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eNOS S-nitrosylation mediated OxLDL-induced endothelial dysfunction via increasing the interaction of eNOS with β-catenin

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ABSTRACT

Protein S-nitrosylation plays an important role in the progression of cardiovascular diseases. eNOS can be S-nitrosylated in endothelial cells, and this modification reversibly attenuates enzyme activity. Under physiological conditions, eNOS directly interacts with β-catenin. However, whether and how eNOS S-nitrosylation regulates the β-catenin signal pathway and participates in endothelial dysfunction remains unknown. Here, we show that OxLDL induces the S-nitrosylation of eNOS, which enhances the interaction between eNOS and β-catenin, transcriptional activity of β-catenin, cell migration and adhesion molecule expression in endothelial cells. In addition, these effects are partially abolished after eNOS is mutated at Cys94 and Cys99, but not Cys441, in endothelial cells. Furthermore, OxLDL increases iNOS expression. The specific iNOS inhibitor 1400 W decreases eNOS S-nitrosylation and the association of eNOS and β-catenin, thereby blocking the β-catenin signal pathway to alleviate OxLDL-induced endothelial dysfunction. Taken together, OxLDL induces eNOS S-nitrosylation at Cys94 and Cys99 via an iNOS-dependent manner, which may increase β-catenin activation and trigger endothelial injury. This study describes a novel mechanism of endothelial dysfunction.

1. Introduction

Atherosclerosis is a progressive disease that is characterized by the accumulation of lipids and fibrous elements in the large arteries. The vascular endothelium is responsible for the regulation of vascular tone and the maintenance of vascular homeostasis [1,2]. Many studies have reported that alterations of endothelial function are early events in atherosclerosis development [3]. During the early stage of atherosclerosis, oxidized low-density lipoprotein (OxLDL) is known to enhance oxidative stress and inflammation, thereby inducing endothelial dysfunction and the formation of atherosclerotic plaques [4,5]. Numerous studies have demonstrated that OxLDL increases endothelial permeability and the expression of adhesion molecules that lead to anabatic adherence and the penetration of monocytes into the vascular endothelium [6–8].

β-catenin is a key modulator in the Wnt signaling pathway, which is involved in vasculogenesis, angiogenesis, intimal thickening and atherosclerosis [9–11]. β-catenin associates with transcriptional T-cell factor/lymphocyte enhancing factor (TCF/LEF), thus regulating the coordination of cell-cell adhesion and the expression of numerous target genes, including cyclin D1, c-Myc and c-Jun. In recent years, several studies have reported that β-catenin plays an important role in OxLDL-induced endothelial dysfunction [12,13]. Nevertheless, how OxLDL regulates the β-catenin pathway remains unclear.

As the first discovered gaseous signaling molecule, nitric oxide (NO) affects a number of cellular processes, including those involving vascular cells [14]. Under physiological conditions, NO is produced mainly from L-arginine by endothelial nitric oxide synthase (eNOS) in the endothelium of blood vessel walls [15]. Recently, eNOS has been demonstrated to interact directly with β-catenin in endothelial cells, and

Abbreviations: OxLDL, oxidized low-density lipoprotein; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; TCF, T-cell factor; LEF, lymphoid enhancer factor; L-NAME, N (ω)-nitro-L-arginine methyl ester hydrochloride

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eNOS activation leads to β-catenin translocation to the nucleus with resultant effects on gene transcription and downstream functional responses [16]. These results indicate that the β-catenin signaling pathway might rely on its interaction with eNOS and eNOS activity.

Protein S-nitrosylation, the covalent modification of a protein cysteine thiol by an NO group to generate an S-nitrosothiol (SNO) [17], plays an important role in the progression of cardiovascular diseases [18–20]. eNOS can be S-nitrosylated in endothelial, cells and this modification reversibly attenuates enzyme activity. These studies have also identified zinc-tetrathiolate cysteine residues as the sites in eNOS that undergo S-nitrosylation in intact endothelial cells [21–23]. To our knowledge, the possibility that S-nitrosylation of cysteine residues on eNOS is involved in the modulation of its interaction with β-catenin remains unexplored.

Inducible nitric oxide synthase (iNOS) is regarded as a principal mediator of NO-dependent S-nitrosylation [24]. However, more investigations are needed to understand the expression of iNOS and the subsequent S-nitrosylation of important proteins in endothelial dysfunction. Several studies have shown that OxLDL elevates iNOS expression in related cardiovascular diseases [25–27]. Therefore, we tested the hypothesis that, in order to cause endothelial dysfunction, OxLDL modulates the β-catenin signaling pathway via eNOS S-nitrosylation induced by iNOS. We report here that OxLDL induces eNOS S-nitrosylation at Cys94 and Cys99 sites. This modification of eNOS enhances its interaction with β-catenin, induces β-catenin nuclear translocation, and promotes the transcriptional activity of β-catenin, thus contributing to OxLDL-induced endothelial dysfunction. Furthermore, the inhibition of iNOS reduces OxLDL-induced eNOS S-nitrosylation and activation of the β-catenin pathway, subsequently attenuating endothelial dysfunction. Altogether, these findings revealed a new mechanism for the regulation of endothelial dysfunction in atherosclerosis.

2. Materials and methods

2.1. Animals

Eight-week-old male C57BL/6 J mice (wild-type mice, WT) and homozygous ApoE$^{-/-}$ mice were purchased from the Animal Center of Nanjing University, Nanjing, China. To accelerate the development of spontaneous atherosclerotic lesions in ApoE$^{-/-}$ mice, animals were fed a high-fat diet (15% fat plus 1.25% cholesterol) for 4 weeks. The animals were housed individually in the specific pathogen free barrier facility at constant temperature (20–22 °C) and humidity (45%–55%) with a 12 h light-dark cycle. ApoE$^{-/-}$ mice were fed with a high-fat diet (15% fat plus 1.25% cholesterol), and C57BL/6 J mice with rodent diet for 4 weeks [28]. The animal experiments were approved by the Committee on Animal Care of Nanjing Medical University and were conducted according to NIH Guidelines for the Care and Use of Laboratory Animals. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals.

