

Native low density lipoprotein promotes lipid raft formation in macrophages

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Abstract. Oxidized low-density lipoprotein (LDL) has an important role in atherogenesis; however, the mechanisms underlying cell-mediated LDL oxidation remain to be elucidated. The present study investigated whether native-LDL induced lipid raft formation, in order to gain further insight into LDL oxidation. Confocal microscopic analysis revealed that lipid rafts were aggregated or clustered in the membrane, which were colocalized with myeloperoxidase (MPO) upon native LDL stimulation; however, in the presence of methyl- β -cyclodextrin (M β CD), LDL-stimulated aggregation, translocation, and colocalization of lipid rafts components was abolished. In addition, lipid raft disruptors M β CD and filipin decreased malondialdehyde expression levels. Density gradient centrifugation coupled to label-free quantitative proteomic analysis identified 1,449 individual proteins, of which 203 were significantly upregulated following native-LDL stimulation. Functional classification of the proteins identified in the lipid rafts revealed that the expression levels of translocation proteins were upregulated. In conclusion, the results of the present study indicated that native-LDL induced lipid raft clustering in macrophages, and the expression levels of several proteins were altered in the stimulated macrophages, which provided novel insights into the mechanism underlying LDL oxidation.

Introduction

Atherosclerosis (AS) is a pathological process associated with the majority of cardiocerebrovascular diseases, and is a predominant cause of morbidity and mortality worldwide (1). Oxidized low-density lipoproteins (ox-LDLs) are known to be of importance in the pathogenesis of AS. However, the precise mechanism underlying LDL oxidation has yet to be fully elucidated (2).

All three major cell types of the human atherosclerotic lesions, particularly macrophages are hypothesized to be the principal mediators of LDL oxidation (3,4). Macrophages have been shown to modulate LDL oxidation through the production of reactive oxygen species (5), lipoxygenase (6), and myeloperoxidase (MPO) (7). MPO is a highly cationic protein that is able to bind to endothelial cells, leukocytes and LDL (2). Sokolov *et al* (8) concluded that the likely site of interaction with MPO is the amino acid stretch 445-456 of apoB-100 though mimicking 3 kinds of apoB-100 fragments. Numerous studies have suggested that MPO adsorbs onto the surface of LDL, promoting the oxidation of amino acid residues and the formation of oxidized lipoproteins (9,10).

In vitro, various antioxidants are able to inhibit LDL oxidation, such as vitamin E and probucol. *In vivo*, serum or interstitial fluid is able to markedly inhibit LDL oxidation by cells due to the presence of vitamin C in human plasma, interstitial fluid and arterial walls (11). As early as 1990, Heiple *et al* (12) hypothesized that macrophages were able to create a closed compartment on the plasma membrane and substrate that excludes proteins in the surrounding medium, thereby protecting cells from external factors. This may explain the ineffectiveness of antioxidants in clinical therapy as compared with *in vitro* studies.

Lipid rafts are membrane microdomains characterized by a high content of sphingolipids and cholesterol, and a low content of protein (13). Lipid rafts have been shown to participate in numerous important steps of atherogenesis, such as inflammation (14), apo-A1-mediated cholesterol efflux (15) and the secretion of pro-inflammatory cytokines by immune cells (16). Lipid rafts in macrophages are important for vesicular trafficking, transmembrane signal transduction,

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Abbreviations: ox-LDL, oxidized low-density lipoprotein; M β CD, methyl- β -cyclodextrin; MPO, myeloperoxidase; ERp29, endoplasmic reticulum protein 29

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protein translocation and cytoskeletal rearrangements (17). In response to various stimuli, numerous molecules move into or out of the lipid rafts. These molecules include, but are not limited to, toll-like receptor 4 (18) and class A scavenger receptor (19), which affect macrophage functions. However, the mechanism underlying the effect of LDL on the translocation and identity of their target molecules in lipid rafts remains unknown.

The present study demonstrated that native-LDL promotes lipid raft clustering in macrophages, and identified lipid raft-associated proteins by label-free quantitative proteomic analysis, in order to gain further insight into LDL oxidation.

