Attenuation by Statins of Membrane Raft-Redox Signaling in Coronary Arterial Endothelium

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Abstract

Membrane raft (MR)—redox signaling platforms associated with NADPH oxidase are involved in coronary endothelial dysfunction. Here, we studied whether statins interfere with the formation of MR-redox signaling platforms to protect the coronary arterial endothelium from oxidized low-density lipoprotein (OxLDL)—induced injury and from acute hypercholesterolemia. In cultured human coronary arterial endothelial cells, confocal microscopy detected the formation of an MRs clustering when they were exposed to OxLDL, and such MR platform formation was inhibited markedly by statins, including pravastatin and simvastatin. In these MR clusters, NADPH oxidase subunits gp91phox and p47phox were aggregated and were markedly blocked by both statins. In addition, colocalization of acid sphingomyelinase (ASM) and ceramide was induced by OxLDL, which was blocked by statins. Electron spin resonance spectrometry showed that OxLDL-induced superoxide (O$_2^-$) production in the MR fractions was substantially reduced by statins. In coronary artery intima of mice with acute hypercholesterolemia, confocal microscopy revealed a colocalization of gp91phox, p47phox, ASM, or ceramide in MR clusters. Such colocalization was rarely observed in the arteries of normal mice or significantly reduced by pretreatment of hypercholesterolemic mice with statins. Furthermore, O$_2^-$ production in situ was 3-fold higher in the coronary arteries from hypercholesterolemic mice than in those from normal mice, and such increase was inhibited by statins. Our results indicate that blockade of MR-redox signaling platform formation in endothelial cell membrane may be another important therapeutic mechanism of statins in preventing endothelial injury and atherosclerosis and may be associated with their direct action on membrane cholesterol structure and function.

Introduction

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are widely used to lower plasma cholesterol level and treat atherosclerotic diseases (Greenwood and Mason, 2007; Wang et al., 2008; Libby et al., 2009). Because approximately 60–70% of serum cholesterol is derived from de novo cholesterol synthesis in the liver, inhibition of HMG-CoA reductase is able to inhibit mevalonate pathway to block cholesterol biosynthesis and, thereby, result in a dramatic reduction in circulating low-density lipoprotein (LDL) cholesterol level (Scandinavian Simvastatin Survival Study, 1994; LIPID Study Group, 1998). Thus, lowering serum cholesterol levels is thought to be the primary mechanism underlying the therapeutic benefits of statin therapy in atherosclerosis and related cardiovascular diseases.

In addition to their cholesterol-lowering effect, statins exhibit non–cholesterol-lowering activity to inhibit inflammatory responses of immune cells, including macrophages and lymphocytes; this has been associated with inhibition of mevalonate pathway to block the synthesis of isoprenoid intermediates, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, and to prevent protein prenylation and consequent membrane trafficking of small G proteins, such as Rho, Ras, and Rac (Bu et al., 2011). Previous studies have demonstrated that both cholesterol-lowering and non–cholesterol-lowering action may contribute to the beneficial effects of statins in protecting endothelial function in endothelial cells (ECs) by inhibiting NADPH oxidase-mediated redox signaling (Mason et al., 2004; Tawfik et al., 2006; Jacobson, 2009). First, statins lower the cholesterol level in ECs, resulting in augmentation of endothelial nitric oxide synthase (eNOS) activation and suppression of superoxide (O$_2^-$) production (Mason et al., 2004; Tawfik et al., 2006; Jacobson, 2009). Under resting condition, eNOS activity is inhibited by binding to a membrane caveolar protein called...
cavolin-1. By interfering with cholesterol biosynthesis and lowering plasma membrane cholesterol levels, statins were shown to decrease the expression of cavolin-1, resulting in eNOS activation and nitric oxide production (Mason et al., 2004). Second, by preventing isoprenylation of Rac1, which is important for NADPH oxidase activation, recent studies have revealed that statins inhibit O$_2^-$ formation in ECs after stimulation of injury factors or under pathologic conditions, such as angiotensin II, homocysteine, and hyperglycemia (Wagner et al., 2000; Vecchione et al., 2007; Briones et al., 2009; Alvarez et al., 2010; Bao et al., 2010c). However, it remains unknown how statins alter the assembling and aggregation of NADPH oxidase subunits and, thereby, affect its activity to produce O$_2^-$ in addition to their effect on Rac1 or eNOS.

