Low density lipoprotein induces eNOS translocation to membrane caveolae: the role of RhoA activation and stress fiber formation

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Abstract

A decrease in the bioavailability of endothelium-derived nitric oxide (NO) is linked to hypercholesterolemia. However, the mechanism by which low density lipoprotein (LDL) mediates endothelial NO synthase (eNOS) dysfunction remains controversial. We investigate the effect of LDL on eNOS regulation in human endothelial cells (ECs). In cultured ECs, a high level of LDL increased the abundance of eNOS and caveolin-1 (Cav-1) in the membrane caveolae and the association of eNOS with Cav-1. Furthermore, it decreased the basal level of NO and blocked NO production stimulated by the calcium ionophore A23187. LDL exposure also increased the formation of stress fibers and the membrane translocation of eNOS. These effects can be blocked by cytochalasin D, an actin cytoskeleton disruptor. In revealing the mechanism underlying the translocation of eNOS, we found that a high level of LDL increased the level of membrane-associated and GTP-formed RhoA and activated the RhoA downstream kinase ROCK-1 activity. Y-27632, a specific inhibitor of ROCK-1, blocked LDL-induced stress fiber formation, eNOS translocation and NO production. In conclusion, a high level of LDL increases the movement of eNOS to membrane caveolae via the increased stress fibers. The RhoA-mediated pathway may play a crucial role in this process in vascular ECs.

Keywords: eNOS; Caveolin-1; RhoA; LDL; Endothelial cell

1. Introduction

Hypercholesterolemia is a central pathogenic factor for atherogenesis, and endothelial dysfunction is one of the earliest events of atherosclerosis. One of the characteristics of endothelial dysfunction is impaired endothelium-dependent vasodilation [1–3]. Both clinical and laboratory studies suggest that high concentrations of low density lipoprotein (LDL) might inhibit endothelium-dependent vascular relaxation through a decrease in the production of bioavailable endothelium-derived nitric oxide (NO). One possible involved mechanism is the impaired activation of endothelial NO synthase (eNOS). In cultured endothelial cells (ECs), LDL or its modified forms or components regulate eNOS at the level of mRNA, protein or activity [4–8]. However, the mechanistic basis for eNOS regulation by LDL remains unclear.

The localization of eNOS within the subcellular compartments determines and regulates its activity [9,10]. One particular cellular site altering eNOS residues is the caveolae. In caveolae, the increased association of eNOS with caveolin-1 (Cav-1) renders eNOS in the inactive state [11–13]. Calcium-dependent calmodulin promotes the dissociation of eNOS from Cav-1 and the subsequent eNOS activation by competitively displacing Cav-1. Conversely, an increased association of eNOS and Cav-1 can alter eNOS activation in response to the agonist stimulation. Recently, we reported that atherogenic concentrations of LDL increased the translocation of Cav-1 to membrane caveolae in human ECs [14]. Thus, the exposure of caveolae-enriched human ECs to high levels of LDL may increase the eNOS caveolae translocation and the association of eNOS with Cav-1, which, in turn, inhibits the function of eNOS.

The endothelial actin cytoskeleton and eNOS activity are co-regulated by diverse stimuli [15,16]. Incubating ECs with...
LDL increases stress fiber formation [17]. Disrupting the actin cytoskeleton by introducing a dominant negative form of RhoA or a cytoskeleton disruptor up-regulates eNOS. However, the role of the actin cytoskeleton in the interaction between eNOS and Cav-1 and their caveolar translocation in ECs have not been demonstrated. Furthermore, it is not known whether LDL-induced eNOS dysfunction is directly linked to LDL-induced stress fiber formation. The purpose of this study, therefore, was to elucidate the mechanism whereby LDL increases the localization of eNOS and Cav-1 in caveolae and whether this localization depends on a change in the actin cytoskeleton in ECs.

2. Materials and methods

2.1. Cell culture and LDL isolation

Human umbilical vein endothelial cells (HUVECs) were isolated and maintained as described [18]. All experiments were performed with cells up to passages three and cultured to confluence before LDL treatment [14]. LDL was isolated from fresh human plasma as described [18]. The oxidation state of LDL was measured by the thiobarbituric acid reactive substances (TBARS) assay. Malondialdehyde (MDA) equivalents were quantified on a spectrophotometer (535 nm). The LDL used in this study contained less than 0.5 nmol of MDA equivalent per milligram of cholesterol. Total cholesterol concentration of LDL was determined by enzymatic assay.