2.2. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords according to a previously described method [29] and cultured in Endothelial Cell Medium (ECM, ScienCell, CA, USA). EA.hy926 endothelial cells and human embryonic kidney 293 (HEK-293) cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, USA) with 10% (v/v) fetal bovine serum (FBS; Gibco, Carlsbad, USA). Confluent cells (80%–90%) were incubated with Sodium nitroprusside (SNP) dehydrate (100 μM, Sigma-Aldrich, St Louis, USA), N (ω)-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μM; Sigma-Aldrich, St Louis, USA) or 1400 W (10 μM; Sigma-Aldrich, St Louis, USA), and oxidized LDL (OxLDL; 100 μg/mL; Yiyuan Biotechnology, China).

2.3. Scratch-wound migration assays

Confluent cells were scratched with a 100 μL pipet tip, and detached cells were removed by PBS washes. The cells were incubated with or without OxLDL (100 μg/mL) for 24 h in serum-free media. Initial and final wound width was measured and quantified using Image-Pro Plus analysis software.

2.4. Isolation of nuclear and cytoplasmic proteins and immunoprecipitation

Whole-cell, cytosolic proteins and nuclear proteins were extracted using RIPA buffer (Sigma Aldrich, St Louis, USA) or a nuclear and cytoplasmic extraction kit (ThermoFisher, San Jose, USA). The supernatants of whole-cell lysates were incubated overnight at 4 °C with mouse anti-eNOS antibody (BD Biosciences, Pharmingen, USA) or normal mouse IgG. Protein G-Sepharose beads (GE Healthcare, Milwaukee, USA) were added for further 2 h incubation at 4 °C, and bound proteins were eluted by being boiled with loading buffer and analyzed by SDS-PAGE.

2.5. Western blotting

Equal amounts of protein were resolved in 8% or 10% SDS-PAGE and western blotting analysis was performed as previously described [29]. Primary antibodies included anti-eNOS antibody (BD, Biosciences Pharmingen, USA), anti-GSNOR antibody, anti-Trx antibody (Abcam, Cambridge, USA), anti-p-eNOS antibody, anti-myc-tag antibody, anti-β-catenin antibody, anti-β-actin antibody, anti-Lamin A/C and anti-Histone H3 (CST, Danvers, USA). Band intensities were analyzed using Image J 1.25 software (National Institutes of Health, Bethesda, USA).

2.6. Immunofluorescence staining

Sections were washed twice with sterile PBS and fixed with ice-cold methanol, permeabilized with 0.01% Triton X-100 and blocked in 3% BSA. The sections were then incubated overnight with primary antibodies, including anti-eNOS (BD Biosciences, Pharmingen, USA), anti-CD31, and anti-β-catenin (CST, Danvers, USA). Secondary fluorescent antibodies (Alexa-488, or -594; Life Technologies, Carlsbad, USA) were added for 1 h and DAPI (Santa Cruz, California, USA) was used for nuclear counterstaining. Samples were imaged through confocal microscope (Zeiss LSM 410, Oberkochen, Germany) and quantified using Image-Pro Plus analysis software.

2.7. Plasmid transfection and RNA analysis

Single mutation at Cys94, Cys99, Cys441 to serine (Hanbio, Shanghai, China) were confirmed by DNA sequencing. EA.hy926 endothelial cells and HEK-293 cells were transfected with expression vectors using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, USA). Intercellular adhesion molecule-1 (ICAM-1, Primerbank ID: 167466197c1) and vascular cell adhesion molecule-1 (VCAM-1, Primerbank ID: 315434270c1) mRNA expression were quantified by real-time PCR with indicated primers.

2.8. Luciferase reporter gene assay

β-catenin/TCF activity reporter assay was performed to assess β-catenin functionality. In 12-well plates, cells grown to 60–80% confluence were transfected with 0.1 μg of pRL-TK vector that provided constitutive expression of Renilla luciferase (Promega, Madison, USA) and 1 μg of either pTOP-FLASH (TOP) or pFOP-FLASH (FOP) containing multimerized wild-type and mutated LEF/TCF binding sites,

respectively, fused to a luciferase reporter gene. Transfections were performed with Lipofectamine 3000 (Invitrogen, Carlsbad, USA). 24 h after transfection, cells were treated with OxLDL (100 μg/mL) or lithium chloride (LiCl) for 12 h. Then the cells were harvested and extracts were prepared in passive lysis buffer (Promega, Madison, USA). The luciferase activity was determined by the Fluoroskan microplate reader (Thermo Labsystems, Helsinki, Finland) according to the instruction of the Dual-Luciferase Reporter assay system (Promega, Madison, USA).

2.9. S-nitrosylation assays

eNOS S-nitrosylation was detected by biotin-switch assay using S-nitrosylated protein detection kit (Cayman Chemical, Ann Arbor, USA) according to the manufacturer. Briefly, protein free thiols were derivitized with a blocking agent. S-nitrosothiols were reduced to yield free thiols which were covalently labelled with maleimide-biotin. Subsequent detection by avidin-coupled reagents could be used to localized the biotinylated proteins. The biotinylated proteins were incubated overnight at 4 °C with Pierce™ NeutrAvidin™ Plus UltraLink™ Resin (ThermoFisher, San Jose, USA), and bound proteins were eluted by being boiled with loading buffer and analyzed by SDS-PAGE.

2.10. Statistical analysis

All data are presented as means ± s.e.m. as indicated. Statistical analysis was performed by a two-tailed Student's *t*-test or one-way ANOVA. For all tests, *P*-values lower than 0.05 were considered statistically significant.