Recent studies have indicated that membrane rafts (MRs, formerly lipid rafts) are of importance in mediating and amplifying a variety of cellular signals (Zhang et al., 2009), which may be a target for the action of statins. MRs are dynamic assemblies of cholesterol; lipids with saturated acyl chains, such as sphingolipids and glycosphingolipids, in the exoplasmic leaflet of the membrane bilayer; and cholesterol in the inner leaflet. MRs clustering is emerging as a novel mechanism mediating the transmembrane signaling in response to various stimuli in a variety of cell types, including lymphocytes, endothelial cells, and neurons (Zhang et al., 2009). Clustered MRs form membrane signaling platforms, in particular, the ceramide-enriched platforms or macromdomains (Zhang et al., 2009). These membrane platforms can recruit or aggregate various signaling molecules, such as small G proteins, tyrosine kinases, and phosphatases, resulting in the activation of different signaling pathways. More recently, there is increasing evidence that MRs clustering on the arterial ECs is an important initiating mechanism in endothelial injury in response to damaging factors, such as death receptor agonists, inflammatory factors, and irradiation (Natoli et al., 1998; Zhang et al., 2006, 2009). It has been shown that MRs clustering recruits or aggregates redox signaling molecules, such as NADPH oxidase subunits, gp91phox, p47phox, and Rac GTPase, resulting in the formation of a membrane signal amplification platform that activates and enhances production of O$_2^-$ (Zhang et al., 2006, 2007). These MR signaling platforms associated with O$_2^-$ production have been referred to as MR-redox signaling platforms. The formation of such MR-redox signaling platforms in the EC membrane is associated with ceramide production through lysosomal acid sphingomyelinase (ASM), which is translocated onto the plasma membrane via membrane proximal lysosome trafficking and fusion after stimulation of death receptors (Jin et al., 2008a; Bao et al., 2010a,b). It has been shown that this lysosomal ASM-mediated formation of redox signaling platforms could be inhibited by cholesterol depletion reagents, methyl-β-cyclodextrin and filipin (Zhang et al., 2006, 2007). Statins have been shown to decreases plasma membrane cholesterol levels in ECs (Mason et al., 2004). In this regard, it is plausible that statins may interfere with MR-redox signaling through their cholesterol-lowering action and, thus, prevent endothelial dysfunction in coronary arteries.

In this study, we first determined whether statins inhibit the formation of MR-redox signaling platforms to decrease O$_2^-$ production in ECs stimulated by a proatherogenic factor, oxidized LDL (OxLDL). Then, we extended our studies to animal experiments to test whether this action of statins on MR clustering can be observed. Using a typical mouse model of acute hypercholesterolemia, we determined the effects of statin treatment on MR clustering, NADPH oxidase assembly, and O$_2^-$ production in the coronary arterial. Our results indicate that blockade of MR-redox signaling platform formation in the EC membrane is another important therapeutic mechanism of statins in preventing reactive oxygen species formation during endothelial injury and atherosclerosis.

### Materials and Methods

**Cell Culture and Stimulation.** Human coronary arterial endothelial cells (HCAECs) were purchased and maintained in commercially available endothelial cell growth medium (Invitrogen, CA), as described previously Tawfik et al. (2006). All studies were performed by using HCAECs of 3–5 passages. OxLDL (Kalen Human Medium OxLDL; Kalen Biomedical, Montgomery Village, MD) was used as proatherogenic stimuli to treat HCAECs. Pravastatin and simvastatin (both statins were purchased from Sigma-Aldrich [St. Louis, MO]) were selected as prototype statins to treat cells and mice, as pravastatin is typical water-soluble statin and simvastatin is a typical lipid-soluble one. Cells were pretreated with pravastatin (10 μM) or simvastatin (5 μM) for 1 hour and then stimulated with OxLDL (100 μg/ml) for 15 minutes in all experiments of the present study, if not otherwise mentioned. Simvastatin was activated by opening the lactone ring by dissolving in 95% ethanol and 0.1 N NaOH, heating at 50°C for 2 hours, and neutralizing with HCl to pH 7.2, as described previously (Gerson et al., 1989; Tawfik et al., 2006). The doses of statins were chosen on the basis of previous studies (Dje N’Guessan et al., 2009; Alvarez et al., 2010) and our observations that statins at these doses did not significantly induced morphologic signs of cytotoxicity in HCAECs. Some groups of cells were pretreated with mevalonate (10 μM), farnesol (10 μM), geranylgeraniol (10 μM), methyl-β-cyclodextrin (1 mM), or filipin (1 μg/ml; all reagents were purchased from Sigma-Aldrich) for 1 hour. Plasmids containing cDNA encoding oncogenic Rac1 gene were used as previously described (Yi et al., 2009). Transfection of cDNA plasmids was performed using the TransFectin Lipid Reagent (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions.