2.2. Cav-1-enriched membrane fractions, immunoprecipitation and Western blotting

Cav-1-enriched membrane fractions were isolated from 2 × 150-mm dishes of cells with use of a modified detergent-free extraction procedure as previously described [14]. The fractions underwent subsequent co-immunoprecipitation (co-IP) and Western blotting with antibodies against eNOS or Cav-1 (BD Transduction Laboratories, San Diego, CA) as described [14].

2.3. Total NO assay and measurement of phosphorylated eNOS

HUVECs in 12-well plates were cultured to confluence in phenol red-free EC medium. After LDL exposure, the cell culture medium was collected, and particulates were removed by centrifugation. All samples were diluted with reaction buffer and ultrafiltered through Ultrafree-MC, a 10,000 molecular weight cutoff filter (Millipore, Bedford, MA), to eliminate proteins. The NO product in the cell culture supernatant was quantitatively measured with use of a kit (R&D System, Minneapolis, MN). Phospho-eNOS levels were determined with use of polyclonal antibodies against phospho-eNOS (Ser1177) (Cell Signaling Technology, Beverly, MA) as described [19]. The total eNOS in the same membranes was also detected afterwards as a control.

2.4. In situ immunofluorescence staining and confocal microscopy

HUVECs, grown to confluence on chamber slides, were exposed to LDL for 2 to 18 h. The cells were fixed and stained under indirect immunofluorescence with rabbit anti-Cav-1 and mouse anti-eNOS IgGs, an FITC-conjugated secondary antibody against rabbit IgG and a Rhodamine-conjugated secondary antibody against mouse IgG, respectively. In the case of F-actin filament staining, FITC-conjugated phalloidin was applied. The results were observed under a Zeiss 510 confocal microscope [14].

2.5. Affinity precipitation of GTP-bound cellular RhoA

The bacterial expression vector GST-TRBD, corresponding to the human RhoA binding domain of Rhotekin (TRBD), was provided by M. Schwartz at the Scripps Institute, La Jolla, CA [20]. A GST-TRBD fusion protein was purified and verified as described [14]. The procedure used to measure GTP-RhoA binding on GST-TRBD was as described. Briefly, cellular proteins were extracted from LDL-treated HUVECs, and 500 µg of protein was incubated with GST-TRBD, sepharose 4B conjugate for 90 min at 4 °C in a binding buffer containing magnesium and protease inhibitors. The sepharose 4B beads were collected and washed. Samples were then resuspended in SDS sample buffer and heated for 10 min. The RhoA proteins in the supernatant were separated on SDS-PAGE and detected by use of an antibody against RhoA (Upstate Biotechnology, Lake Placid, NY). The amount of TRBD-bound RhoA was normalized to the total amount of RhoA in cell lysates for comparing RhoA activity.

2.6. Rho-associated protein kinase-1 (ROCK-1) kinase activity assay

The procedure to measure ROCK-1 kinase activity was as previously described [21,22]. Briefly, cells were lysed and the ROCK proteins immunoprecipitated with use of an anti-ROCK-1 antibody (Santa Cruz Biotech, Santa Cruz, CA). The pelleted immunocomplex was washed with a ROCK assay buffer then resuspended in 30 µl of the kinase buffer containing 25 µCi of [γ-32P] ATP, 25 µM ATP, and 5 µg of myelin basic protein (MBP) and incubated at 30 °C for 30 min [22]. The kinase reaction was stopped by adding SDS sample buffer; the mixture was resolved on 10% SDS-PAGE, and phosphorylated MBP was detected by autoradiography.

2.7. Statistics

Quantitative data were expressed as mean ± S.D. Statistical significance of the data was evaluated with use of the
Student’s t test. P values less than 0.05 were considered significant. For nonquantitative data, the results were expressed as being representative of at least three independent experiments.

