

ox-LDL downregulates eNOS activity via LOX-1-mediated endoplasmic reticulum stress

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Abstract. Vascular endothelial injury induced by oxidized low-density lipoprotein (ox-LDL) has been implicated in the early stages of the pathogenesis of atherosclerosis. In this study, we incubated bovine aortic endothelial cells (BAECs) with ox-LDL (100 μ g/ml) in order to induce endoplasmic reticulum (ER) stress and to investigate the regulation of endothelial nitric oxide synthase (eNOS). Within 4 h of exposure, ox-LDL rapidly induced ER stress, as demonstrated by the measurements of proline-rich extensin-like receptor kinase (PERK) and glucose-regulated protein (GRP)78. ox-LDL induced the rapid dephosphorylation of eNOS at Ser1179 and a subsequent decrease in eNOS activity. This effect appeared to be highly specific as no change was observed in the levels of phosphorylated eNOS at Thr497 or eNOS protein. Of note, a simultaneous decrease was also observed in the active (phosphorylated) form of Akt (Thr308/Ser473), which has been demonstrated to phosphorylate eNOS at Ser1179. Further analysis indicated that Brefeldin A (BFA), an ER stress-inducing reagent, induced the rapid dephosphorylation of Akt and eNOS at Ser1179. 4-Phenylbutyric acid (PBA), an inhibitor of ER stress, blocked the ox-LDL-induced dephosphorylation of Akt and eNOS. Furthermore, JTX20, a lectin-like ox-LDL receptor-1 (LOX-1) blocking antibody significantly eliminated the ability of ox-LDL to mediate the dephosphorylation of eNOS and Akt. Our results indicate that the downregulation of eNOS by ox-LDL, as driven by LOX-1-mediated ER stress, is associated with the PI3K-Akt-eNOS signaling pathway.

Introduction

Vascular endothelial dysfunction is implicated in the initial development of atherosclerosis (1,2). Oxidized low-density lipoprotein (ox-LDL) inhibits nitric oxide (NO) production and impairs endothelial function (1,2). In endothelial cells (ECs), NO is derived from L-arginine in a reaction catalyzed by endothelial NO synthase (eNOS) (3). Previous studies have demonstrated that decreased eNOS activity is an important feature in endothelial dysfunction (4). The lectin-like ox-LDL receptor-1 (LOX-1) with a type II membrane protein structure has been identified as a major ox-LDL receptor that is expressed in ECs (5). Relatively long incubation periods with ox-LDL (12-24 h) have been determined to induce endothelial dysfunction by upregulating the expression of LOX-1 and inhibiting the function of eNOS (6). Thus, an ox-LDL-LOX-1-eNOS pathway appears to play a crucial role in the pathogenesis of endothelial dysfunction. Akt has been indicated to phosphorylate eNOS at Ser1179, and the activation of the PI3K-Akt-eNOS signaling pathway appears to be an important mechanism for maintaining the integrity of the endothelium of the arteries. In previous studies, ox-LDL has been reported to inactivate Akt through the activity of LOX-1 (7) and subsequently, to dephosphorylate the Ser1179/1177 (bovine/human) residue of eNOS. The Ser1179/1177 dephosphorylation of eNOS decreases eNOS activity (8,9). However, the mechanisms through which LOX-1 interrupts the AKT signaling cascade, leading to the dephosphorylation of eNOS at Ser1179/1177, have not yet been fully elucidated.

Previous studies have demonstrated that endoplasmic reticulum (ER) stress induced by ox-LDL in human vascular cells modulates the balance between survival and apoptosis induced by ox-LDL (10-12). Other stimuli that can trigger ER stress include high glucose levels, oxidative stress, Ca²⁺ overload, ischemia and hypoxia. Evidence of ER stress has been detected in atherosclerotic lesions in hypercholesterolemic mice (13) and ER stress has been suggested to occur in acute coronary syndrome (14). ER stress can lead to the accumulation of unfolded and misfolded proteins, causing an 'unfolded protein response' (UPR). The UPR in turn prompts adaptive responses to ER dysfunction and emerges as a new adaptive system that determines the fate of cells (survival or death) (15) and thus, the UPR may play a role in endothelial dysfunction.

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tion (16). A previous study indicated that the inactivation of Akt is involved in ER stress-mediated signaling, which can occur upon exposure to ER stress (17). This result led us to investigate whether ER stress initiated by LOX-1 is associated with ox-LDL-induced eNOS dysfunction in ECs.

In the present study, we found that ER stress is an important trigger for the Akt and eNOS downregulation induced by ox-LDL in ECs. Short-term treatment with ox-LDL immediately induced ER stress and caused the dephosphorylation of Akt and eNOS in bovine aortic ECs (BAECs) prior to changes in the expression of the eNOS and LOX-1 proteins. Of note, treatment with JTX20, a LOX-1 blocking antibody, or 4-phenylbutyric acid (PBA), a chemical chaperone facilitating the correct folding of proteins, partly rescued the dephosphorylation of Akt/eNOS during a short-term (0-4 h) ox-LDL treatment. Our findings demonstrated that ER stress preferentially regulated Akt/eNOS dephosphorylation prior to the alteration of eNOS and LOX-1 expression during the early phase of ox-LDL treatment. The present study provides new data regarding the inhibition of eNOS induced by ox-LDL through LOX-1-mediated ER stress, which may be implicated in endothelial dysfunction reported in coronary artery disease.