**Confocal Microscopy of MRs and Related Proteins in HCAECs.** For confocal detection of MR platforms or MR-associated proteins, HCAECs were grown on poly-L-lysine–coated chamber slides, stimulated or remain unstimulated, and fixed in paraformaldehyde fixation buffer/phosphate-buffered saline for 10 minutes. Detection of MR clusters was performed as described previously (Jin et al., 2008a). In brief, cells were fixed, unpermeabilized, and stained with Alexa Fluor 488-conjugated cholera toxin B (A1488-CTXB, 2 μg/ml) and Molecular Probes (Life Technologies), Carlsbad, CA), which specifically binds to G$_{12/13}$ gangliosides enriched in MRs. The patch formation of A1488-CTXB–labeled gangliosides complex represented the MR clusters. Clustering was defined as one or several intense spots of fluorescence on the cell surface, whereas unstimulated cells showed a homogenous distribution of fluorescence throughout the membrane. In each experiment, the presence or absence of clustering in samples of 200 cells was scored by two independent observers after specifying the criteria for positive spots of fluorescence. Cells displaying a homogenous distribution of fluorescence were marked negative. Results were given as the percentage of cells showing one or more clusters after the indicated treatment as described.

To detect the colocalization of ASM with ceramide in HCAECs, the cells were incubated with rabbit anti-ASM (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-ceramide antibodies (1:200; Alexis Biochemicals [Enzo Life Scienes], Farmingdale, NY).
Cells then were stained for another hour with Alexa Fluor 488 (Ab488)–labeled donkey anti-rabbit and Texas Red (TR)–labeled donkey anti-mouse antibodies (1:500; Invitrogen [Life Sciences], Carlsbad, CA). Similarly, for detection of the colocalization of MRs with gp91phox or p47phox, HCAECs were first incubated with Ab488-CTxB (2 μg/ml) and mouse monoclonal anti-gp91phox or anti-p47phox (1:200; BD Biosciences, San Jose, CA), followed by incubation with TR-conjugated anti-mouse (1:500; Invitrogen). Then, the slides were washed, mounted, and visualized through sequentially scanning on an Olympus laser scanning confocal microscope (Fluoview FV1000; Olympus, Tokyo, Japan). Colocalization was analyzed using Image Pro Plus software (Media Cybernetics, Inc, Bethesda, MD), and the colocalization coefficient was represented by Pearson’s correlation coefficient. All antibodies and probes were incubated at room temperature for 1 hour if not mentioned.

**Electronic Spin Resonance Spectrometric Detection of O2**−. Electronic spin resonance (ESR) detection of O2− was performed as described previously (Zhang et al., 2007). In brief, HCAECs were gently collected and suspended in modified Krebs-HEPES buffer containing deferoxamine (100 μM, metal chelator). Approximately 1 × 106 HCAECs were incubated with OxLDL (100 μg/ml) or bacterial sphingomyelinase (0.01 U/ml; Sigma-Aldrich) for 30 minutes in the absence or presence of statins (pravastatin, 10 μM; simvastatin, 5 μM), then mixed with 1 mM spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH; Noxygen, Elzach, Germany) in the presence or absence of 100 units/ml polyethylene glycol–conjugated superoxide dismutase (SOD). The cell mixture loaded in glass capillaries was immediately analyzed for O2− production at each minute for 10 minutes with use of a Miniscope MS200 ESR spectrometer (Magnettecht, Berlin, Germany). The ESR settings were as follows: biofield, 3350; field sweep, 60G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3G; 4096 points of resolution; receiver gain, 100; and kinetic time, 10 minutes. The SOD-inhibitable signals were normalized by protein concentration and compared among different experimental groups.

