodies were washed five times with ice-cold TBS containing 2.5 mM Ca²⁺ or three times with ice-cold TBS containing 2.5 mM EDTA, followed by two washes with ice-cold TBS containing 2.5 mM Ca²⁺. AlexaFluor 594-labelled annexin V (Invitrogen) was diluted 1:100 in binding buffer [10 mM Hepes (pH 7.5)/140 mM NaCl/2.5 mM CaCl₂] and incubated with cells for 15 min at 4 °C to visualize apoptotic bodies. After washing three times with ice-cold binding buffer, cells were fixed and processed for indirect immunofluorescence microscopy [30]. To quantify apoptotic body binding, 100 LOX-1-FLAG-transfected cells were counted to assess the number of LOX-1-transfected cells with apoptotic cells (annexin V-positive bodies containing DNA) attached.

Immunofluorescence microscopy

Immunofluorescence was performed as described previously [30] (see the supplementary material at <http://www.BiochemJ.org/bj/393/bj3930107add.htm>). Samples were viewed using a Zeiss Axioplan II epifluorescence microscope linked to a Digital Pixel Systems CCD (charged coupled device) camera. High-resolution images were collected using a DeltaVision Optical Restoration Microscopy System (Applied Precision Inc.) and an Olympus IX-70 epifluorescence microscope. Approx. 15–20 0.2- μ m-thick optical sections were collected and datasets deconvolved using the SoftWorX deconvolution algorithm. Optical sections were

projected as two-dimensional images and saved as 24-bit RGB TIFF files.

RESULTS

Recombinant LOX-1 expression

The extracellular domain of human LOX-1 (Figure 1A; residues 68–273) was expressed in both bacteria and Sf9 insect cells as His₆-tagged proteins. Recombinant bacterial LOX-1 expression was induced by IPTG (Figure 1B, lane 2) and purified as an insoluble protein (ec-LOX-1) from inclusion bodies (Figure 1B, lane 3). In contrast, insect-cell expression of bv-LOX-1 produced a soluble protein that was secreted into the extracellular medium (Figure 1B, lane 5). Secreted bv-LOX-1 was evident as a doublet on SDS/PAGE (Figure 1B, lane 5), probably corresponding to glycosylation-linked heterogeneity. Both bacterially and baculovirus-expressed recombinant LOX-1 proteins were greater than 99% pure as judged by SDS/PAGE after affinity purification on Ni-NTA-agarose resin.

Rabbit polyclonal antibodies raised to a LOX-1 peptide (residues 107–120 in the extracellular ‘neck’ domain) detected 0.4 pmol (10 ng) of ec-LOX-1, whereas sheep polyclonal antibodies raised to ec-LOX-1 displayed 10-fold greater sensitivity and detected 0.04 pmol (1 ng) of ec-LOX-1 (Figure 1C). The sheep antibodies also recognized bv-LOX-1 and native LOX-1 from primary human umbilical-vein endothelial cells (results not shown). We expressed the tagged hLOX-1-FLAG protein in mammalian HeLa cells with a transfection efficiency of approx. 15–20%. The transfected LOX-1-FLAG protein was simultaneously detected by both affinity-purified sheep anti-LOX-1 and mouse monoclonal anti-FLAG antibodies (Figure 1D; a colour overlay is shown in supplementary Figure S1 at <http://www.BiochemJ.org/bj/393/bj3930107add.htm>).

Recombinant LOX-1 folded and glycosylation state

Far-UV CD showed that the native bv-LOX-1 molecule had a folded state with pronounced minima at 208 and 222 nm (Figure 2A), indicating α -helical content. Remarkably, little difference could be detected between the CD spectra of the native bv-LOX-1 protein and the heat denatured–renatured protein (Figure 2A), indicating substantial refolding. CD analyses at increasing 5°C intervals from 25 to 90°C (Figure 2B) showed that the intensity of the bands at 208 and 222 nm slowly decreased with increasing temperature. However, bv-LOX-1 still exhibited a pronounced spectrum, including the 208 nm minimum (Figure 2B) at 90°C, indicating substantial thermal stability.

PNGase F digestion showed that bv-LOX-1 is N-glycosylated at four distinct sites. PNGase F cleaves simple and complex asparagine-linked sugar moieties from a polypeptide backbone. Ec-LOX-1 and bv-LOX-1 showed an approx. 5 kDa apparent molecular mass difference on SDS/PAGE (Figure 3A). SDS/heat denaturation and PNGase F treatment of bv-LOX-1 resulted in increased SDS/PAGE mobility identical with that of ec-LOX-1, indicating that differential N-glycosylation of bv-LOX-1 produces the two polypeptides secreted into the medium (Figure 3A). Titration of PNGase F digestion caused progressively greater SDS/PAGE mobility of bv-LOX-1 (Figure 3B); partial PNGase F digestion revealed five differentially glycosylated bv-LOX-1 polypeptides (Figure 3B). Each N-linked carbohydrate attached to bv-LOX-1 effects an approx. 1–2 kDa decrease in SDS/PAGE mobility; thus the fastest migrating LOX-1 polypeptide must correspond to deglycosylated bv-LOX-1, whereas slower-migrating bv-LOX-1 forms must contain between one and four N-linked sugars.