Materials and methods

Materials. The cell culture materials used in the present study were obtained from Invitrogen (Carlsbad, CA, USA). BAECs and growth medium were purchased from Cell Systems Corp. (Kirkland, WA, USA). 2',5'-ADP-Sepharose 4B was produced by Amersham Biosciences (Piscataway, NJ, USA). The antibody against eNOS was purchased from the BD Transduction Laboratories/BD Biosciences (San Jose, CA, USA). Antibodies against phosphorylated eNOS (p-eNOS; at serine 1179), Akt, phosphorylated Akt (p-Akt; at serine 473) and p-Akt (at threonine 308) were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody against p-eNOS (at threonine 497) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies against protein phosphatase (PP)2A, glucose-regulated protein (GRP)78, p-proline-rich extensin-like receptor kinase (PERK) and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ^3H -arginine was purchased from PerkinElmer Life Sciences (Waltham, MA, USA). The protease inhibitor tablet was purchased from Roche Applied Science (Indianapolis, IN, USA). Calmodulin, NADPH, tetrahydrobiopterin, L-arginine, *N*-nitro-L-arginine methyl ester, LY294002 and recombinant human insulin-like growth factor-1 (IGF-1) were purchased from Sigma (St. Louis, MO, USA). PBA was obtained from Sigma-Aldrich (Strasbourg, France). ox-LDL was obtained from Guangzhou Yiyuan Biotech Co. Ltd. (Guangzhou, China). The JTX20 antibody was obtained from Wuhan Sanying Biotechnology, Inc. (Wuhan, China) and all the other reagents used were purchased from Sigma, unless otherwise indicated.

Cell culture. The BAECs were cultured in endothelial growth medium supplemented with 10% fetal bovine serum, 10 $\mu\text{g}/\text{ml}$ human recombinant epidermal growth factor, 1 mg/l hydrocortisone, 50 $\mu\text{g}/\text{ml}$ gentamicin, 50 ng/ml amphotericin B

and 12 $\mu\text{g}/\text{ml}$ bovine brain extracts. The BAECs between passages 4 and 10 were employed for the experiments.

Western blot analysis. The cells were harvested and lysed on ice for 30 min in a modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 1 mM Na_3VO_4 , 5 mM sodium pyrophosphate and a protease inhibitor tablet). The cell lysates were centrifuged at 14,000 \times g for 15 min, and the supernatant was recovered. The total protein concentration was determined using BCA protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL, USA). The lysates were denatured by boiling in SDS sample buffer. The proteins were separated through gradient SDS/PAGE on a 4-20% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with a semi-dry transfer cell (Bio-Rad, Hercules, CA, USA). After being blocked with 3% BSA in TBS for 1 h, the membranes were probed with the appropriate primary antibodies. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with horseradish peroxidase. The western blots were then visualized through enhanced chemiluminescence detection reagents (Sigma) according to the instructions provided by the manufacturer. The protein bands were quantified by scanning with the Bio-Rad GelDocTM XR and ChemiDocTM XRS systems and analyzed with Quantity One 1-D analysis software version 4.6.3.

Measurement of eNOS activity. The effect of ox-LDL on the eNOS-mediated metabolism of ^3H -arginine to ^3H -citrulline was determined, and the assay was performed under apparent V_{max} conditions, as previously described (18-20). Briefly, BAEC lysates were suspended in cold lysis buffer (0.3 M sucrose, 10 mM HEPES, 1% Nonidet P-40, 0.1 mM EDTA, 1 mM dithiothreitol, 10 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor and 50 μM phenylmethylsulfonyl fluoride, pH 7.4) through vigorous vortexing. The cell lysates (150-250 μg of protein) were combined with NADPH (2 mM), CaCl_2 (230 μM), tetrahydrobiopterin (3 μM) and ^3H -arginine (0.2 μCi , 10 μM) for 20 min at 37°C in an assay volume of 100 μl . The reaction products were measured using a liquid scintillation counter. To determine whether ox-LDL altered the activity of eNOS, calcium was replaced with EDTA (1.7 mM) in the assay buffers.

Pull-down assay. Cells were harvested and lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4 , 5 mM sodium pyrophosphate and a protease inhibitor tablet). The cell lysates were centrifuged at 14,000 \times g for 15 min, and the supernatants were recovered. The supernatants, which contained equal quantities of protein, were incubated with 2',5'-ADP-Sepharose 4B resins (50 μl in a 50% slurry) overnight at 4°C as previously described (21). The resins were washed twice with a standard washing buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA and 0.5% Nonidet P-40), 3 times with high-salt washing buffer (50 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA and 0.5% Nonidet P-40) and once with a standard washing buffer. The proteins pulled down were then eluted by boiling the beads for