3. Results

3.1. LDL increases eNOS membrane translocation

We first exposed ECs to LDL to investigate whether LDL regulates eNOS at the protein level. Western blotting revealed that ECs exposed to LDL for 6 h did not cause a significant change in the level of eNOS protein in whole cell lysates. The same level of Cav-1 detected in the same blot indicated that LDL exposure did not regulate the Cav-1 protein in ECs (Fig. 1A) [14]. However, the eNOS level in the cellular membrane from LDL-exposed cells markedly increased between 4 and 6 h and decreased in the cytosol portions correspondingly (Fig. 1B). Thus, LDL appears to regulate eNOS in ECs by translocating eNOS from the cytoplasm to the cellular membrane rather than by increasing de novo protein synthesis. Furthermore, the exposure of ECs to different concentrations of LDL increased the level of eNOS in the cellular membrane in a dose-dependent fashion: the concentration producing the highest level was 240 mg/dl (Fig. 1C). Therefore, LDL at a concentration of 240 mg/dl was used in the rest of the experiments.

Fig. 1. Effect of LDL on eNOS membrane translocation. ECs were incubated with 240 mg/dl of LDL for different times or different concentrations of LDL for 4 h as indicated. (A) Cell lysates were extracted and equal amounts of total cellular proteins (15 μg/lane) separated on SDS-PAGE. The eNOS protein was detected on Western blotting, and then Cav-1 protein was detected with the same blot. (B) The membrane proteins and cytosol proteins were isolated, and 5 μg/lane of membrane proteins and 15 μg/lane of cytosol proteins were separated on SDS-PAGE. (C) The membrane proteins were isolated and equal amounts of proteins (5 μg/lane) separated on SDS-PAGE. The eNOS protein was detected on Western blotting. The relative density of each band was quantified and labeled as a multiple of the control. Results represent three independent experiments.

Fig. 2. Distributions and the interaction between eNOS and Cav-1. (A) After exposing ECs to LDL for 4 h, cellular fractions were isolated by use of a detergent-free, sucrose-gradient separation method. The fractions were numbered from top to bottom. Fractions of equal volume (30 μl/lane) were separated on SDS-PAGE, and the Cav-1 protein was first detected on Western blotting. eNOS protein levels were detected on the same blot without stripping. The relative density of each band was quantified and labeled as a percentage of the total amount of eNOS protein (sum of the density of all bands). (B) The caveolin-enriched fractions (lanes 4–6) were pooled and the proteins concentrated. The same amount of protein (10 μg/lane) was separated on SDS-PAGE, and both eNOS and Cav-1 proteins were detected. (C) The Cav-1 proteins were immunoprecipitated with the corresponding antibody from whole cell lysates. Aliquots of the whole cell lysates (15 μg/lane) and the Cav-1-antibody-precipitated proteins were separated on SDS-PAGE, and both eNOS and Cav-1 proteins were detected. Results represent three independent experiments.

3.2. LDL increases coupling of eNOS and Cav-1 and eNOS caveolae translocation

To explore further whether LDL affects the association of eNOS with Cav-1, we isolated cellular fractions using the detergent-free sucrose gradient method and detected both eNOS and Cav-1 by Western blotting. In intact ECs, eNOS could be detected in the caveolin-enriched fractions (lanes
4–6, caveolae) and the bottom fractions (lanes 10–13). However, LDL exposure increased the level of eNOS in caveolae fractions from 8.6% of total eNOS in controls to 22.3% in LDL-exposed cells (see Fig. 2A). In order to compare the abundance of caveolae-associated eNOS in control and LDL-exposed cells, we then pooled caveolae fractions (lanes 4–6) and then concentrated the proteins. The eNOS protein in equal amounts of total protein from control and LDL-exposed cells was analyzed by Western blotting. As shown in Fig. 2B, LDL exposure increased the presence of eNOS and Cav-1 proteins in caveolae. To ascertain the association of eNOS with Cav-1 in ECs, we immunoprecipitated Cav-1 from whole-cell lysates and then detected the co-immunoprecipitated eNOS. LDL exposure increased the level of eNOS protein in the Cav-1 immunoprecipitated samples (Fig. 2C). Thus, LDL exposure increases both the abundance of eNOS in caveolae and its direct association with Cav-1.