Materials and methods

Materials. Methyl- β -cyclodextrin (M β CD) and anti-neutrophil myeloperoxidase [MPO; mouse anti-goat polyclonal IgG (H+L)] antibodies were obtained from Santa Cruz Biotechnology, Inc. (1:100, cat. no. sc-16129, Dallas, TX, USA), along with lipid-raft disruptor filipin. Alexa Fluor 488-cholera toxin subunit B (CTXB) and Alexa Fluor 594-labeled chicken anti-Goat IgG (H+L) secondary antibody were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Anti-flotillin-1 antibody was purchased from BD Biosciences (1:1000, cat no. 61802; Franklin Lakes, NJ, USA). Polyclonal anti-ERp29 rabbit anti-mouse antibody was obtained from Abcam (1:3000, ab11420; Cambridge, MA, USA). Optiprep was obtained from Axis-Shield, Inc. (Norton, MA, USA). High glucose Dulbecco's modified Eagle's medium (DMEM) was purchased from GE Healthcare Life Sciences (Logan, UT, USA). An Enhanced Chemiluminescence (ECL) kit was obtained from PerkinElmer Inc. (Waltham, MA, USA). Human LDL was purchased from ProSpec-Tany TechnoGene, Ltd. (Ness Ziona, Israel).

Cell culture and oxidation of native LDL in Raw264.7 cells. Raw264.7 mouse macrophages were purchased from the China Centre for Type Culture Collection (Wuhan, China). The cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (both from Thermo Fisher Scientific, Inc.) at 37°C and in 5% CO₂. When cell density reached 70-80% confluence, the cells were washed three times with phosphate-buffered saline and pre-incubated for 2 h in serum-free DMEM for LDL oxidation. The Raw264.7 cell line was incubated with native-LDL (100 μ g/ml) at 37°C for 3, 6, 12 and 24 h.

Cholesterol depletion. To disrupt lipid raft membrane domains, membrane cholesterol was depleted by treating the Raw264.7 cells with DMEM supplemented with 5 mM M β CD for 30 min or 100 nM filipin for 15 min at 37°C.

Thiobarbituric acid assay (TBA). The TBA assay was used to assess the extent of cell-mediated LDL oxidation as described previously (20). TBA reacts with malondialdehyde (MDA) and MDA-like derivatives to form TBA reactive species, which may be quantified by spectrophotometry at 535 nm using a UV-2000 spectrophotometer [UNICO (Shanghai) Scientific

Instrument Co., Ltd.]. Data are presented as MDA equivalents (nM MDA/mg protein).

Confocal analysis of lipid rafts and their colocalization with LDL and MPO in Raw264.7 cells. Detection of lipid rafts was performed as described previously (21). Briefly, the Raw264.7 cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and double stained with Alexa Fluor 488-CTXB (which attaches to ganglioside GM1 and thus does not require a second antibody), or incubated with anti-MPO antibody followed by Alexa Fluor-594-labeled secondary antibody, prior to being visualized under a DM6000 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Isolation of detergent-free lipid rafts. Detergent-free rafts were prepared using the OptiPrep gradient method previously described by Macdonald and Pike (22). Following centrifugation at 52,000 x g for 90 min, cloudiness was observed throughout the gradient. A diffuse band was observed about one-third of the way down the gradient, and a distinct band was apparent at the interface of the 20% end of the gradient and the 25% OptiPrep bottom layer. The gradients were fractionated into 0.67 ml fractions (each in a new tube), and the distribution of various proteins was assessed by western blotting as described below.

Western blotting. Total protein from each fraction was determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.). The proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis prior to being electrophoretically transferred onto nitrocellulose membranes (GE Healthcare Life Sciences). The membranes were blocked using 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) for 1 h, incubated with primary antibodies against anti-flotillin-1, ERp29 and transferrin (1:2,000; cat. no. ab84036, Abcam) in TBST overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit/anti-mouse secondary antibodies [1:1,000; cat. nos. A0208, A0216; IgG (H+L); Beyotime Institute of Biotechnology, Haimen, China]. Prior to being washed three times with TBST. Protein bands were visualized on X-ray film (Kodak, Rochester, NY, USA) and quantified using Image J software (version 1.48U; National Institutes of Health, Bethesda, MA, USA).