3. Results

3.1. OxLDL promotes β-catenin transcriptional activity by increasing its association with eNOS and nuclear translocation

To determine whether OxLDL influences the association of eNOS and β-catenin, eNOS was immunoprecipitated from HUVECs lysates, and the resulting immunoprecipitates were subjected to western blotting for both eNOS and β-catenin analysis. As shown in Fig. 1A, treatment of HUVECs with 100 μg/mL OxLDL for 12 h promoted the interaction between eNOS and β-catenin. Then, we further examined the nuclear localization of β-catenin and eNOS. Western blotting analysis of nuclear protein extracts indicated that the nuclear accumulation of eNOS and β-catenin protein was significantly elevated after treatment with OxLDL or LiCl, which was used as a positive control to confirm nuclear translocation of β-catenin (Fig. 1B). Subsequently, we studied

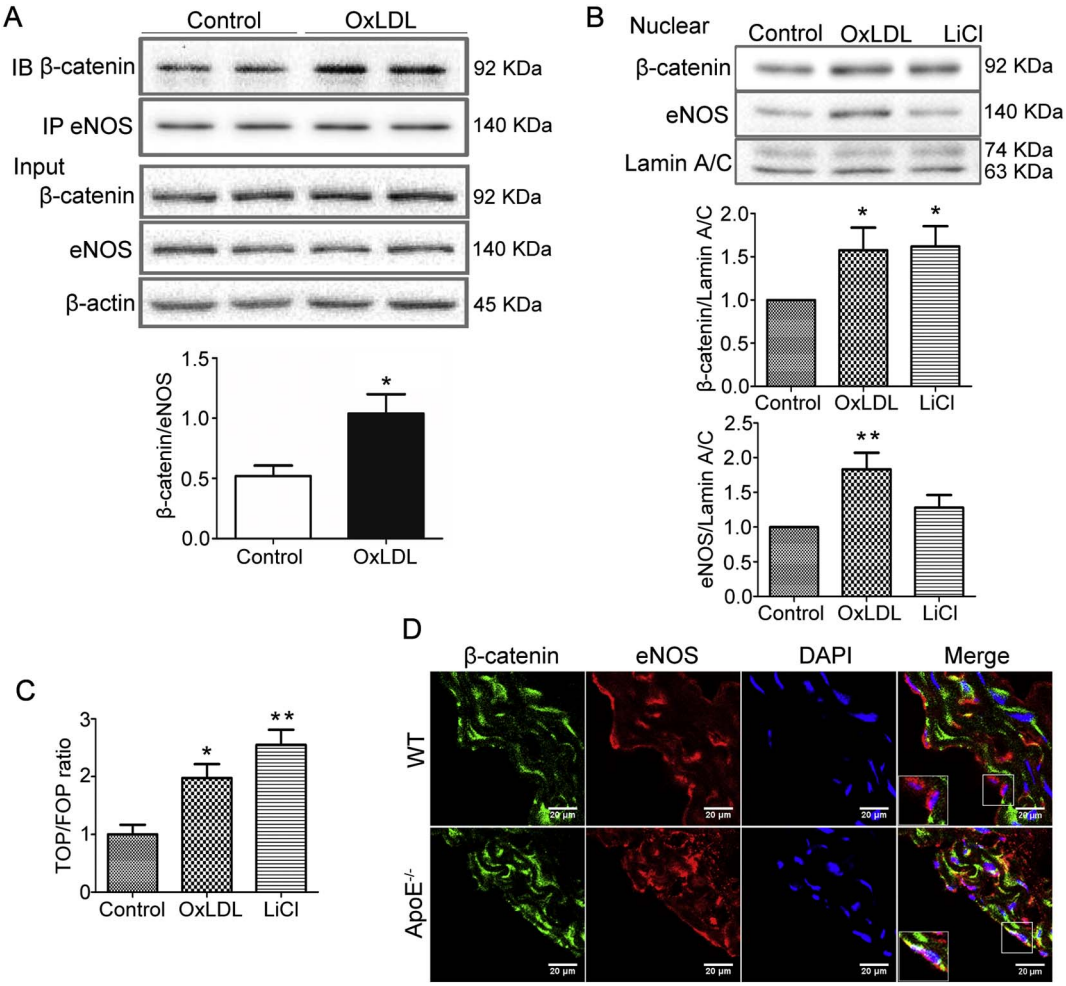

Fig. 1. OxLDL promotes β-catenin transcriptional activity by increasing its association with eNOS and nuclear translocation. (A) Human umbilical vein endothelial cells (HUVECs) were treated with OxLDL (100 μg/mL) for 12 h, cell lysates were subjected to immunoprecipitation (IP) using anti-eNOS antibody, and blotted with anti-β-catenin antibody. $n = 7$, $^{*}P < 0.05$ vs. control. An aliquot of total lysate was analyzed for eNOS, β-catenin and β-actin expression (lower panels). (B) Western blotting analysis and quantification of nuclear β-catenin and eNOS protein. Lamin A/C was used for normalization for nuclear proteins. Treatment of cells by LiCl (10 mM, 12 h) as a positive control of β-catenin translocation. $n = 7$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. (C) HUVECs were transfected with TOP flash or FOP flash plasmids accompanied with Renilla plasmid for 24 h, then treated with OxLDL (100 μg/mL) for 12 h. TOP/FOP activity was detected. $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. (D) Representative immunostaining for β-catenin (green), eNOS (red) and DAPI (blue) of aorta from C57BL/6 (WT) or high-fat fed ApoE$^{-/-}$ mice. Scale bars, 20 μm.