3.3. LDL alters eNOS function

To determine the effect of LDL on the function of eNOS, we determined the total NO released into the culture medium in LDL-exposed cells with or without stimulation of the calcium ionophore A23187. LDL exposure for 6 h decreased 13 ± 8% of the total NO level, and A23187 stimulation increased 45 ± 18% of NO in the culture media. LDL exposure blocked the stimulatory effects of A23187. As a control, the pre-incubation of ECs with a specific eNOS inhibitor, L-NAME, at 10 μmol/l for 30 min also inhibited the A23187-stimulated increase of NO (Fig. 3A). However, LDL exposure seemed not to alter the phosphorylation status of eNOS when the level of phosphorylated eNOS was determined by use of an antibody against phospho-eNOS (Ser1177). The total eNOS in the same membranes was detected afterwards to show the same amount of protein loading. Results represent three independent experiments.

Fig. 3. LDL decreases eNOS activity but does not alter eNOS phosphorylation. (A) After exposing ECs to LDL (240 mg/dl), L-NAME (50 μmol/l) or the calcium ionophore A23187 (5 μmol/l) for 6 h, the cell culture medium was collected. Samples were diluted and ultrafiltered. The NO product in the cell culture supernatant was quantitatively measured. Data are mean ± S.D. of the released NO in four independent experiments, each performed in triplicate. (B) ECs cultured in six-well plates were incubated with LDL (240 mg/dl) for different times. Levels of phosphorylation of eNOS were determined with use of an antibody against phospho-eNOS (Ser1177). The total eNOS in the same membranes was detected afterwards to show the same amount of protein loading. Results represent three independent experiments.

A. LDL-induced stress fiber formation

![ LDL-induced stress fiber formation ]

B. Effect of cyto D on stress fiber formation

![ Effect of cyto D on stress fiber formation ]
displayed a lower level of phosphorylation of eNOS detected with this antibody, as the band of the phospho-eNOS in HUVECs detected was faint (data not shown).

### 3.4. LDL induces endothelial stress fiber formation

We previously reported that incubation of ECs with LDL increases stress fiber formation [17]. Here, we observed the acute effect as early as 2 h after LDL exposure. We stained the cytoskeleton in controls and cells exposed to LDL from 2 to 18 h with FITC-phalloidin. Typically, the filaments are located in the peripheral cytoplasm adjacent to the cell margins. LDL exposure increased the formation of stress fibers seen throughout the cellular cytoplasm (Fig. 4A). Cyto D, an actin polymerization inhibitor, blocks increased endothelial endocytosis by altering the cytoskeletal structure [23]. The formation of stress fibers was blocked with the addition of 1.25 μg/ml of cyto D (Fig. 4B). The cell shape became round, and the actin filaments were replaced by many small dots along the cell membrane.

### 3.5. Cyto D interrupts the coupling of eNOS with Cav-1 and blocks the translocation of eNOS to the plasma membrane

To explore further the relationship between cytoskeletal change and the LDL-increased association of eNOS with

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**Fig. 5.** Effect of cyto D on Cav-1 and eNOS localization. ECs grown on slide chambers were treated with cyto D (1.25 μg/ml), LDL (240 mg/dl), or both, for 6 h. The untreated cells served as controls. After fixation, the cells were double immunostained with primary antibodies of rabbit IgG against Cav-1 and mouse IgG against eNOS. The secondary antibodies were FITC-conjugated anti-rabbit IgG and Texas red-conjugated anti-mouse IgG. The results were observed under a confocal fluorescence microscope. Results shown are the images for FITC (green), Texas red (red) and their merged images.
Cav-1, we studied the effect of cyto D on both eNOS and Cav-1 localization. ECs were exposed to LDL, cyto D, or both, for 6 h, and then double immunostained with both anti-Cav-1 and anti-eNOS antibodies. Fig. 5 shows the images of FITC-phalloidin-stained Cav-1, Texas-red-stained eNOS and the merged images. LDL-exposed cells show increased co-localization of eNOS and Cav-1 in the cytoplasm membrane. In cells treated with cyto D, most of the eNOS proteins were retained in the cellular cytoplasm and were disassociated with Cav-1, which was distributed along the cellular membrane. Further, cyto D inhibited the LDL-increased abundance of eNOS in the caveolae fractions, but the distribution of Cav-1 in the cells was almost unchanged (data not shown). The eNOS level in the cellular membrane in LDL-exposed cells was markedly decreased after cyto D treatment (Fig. 6A). In order to compare the abundance of caveolae-associated eNOS in cyto D-treated cells, we pooled caveolae fractions (lanes 4–6) and concentrated the proteins. The level of eNOS protein was analyzed by Western blotting as described in Fig. 2B. As shown in Fig. 6B, cyto D inhibited the caveolae translocation of eNOS in both controls and LDL-exposed ECs. After immunoprecipitating Cav-1 from whole-cell lysates, we then detected eNOS in the Cav-1 immunoprecipitated samples. We found that cyto D inhibited both the basal and LDL-increased association of eNOS and Cav-1 (Fig. 6C). Thus, cyto D inhibits the translocation of eNOS to membrane caveolae and the movement of Cav-1 between