Mass spectrometry analysis. In-gel digestion with trypsin was performed as previously described (23-25). Peptide samples were resuspended in loading buffer (containing 0.1% formic acid, 0.03% trifluoroacetic acid and 1% acetonitrile) and loaded onto a 15 cm nano-HPLC column (internal diameter 100 μ m) packed with Reprosil-Pur 120 C18-AQ 1.9 μ m beads (Dr Maisch HPLC GmbH, Ammerbuch, Germany), and eluted for 120 min with 4-80% buffer B reverse phase gradient (buffer A, 0.1% formic acid and 1% acetonitrile in water; buffer B, 0.1% formic acid in acetonitrile) generated by a NanoAcquity UPLC system (Waters Corporation, Milford, MA, USA). The peptides were ionized with 2.0 kV electrospray ionization voltage from a nano-ESI source (Thermo Fisher Scientific, Inc.) on a hybrid LTQ XL Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc.).

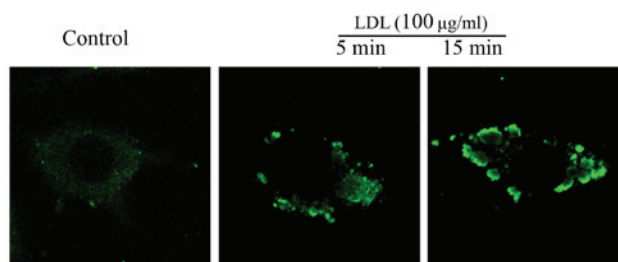


Figure 1. Native-LDL-induced lipid raft clustering in Raw264.7 cells. Raw264.7 cells were stimulated with LDL (100 $\mu\text{g/ml}$) or without (100 $\mu\text{g/ml}$ PBS as control), and stained with Alexa 488-cholera toxin subunit B lipid raft probe. LDL, low-density lipoprotein. Magnification, $\times 1,000$.

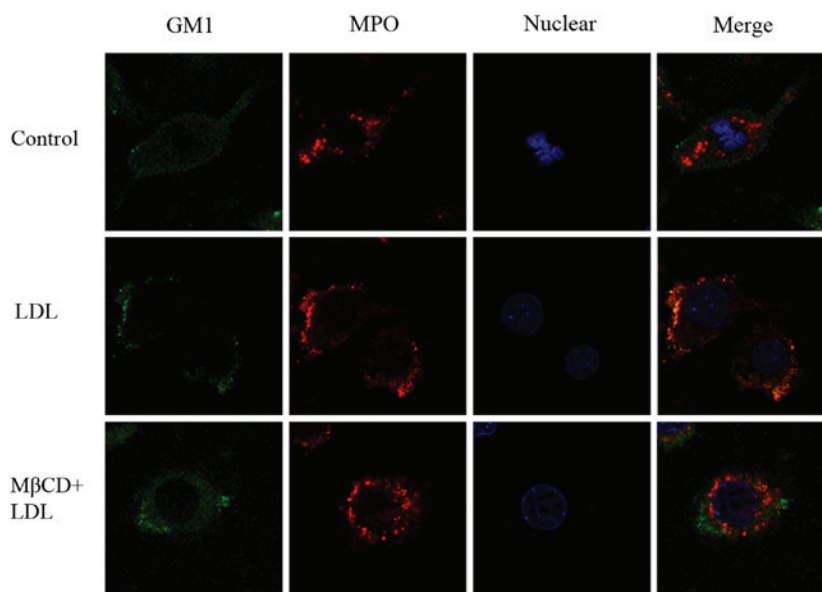


Figure 2. Representative confocal microscopy images of lipid rafts and MPO in Raw264.7 cells. Raw264.7 cells were stained with Alexa 488-cholera toxin subunit B (green) and Alexa 594-conjugated anti-MPO antibody (red), the cell nuclei are shown in blue, with the overlaid images shown on the right. Yellow spots in the overlaid images were defined as colocalization of both molecules. The control was 100 $\mu\text{g/ml}$ of PBS. MPO, myeloperoxidase; LDL, low-density lipoprotein; M β CD, methyl- β -cyclodextrin. Magnification, $\times 1,000$.

