

ABSTRACT

Recent studies have reported that lipid raft (LR) redox signaling platforms associated with NADPH oxidase are involved in coronary endothelial dysfunction upon different injury factors. The present study was designed to test whether statins could interfere with the formation of LR redox signaling platforms to protect the coronary arterial endothelium from injury. By confocal microscopy, we first detected the formation of LRs clustering in human coronary arterial endothelial cells (HCAECs) exposed to oxLDL treatment, while such LRs formation was inhibited markedly by statins (lovastatin, pravastatin and simvastatin). In these LR clusters, NADPH oxidase subunits, gp91^{phox} and p47^{phox} were aggregated with LR, which was almost completely blocked by statins. Besides that, colocalization of ASM and ceramide was induced under oxLDL treatment while it was blocked with pretreatment of statins. To further explore the functional relevance of this action, we performed detergent resistant membrane flotation to isolate LRs. Electron spin resonance spectrometry showed that superoxide (O₂⁻) production was 2.5-fold higher in the LR fractions from oxLDL-treated HCAECs than that from untreated cells. This oxLDL-induced enhancement of O₂⁻ production in LR fractions was substantially blocked by pretreatment with statins. Our results indicate that blockade of LR redox signaling platform formation in endothelial cell membrane may be another important therapeutic mechanism of statins in preventing endothelial injury and atherosclerosis. (Supported by NIH grants HL057244, HL075316, and DK054927)

METHODS

Cell Culture: HCAECs were cultured and maintained in HCAEC growth medium (Genlantis, San Diego, California) at 37 °C under a humidified atmosphere of 5% CO₂/95% air. Chemical reagent treatment: oxLDL 100 µg/ml 30min (KALEN Biomedical, Montgomery Village, MD) with or without 16 h pretreatment of lovastatin 10 µmol, pravastatin 20µmol, simvastatin 1µmol.

Immunofluorescence and confocal Microscope Analysis. For microscopic detection of LR platforms or LR-associated proteins, cells grown on poly-L-lysine coated glass coverslips were treated as indicated, washed, fixed in 4% paraformaldehyde, and blocked with 1% BSA for 30 min. Cells were incubated for 1 h with indicated primary antibodies followed by incubation with 5 µg/mL FITC- or Texas Red-conjugated secondary antibodies for an additional 2 h. Cells were washed and mounted on glass slide with mounting media (Vector Laboratories, Inc.). Staining was visualized using a conventional Zeiss fluorescence microscope or a Leica TCS SP2 scanning confocal microscope. GM1 gangliosides enriched in lipid rafts can be stained by FITC- or Alexa-labeled cholera toxin (CTX) (Molecular Probes). The patch formation of FITC-labeled CTX and gangliosides complex represented the clusters of lipid rafts.

Isolation of Lipid Raft-Microdomain by Gradient Centrifugation. Cells were lysed in MBS buffer with 1% Triton X-100. Cell extracts were homogenized and homogenates were applied for gradient centrifugation in a discontinuous 40%/30%/5% OptiPrep Density Gradient Medium (Sigma). Samples were centrifuged at 32,000 rpm for 30 h at 4 °C. 12 fractions were collected from the top to the bottom (fraction number 1-12). An immunoblot analysis was performed to confirm LRs were pooled at fraction 4-6. Then ESR analysis was performed to detect superoxide production of the fractions containing lipid raft microdomains and of other fractions without LRs.

ESR Detection of Superoxide Production. Gently collected HCAEC fractions were suspended in modified Krebs/HEPES buffer containing deferoximine (25 µmol/L, metal chelator), HCAECs were mixed with 1 mmol/L spin trap 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethyl-pyrrolidine (CMH) in the presence or absence of 100 units/ml polyethylene glycol (PEG)-conjugated superoxide dismutase (SOD). The cell mixture loaded in glass capillaries was immediately analyzed by ESR (Noxygen Science Transfer & Diagnostics GmbH, Denzlingen, Germany) for production of O₂⁻ at each minute for 10 min.

BACKGROUND

- It is well known that hyperlipidemia and associated oxidase stress are important detrimental factors for a variety of cardiovascular diseases, such as hypertension, diabetes and atherosclerosis. Clinically, Statins, the lipid lowering drugs, have been shown not only to improve the serum lipid profile, but also exert non-lipid lowering benefits by reducing hyperlipidemia-related superoxide production. However, the underlying mechanism of how Statins attenuate O₂⁻ generation remains unknown.
- It has been reported that NADPH oxidase is a major source for the O₂⁻ production in the coronary artery, and recent studies in our laboratory have demonstrated that clustering of ceramide-enriched lipid rafts (LRs) could assemble and activate NADPH oxidase to generate O₂⁻ in endothelial cells (Li PL, et al. *Arterioscler Thromb Vasc Biol.* 2008 Mar;28(3):485-90).
- In the present study, we proposed that Statins disrupt lipid raft formation, impede NADPH Oxidase activation, and consequently deprive hyperlipidemia-associated oxidase stress in coronary artery endothelial cells.