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Fig. 6. Effect of cyto D on eNOS membrane translocation. (A) Confluent ECs were treated with cyto D (1.25 μg/ml), LDL (240 mg/dl), or both, for 2 and 4 h. Cell membrane proteins were isolated and quantified. Equal amounts of protein (5 μg/lane) were separated on SDS-PAGE. (B) ECs were treated with cyto D (1.25 μg/ml), LDL (240 mg/dl) or both, for 4 h. Cellular fractions were isolated, and the caveolin-enriched fractions (lanes 4–6) were pooled and the proteins concentrated as described in Fig. 2B. The proteins (10 μg/lane) were separated on SDS-PAGE, and eNOS proteins were detected on Western blotting. (C) ECs were exposed to LDL or cyto D for 4 h. The Cav-1 proteins were immunoprecipitated with the corresponding antibody from whole-cell lysates. The precipitated-Cav-1 complex was separated on SDS-PAGE, and the eNOS and Cav-1 proteins were detected on Western blotting. The relative density of each band was quantified and labeled as a multiple of the controls. Results represent three independent experiments.
membrane and cytoplasm organelles such as the endoplasmic reticulum.

3.6. LDL activates the RhoA-mediated pathway

The RhoA-mediated signaling pathway was reported to play an important role in the regulation of actin cytoskeleton [24]. To dissect the mechanism underlying the caveolae translocation of Cav-1 and eNOS and stress fiber formation, the activation of RhoA in ECs was investigated. The level of RhoA in the subcellular localization was determined by Western blotting. LDL increased the level of RhoA in the membrane portion and decreased it in the cytosol portions simultaneously after 1 h (Fig. 7A). However, LDL exposure up to 4 h did not increase the total amount of RhoA in whole cell lysates (Fig. 7B, lower panel). To test RhoA activity, we used GST-TRBD conjugated with sepharose 4B to pull down the GTP-bound form of RhoA [20]. As shown in Fig. 7B, LDL exposure increased the TRBD-bound RhoA (GTP-RhoA) beginning at 30 min, peaking at 1 h and returning to the basal level at 4 h. Collectively, data presented in Fig. 7A and B suggest that LDL transiently increases RhoA activity in ECs.

Furthermore, we examined LDL activation of ROCK-1, the downstream kinase directly regulated by RhoA. LDL increased the phosphorylation of MBP by immunoprecipitating ROCK-1 after 1 h of exposure (Fig. 7C). Thus, these data demonstrate the LDL transiently activates RhoA and its downstream signaling in human ECs.

3.7. Y-27632 interrupts stress fiber formation and blocks the translocation of eNOS to the plasma membrane

To explore further the role of ROCK activation on cytoskeletal change and LDL-increased eNOS translocation, we studied the effect of Y-27632, a specific ROCK kinase inhibitor, on stress fiber formation and eNOS localization. ECs were exposed to Y-27632, LDL, or both, for 6 h, and