Data-dependent acquisition of centroid MS spectra at 30,000 resolution and MS/MS spectra were obtained in the LTQ following collision-induced dissociation (collision energy, 35%; activation Q, 0.25; and activation time, 30 msec) for the top 10 precursor ions, with charge determined by the Sage Sorcerer SEQUEST (version 3.5; Sage-N Research, Inc., Milpitas, CA, USA) acquisition software to be ≥ 2 . Dynamic exclusion of peaks already sequenced at 30 sec with early expiration for two count events with signal-to-noise > 2 . Automatic gating control was set to 150 msec maximum injection time. The Sage Sorcerer SEQUEST (version 3.5; Sage-N Research, Inc.) was used to search and match MS/MS spectra to a complete semi-trypsin mouse proteome database (NCBI reference sequence revision 54, with 34,421 target entries; <http://www.ncbi.nlm.nih.gov/refseq/>) and a pseudo-reversed decoys sequences (26,27) with a 20 ppm mass accuracy threshold. Static modifications for cysteine carbamidomethyl (+57.021465) and dynamic modifications for oxidized methionine (+15.99492) were included. Only b and y ions were considered for scoring (Xcorr), and Xcorr along with ΔCn were dynamically increased for groups of peptides organized by a combination of trypticity (fully or partial) and

precursor ion charge state in order to remove false positive hits along with decoys until a false discovery rate (FDR) of $< 1\%$ was achieved.

The FDR was estimated by the number of decoy matches (nd) and total number of assigned matches (nt): $\text{FDR} = 2\text{nd}/\text{nt}$, assuming mismatches in the original database were the same as in the decoy database (28). Quantification of proteins was based on the comparison of extracted ion current intensities for identified peptides as previously described (29).

Protein-protein interaction analysis. The upregulated proteins were analyzed using the Panther classification system (<http://www.pantherdb.org/>) and were assigned to various functional groups.

Statistical analysis. All values are presented as the mean \pm standard deviation. Statistical analyses were performed using GraphPad Prism software (version 5; GraphPad Software, Inc., La Jolla, CA, USA). The paired, two-tailed Student's t-test was used to analyze the significance of the difference between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Native-LDL promotes lipid raft clustering in macrophages. Previous studies have demonstrated that ox-LDL induced aggregation of gp^{91phox} (30) and Fas (31) in lipid rafts. In order to examine whether native-LDL induces lipid raft clustering, GM1 ganglioside (the predominant lipid raft component) was stained using Alexa-488-labeled CTXB. As shown in Fig. 1, native-LDL stimulation caused an aggregation of GM1, exhibited as green dots or patches. These results suggest that treatment with native-LDL led to the formation of lipid rafts in Raw264.7 cells.

Native-LDL-induces aggregation of MPO in lipid raft clusters. To examine whether MPO was able to aggregate in lipid raft clusters following native-LDL stimulation, Raw264.7 cells were stained with Alexa-488 CTXB and Alexa-594-conjugated anti-MPO, and the distribution of MPO in lipid raft clusters was visualized by confocal microscopy. As shown in Fig. 2, MPO, an important enzyme involved in the oxidation of LDL, was distributed in both the membrane and cytosol of cells. After LDL treatment, MPO was redistributed in the plasma membrane and colocalized with GM1, so LDL increased MPO translocation into lipid rafts. This colocalization was blocked in MβCD-treated cells.

Lipid raft disruptors attenuate LDL oxidation in Raw264.7 cells. The role of lipid rafts in macrophage-mediated LDL oxidation was investigated. Fig. 3 shows that Raw264.7 cells oxidized human LDL over a 24 h time period, which was significantly decreased following treatment with MβCD and filipin compared with the controls.