**Acute Hypercholesterolemia in Mice.** Poloxamer 407 (P407; Sigma-Aldrich) was used to induce acute hypercholesterolemia in mice as described previously (Johnston et al., 2006). In brief, twenty 6-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were randomly divided into four groups, with 5 mice for each group: control group, P407 group (hypercholesterolemia), statin group, and P407 plus statin group. The mice from statin group and P407 plus statin group were intragastrically fed statins (pravastatin, 140 mg/kg; simvastatin, 5 mg/kg) or bacterial sphingomyelinase subunits in the coronary arterial intima were detected as described previously with some modifications (Jankov et al., 2005). In brief, the mouse hearts were frozen in Tissue-Tek OCT and cut by cryostat into 10-μm sections and mounted on Superfrost/Plus slides. After fixation with acetone, the frozen section slides were incubated with rabbit anti-flotillin-1 antibody (1:50; Cell Signaling Technology, Danvers, MA) and mouse anti-gp91phox or anti-p47phox antibody overnight at 4°C (BD Biosciences). Similarly, detection of the colocalization of MR clusters with ASM or ceramide was performed by incubating section slides with the same rabbit anti-flotillin-1 as described above and mouse anti-ceramide (BD Biosciences) antibodies or with a mouse anti-flotillin-1 (BD Biosciences) and rabbit anti-ASM antibodies (Santa Cruz Biotechnology). After incubation with primary antibodies, the slides were washed and labeled with corresponding Alexa Fluor-488– and Alexa Fluor-555–conjugated secondary antibodies (Invitrogen). All primary antibodies were used at a dilution of 1:50, and all secondary antibodies were used at a dilution of 1:200. Then, the slides were washed, mounted, and subjected to confocal microscopic analysis (Fluoview FV1000; Olympus).

**In Situ Detection of O2**− Production in Mouse Coronary Arteries. Dihydroethidium (DHE) is a lipophilic cell-permeable dye that can be oxidized by O2− to form ethidium bromide. Ethidium then binds irreversibly to the double-stranded DNA (such as chromosomal DNA), causing amplification of a red fluorescent signal at 480/610 nm excitation-emission. The O2− produced in the arterial wall was detected in situ by using DHE as probes (Molecular Probes) as described previously (Jankov et al., 2008; Nijmeh et al., 2010). In brief, the unfixed tissue slides from different groups were incubated with DHE (10 μM) in phosphate-buffered saline at room temperature for 30 minutes. Then, the slides were washed, fixed, mounted, and subjected to confocal microscopic analysis (Fluoview FV1000; Olympus). In some experiments, the tissue slides were incubated at room temperature for 30 minutes in the presence of NADPH oxidase–specific inhibitor gp91 day-tat peptide (5 μM; AnaSpec, Fremont, CA) or O2− scavenger polyethylene glycol–SOD (100 U/ml; Sigma-Aldrich).

**Statistics Analysis.** Data are presented as mean values ± S.E.M. Significant differences between and within multiple groups were examined using one-way analysis of variance test, followed by Duncan’s multiple-range test. A Student’s t test was used to detect significant differences between two groups. The statistical analysis was performed using SigmaStat 3.5 software (Systat Software, San Jose, CA). P ≤ 0.05 was considered to be statistically significant.

**Results**

**Statins Inhibit OxLDL-Induced MR Clustering in HCAECs.** Figure 1A presents the representative fluorescent confocal microscopic images showing Alexa488-CTxB–labeled patches in the membrane of HCAECs. Under resting conditions (control), there was only a diffuse fluorescent staining in the cell membrane, indicating possibly evenly distributed single MRs. CTxB specifically binds with ganglioside GM1 enriched in MRs. When HCAECs were incubated with OxLDL, some large fluorescent dots or patches were observed in the membrane, indicating that MRs aggregated on the cell membrane after OxLDL treatment. In statin-pretreated (pravastatin and simvastatin) groups, the number
of green spot or patches was decreased, indicating that the MR clustering is attenuated. Figure 1B summarized the effects of different doses of OxLDL on the MR clustering by counting the percentage of cells with these MR clusters or patches. We found that control cells displayed a small percentage with MR clustering (26.4% ± 5.8%). After these cells were stimulated with OxLDL, MR-clustered positive cells increased significantly with a maximum response of 70.1% ± 6.1% at 100 μg/ml. When these cells were pretreated with pravastatin or simvastatin, OxLDL-induced MR clustering was significantly inhibited. These inhibitory effects of statins could be reversed by treating the cells with mevalonate or farnesol, but not with geranylgeraniol or by overexpression of oncogenic Rac1 GTPase.