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**Fig. 7. Effect of LDL on RhoA membrane translocation and activation.** Confluent ECs were exposed to 240 mg/dl of LDL for different times as indicated. Cell lysates were extracted and quantified. (A) The cytosol and membrane proteins were isolated. Equal amounts of protein (15 μg/lane for cytosol proteins, 5 μg/lane for membrane proteins) were separated on SDS-PAGE. (B) Cellular proteins (500 μg) were incubated with sepharose 4B-bound GST-TRBD for 90 min at 4°C in a binding buffer containing magnesium and protease inhibitors. The protein complex on sepharose 4B beads was collected and separated on SDS-PAGE. The total amount of RhoA in cell lysates was used as a control for the comparison of RhoA activity. The blots in (A) and (B) were analyzed on Western blotting with antibodies against RhoA. (C) ROCK-1 proteins from the total cell lysates (500 μg) were immunoprecipitated with an anti-ROCK-1 antibody. The pelleted immunocomplexes were washed and then resuspended in 30 μl of kinase buffer containing 25 μCi of [γ-32P]ATP, 25 μM ATP, and 5 μg of MBP and incubated at 30°C for 30 min. The kinase reaction was stopped by adding SDS sample buffer, the mixture was resolved on 10% SDS-PAGE, and phosphorylated MBP was detected on autoradiography. The relative density of each band was quantified and labeled as a multiple of the controls. Results represent three independent experiments.
then were double immunostained with anti-eNOS antibodies and FITC-phalloidin. As shown in Fig. 8A, LDL-exposed cells show increased eNOS membrane localization. In cells treated with Y-27632, the formation of stress fibers was blocked, and most of the eNOS proteins were retained in the cellular cytoplasm. Further, we determined the total level of NO released into the culture medium in LDL-exposed cells with or without Y27632. As shown in Fig 8B, Y27632 reversed the inhibitory effect of LDL on NO release in the culture media. These results suggest that ROCK activation, at least in part, plays an important role in the formation of stress fiber, cellular membrane translocation of eNOS and production of NO.

4. Discussion

The major findings of the present study are that atherogenic concentrations of LDL increase the translocation of Cav-1-associated eNOS to membrane caveolae in ECs, which is dependent upon actin-based cytoskeleton. Disrupting this stress fiber formation can block the caveolar

![Image of Fig. 8](image-url)

Fig. 8. Effect of Y-237623 on stress fiber formation, eNOS localization and activity. ECs grown on slide chambers were treated with Y-27632 (5 μM), LDL (240 mg/dl), or both, for 6 h. The untreated cells served as controls. (A) After fixation, the cells were immunostained with primary antibodies of mouse IgG against eNOS and the secondary antibody of Texas red-conjugated anti-mouse IgG. Then, the cells were stained with FITC-phalloidin. The results were observed under a confocal fluorescence microscope. Results shown are the images for F-actin (green) and eNOS (red). (B) The cell culture medium was collected and the NO product measured as described in Fig. 3A. Data are mean ± S.D. of the released NO in three independent experiments, each performed in triplicate.
localization of eNOS. Functionally, LDL exposure decreases the basal level of NO and blocks A23187-stimulated NO production. Furthermore, a high level of LDL activates RhoA and its downstream kinase ROCK-1. A specific inhibitor of ROCK-1 could block LDL-induced stress fiber formation and eNOS translocation. Thus, a high level of LDL increases the movement of eNOS to membrane caveolae via the increased stress fibers. The RhoA-mediated pathway may play a crucial role in this process in vascular ECs.

A decrease in the bioavailability of endothelium-derived NO is linked to hypercholesterolemia. However, the mechanism by which LDL mediates eNOS dysfunction remains controversial. Long-term incubation of atherogenic concentrations of LDL in ECs was reported to down-regulate eNOS mRNA and protein levels [7]. We found an increase in membrane-associated eNOS as early as 4 to 6 h after LDL exposure, with a minimal change in eNOS protein levels. Our results emphasize the role of LDL in the early and acute dysfunction of eNOS in ECs. eNOS localization in caveolae is an essential step in the regulation of this enzyme. This compartmentalization facilitates dynamic protein–protein interactions and calcium- and phosphorylation-dependent signal transduction events that modify eNOS activity (see review in Ref. [10]). In caveolae, Cav-1 inhibits eNOS activity by directly interacting with eNOS via its scaffolding domain [11,12]. In BAECs, high levels of LDL cholesterol increased the production of Cav-1 and the interaction between eNOS and Cav-1, which might contribute to impaired NO production [8]. Direct loading of cholesterol to the same type of cells increased eNOS production, number of caveolae and NO production, which was modulated by oxidative stress [25]. Our current findings support in part the observation that a high level of LDL increases the localization of eNOS in caveolae and the association of eNOS with Cav-1. However, we did not observe a change in Cav-1 in the mRNA or protein levels in early passage HUVECs within 24 h of exposure [14]. The increase in caveolar eNOS does not appear to be due to an increase in de novo Cav-1 protein synthesis. Instead, LDL exposure increases the accumulation of Cav-1 protein in membrane caveolae [14]. A possible explanation for the controversial result of eNOS activation in ECs is a difference in cell type, culture conditions and methods to detect eNOS activation. Notably, the abundance of Cav-1 is higher in HUVECs than in BAECs and that of phosphorylated serine-1177 in eNOS recognized by this antibody is lower in HUVECs than in BAECs. Using this antibody, we found that LDL exposure does not affect the phosphorylation of eNOS in HUVECs.