Total protein concentration in lipid rafts was increased following treatment with LDL in Raw264.7 cells. Lipid rafts were isolated and purified from non-treated and LDL-stimulated Raw264.7 cells using OptiPrep gradient centrifugation. As shown in Fig. 4, low protein concentration levels were present in the lipid raft fractions (fraction 6-8). The highest protein concentration levels were located at the bottom of the gradient. However, an increase in total protein concentration levels was observed in fraction 7, and the total protein levels in the lipid rafts fractions significantly increased following treatment with LDL ($P < 0.05$).

Quantitative proteomics of lipid raft-associated proteins induced by LDL in Raw264.7 cells. To identify differentially regulated lipid raft-associated proteins following LDL stimulation, label-free quantitative proteomics analysis was performed on lipid raft fractions of macrophages. The distribution of the ratios of protein abundance between the LDL-stimulated macrophages and the resting state is shown in Fig. 5A. The present study identified 1,449 proteins with ≥ 1 unique peptides with quantifiable abundance measurements, of which many were lipid raft marker proteins, including flotillins and glycosylphosphatidylinositol-anchored proteins. From the identified proteins, a fold change of ≥ 2 was used to define differential regulation. Out of the 1,449 proteins, 204 and 203 proteins were shown to be downregulated and upregulated following LDL stimulation, respectively. Protein groups were then sorted according to biological processes,

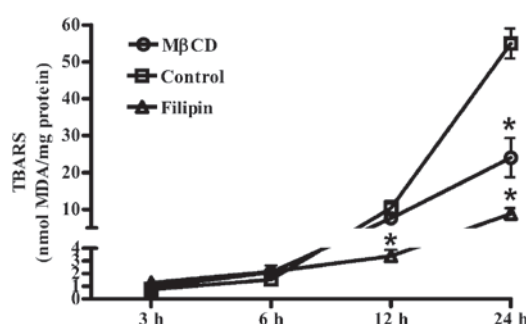


Figure 3. Effect of lipid raft disruptors on cell-mediated LDL oxidation. Raw264.7 cells were pre-treated with serum-free medium in the presence or absence of 5 mM MβCD for 30 min or filipin for 15 min, prior to being incubated with LDL for the indicated time periods. Lipid oxidation was assessed using a thiobarbituric acid assay. Each data point represents the mean of triplicate measurements. * $P < 0.05$, vs. the control. LDL, low-density lipoprotein; MβCD, methyl-β-cyclodextrin; TBARS, thiobarbituric acid reactive substances.

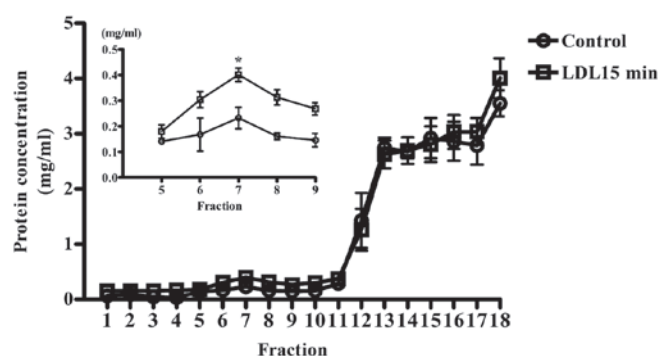


Figure 4. Total protein concentration levels in lipid rafts increases following treatment with LDL in Raw264.7 cells. Protein concentration in each fraction was measured using a bicinchoninic acid assay. The experiment was repeated four times. LDL, low-density lipoprotein.

cellular components and molecular function GO categories (Fig. 5B). Upregulated proteins comprised GO terms associated with metabolic processes and response to stimuli. Notably, the results also demonstrated enrichment for biological adhesion, localization, and enzyme regulator activity. Furthermore, apoB100 was identified in lipid rafts when the protein spectra were matched to a human proteome database, which indicated the human origin of LDL.

The 203 upregulated proteins were analyzed using STRING. Numerous interaction groups were apparent, such as Hmx-1 - Bax, Pfn - Cap-1. Notably, ERp29 was associated with calreticulin.