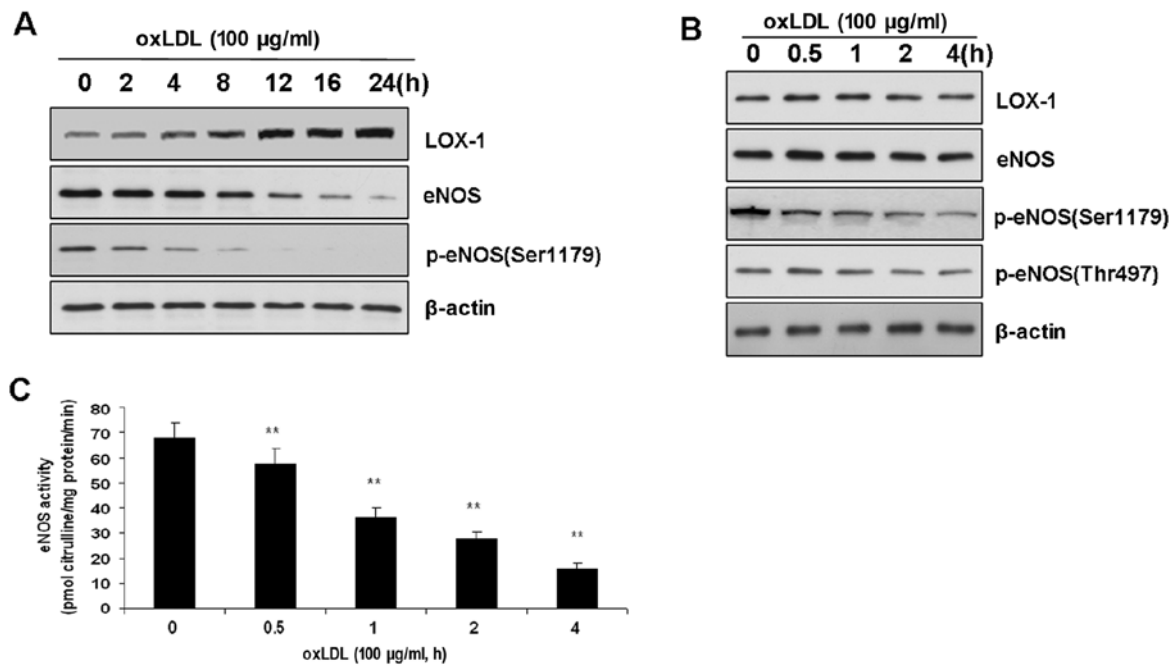


Figure 1. (A and B) Effects of oxidized low-density lipoprotein (ox-LDL) (100 µg/ml) on the expression of lectin-like ox-LDL receptor-1 (LOX-1) and endothelial nitric oxide synthase (eNOS) and on the phosphorylation status of eNOS at Ser1179 and Thr497. (C) The activity of phosphorylated eNOS (p-NOS) was assayed by measuring the conversion of [³H]-arginine to [³H]-citrulline. Results are expressed as pmol citrulline/mg protein/min. The data are presented as the means ± SE (**P<0.01 vs. control, n=5). Representative blots are depicted from 3 independent experiments.

5 min in SDS sample buffer and the proteins were analyzed by western blot analysis.

Statistical analysis. Data are expressed as the means ± SD and were analyzed using SPSS 10.0 statistical software (SPSS Inc., Chicago, IL, USA). A one-way ANOVA, followed by LSD post hoc tests were employed to determine the significance of differences among the groups (P-values <0.01 and <0.05, respectively, were considered to indicate statistically significant differences).

Results

Effects of ox-LDL on LOX-1, eNOS and ER stress sensors in BAECs. LOX-1 has been demonstrated to be a major endothelial receptor for ox-LDL in ECs. Therefore, we investigated whether LOX-1 expression is altered following treatment with ox-LDL. The incubation of BAECs with ox-LDL (100 µg/ml) for 8 to 24 h increased the expression of LOX-1 compared with that of the control (P<0.01). These results are consistent with those of previous studies (6). However, the expression of LOX-1 was not altered during short-term treatment with ox-LDL prior to a 4-h incubation.

Previous studies have indicated that long-term treatment with ox-LDL (from 12 to 24 h) induces endothelial dysfunction by upregulating the expression of LOX-1 and inhibiting the activity of eNOS. In this study, eNOS expression was also found to be decreased following the treatment of BAECs with ox-LDL for 12 h (Fig. 1). Therefore, we investigated whether the changes in LOX-1 expression accounted for the alterations in eNOS phosphorylation and function. To determine the effects of ox-LDL on eNOS phosphorylation, we

added ox-LDL (100 µg/ml) to the medium, which exerted a time-dependent effect on the dephosphorylation of eNOS at Ser1179 (Fig. 1). In other words, ox-LDL (100 µg/ml) reduced the level of eNOS phosphorylation. This time-dependent effect of ox-LDL was specific to Ser1179, as neither eNOS at Thr497 phosphorylation nor the level of total eNOS in the BAECs was affected (Fig. 1). Accordingly, eNOS activity was also reduced in a time-dependent manner (Fig. 1).

Effects of ox-LDL on ER stress sensors in BAECs. As ox-LDL can trigger ER stress in ECs, and ER stress is characterized by the activation of ER stress sensors, which leads to UPR induction, we also investigated alterations in ER stress sensors in BAECs. The incubation of BAECs with ox-LDL (100 µg/ml) induced the time-dependent activation of ER stress sensors, as assessed by the phosphorylation of PERK. This activation was particularly noticeable immediately after 2 h following treatment with ox-LDL (Fig. 2). As expected, ox-LDL-activated PERK induced GRP78 expression (Fig. 2) and GRP78 has been reported to regulate protein biosynthesis (22).

Taken together, these data indicate that short-term treatment with ox-LDL immediately activates ER stress and induces eNOS dephosphorylation in BAECs prior to the alteration of total eNOS and LOX-1 expression.