The endothelial actin cytoskeleton and eNOS activity are co-regulated by diverse stimuli [9]. The disruption of the actin cytoskeleton leads to the up-regulation of eNOS expression, which is not due to an increase in eNOS gene transcription but to prolongation of the eNOS mRNA half life [15]. Here we show that LDL exposure increases stress fiber formation and the translocation of eNOS into caveolae. LDL-increased eNOS in the cytoplasm membrane and caveolar fractions were abolished by the addition of cyto D. This result suggests a novel mechanism whereby RhoA activation and stress fiber formation may mediate eNOS caveolar translocation. However, it is not clear how the stress fiber mediates eNOS movement. We observed that disruption of the actin cytoskeleton by cyto D caused a dissociation of eNOS and Cav-1. The presence of cyto D also decreased the level of eNOS but not Cav-1 in caveolae fractions. Thus, there are at least two possibilities for this scenario: (1) two different mechanisms may be involved in the eNOS and Cav-1 intracellular trafficking since disrupting the formation of stress fiber inhibits the translocation of eNOS only; or (2) Cav-1 moves between membrane caveolae and cytoplasm organelles such as the Golgi network along actin filaments. The F-actin cross-linking protein filamin is a ligand for Cav-1, which suggests that the actin network is directly involved in the spatial organization of Cav-1-associated membrane domains [26]. In normal quiescent fibroblasts, the intact actin cytoskeleton is necessary for the growth factor-mediated transport of caveolin from the cell surface to the early endocytic compartment and the activation of the MAP kinase pathway [27]. Cav-1 serves as a vehicle to move certain Cav-1 binding proteins, including eNOS, to the membrane caveolae. Thus, disrupting the formation of stress fiber inhibits the translocation of eNOS and the movement of Cav-1 between the membrane and cytoplasm organelles.

Changes in actin cytoskeleton are mediated, in part, by the small GTP-binding protein Rho-mediated pathway [24]. Signaling from Rho to the actin cytoskeleton is through the protein kinases ROCK and LIMK [21]. Increasing evidence has suggested the close relation between the activation of RhoA and the dysfunction of eNOS [28]. The anti-atherosclerotic effects of statins involve both an increase in eNOS activity and inhibition of Rho activity [29]. Laufs et al. [15] reported that the activation of Rho and stress fiber formation negatively regulates eNOS. The inhibition of RhoA and ROCK prevents the down-regulated expression and activity of eNOS in response to thrombin and hypoxia [30,31]. Treatment of endothelium-intact rat aorta with Y-27632 resulted in an attenuation of maximal force generated in response to phenylephrine [32]. This inhibition was significantly less effective in the presence of eNOS inhibitors. Overexpression of active RhoA and ROCK in human ECs negatively regulates eNOS activity [33]. In the present report, our findings suggest that RhoA-mediated stress fiber formation in the endothelium plays a critical role in mediating eNOS activity. This is the first evidence that a high level of LDL increases the activation of RhoA and its downstream ROCK-1. The inhibition of RhoA reduces the formation of stress fibers and the caveolar localization of eNOS and NO production. Thus, agents that inhibit RhoA activity and modulate the endothelial actin cytoskeleton may be beneficial for treating endothelial dysfunction in cardiovascular diseases including atherosclerosis.
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