**Statins Inhibit OxLDL-Induced ASM Translocation and Ceramide Production in HCAECs.** Previous studies have shown that lysosomal trafficking and translocation of ASM into MRs result in ceramide production, MR clustering, and formation of ceramide-enriched macrodomains (Jin et al., 2008a). To examine whether ASM/ceramide is involved in OxLDL-induced MR clustering and whether OxLDL-induced MR clustering forms ceramide-enriched macrodomains, we stained HCAECs with Alexa488-conjugated anti-ASM and TR-labeled anti-ceramide. As shown in Fig. 2A, OxLDL stimulation caused an aggregation of ASM in ceramide-enriched macrodomains, which exhibited as yellow dots or patches. When these cells were pretreated with pravastatin or simvastatin, OxLDL-induced ASM aggregation in ceramide clusters was significantly blocked. This result means that Ox-LDL led to ASM translocation and ceramide production in the cell membrane, especially in the MR domain (see below), which can be blocked by statins. Summarized colocalization coefficient shown in Fig. 2B suggests that statins markedly block OxLDL-induced ASM translocation, ceramide production, and subsequent formation of ceramide-enriched macrodomains.

**Statins Prevent OxLDL-induced NADPH Oxidase Subunit Aggregation in MR Clusters.** To examine whether NADPH oxidase subunits are able to aggregate in MR clusters after OxLDL stimulation, we stained HCAECs with Alexa488-conjugated anti-ASM and TR-conjugated anti-ceramide antibodies. (A) Representative images show the colocalization (yellow) of ASM (green, Alexa488-anti-ASM) and ceramide (red, TR-anti-ceramide) under the stimulation of OxLDL (100 μg/ml) with or without pretreatment of statins. (B) Summarized data show the colocalization coefficient indicating the relative ratio of cells with colocalized yellow spots (n = 5). *P < 0.05, versus control; #P < 0.05, versus vehicle.
with both Al488-CTXB and TR-conjugated anti-gp91\textsuperscript{phox} or anti-p47\textsuperscript{phox} antibodies, and the distribution of gp91\textsuperscript{phox} or p47\textsuperscript{phox} in MR clusters was visualized by confocal microscopy. As shown in Fig. 3A, gp91\textsuperscript{phox}, a membrane-associated subunit of NADPH oxidase, was evenly distributed throughout the whole cell under control condition, and no colocalization of gp91\textsuperscript{phox} or CTXB-positive MR dots or patches was observed. When HCAECs were stimulated with OxLDL, gp91\textsuperscript{phox} was aggregated in MR clusters, as shown by strong yellow fluorescent dots or patches. In Fig. 3B, p47\textsuperscript{phox}, a cytosolic subunit of NADPH oxidase was also found to evenly spread throughout the whole cell, mainly in cytosol under control condition. OxLDL induced translocation of p47\textsuperscript{phox} into MR clusters, as shown by colocalization of p47\textsuperscript{phox} in CTXB-positive yellow dots or patches. In pravastatin- and simvastatin-pretreated HCAECs, however, OxLDL-induced aggregation of positive yellow dots or patches. In pravastatin- and simvastatin-statins. Moreover, these effects of OxLDL were inhibited by MR-redox signaling platforms) that possess redox signaling function. Moreover, these effects of OxLDL were inhibited by statins.

Effects of Statins on OxLDL-Induced O$_2^-$ Production in HCAECs. Using ESR analysis, we determined the production of O$_2^-$ in HCAECs induced by OxLDL in the absence or presence of statins. Figure 4A depicts representative ESR spectrographs of O$_2^-$ production as trapped by CMH under different treatments. As shown in summarized data in Fig. 4B, OxLDL alone increased O$_2^-$ production by 2.7 ± 0.2-fold, compared with control. When these cells were pretreated with pravastatin and simvastatin, OxLDL-induced O$_2^-$ production was reduced. Methyl-$\beta$-cyclodextrin (MCD) and filipin are potent chemical chelators for cholesterol and are used as MR disruptors. Similar to statins, these two MR disruptors inhibited OxLDL-induced O$_2^-$ production. Moreover, sphingomyelinasen-induced production of O$_2^-$ could be inhibited by pravastatin, simvastatin, and MCD (Fig. 4C).