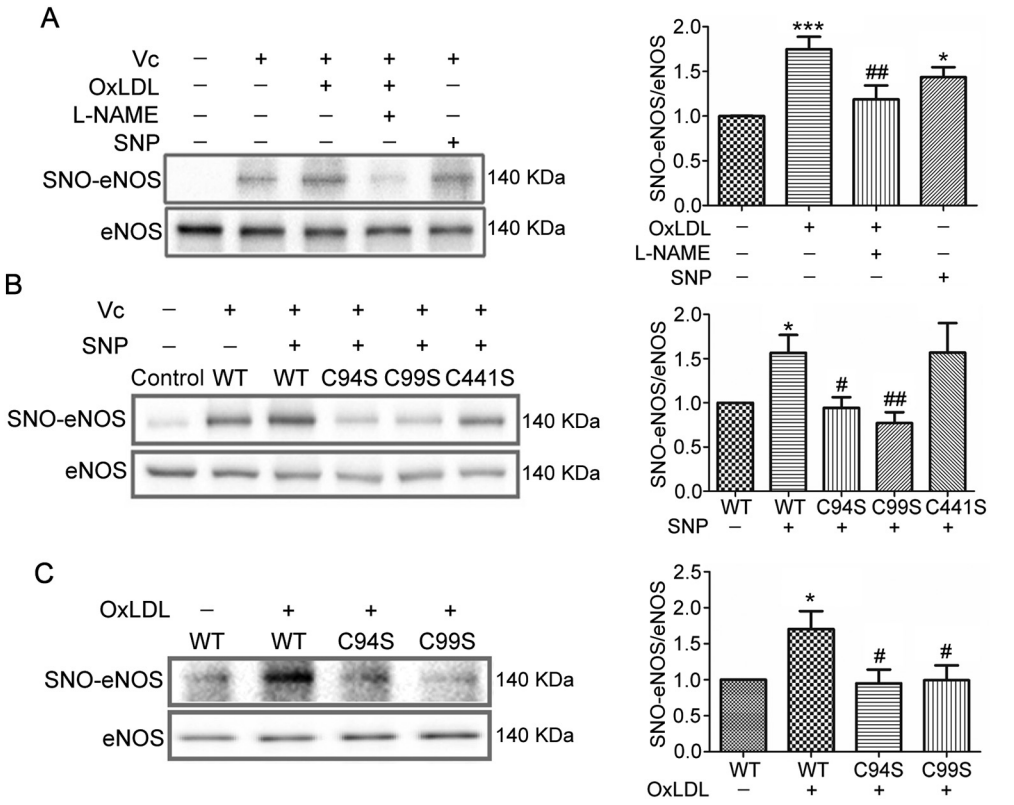

Fig. 2. OxLDL induces eNOS S-nitrosylation at Cys94 and Cys99 sites. (A) EA.hy926 endothelial cells were treated with OxLDL (100 μg/mL) for 12 h with or without L-NAME (100 μM) pretreated for 1 h, then lysates of cells were subjected to biotin-switch assay. No Vitamin C (Vc) as negative control. Cells were treated with SNP (100 μM) for 2 h as positive control. S-nitrosylated eNOS were quantified and normalized against total eNOS protein (right panel). $n = 7$, $^{*}P < 0.05$, $^{***}P < 0.001$ vs. control, $^{\#\#}P < 0.01$ vs. OxLDL. (B) HEK-293 cells were transfected with myc-tagged wild-type or mutated eNOS (C94S, C99S, and C443S) respectively for 24 h. Then cells were treated with SNP (100 μM) for 2 h. Lysates of HEK-293 cells were subjected to biotin-switch assay. S-nitrosylated eNOS were quantified and normalized against total eNOS protein (right panel). $n = 7$, $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. WT + SNP. (C) HUVECs were transfected with wild-type or mutated eNOS (C94S and C99S) respectively at least for 24 h. Then treated with OxLDL (100 μg/mL) for 12 h. Lysates of HUVECs were subjected to biotin-switch assay. S-nitrosylated eNOS were quantified and normalized against total eNOS protein (right panel). $n = 5$, $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.05$ vs. WT + OxLDL.

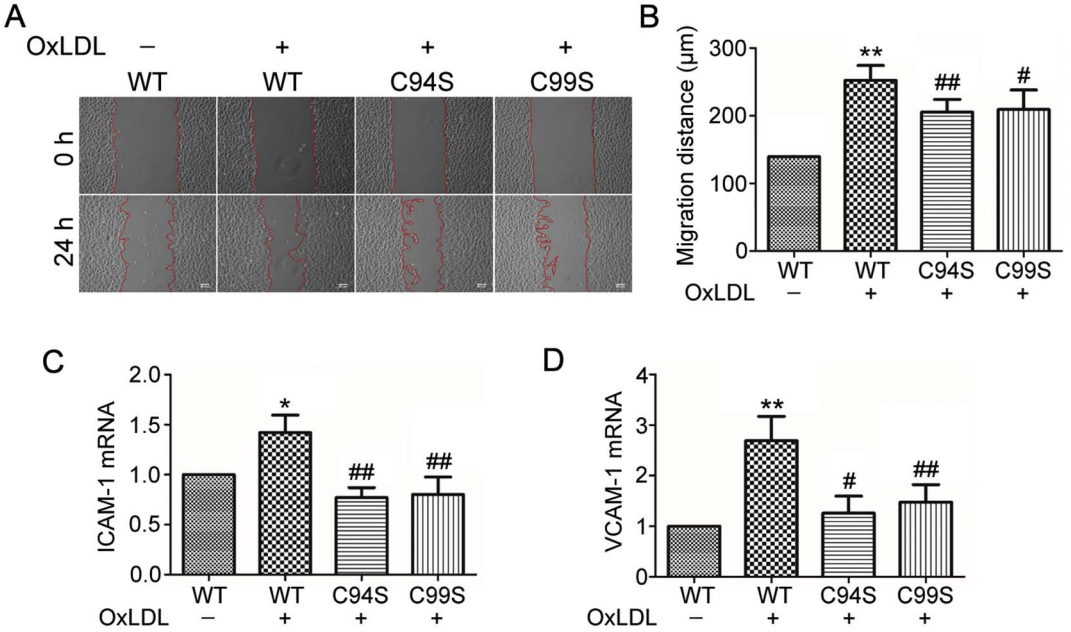

Fig. 3. eNOS S-nitrosylation is involved in OxLDL-induced endothelial dysfunction. (A) HUVECs were transfected with WT or mutated eNOS (C94S and C99S) respectively for 24 h, which were then scratched and incubated in DMEM containing OxLDL (100 μg/mL) for 24 h. Scale bars, 50 μm. (B) The quantification of endothelial cells migrated width. $n = 6$, $^{**}P < 0.01$ vs. WT; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ vs. WT + OxLDL. (C) and (D) HUVECs were transfected with WT or mutated eNOS (C94S and C99S) for 24 h. Then treated with OxLDL (100 μg/mL) for 12 h. mRNA levels of ICAM-1 and VCAM-1 were analyzed by real-time PCR. $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. WT; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ vs. WT + OxLDL.