Validation of the label-free quantification of lipid raft proteins by immunoblotting. To further validate the proteomic identification results, the expression levels of one of the upregulated proteins, ERp29, were analyzed by western blotting. The floated low density fractions 6, 7 and 8 (lipid rafts observed one-third of the way down the gradient) represented the lipid raft fractions following gradient ultra-centrifugation of the cell lysates. As shown in Fig. 6A, the proteins from various fractions were immunoblotted for flotillin-1, a well-documented marker protein of lipid rafts using an

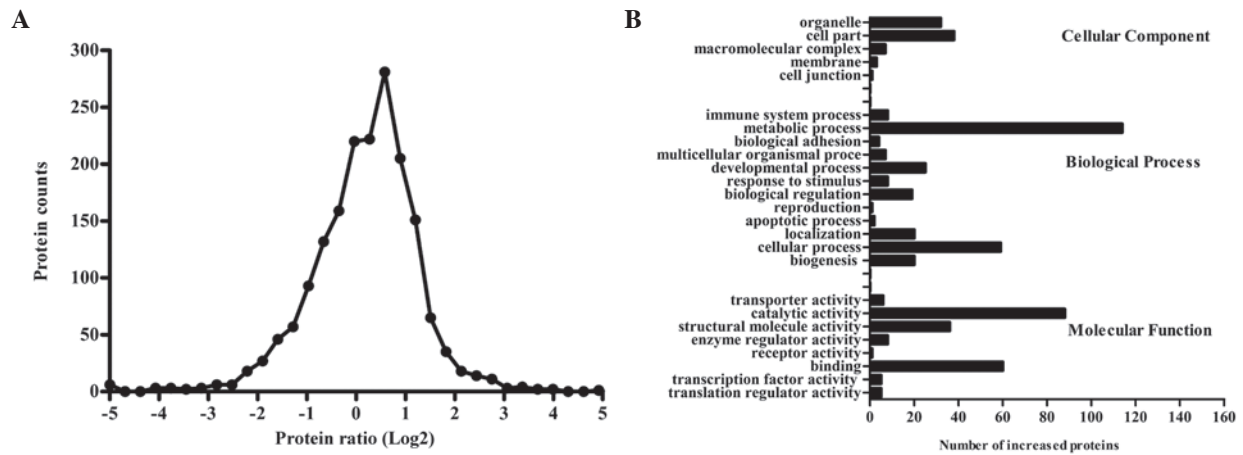


Figure 5. Proteome analysis of macrophages lipid rafts (A) The protein ratio distribution of LDL/control. (B) Functional classification analysis of upregulated proteins.