Blockade of P407-Induced Increase in Plasma Cholesterol Levels in Mice by Statins. To determine the inhibitory role of statins on OxLDL-induced NADPH oxidase activation and O$_2^-$ production in vivo, we pretreated mice with vehicle or a cocktail of statins and then induced acute hypercholesterolemia by treating mice with P407 for 24 hours. As shown in Fig. 5, P407 caused a 9.8-fold increase in plasma cholesterol concentration in control mice, suggesting that these P407-treated mice experienced acute hypercholesterolemia. In mice treated with statins alone, the basal cholesterol level decreased significantly by 34%, compared with control mice. In mice pretreated with statins, P407-induced increase in cholesterol level was reduced by 64%, compared with that of vehicle-pretreated mice.

Colocalization of gp91\textsuperscript{phox} and p47\textsuperscript{phox} in MRs in Arterial Intima of Hypercholesterolemic Mice. To determine whether NADPH oxidase subunits aggregate in MR clusters in the arterial intima exposed to high cholesterol concentration, we stained frozen sections of mouse hearts with the MR marker protein, Alexa488-conjugated anti-flotillin-1, and TR-conjugated anti-gp91\textsuperscript{phox} or anti-p47\textsuperscript{phox} and then examined the colocalization of flotillin-1 with gp91\textsuperscript{phox} or p47\textsuperscript{phox} in the coronary arteries. In Fig. 6, A and B, typical merged images show strong yellow patches in the edge of arterial lumen in P407-treated mice, indicating the colocalization of gp91\textsuperscript{phox} or p47\textsuperscript{phox} in MR clusters in arterial intima. No such yellow patches were observed in the arteries of control mice or mice treated with statins alone. Furthermore, pretreatment of mice with statins significantly reduced the formation of yellow patches in the arterial intima of P407-treated mice. Figure 6C shows summarized colocalization coefficient indicating the relative ratio of cells with colocalized yellow spots (n = 5). *P < 0.05, versus control; #P < 0.05, versus vehicle.

Colocalization of ASM or Ceramide in MRs in Artery Intima of Hypercholesterolemic Mice. As shown in Fig. 7, colocalization of ceramide or ASM in MRs was examined in mouse arteries, similar to Fig. 6. We found no colocalization of either ASM or ceramide in MRs in arteries from control mice; however, strong colocalization of ASM or ceramide was observed (shown as yellow patches) in the edge of arterial lumen from P407-treated mice. Pretreatment of mice with statins abolished such colocalization, because yellow fluorescence was markedly reduced. Figure 7C shows summarized colocalization coefficient between flotillin-1 and ASM or ceramide. These results suggest that acute hypercholesterolemia...
in mice causes translocation of ASM into MRs and consequent activation of this enzyme, resulting in ceramide production, which promotes MR clustering and formation of ceramide-enriched platforms. This high plasma cholesterol-induced ASM translocation/activation and ceramide production could be blocked by statins.

Statins Inhibit Oxidase–Associated with Membrane Rafts

Although statins are known to inhibit NADPH oxidase assembly and activation in MR-redox signaling platforms in ECs induced by OxLDL and in the coronary arterial endothelium of mice with acute hypercholesterolemia, the present study demonstrated that statins inhibit NADPH oxidase assembly and activation in MR-redox signaling platforms in ECs induced by OxLDL and in the coronary arterial endothelium of mice with acute hypercholesterolemia. Our results prove a hypothesis that OxLDL induces the generation of ROS through MR aggregation and MR-redox signal platform formation, which are mediated by ASM translocation and activation. Statins may improve the endothelial function through blocking this MR signaling pathway by blockade of ASM activation, MR clustering, and MR-redox platform formation.