β-catenin/TCF/LEF signaling using the TOP-FOP TCF/LEF luciferase reporter constructs, a well-established assay to measure the transcriptional activity of β-catenin. The results showed that the relative transcriptional activity of β-catenin was significantly increased in HUVECs treated with either OxLDL or LiCl (Fig. 1C). Moreover, eNOS showed significant nucleus distribution in high-fat fed ApoE$^{-/-}$ mice aortic endothelial cells (Suppl. Fig. 1). Additionally, the association and nuclear translocation of eNOS and β-catenin were also enhanced in aortic endothelial cells from the high-fat fed ApoE$^{-/-}$ mouse model (Fig. 1D).

3.2. OxLDL induces eNOS S-nitrosylation at cysteine 94 and 99 sites

Previous studies have shown that S-nitrosylation, a type of post-translational modification, plays a vital role in regulating the function of eNOS [30]. Therefore, we examined the S-nitrosylation of eNOS in EA.hy926 cells treated with OxLDL by a biotin-switch assay. As

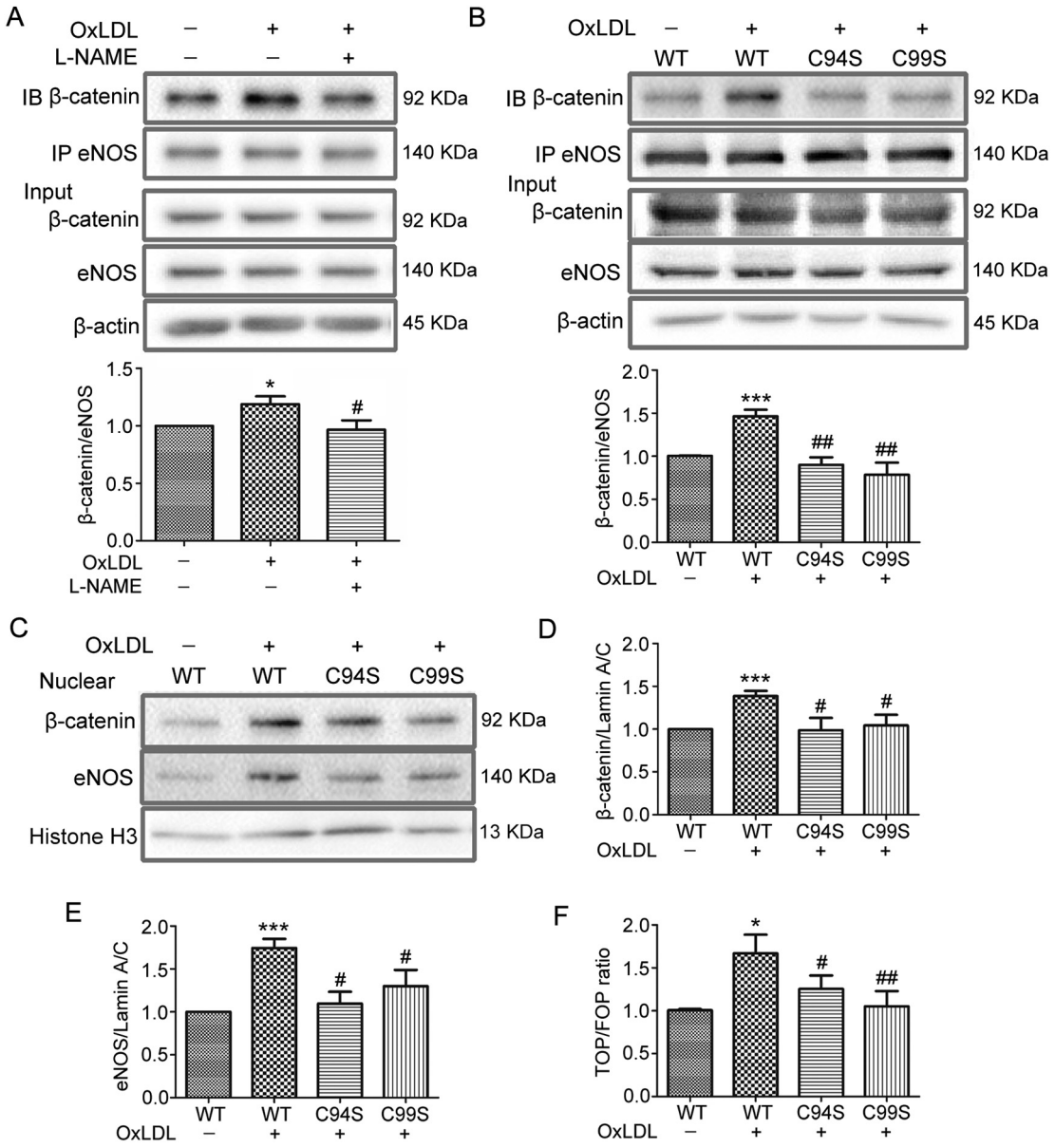

Fig. 4. S-nitrosylation of eNOS regulates the transcriptional activity of β-catenin via increasing the association of eNOS and β-catenin. (A) HUVECs were treated with OxLDL (100 μg/mL) for 12 h with or without L-NAME (100 μM) pretreated for 1 h, cell lysates were immunoprecipitated with an anti-eNOS antibody, both β-catenin and eNOS were detected by western blotting. An aliquot of total lysate was analyzed for eNOS, β-catenin and β-actin expression. $n = 6$, $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. OxLDL. (B-E) HUVECs were transfected with WT or mutated eNOS (C94S and C99S) for 24 h. (B) Then treated with OxLDL (100 μg/mL) for 12 h, cell lysates were immunoprecipitated with an anti-myc-tag antibody, and blotted with anti-β-catenin antibody (upper panel). An aliquot of total lysate (Input) was analyzed for eNOS (myc-tag), β-catenin and β-actin expression (lower panel). $n = 7$, $^{***}P < 0.001$ vs. WT; $^{\#\#}P < 0.01$ vs. WT + OxLDL. (C) OxLDL (100 μg/mL) treated for 12 h, western