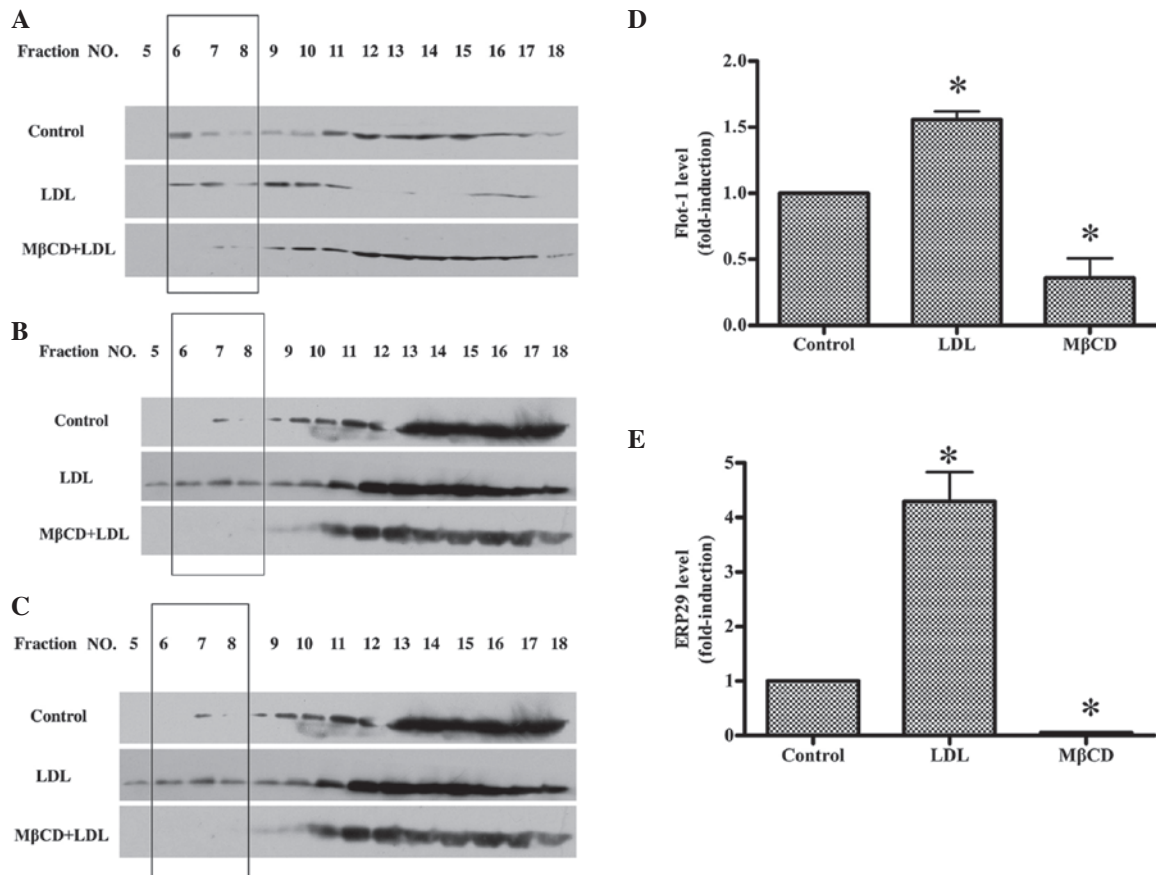


Figure 6. Western blot analysis of the distribution of flotillin-1 and ERp29 in lipid raft fractions isolated from Raw264.7 cells stimulated with native-LDL alone or with 15 min pre-treatment with MβCD as indicated. The expression of (A) flotillin-1, the protein marker for lipid rafts; (B) ERp29; and (C) transferrin receptor, a non-raft plasma membrane protein. Quantitative analysis of the ratio of the relative fold increase over the control for (D) flotillin-1 and (E) ERp29. The results represent the mean value of three independent experiments. *P<0.05, vs. the control. Flot-1, flotillin-1; ERp29, endoplasmic reticulum protein 29; LDL, low-density lipoprotein; MβCD, methyl-β-cyclodextrin.

anti-flotillin-1 antibody. It was demonstrated that flotillin-1 localized not only in lipid raft fractions, but also in non-raft fractions (high density fractions). It should be noted that this was not due to unsuccessful isolation of the lipid raft, but a phenomenon observed in previous studies (22,32). LDL induced a significant increase of the flotillin-1 into lipid raft, however, pre-treatment with MβCD almost entirely disrupted the lipid

raft fractions, as determined by the lack of flotillin-1 in raft fractions. As shown in Fig. 6B, the expression levels of ERp29 in lipid rafts were significantly increased following treatment with LDL. This increase in expression levels was suppressed by MβCD pre-treatment. Notably, the transferrin receptor, a marker for the non-raft plasma membrane, was distributed at the bottom of the gradient (Fig. 6C), indicating that non-raft

plasma membrane did not significantly contaminate the major lipid raft fractions. Bar graphs show the band density ratio of Flot-1 (Fig. 6D) and ERp29 (Fig. 6E), respectively.

Discussion

It has been demonstrated that ox-LDL-induces lipid raft-redox signaling in the coronary arterial endothelium (30). The present study demonstrated that LDL increases the formation of lipid rafts. Numerous changes occurred in lipid rafts following treatment with LDL in macrophages.