Changes in Akt and protein phosphatase activation in ox-LDL-treated BAECs. Akt has been described as a kinase responsible for the phosphorylation of eNOS at Ser1179 under various conditions. Therefore, we investigated whether Akt activity is altered by a short-term treatment with ox-LDL and if so, whether this change is associated with the changes in the phosphorylation of eNOS at Ser1179. Our results revealed

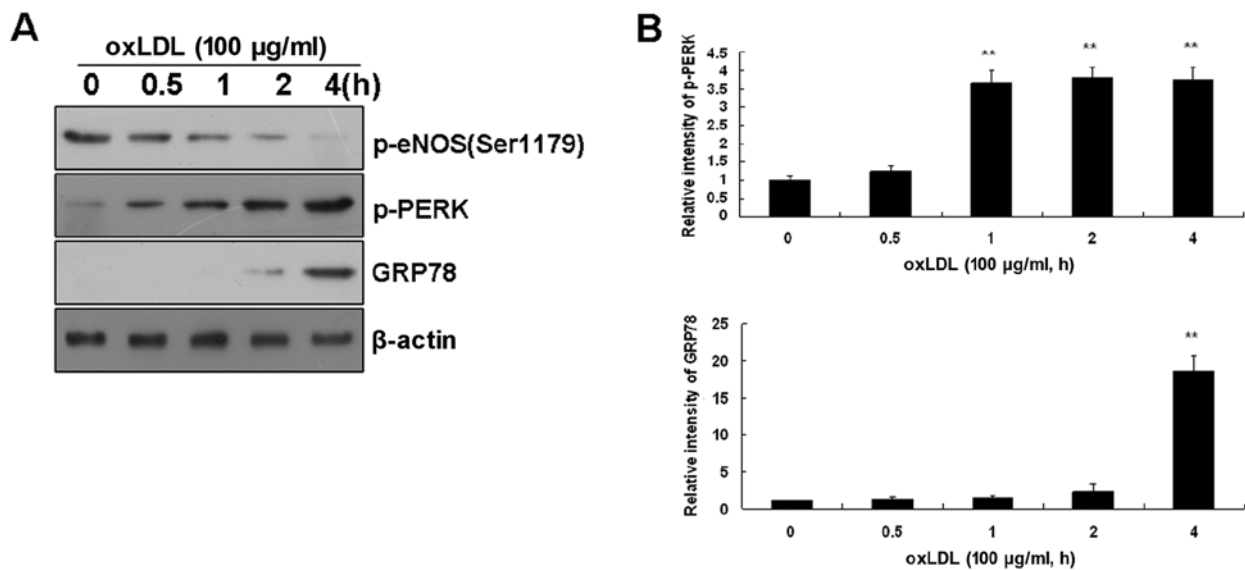


Figure 2. Induction of endoplasmic reticulum (ER) stress by oxidized low-density lipoprotein (ox-LDL) in bovine aortic endothelial cells (BAECs). (A) Time course of the ER stress sensors, phosphorylated proline-rich extensin-like receptor kinase (p-PERK) and glucose-regulated protein (GRP78), in BAECs treated with ox-LDL (100 µg/ml). Western blot analysis was performed on total protein extracts using β -actin protein levels as the control. (B) Quantitative densitometry analyses of p-PERK and GRP78 in the presence of ox-LDL. Data are presented as the means \pm SE (** P <0.01 vs. control, n =4).

that Akt was activated, as demonstrated by the phosphorylation of its Thr308 and Ser473 residues. As depicted in Fig. 3A, the dephosphorylation of Akt also occurred in the BAECs subjected to short-term treatment with ox-LDL. The time course of p-Akt levels was largely parallel with that of p-eNOS Ser1179 levels. These data suggested that the failure of Akt phosphorylation at Thr308 and Ser473 contributed to the dephosphorylation of eNOS Ser1179 during the short-term treatment of BAECs with ox-LDL.

Both Akt and eNOS at Ser1179 have been reported to be dephosphorylated by PP2A (23,24). To determine whether the loss of Akt and eNOS phosphorylation at Ser1179 was due to the increased PP2A and PP1 activity, we measured the effects of ox-LDL on the expression levels of PP2A and PP1. PP2A is a heterotrimeric enzyme consisting of a catalytic subunit C, a structural subunit A and a regulatory subunit B (24). As depicted in Fig. 3A, none of the PP2A subunits exhibited changes in expression levels in the ox-LDL-treated BAECs. These data indicated that the inactivation of Akt, as well as the loss of eNOS phosphorylation at Ser1179 in the short-term ox-LDL-treated BAECs, was not due to the elevated expression of PP2A and PP1.

Effects of ox-LDL on the association of PP1, PP2A and Akt with eNOS. Akt and PP2A have been reported to be associated with eNOS (21), raising the possibility that the increased association with protein phosphatases induced the dephosphorylation of Akt and eNOS at Ser1179. To investigate this hypothesis, we pulled down eNOS from BAECs after 2 and 4 h of ox-LDL treatment and measured the levels of eNOS-associated Akt, PP2A and PP1. As depicted in Fig. 3B, whereas the levels of p-Akt and p-eNOS (Ser1179) were both reduced, the quantities of Akt, PP1 and PP2A associated with eNOS were not significantly affected by ox-LDL treatment. These results

indicated that the loss of Akt and eNOS phosphorylation at Ser1179 following short-term treatment with ox-LDL was not due to the increased association of PP1 and PP2A with eNOS.

ER stress downregulates eNOS and Akt phosphorylation in BAECs. To further understand the mechanisms through which treatment with ox-LDL leads to the loss of Akt phosphorylation and eNOS phosphorylation at active sites, we investigated whether ER stress affects eNOS phosphorylation in BAECs. We treated the BAECs with an ER stress-inducing reagent, Brefeldin A (BFA; which inhibits ER-Golgi transport, at a concentration of 5 µg/ml), to induce UPR signaling. As assessed by western blot analysis, further evaluation of the phosphorylation of eNOS indicated that BFA induced the rapid dephosphorylation of eNOS at Ser1179 (Fig. 4A and B). Surprisingly, this time-dependent result of ER stress was also specific, as neither eNOS phosphorylation at Thr497 nor the level of total eNOS protein in the BAECs was affected.