blotting analysis and quantification (D, E) of nuclear eNOS (myc-tag) and β-catenin protein. Histone was used for normalization for nuclear proteins respectively. $n = 6$, $^{***}P < 0.001$ vs. WT; $^{\#}P < 0.05$ vs. WT + OxLDL. (F) HUVECs were transfected with TOP flash or FOP flash accompanied with Renilla at least for 24 h, then treated with OxLDL (100 μg/mL) for 12 h. TOP/FOP activity was detected. $n = 6$, $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. WT + OxLDL.

expected, OxLDL significantly increased the S-nitrosylation of eNOS in endothelial cells. Additionally, L-NAME and SNP treatment were used as negative and positive controls, respectively (Fig. 2A). To identify the S-nitrosylated cysteine residue, we used site-directed mutagenesis to change the three predicted cysteines of eNOS individually to serines (C94S, C99S, and C441S). SNP treatment still enhanced the S-nitrosylation of eNOS after overexpression of wild-type or eNOS mutated at cysteine 441, but not at cysteine 94 or 99 (Fig. 2B). Similarly, after eNOS mutation at cysteine 94 and 99, OxLDL no longer increased the S-nitrosylation of eNOS in HUVECs (Fig. 2C). These findings indicated that eNOS S-nitrosylation at cysteine 94 and 99 is critical for eNOS activity in OxLDL-treated endothelial cells.

3.3. eNOS S-nitrosylation is involved in OxLDL-induced endothelial dysfunction

To determine whether eNOS S-nitrosylation is involved in OxLDL-induced endothelial dysfunction, wild type and mutant (C94S and C99S) eNOS constructs were transfected into HUVECs. Scratch-wound migration assays showed that cell migration was increased in wild type eNOS-transfected HUVECs cultured with OxLDL, but migration was partially restored to control levels in the C94S and C99S-transfected groups (Fig. 3A and B). Moreover, after eNOS mutation at Cys94 and Cys99, OxLDL failed to increase the level of VCAM-1 and ICAM-1 mRNA, as measured by real-time PCR (Fig. 3C and D). These results suggested that eNOS S-nitrosylation participates in OxLDL-induced