OxLDL is a proatherogenic lipoprotein that leads to vascular dysfunction at the early stage of atherosclerosis. It
has been reported that elevated serum levels of OxLDL are associated with increased risk of endothelial dysfunction and coronary artery diseases (Steinberg and Witztum, 2002; Li and Mehta, 2005; Zhu et al., 2005; Heinecke, 2006), where NADPH oxidase activity and \( \text{O}_2^{\cdot-} \) production were found to be significantly enhanced (Li and Mehta, 2005; Zhu et al., 2005; Chow et al., 2007). In this regard, accumulating evidence suggests that MR clustering promotes aggregation or translocation of NADPH oxidase subunits and, thereby, forms MR-redox signaling platforms in ECs after death-receptor activation or after stimulation of various endothelial injury factors, including FasL, tumor necrosis factor-\( \alpha \), and endostatin (Zhang et al., 2006). In the present study, we demonstrated that OxLDL also induced the formation of a large number of MR-redox signaling platforms (characterized by gp91\(_{\text{phox}}\) aggregation and p47\(_{\text{phox}}\) translocation in MR clusters) and consequent production of \( \text{O}_2^{\cdot-} \) in ECs. These results support the view that the MR clustering induced by OxLDL serves as a membrane platform for assembly of NADPH oxidase subunits to form an active enzyme complex. To our knowledge, our findings for the first time reveal the role of MR-redox signaling platform associated with NADPH oxidase in OxLDL-induced \( \text{O}_2^{\cdot-} \) production in coronary ECs. Targeting the formation of this MR-redox signaling platform may be an important therapeutic strategy for improvement of endothelial function and prevention of atherosclerosis. The present study explores this possibility by using statins, a group of commonly used cholesterol-lowering compounds.

MRs are dynamic assemblies of cholesterol, lipids with saturated acyl chains, such as sphingolipids and glycosphingolipids. Because the integrity of MRs is highly dependent on the cholesterol level in the plasma membrane and several chemical cholesterol chelators, including MCD and filipin, are MR disruptors, MRs may serve as potential targets for the classic cholesterol inhibition action of statins. In the present study, we demonstrated that pravastatin and simvastatin markedly attenuated MR clustering induced by OxLDL in HCAECs and mevalonate could significantly reverse the inhibitory effects of statins, indicating that mevalonate pathway–mediated cholesterol may be important for OxLDL-induced MR clustering. Moreover, MR-mediated \( \text{O}_2^{\cdot-} \) production induced by OxLDL was also abolished by pravastatin and simvastatin in an action similar to that of the MR disruptors MCD and filipin, which deplete cholesterol in the plasma membrane. Thus, our results suggest that statins may inhibit MR clustering by decreasing cholesterol levels in ECs. In line with our view, a recent study has reported that statins (lovastatin and atorvastatin) disrupt MRs, leading to decreased MR expression of lectin-like oxidized low-density

![Fig. 6. Statins block the NADPH oxidase subunits clustering in MR clusters in coronary arteries of mice with acute hypercholesterolemia. Frozen sections of mouse hearts were stained with Al488-anti-flotillin-1 and TR-anti gp91\(_{\text{phox}}\) or TR-anti-p47\(_{\text{phox}}\). Representative merged images displayed yellow dots or patches indicating the colocalization of MR marker protein flotillin-1 with NADPH oxidase subunit gp91\(_{\text{phox}}\) (A) or p47\(_{\text{phox}}\) (B). Each image includes an enlarged view of region of interest at the lower right corner. Scale bar, 50 \( \mu \)m. (C) Summarized data show the colocalization coefficiency (\( n = 5 \)). * \( P \) < 0.05, versus control; \# \( P \) < 0.05, versus vehicle.](image1)