Figure 2 Far-UV CD analysis of recombinant bv-LOX-1

(A) Native bv-LOX-1 CD spectrum (continuous line) was measured by scanning from 200 to 250 nm at 25°C; the ‘denatured’ (90°C) and ‘refolded’ bv-LOX-1 CD spectrum (broken line) was measured by scanning from 200 to 250 nm at 25°C. (B) Bv-LOX-1 CD spectra were measured by scanning from 200 to 250 nm at 25°C and subsequently at increasing 5°C intervals until 90°C was reached. Each spectrum in (A) represents an average of four scans; each spectrum in (B) is an average of two scans. The units of $[\theta]_{\text{MRW}}$ are degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$.

LOX-1 binding to phosphatidylserine is Ca^{2+} -dependent

Native LOX-1 is implicated in the recognition of apoptotic cells and activated platelets via a PS-dependent mechanism [12,13]. We tested whether this recognition is mediated by a direct interaction between LOX-1 and PS using recombinant soluble bv-LOX-1 as a probe in a protein–phospholipid overlay assay. The data showed that LOX-1 bound directly to PS (Figure 4). Comparison with a positive-control and Ca^{2+} -dependent phospholipid-binding protein, annexin VI, and a negative-control Golgi protein (sTGN46) (Figure 4A) further supported this finding. Bv-LOX-1 exhibited specificity for PS, with no binding detected to other phospholipids, including PC, PE (phosphatidylethanolamine) and PI (phosphatidylinositol) (Figure 4B).

The LOX-1 protein is part of the superfamily of C-type lectins, but there was no evidence that Ca^{2+} ions regulated ligand recognition. Binding experiments were carried out in buffers containing 2 mM Ca^{2+} to mimic physiological extracellular conditions. When we tested the Ca^{2+} -dependence of LOX-1-mediated PS recognition, we found that bv-LOX-1 bound to PS only in the presence of millimolar Ca^{2+} levels (Figure 5A). Bivalent Mg^{2+} could not substitute for Ca^{2+} in this assay (Figure 5B), further supporting the notion of a Ca^{2+} -dependent

Figure 3 Glycosylation of bv-LOX-1

(A) Glycosylation of bv-LOX-1 was analysed using PNGase F. A 10 μ g portion of SDS- and heat-denatured bv-LOX-1 and heat-denatured bv-LOX-1 were incubated for 16 h with 500 units of PNGase F, fractionated by SDS/12 % (w/v) PAGE and visualized using Coomassie Blue staining. Mock-treated SDS- and heat-denatured bv-LOX-1, untreated native bv-LOX-1 and ec-LOX-1 were also run as controls. (B) Samples (10 μ g) of heat-denatured bv-LOX-1 were partially deglycosylated using different amounts of PNGase F (0–500 units) for 2 h and compared with untreated native bv-LOX-1 and ec-LOX-1. Samples were analysed by SDS/12 % (w/v) PAGE and visualised using Coomassie Blue staining. The predicted molecular mass of unglycosylated LOX-1 is 26 kDa.

mechanism of LOX-1 recognition of PS. Ca²⁺ titration in the phospholipid overlay assay showed a sigmoidal relationship, whereas control titration of Mg²⁺ (0–10 mM) showed little effect (Figure 5C). Bv-LOX-1 binding to PS was saturated in the presence of 10 mM Ca²⁺; half-maximal binding was observed at 1.4 mM Ca²⁺ (Figure 5C).

LOX-1-mediated recognition of apoptotic cells is PS- and Ca²⁺-dependent

Human LOX-1 is implicated in PS-mediated recognition of apoptotic bodies [12], and we thus tested the Ca²⁺-dependence of this phenomenon using transfected HeLa cells expressing LOX-1-FLAG (Figure 6). Labelled annexin V detected external PS on apoptotic cells bound to HeLa cells expressing LOX-1-FLAG (Figure 6A). Apoptotic bodies were revealed by staining for both DNA and annexin V (Figure 6A). However, relatively small membrane fragments lacking DNA but derived from apoptotic cells also bound to both non-transfected and transfected HeLa cells in a non-specific manner (Figure 6A). To assess apoptotic-body binding to human LOX-1, the number of LOX-1-FLAG-expressing HeLa cells with one or more annexin V-positive apoptotic bodies containing DNA bound were counted under different incubation conditions (Figures 6B and 6C). The number of apoptotic bodies bound to each cell expressing LOX-1 ranged