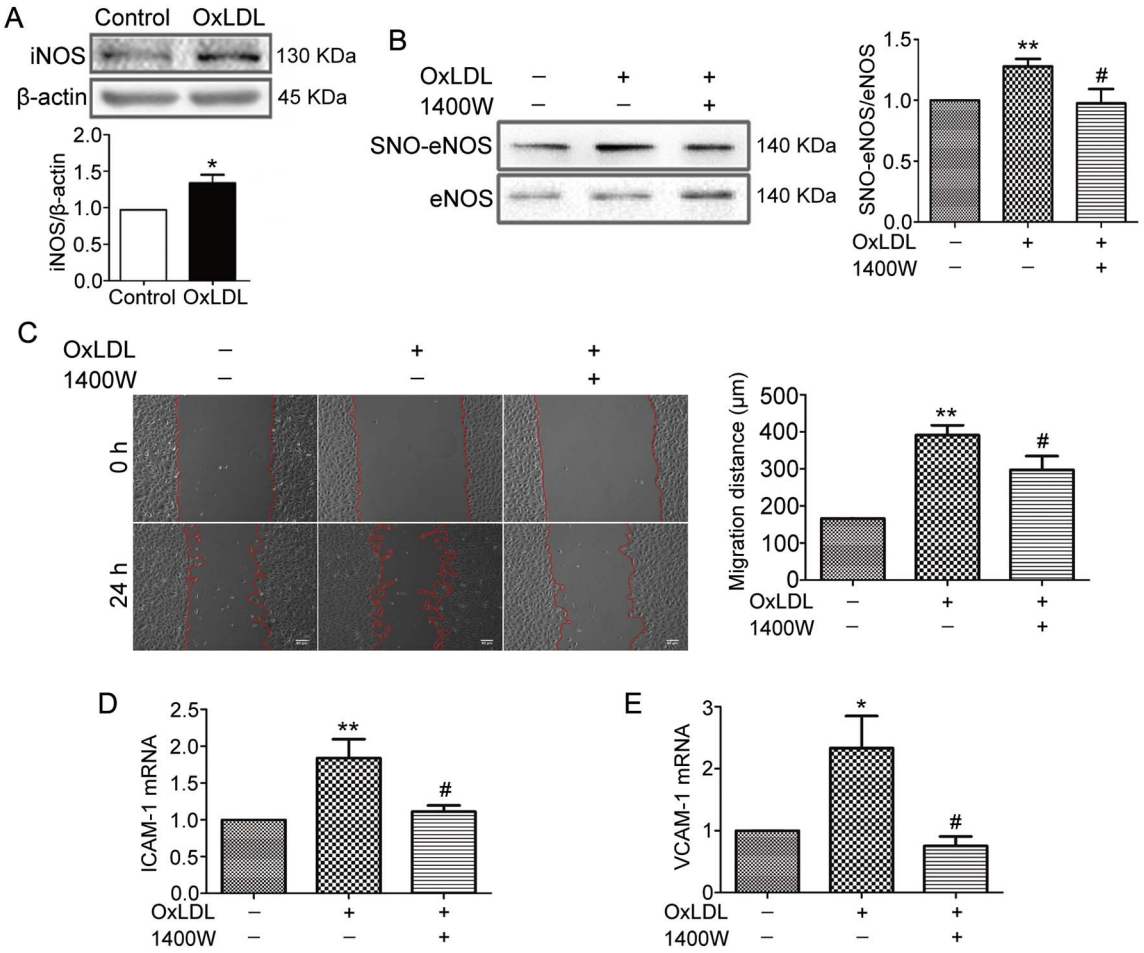

Fig. 5. iNOS mediates OxLDL-induced eNOS S-nitrosylation and endothelial dysfunction. (A) HUVECs were treated with OxLDL (100 μg/mL) for 12 h. Western blotting analysis and quantification of iNOS protein. $n = 6$, $^{*}P < 0.05$ vs. control. (B) HUVECs were treated with OxLDL (100 μg/mL) for 12 h with or without 1400 W (10 μM) pretreated for 1 h. Cell lysates were subjected to S-nitrosylation assay. S-nitrosylated eNOS were quantified and normalized against total eNOS protein (right panel). $n = 5$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. OxLDL. (C) HUVECs were pretreated with or without 1400 W (10 μM) for 1 h, which were then scratched and incubated in DMEM containing OxLDL (100 μg/mL) for 24 h. Scale bars, 50 μm. The quantification of endothelial cells migrated width (right panel). $n = 5$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. OxLDL. (D) and (E) HUVECs were pretreated with or without 1400 W (10 μM) for 1 h, then treated with OxLDL (100 μg/mL) for 12 h. mRNA levels of ICAM-1 and VCAM-1 were analyzed by real-time PCR. $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. OxLDL.

endothelial dysfunction, and Cys94 and Cys99 are critical for this effect.

3.4. S-nitrosylation of eNOS regulates the transcriptional activity of β-catenin via increasing the association of eNOS and β-catenin

To explore whether eNOS S-nitrosylation affects OxLDL-induced endothelial dysfunction via the transcriptional activity of β-catenin, a series of experiments were performed in HUVECs transfected with wild type and mutant eNOS. The increased interaction between eNOS and β-catenin in HUVECs treated with OxLDL was abolished with pretreatment with L-NAME, a NOS inhibitor (Fig. 4A). Additionally, the interaction between eNOS and β-catenin was decreased in C94S- and C99S-transfected HUVECs treated with OxLDL compared with wild type eNOS-transfected cells (Fig. 4B). Similarly, the expression of eNOS and β-catenin was reduced in the nucleus fraction of both C94S- and C99S-transfected cells compared with wild type eNOS-transfected cells (Fig. 4C, D and E). Furthermore, Cys94- and Cys99-transfected cells displayed decreased β-catenin transcriptional activity, as measured by TOP-FOP luciferase reporter constructs (Fig. 4F). These results suggested that eNOS S-nitrosylation contributes to OxLDL-induced endothelial dysfunction via increasing its interaction with β-catenin and promoting β-catenin translocation and transcriptional activity.

3.5. iNOS mediates OxLDL-induced eNOS S-nitrosylation and endothelial dysfunction

iNOS plays a significant role in OxLDL-induced endothelial dysfunction and is regarded as a principal mediator of NO-dependent S-nitrosylation [24]. To explore whether OxLDL-induced eNOS S-nitrosylation is dependent on iNOS in endothelial cells, we measured the expression of iNOS in HUVECs using a western blotting assay. Pretreatment with 1400 W, a specific iNOS inhibitor, down-regulated the S-nitrosylation of eNOS caused by OxLDL in EA.hy926 cells (Fig. 5B). Similarly, the increased migration and ICAM-1 and VCAM-1 mRNA levels were partially restored to control levels with 1400 W pretreatment (Fig. 5C–E). These results suggested that iNOS is involved in OxLDL-induced eNOS S-nitrosylation and endothelial dysfunction.

3.6. Inhibition of iNOS reduces the OxLDL-induced interaction and nuclear translocation of eNOS and β-catenin

To determine whether iNOS-induced eNOS S-nitrosylation regulates the interaction of β-catenin and eNOS, a series of experiments were performed in HUVECs pretreated with 1400 W. As expected, immunoprecipitation quantification showed that pretreatment with 1400 W reduced the interaction of eNOS and β-catenin, which was caused by OxLDL, in EA.hy926 cells (Fig. 6A and B). Similarly, the level