To explore the possible role of Akt in ER stress, we then examined Akt phosphorylation in the stressed cells by western blot analysis. We determined that the basal level of p-Akt was gradually dephosphorylated upon exposure to ER stress in the BAECs. We observed a slight decrease in the level of phosphorylation at 2 h, and Akt at Thr308 and Ser473 was significantly dephosphorylated at 4 h in response to BFA (5 µg/ml). Conversely, the expression level of total Akt protein was not affected by ER stress (data not shown), indicating that the downregulation of Akt phosphorylation was not induced by the downregulation total Akt protein.

Previous studies have indicated that phosphorylated PERK (p-PERK) and GRP78 are induced in response to ER stress (25). Since we observed that ER stress downregulated Akt activation, we investigated whether BFA affects the PI3K/Akt pathway in BAECs. BFA, at a concentration of 5 µg/ml,

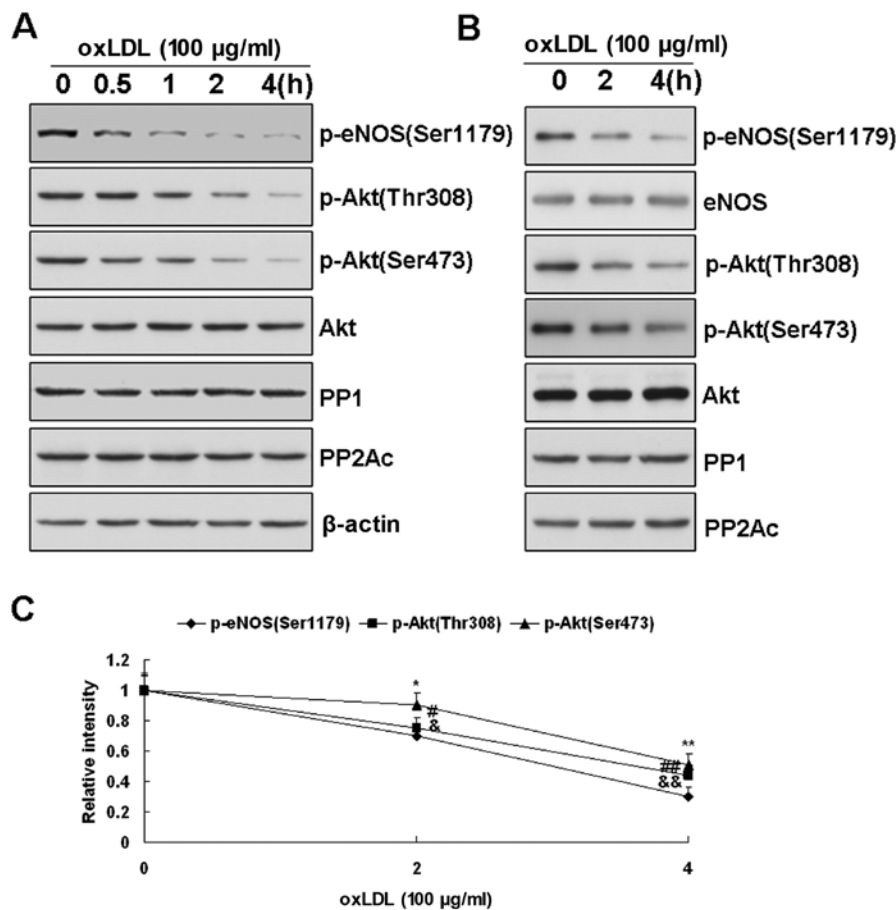


Figure 3. (A) Changes in Akt, protein phosphatase (PP)2A and PP1 in oxidized low-density lipoprotein (ox-LDL)-treated bovine aortic endothelial cells (BAECs) measured by western blot analysis. Effects of ox-LDL (100 μ g/ml) on Akt phosphorylation at Thr308 and Ser473 in BAECs. As the phosphorylation of endothelial nitric oxide synthase (eNOS) at Ser1179 decreased upon ox-LDL treatment, the phosphorylated form of Akt at Thr308 and Ser473 was also reduced in a time-dependent manner. However, the Akt protein levels depicted did not change according to the results presented here. (B) Effects of ox-LDL (100 μ g/ml) on the expression of PP2A and PP1 in BAECs after treatment for 0, 2 and 4 h. No significant change was observed in the levels of PP2A and PP1 in the BAECs before and after ox-LDL treatment. (B and C) Representative blots are presented from 3 independent experiments. (B) Effects of ox-LDL on the association of PP1, PP2A and Akt with eNOS. After 2 or 4 h of treatment of ox-LDL (200 μ g/ml), eNOS in the BAECs was pulled down with the 2',5'-ADP-Sepharose beads, and eNOS-associated proteins were measured by western blot analysis with the corresponding antibodies. Consistent with the results obtained with the cell lysates, ox-LDL treatment decreased the phosphorylation of Akt (at Ser473 and Thr308) and eNOS (at Ser1179). However, in the samples containing equal amounts of eNOS input, the levels of PP1, PP2A and Akt associated with eNOS were not significantly altered by ox-LDL treatment. All the data presented are representative of 3 independent experiments. (C) Quantitative densitometry analyses of Akt phosphorylation at Thr308 and Ser473 associated with eNOS. Data are presented as the means \pm SE (*, &#x26;P<0.05; **, &#x26;##P<0.01 vs. the levels observed prior to treatment with ox-LDL, n=3).

rapidly reduced the level of Akt and eNOS phosphorylation, but had no effect on the quantity of Akt (data not shown), and treatment with 100 ng/ml of IGF-1, an activator of PI3K, reversed the BFA-induced decrease in Akt and eNOS phosphorylation (Fig. 4C).