between 0 and 4. Binding of apoptotic bodies was decreased to 36 % (of control) in the presence of PS liposomes, but was not affected by PC liposomes, indicating PS-mediated recognition of apoptotic bodies (Figure 6B). Apoptotic-body binding to LOX-1-FLAG-expressing HeLa cells was decreased to 42 % (of control) in the presence of millimolar levels of EDTA (Figure 6C). Pre-incubation of LOX-1-FLAG-transfected cells with the JTX92 monoclonal antibody, which blocks LOX-1 binding to OxLDL [9,31,32], decreased apoptotic body binding to 32 % of control (Figure 6C). Pre-incubation with affinity-purified sheep anti-LOX-1 had a similar effect and reduced apoptotic body binding to 45 % (of control) (Figure 6C). We thus observed similar reductions in apoptotic-body binding in the presence of EDTA or LOX-1-specific antibodies, suggesting that Ca²⁺ is necessary for LOX-1-dependent binding to apoptotic bodies. Taken together, these data indicate that cellular LOX-1-mediated recognition of PS in biological membranes or particles is Ca²⁺-dependent.

DISCUSSION

The present study shows that the LOX-1 scavenger receptor directly binds a key cellular phospholipid, PS, in a Ca²⁺-dependent manner. This binding also occurs in biological membranes, as human cells expressing LOX-1 can recognize apoptotic bodies via

Figure 4 LOX-1 binding to phospholipids

(A) A protein lipid overlay assay was used to assess binding of bv-LOX-1 and control proteins to PC and PS. Serial dilutions (500, 250, 100, 50, 25, 10 and 5 pmol) of phospholipids were probed with 100 nM bv-LOX-1, 100 nM annexin VI or 100 nM control protein (His₆-tagged sTGN46), all in the presence of 2 mM Ca²⁺. Bound protein was detected using affinity-purified antibodies, followed by species-specific HRP-conjugated antibodies and enhanced chemiluminescence. (B) Serial dilutions of PC, PS, PE and PI were probed with 100 nM bv-LOX-1 in the presence of 2 mM Ca²⁺.

LOX-1 in a PS- and Ca²⁺-dependent mechanism. The recognition of PS by LOX-1 was maximal at physiological levels (> 1 mM) of Ca²⁺ ions, suggesting that native LOX-1 binds PS under extracellular conditions where millimolar Ca²⁺ levels are present. Bivalent Mg²⁺ was unable to substitute for Ca²⁺, supporting the specificity for Ca²⁺ in ligand recognition.

How is PS bound by LOX-1? The C-type lectin-like domain of LOX-1 is the OxLDL-binding domain [16,21–24] and is highly homologous with the lectin-like domains of NK-cell receptors [16]. Our engineered recombinant, secreted, folded and N-glycosylated bv-LOX-1 protein, which lacks the cytoplasmic and transmembrane domains, contains both a 'neck' domain and the C-type lectin fold. Although it is possible the 80-residue 'neck' domain mediates PS binding rather than the 130-residue C-type lectin fold, the neck domain has no known homology with other proteins that mediate phospholipid recognition. It is more likely that the LOX-1 C-type lectin fold mediates PS recognition. Although the closely related NK cell receptors lack the Ca²⁺-binding residues found in archetypal C-type lectins such as mannose-binding protein, it has been reported that the NK-cell receptor CD69 binds carbohydrate in a Ca²⁺-dependent manner [33]. This, together with the fact that LOX-1 binds to PS in a Ca²⁺-dependent manner, indicates that ligand recognition and Ca²⁺ binding to C-type lectins is more complex than predicted.

Our findings thus support a novel mechanism for LOX-1-mediated recognition of apoptotic bodies [12,13,34]. How is this linked with the well-characterized role for LOX-1 in high-affinity recognition of OxLDL? One possibility is that LOX-1 is a multifunctional sensor that recognizes both apoptotic bodies and OxLDL. The LOX-1 'neck' domain is unlikely to participate in

Figure 5 LOX-1 binds to PS in a Ca²⁺-dependent manner

(A) Bv-LOX-1 (in the presence of 0–5 mM Ca²⁺) was used to detect immobilized PS. Buffer containing no Ca²⁺ also contained 2 mM EDTA. (B) 100 nM bv-LOX-1 in the presence of 2 mM Mg²⁺ was tested for binding to immobilized PS. (C) Ca²⁺ and Mg²⁺ titration to quantify bv-LOX-1 binding to immobilized PS. Protein bound was quantified using densitometry. Results are means ± S.E.M. (*n* = 3) for three independent experiments.