Oxidation of LDL has an important role in the pathogenesis of atherosclerosis and vascular diseases. However, the precise mechanisms underlying the role of LDL remain to be elucidated, and the enzymes responsible for these mechanisms also have yet to be identified (2). Certain studies have suggested that LDL oxidation does not take place in the blood circulation, and must occur in the arterial wall due to the fact that blood contains numerous antioxidant molecules (33,34). However, a previous study recently reported results that supported the possibility of LDL oxidation in the circulation (35). There may be two major ways in which cell-mediated LDL oxidation may occur in cells: i) Cell oxidative stress activated by microbial infection causes normal levels of LDL oxidative damage, termed passive oxidation; and ii) cell oxidative stress activated by high-level LDL, termed active LDL oxidation (36). The results of the present study demonstrated that LDL induced lipid raft clustering in macrophages. These data suggested that LDL signaling may be associated with lipid rafts.

MPO is an important enzyme in innate immunity and defense against pathogens (37). The first study to suggest that MPO is implicated in atherogenesis was conducted in 1994 (38). Numerous reactive oxygen species generated by MPO oxidize LDL, and the interaction between MPO and LDL may enhance LDL oxidation (39). The present study demonstrated that MPO was aggregated in lipid rafts following LDL cultivation with macrophages for 9 h. However, following pretreatment with M β CD, a lipid raft disruptor, the colocalization signals on the cell membrane were inhibited. These results indicated that lipid rafts may be involved in LDL oxidation. To further demonstrate this hypothesis, two lipid raft disruptors were used to examine the association between lipid rafts and LDL oxidation. It was demonstrated that lipid raft disruption significantly inhibited LDL oxidation by macrophages.

Detergent-free and low density lipid rafts were isolated from LDL-treated or untreated Raw264.7 cells following density gradient centrifugation. The data demonstrated that the detergent-free lipid rafts contained higher protein concentration levels in the LDL-treated group, compared with the control group. This suggested that certain proteins may translocate into lipid rafts under LDL stimulation. To examine the possibility that LDL induces the recruitment of non-lipid raft proteins in lipid rafts, label-free quantitative proteomic analysis was undertaken to profile the lipid raft proteome in control and LDL-treated Raw264.7 macrophages. A total of 203 significantly upregulated lipid raft-associated proteins were identified. Functional classification of the identified proteins in the lipid rafts revealed an increase in proteins involved in biological adhesion, localization, and enzyme

regulator activity. These processes are closely associated with LDL metabolism, including LDL oxidation.

Endoplasmic reticulum protein 29 (ERp29) is ubiquitously expressed and has been characterized as a luminal ER protein (40). The C-terminal domain of ERp29 contains a novel helical fold which is able to directly bind certain membrane proteins or hydrophobic secretory proteins (41). Furthermore, ERp29 is a 4PBA-regulated ER chaperone that regulates wild-type cystic fibrosis transmembrane conductance regulator (CFTR) biogenesis and is able to promote F508-CFTR trafficking to the plasma membrane in CF epithelial cells (42). The results of the present study demonstrated that the levels of ERp29 increased in macrophage lipid rafts following LDL treatment. Through its protein binding and protein translocation functions, ERp29 may participate in LDL oxidation by mediating associated enzyme secretion or translocation into lipid rafts. A further possibility is that ERp29 may provide binding sites for LDL or LDL oxidation-associated proteins in lipid rafts. A previous study determined by protein-protein interaction analyses that ERp29 was associated with calreticulin, and the biosynthetic precursor, apoproMPO, had transient interactions with the molecular chaperone calreticulin (43). ERp29 may participate in LDL oxidation through calreticulin and MPO. However, the precise mechanism underlying ERp29 participation in LDL oxidation through lipid rafts requires further investigation.

In conclusion, the present study demonstrated that LDL induced lipid raft clustering in macrophages, and MPO aggregation into lipid rafts. The inhibitory effect of LDL oxidation is associated with the disruption of lipid rafts, thus lipid raft disruptions attenuate LDL oxidation by macrophages. Label-free quantitative proteomics analysis used in this study showed that LDL induced the translocation of numerous proteins in to and out of macrophage lipid rafts. These findings may provide novel insights into the mechanism underlying LDL oxidation.

Acknowledgements

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