The chemical chaperone, PBA, reduces ER stress and prevents the loss of Akt and eNOS phosphorylation in BAECs. To investigate the hypothesis that the downregulation of eNOS phosphorylation is associated with the Akt signaling pathway through the induction of ER stress, the BAECs were treated with ox-LDL in the presence or absence of PBA in order to inhibit ER stress. The induction of p-PERK and GRP78 by ox-LDL was significantly reduced (P<0.01) in the BAECs pre-treated with PBA (10 mM) for 4 h (Fig. 5). Further evaluation of the phosphorylation of eNOS revealed that PBA reversed the dephosphorylation of Akt and eNOS induced by ox-LDL (P<0.01). Again, this time-dependent effect of PBA did not

affect the level of total Akt or the level of total eNOS in the BAECs.

The anti-LOX-1 blocking antibody, JTX20, reduces ER stress and inhibits the loss of Akt and eNOS phosphorylation in BAECs. We then investigated the hypothesis that the downregulation of eNOS phosphorylation is associated with the Akt signaling pathway through the induction of ER stress by LOX-1 (Fig. 6). The BAECs were treated with ox-LDL in the presence or absence of the anti-LOX-1 blocking antibody, JTX20, to block eNOS dephosphorylation. The induction of eNOS and Akt dephosphorylation by ox-LDL was significantly reduced (P<0.01) in the BAECs pre-treated with JTX20 (40 μ g/ml) for 1 h (Fig. 6). Further evaluation of ER stress indicated that JTX20 reversed the ox-LDL-induced expression of p-PERK and GRP78 (P<0.01). These data strongly suggest that the effects of ox-LDL on the PI3K-Akt-eNOS signaling pathway or the induction of ER stress are mediated through

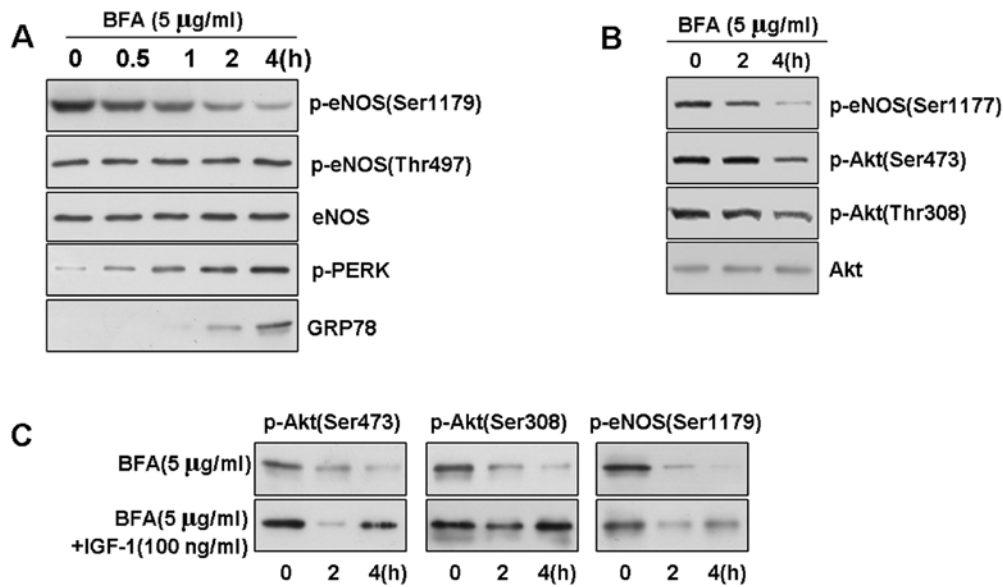


Figure 4. (A and B) Effects of the endoplasmic reticulum (ER) stress-inducing reagent, Brefeldin A (BFA), on endothelial nitric oxide synthase (eNOS) phosphorylation in bovine aortic endothelial cells (BAECs). (A) eNOS phosphorylation at Ser1179 decreased as early as 0.5 h, as phosphorylated proline-rich extensin-like receptor kinase (p-PERK) and glucose-regulated protein (GRP)78 expression increased in the BAECs treated with BFA (5 µg/ml). (B) Quantitative densitometry analyses of the data in (A) were conducted. Data are presented as the means ± SE. (C) Effects of BFA (an inducer of ER stress) and insulin-like growth factor-1 (IGF-1) (an activator of PI3K) on the status of Akt and eNOS phosphorylation in BAECs. The levels of phosphorylated Akt and eNOS were determined by western blot analysis at the indicated times after stimulation with 5 µg/ml of BFA. Selected wells were also stimulated simultaneously with 100 ng/ml of IGF-1 and 5 µg/ml of BFA, as depicted in the figure. All the experiments were performed 4 times, and similar results were obtained.

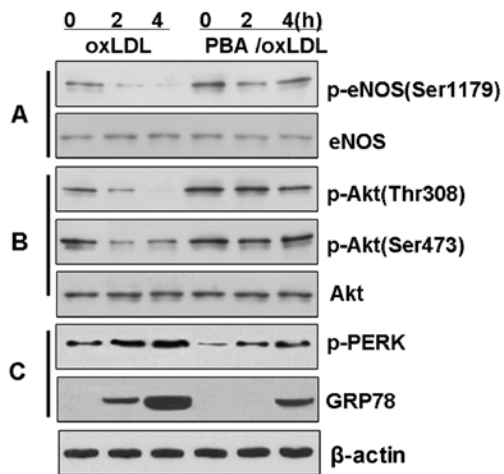


Figure 5. The chemical chaperone, 4-phenylbutyric acid (PBA), reduces endoplasmic reticulum (ER) stress and prevents the loss of Akt and endothelial nitric oxide synthase (eNOS) phosphorylation in bovine aortic endothelial cells (BAECs). The expression of (A) eNOS, (B) Akt and (C) ER stress sensors in BAECs pre-treated with PBA (10 mM) for 14 h. Representative blots are presented from 3 independent experiments. ox-LDL, oxidized low-density lipoprotein.