![Fig. 7. Statins block ceramide or ASM clustering in MR clusters in coronary arteries of mice with acute hypercholesterolemia. Frozen sections of mouse hearts were stained with Al488-anti-flotillin-1 and TR-anti-ASM or TR-anti-ceramide. Representative merged images displayed yellow dots or patches indicating the colocalization of MR marker protein flotillin-1 with ASM (A) or ceramide (B). Each image includes an enlarged view of region of interest at the lower right corner. Scale bar, 50 \( \mu \)m. (C) Summarized data show the colocalization coefficiency (\( n = 5 \)). * \( P \) < 0.05, versus control; \# \( P \) < 0.05, versus vehicle.](image2)
signaling in ECs (Zhang et al., 2006, 2007). The present study clusters and amplifies MR-NADPH oxidase essential for aggregating NADPH oxidase subunits in MR. Studies have demonstrated that ASM-ceramide signaling is production in MR clusters induced by OxLDL. Our previous statins blocked the translocation of ASM to and ceramide domains, which can serve as MR-redox signaling platforms previously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b). Ceramides spontaneously fuse MRs into large ceramide-enriched membrane (Jin et al., 2008b; Bao et al., 2010a,b).
marker and colocalization with NADPH oxidase subunits were substantially blocked by pretreatment of mice with statins. In all groups of mice, statins had no effect on mRNA levels of gp91phox or p47phox (Supplemental Fig. 1), confirming that statins inhibit NADPH oxidase activation rather than gene expression. Furthermore, double staining of MR marker flotillin-1 with ASM or ceramide showed that hypercholesterolemic mice also exhibited enhanced formation of ceramide-enriched platforms in the intima of coronary arteries. In mice receiving statins, the formation of ceramide-enriched platforms was almost completely blocked. Moreover, our preliminary study demonstrated that P407 had no effect on MR clustering in HCAECs, suggesting that MR clustering and formation of redox signaling platforms in vivo are associated with P407-induced cholesterol increase rather than direct interaction between P407 and MRs. Taken together, these results demonstrate that the ceramide-enriched MR-redox signaling platforms associated with NADPH oxidase are increasingly formed in the intact arterial endothelium of hypercholesterolemic mice and that statins block the formation of this ceramide-enriched MR-redox signaling platform.

The next question addressed in the present study was whether assembled NADPH oxidase in such MR-redox signaling platforms is activated to produce O₂⁻ in the coronary arterial endothelium in mice with hypercholesterolemia and whether statins interfere with NADPH oxidase activation in vivo. As measured by DHE fluorescence analysis, O₂⁻ production was indeed increased in the coronary arteries from hypercholesterolemic mice, compared with normal mice, which was attenuated by pretreatment of mice with statins. It is obvious that MR clustering—associated assembly of NADPH oxidase in the cell membrane leads to activation of this O₂⁻-producing enzyme.

In summary, the present study demonstrated that acute treatment of coronary arterial ECs with statins inhibited OxLDL-induced MR clustering, ASM translocation into membrane, ceramide production, and the formation of MR-redox signaling platforms in these ECs in vitro. The inhibitory effect of statins on MR-redox signaling is associated with their direct inhibitory effects on NADPH oxidase activation and expression.

**Authorship Contributions**

**Participated in research design:** Wei, P.-L. Li, Zhang.

**Conducted experiments:** Wei, X. Li, Xiong, Xia, Abais, Boini, Zhang.

**Performed data analysis:** Wei, X. Li, Zhang.

**Wrote or contributed to the writing of the manuscript:** Wei, X. Li, P.-L. Li, Zhang.

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Supplementary Material

Attenuation by Statins of Membrane Raft-Redox Signaling in Coronary Arterial Endothelium

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Real-time reverse transcription polymerase chain reaction (RT-PCR): Total RNA from isolated mouse coronary arteries was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol described by the manufacturer. Aliquots of total RNA (1 µg) from each sample were reverse-transcribed into cDNA according to the instructions of the first-strand cDNA synthesis kit manufacturer (Bio-Rad, Hercules, CA, USA). Equal amounts of the reverse-transcriptional products were subjected to PCR amplification using SYBR green as the fluorescence indicator on a Bio-Rad iCycler system (Bio-Rad). The mRNA levels of target genes were normalized to the β-actin mRNA levels. The primers used in this study were synthesized by Operon (Huntsville, AL, USA) and the sequences were: for gp91\textsubscript{phox} sense TGGCACATCGATCCCCTCAGAA, antisense GGTCATGCACTCAAGGCAACCT; for p47\textsubscript{phox} sense CCA CAC CTG CTG GAC TTC TT, antisense ATC TTT GGG CAC CAG GTA TG; for β-actin sense TCGCTGCCTGGTACCAGAC; antisense GGCCTGCCTACCCACCATAGGA.

![Figure 1. Statins had no effect on mRNA levels of gp91\textsubscript{phox} or p47\textsubscript{phox} in coronary arteries as determined by real-time RT-PCR (n=4).](image-url)