ligand recognition, owing to its juxtaposition between the transmembrane domain (within the lipid bilayer) and the C-type lectin-like domain. Thus it is more likely that the LOX-1 C-type lectin fold binds to both PS and OxLDL; it has been shown that antibodies to this domain inhibit OxLDL recognition by LOX-1 [9,31,32]. It is well established that the oxidation or modification of LDL leading to formation of pro-atherogenic particles results in chemical changes on both the ApoB-100 (apolipoprotein B-100) protein moiety and phospholipids that form the particle [35,36]. Steric hindrance within a native LDL particle may prevent LOX-1-mediated recognition of PS moieties, thus contributing to a masking effect. However, oxidation of LDL particles could lead to a reorganization of ApoB-100 and phospholipid constituents, leading to presentation of hitherto 'masked' phospholipids to the aqueous medium, thus facilitating LOX-1-mediated recognition.

Figure 6 LOX-1-dependent binding to apoptotic bodies is Ca²⁺-dependent

(A) Apoptotic-body binding to untransfected and LOX-1-FLAG transfected HeLa cells was analysed by immunofluorescence microscopy. Apoptotic bodies were incubated with untransfected or LOX-1-FLAG-transfected HeLa cells (plus Ca²⁺) and exposed PS on bound apoptotic bodies was detected using AlexaFluor 594-labelled annexin V (red) prior to cell fixation. LOX-1-FLAG was detected using mouse anti-FLAG and secondary anti-mouse FITC conjugate (green). Nuclei were stained with 4',6-diamidino-2-phenylindole ('DAPI'; blue). The asterisk indicates a bound apoptotic body. Cell images represent projections of deconvolved three-dimensional stacks of 25–30 optical sections. The scale bar represents 10 μ m. (B) HeLa cells expressing LOX-1-FLAG were pre-incubated with 100 μ M PS or PC liposomes and assayed for apoptotic-body binding (in the presence of Ca²⁺). The baseline control (100 %) is taken as apoptotic bodies bound to transfected cells in the absence of competing liposomes $\pm$ S.E.M. ($n = 3$ independent experiments). (C) HeLa cells expressing LOX-1-FLAG were assayed for apoptotic-body binding in the presence of EDTA (to complex bivalent cations), or the LOX-1-specific monoclonal antibody JTX92 (10 μ g/ml) or purified sheep anti-LOX-1 (10 μ g/ml). The baseline control (100 %) is taken as apoptotic bodies bound to transfected cells in the absence of competing EDTA or competing antibodies $\pm$ S.E.M. ($n = 6$ independent experiments). * $P < 0.05$ versus control; ** $P < 0.01$ versus control.

The PS phospholipid is known to be a constituent of the OxLDL particle [37,38], indicating that LOX-1-mediated recognition of PS-containing lipid particles is a likely possibility *in vivo*. LOX-1 bears no sequence similarity to annexins, indicating that PS binding is mechanistically different between these two proteins.

Is there a role for LOX-1-mediated recognition of apoptotic bodies *in vivo*? Programmed cell death is an essential feature of

multicellular development in eukaryotes [39]. Apoptotic-body clearance is critical during body and tissue development in multicellular organisms. Macrophages and dendritic cells are key mediators of apoptotic-cell recognition and clearance. Interestingly, many of the mammalian scavenger receptors confer the ability to recognize apoptotic cells, especially in myeloid cells such as macrophages and monocytes [15]. The LOX-1 molecule is

found both on endothelial cells [1] and myeloid cells [4,40] raising the possibility that this receptor may also mediate recognition and clearance of apoptotic bodies by different cell types in mammals. The role of the endothelium in the recognition of apoptotic bodies has not been studied in depth, but this property appears to be conserved during mammalian evolution, since mouse, bovine and human endothelial cells all have this capacity [12,41–43]. It is clear that primary endothelial cells can bind apoptotic bodies in a LOX-1-dependent manner [12]. It is thus likely that LOX-1 is the receptor that mediates such endothelial recognition and clearance (phagocytosis) of apoptotic bodies.

In conclusion, our results show that the LOX-1 mammalian lectin that regulates vascular function can recognize a key cellular phospholipid, PS, *in vitro* on immobilized surfaces, but only in the presence of millimolar levels of Ca^{2+} . Human cells expressing LOX-1 also bind PS exposed on apoptotic bodies in a Ca^{2+} -dependent manner. Future studies will aim to map the site in LOX-1 that binds to Ca^{2+} and PS and determine how this can be reconciled with the structure of the extracellular domain.

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