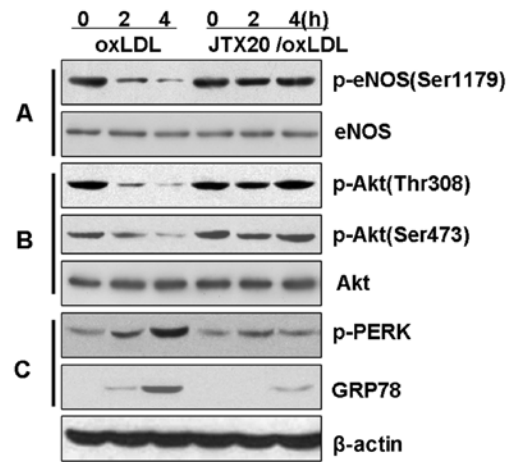


Figure 6. The anti-LOX-1 blocking antibody, JTX20, reduces endoplasmic reticulum (ER) stress and prevents the loss of Akt and endothelial nitric oxide synthase (eNOS) phosphorylation in bovine aortic endothelial cells (BAECs). The expression of (A) eNOS, (B) Akt and (C) ER stress sensors in BAECs pre-treated with JTX20 (40 µg/ml) for 30 min. Representative blots are presented from 3 independent experiments. ox-LDL, oxidized low-density lipoprotein; LOX-1, lectin-like ox-LDL receptor-1.

the activation by ox-LDL of the LOX-1 receptor and subsequent intracellular signaling pathways.

Discussion

ox-LDL has been demonstrated to be an important pathogenic factor in the formation of atherosclerotic plaque. Endothelial dysfunction is the initial change that occurs in the vascular wall during the course of atheroma formation. LOX-1 has been

identified as the major receptor for ox-LDL in ECs. As one of the key intrinsic molecules, LOX-1 has been reported to induce endothelial dysfunction after being triggered by ox-LDL (5). When bound to and activated by ox-LDL, LOX-1 enhances NO catabolism as a result of superoxide generation and decreases the release of NO through reduced eNOS activity. LOX-1 has been shown to be associated with the reduced expression of constitutive eNOS (26). As previously described,

the incubation of human coronary artery ECs (HCAECs) with ox-LDL for 24 h increased the expression of LOX-1 (mRNA and protein) compared with the control. The incubation of HCAECs with ox-LDL markedly reduced eNOS expression (as measured by RT-PCR and western blot analysis) (26).

In the present study, we examined the effects of LOX-1 in eNOS expression and activity in ox-LDL-treated BAECs. Our results demonstrated that ox-LDL did not upregulate the expression of LOX-1 until after 4 h (Fig. 1), and the level of LOX-1 began to increase after 4 h of treatment with ox-LDL. At the same time, a significant reduction in total eNOS expression was observed after 12 h of ox-LDL treatment in the BAECs. These results are consistent with those of a previous study (26). Of note, a significant decrease was observed in eNOS phosphorylation at Ser1179 following short-term (<4 h) treatment with ox-LDL, and this change was independent of the expression of LOX-1 protein and the level of total eNOS protein in the BAECs (Fig. 1). These findings indicated that the changes in the phosphorylation of eNOS occurred more rapidly than the alteration of its protein level. Thus, ox-LDL mediated the reduction of eNOS activity through two separate mechanisms, one being a mechanism with rapid effects without an increase in LOX-1 expression and the other being a mechanism with relatively slow effects with increased LOX-1 expression. The former mechanism required a decrease in eNOS phosphorylation at Ser1179, and the latter involved a decrease in the expression of eNOS.

LOX-1 is a 50 kDa type II transmembrane glycoprotein comprising 273 amino acids (5). The protein contains a short N-terminal cytoplasmic domain, a single transmembrane domain and an extracellular domain comprising a neck domain followed by a C-terminal, C-type, lectin-like, ligand-binding domain (27). Previous studies have indicated that the biological effects of ox-LDL, exerted through LOX-1 binding, involve a number of signaling pathways, including the Akt, tyrosine kinase and mitogen-activated protein kinase (MAPK) pathways (28). The Akt cascade is a signal transduction pathway that mediates the activation of eNOS. eNOS at Ser1179 was first reported to be phosphorylated by Akt (29,30). Since then, Akt has remained the key kinase that phosphorylates eNOS at Ser1179 under various circumstances, although a number of other kinases, including 5' adenosine monophosphate-activated protein kinase (AMPK), protein kinase G (PKG), protein kinase A (PKA), extracellular signal-regulated kinase (ERK)1/2 and Ca²⁺/calmodulin-dependent kinase II (5,31), have been reported to phosphorylate eNOS at Ser1179 (32).