RESULTS

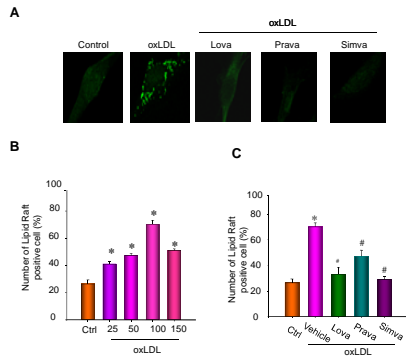


Figure 1. Panel A: Confocal fluorescence images showed that Cholera Toxin B (Green spots), a LR marker, was aggregated on cell membrane upon oxLDL treatment. In Statins (lovastatin, pravastatin, and simvastatin) pretreated groups, the green spot intensity was decreased, which indicated the LR formation was attenuated. Panel B: Summarized dose effects of oxLDL on the LR formation. The percentage of LR clustering positive cells was increased from 41% to 70% upon oxLDL stimulation from 25µg/ml to 100 µg/ml, correspondingly (n = 4). Panel C: Statins pretreatment significantly alleviated LR formation induced by oxLDL compared with vehicle group (n = 4). These results suggest that Statins have inhibitive effects on oxLDL-induced LR formation in HCAECs.

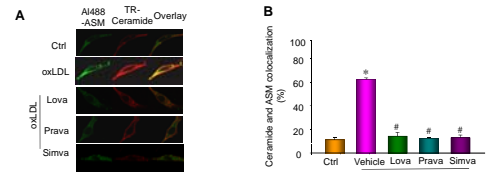


Figure 2. A: Confocal fluorescence image showed the colocalization (Yellow) of ASM (Green) and ceramide (Red) under the stimulation of oxLDL, while the colocalized yellow spots were almost completely abolished by the pre-treated Statins (lovastatin, pravastatin, and simvastatin). B: Summarized data showed the colocalized yellow spots density in different groups. These results suggest that oxLDL activates ASM, generates ceramide and subsequently forms LR clusters, and Statins can block the activation of ASM by oxLDL and LR formation (* p<0.05 vs. control group, # p<0.05 vs. oxLDL treatment groups, n=5).

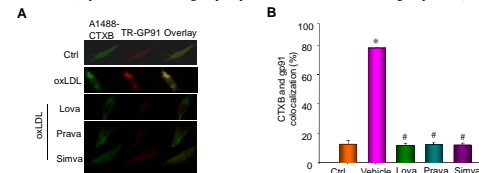


Figure 3. Since the activation of NADPH oxidase is usually associated with the LR formation, the role of gp91^{phox}, a NADPH catalytic subunit, was detected upon the oxLDL treatment and the effect of Statins (lovastatin, pravastatin, and simvastatin) was examined by confocal fluorescence image assay. A: The clustering of Alexa-488-conjugated CTXB (Green) in oxLDL treated group, an indication of LR formation, is colocalized with Texas Red-conjugated gp91^{phox} (Red) to produce yellow spots in the corresponding overlaid image. In contrast, the intensity of both the aggregated lipid green spots and the gp91^{phox} red stains were decreased in either control or Statins pre-treated groups. B: Summarized results show that the percentage of oxLDL-induced colocalization (yellow) of LR (green) and gp91^{phox} (Red) by oxLDL was significantly attenuated by pre-treated Statins. These results suggest that Statins can inhibit NADPH oxidase catalytic unit of gp91 assembled to LR, a key step for the activation of NADPH oxidase. (* p<0.05 vs. control, # p<0.05 vs. oxLDL treatment groups, n=5).

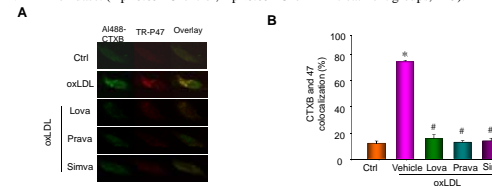


Figure 4. In order to explore whether p47, another NADPH oxidase cytosolic subunit, is translocated to LR platform and the effects of Statins on the p47 translocation process upon oxLDL stimulation, a similar confocal fluorescence image analysis was performed and similar results obtained as stated in figure 3. A: The clustering of Alexa-488-conjugated CTXB (Green) in oxLDL treated group is colocalized with the translocated TR-p47 (Red) and yellow spots produced in the corresponding overlaid image, and these colocalized yellow spots were almost invisible in control or Statins pretreated groups. B: Summarized results show that the percentage of oxLDL-induced colocalization (yellow) of LR (green) and p47 (Red) by oxLDL was significantly attenuated by pre-treated Statins. These results suggest that Statins can inhibit NADPH oxidase cytosolic subunit of p47 assembled to LR. (* p<0.05 VS. Control, # p<0.05 VS. oxLDL treatment group, n =5).

CONCLUSION

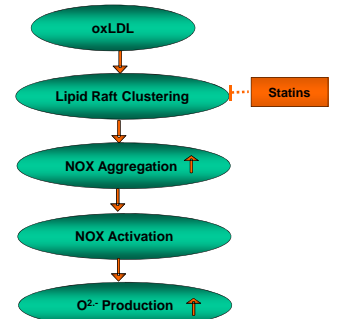


Figure 5. The confocal fluorescence images results have demonstrated that Statins could prevent oxLDL-stimulated NADPH oxidase subunits of gp91 and p47 recruiting into LR platform, a key process of NADPH oxidase activation. In order to clarify whether Statins can functionally decrease oxLDL induced superoxide production, electron spin resonance spectrometry was performed with two well-known LR disrupters, MCD and Filipin, as positive controls. A: representative ESR spectrophotograph of O₂⁻ trapped by 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) in the reaction mixture with NADH as substrate. B: Summarized data shows that O₂⁻ production was 2.5-fold higher in the LR fractions from oxLDL-treated HCAECs compared with control. And this oxLDL-induced enhancement of O₂⁻ production in LR fractions was substantially alleviated by pretreatment with Statins (n = 3).