Therefore, we investigated the hypothesis that changes in Akt activity may account for the alteration of eNOS phosphorylation at Ser1179 following short-term treatment with ox-LDL. Akt has been reported to be activated following its phosphorylation at the Thr308 and Ser473 residues. As depicted in Fig. 3A, Akt was dephosphorylated at the Thr308 and Ser473 residues during a short-term treatment of BAECs with ox-LDL. The time course for Akt dephosphorylation was largely parallel with that of eNOS dephosphorylation at Ser1179. However, these dephosphorylation events may result from the activity of a phosphatase. To characterize the eNOS dephosphorylation at Ser1179 and determine whether it was the result of the cleavage of phosphates by phosphatases, we examined the expression of PP2A and PPI and their association with eNOS

by pull-down assay during the short-term treatment of BAECs with ox-LDL. Our data indicated that the inactivation of Akt, as well as the loss of eNOS phosphorylation at Ser1179, was not accompanied by the elevated expression of PP2A and PPI and an increase in their association with eNOS (Fig. 3). LOX-1 exhibits a short 36-amino acid cytosolic tail with no homology to known catalytic or signaling domains with the purpose of transducing signals to activate downstream functions. One gap in the current understanding of LOX-1 biology is the identity of the downstream mediating signals activated by LOX-1. However, to date, the correlation between the activation or the inactivation of the Akt-eNOS pathway and the activation of LOX-1 has not been determined. Our data demonstrated that the short-term treatment of BAECs with ox-LDL activated Akt by phosphorylating its Thr308 and Ser473 residues, thus inducing eNOS phosphorylation at Ser1179. However, this transient event was not associated with LOX-1 expression, and did not induce a change in eNOS protein levels and did not stimulate the activities of PP2A and PPI.

ER stress has been demonstrated to be induced by ox-LDL in human vascular cells (10). Once stimulated, ER stress can modulate the balance between cell survival and apoptosis. When the ER environment is perturbed and the folding of nascent proteins is impaired, a quality control system termed the UPR is mobilized (33-35). Initially, the UPR is an adaptive response in which affected cells attempt to overcome the accumulation of misfolded proteins by increasing their protein-folding capacity. However, when ER stress is excessive and prolonged, cells undergo apoptotic cell death. Thus, the ER stress response exhibits a conditional ability to protect cells against offensive agents or to activate the cell death program. The extent to which ER stress following stimulation with ox-LDL correlates with the modification of eNOS activity has not yet been fully elucidated.

The levels of ox-LDL-induced ER stress and the UPR can be assessed by the phosphorylation of an ER stress sensor (p-PERK) and the expression of ER-resident chaperones (GRP78) (36). The UPR is an adaptive response that first tends to restore ER activity and cellular homeostasis but preferentially induces apoptosis when ER stress is prolonged, depending on the nature of the agent and the intensity of the stress (37). In this study, to our knowledge, we provide the first evidence of the induction of ER stress by ox-LDL and its potential involvement in the dephosphorylation of eNOS during the short-term treatment of BAECs with ox-LDL. We observed that the incubation of BAECs with ox-LDL immediately induced the phosphorylation of PERK, which was observed as early as 1 h after the initiation of ox-LDL treatment (Fig. 2). This finding indicated that the level of p-PERK began to increase as soon as ox-LDL treatment was initiated. Furthermore, as p-PERK levels began to increase rapidly, the level of phosphorylated eNOS at Ser1179 began to decrease rapidly, as observed between 0.5 and 4 h. The most significant evidence indicating the ox-LDL-dependent switch of ER stress was provided by the persistent expression of GRP78, an ER-localized chaperone stimulated through the ATF6 and PERK pathways (38,39), which markedly increased at 4 h after the initiation of treatment.

Taken together, these data demonstrated that short-term treatment with ox-LDL immediately induced ER stress and

caused the dephosphorylation of eNOS in BAECs prior to the alteration of total eNOS and expression of LOX-1. Based on these data, we performed a series of experiments to investigate these cellular pathways.

A previous study indicated that the inactivation of Akt is involved in ER stress-mediated signaling. As demonstrated, Akt was gradually inactivated in response to exposure to ER stress (17). In this study, our data demonstrated that BFA, an ER stress-inducing reagent, induced the rapid dephosphorylation of Akt and eNOS at Ser1179 (Fig. 4). However, IGF-1 (an activator of PI3K) partially reversed the BFA-induced increase in Akt and eNOS dephosphorylation (Fig. 4C). In addition, the induction of p-PERK and GRP78 by ox-LDL was observed to be significantly reduced in the BAECs pre-treated with PBA (a chemical chaperone that has the ability to decrease levels of ER stress) for 14 h. PBA also reversed the ox-LDL-induced decrease in Akt and eNOS phosphorylation (Fig. 5). Taken together, these data indicate that the ER stress induced by ox-LDL induces the dephosphorylation of eNOS at Ser1179 in BAECs, and that this induction is associated with Akt dephosphorylation.

This finding led us to investigate whether the inhibition of eNOS activity induced by ER stress mediated by ox-LDL is associated with LOX-1 in BAECs. Accordingly, we treated BAECs with ox-LDL in the presence or absence of the anti-LOX-1 blocking antibody, JTX20, and subsequently examined the level of eNOS phosphorylation. The JTX20 antibody reversed the ox-LDL-induced increase in p-PERK and GRP78 expression. JYX20 also significantly reduced the ox-LDL-induced eNOS and Akt dephosphorylation observed in the BAECs (Fig. 6).

In conclusion, although long-term treatment with ox-LDL upregulated LOX-1 expression and downregulated eNOS expression, thereby mediating eNOS activity, our results also indicated that eNOS was downregulated through dephosphorylation at Ser1179 through a rapid regulatory mechanism associated with the Akt signaling pathway. This mechanism was induced by LOX-1-triggered ER stress during the short-term treatment of BAECs with ox-LDL, and the mechanism involved was independent of the stimulation of LOX-1 expression and independent of changes in eNOS enzyme levels.

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