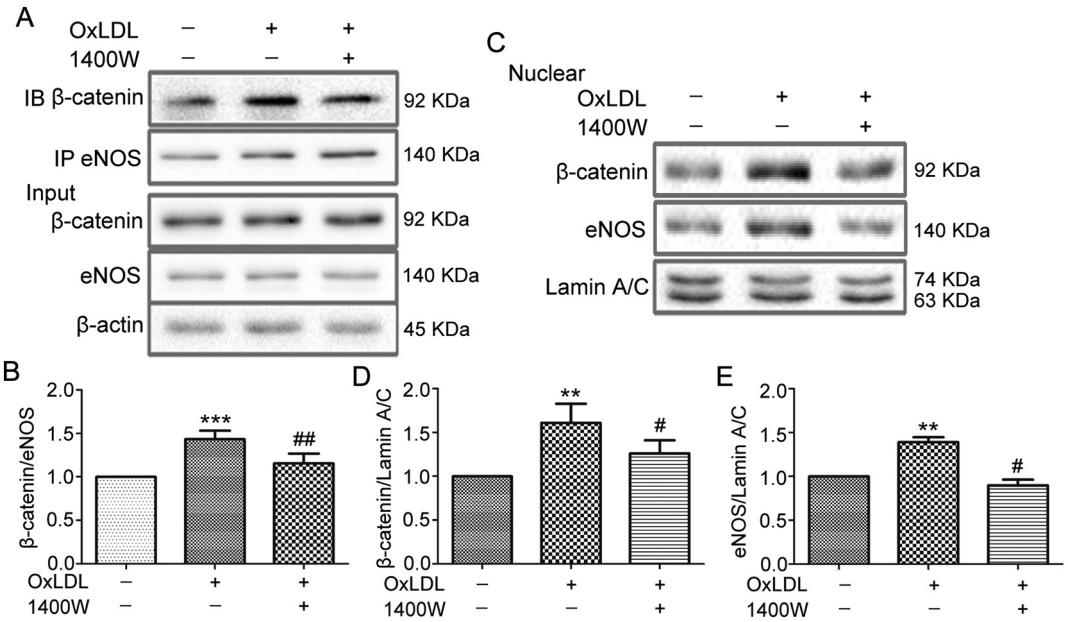

Fig. 6. Inhibition of iNOS reduces OxLDL-induced the interaction and nuclear-translocation of eNOS and β-catenin. HUVECs were treated with OxLDL (100 μg/mL) for 12 h with or without 1400 W (10 μM) pretreated for 1 h. (A, B) HUVECs lysates were immunoprecipitated with anti-eNOS antibody, and blotted with anti-β-catenin antibody. An aliquot of total lysate (Input) was analyzed for eNOS, β-catenin and β-actin expression. $n = 7$, $^{***}P < 0.001$ vs. control; $^{\#\#}P < 0.01$ vs. OxLDL. (C, D, E) Western blotting analysis and quantification of nuclear eNOS, β-catenin and Lamin A/C proteins. $n = 7$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. OxLDL.

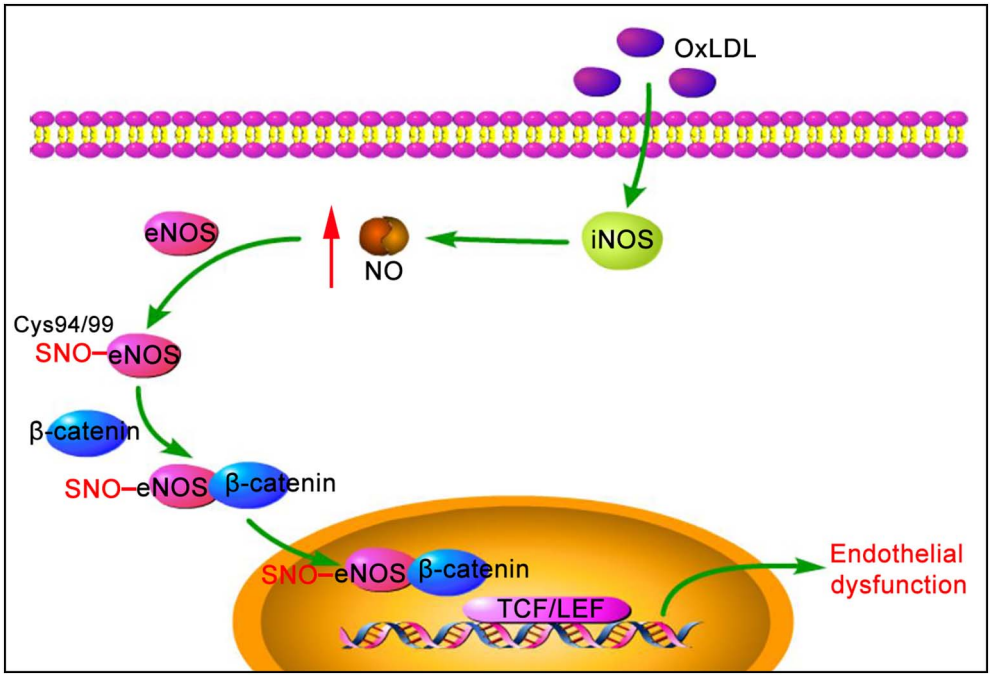

Fig. 7. Schematic diagram illustrated that OxLDL increases iNOS-mediated S-nitrosylation of eNOS at Cys94 and Cys99, which promotes the interaction of eNOS and β-catenin and nuclear translocation, thereby elevating the transcriptional activity of β-catenin, eventually lead to endothelial dysfunction.

of eNOS and β-catenin was also reduced in the nuclear fraction in 1400 W-pretreated HUVECs (Fig. 6C, D and E).

4. Discussion

Endothelial cells are crucial for both vascular homeostasis and protecting the vasculature against atherogenic insults [3]. OxLDL-mediated injury to endothelial cells is crucial for endothelial dysfunction in the pathogenesis of atherosclerosis and atherosclerotic plaque rupture at advanced stages [6]. We confirmed that the phosphorylation of eNOS (Ser1177) was decreased in OxLDL-treated endothelial cells (Suppl. Fig. 2). However, the precise mechanism of OxLDL on endothelial dysfunction remains to be explored. Under physiological conditions, eNOS can bind to β-catenin in endothelial cells to regulate the downstream β-catenin signal pathway [16]; therefore, we wondered whether OxLDL could affect the association and nuclear translocation of the eNOS/β-catenin complex. Our data showed that OxLDL could increase the association and nuclear translocation of eNOS and β-catenin in endothelial cells, thereby promoting the transcriptional activity of β-catenin. Furthermore, the association and nuclear translocation of

eNOS and β-catenin were also enhanced in aortic endothelial cells in an atherosclerosis mouse model (Fig. 1).

Previous reports demonstrated that eNOS is S-nitrosylated at Cys94 and Cys99 in endothelial cells, and eNOS S-nitrosylation is inversely related to eNOS activation (phosphorylation at Ser1179) [21,22]. S-nitrosylation is a dynamic post-translational modification for the regulation of protein function [31]. Cys94 and Cys99 are widely investigated sites that have been shown to be S-nitrosylation cysteine sites of eNOS, and they form a zinc-tetrathiolate cluster at the eNOS homodimer interface, which is responsible for dimer formation of the active enzyme [21,22]. Cys441 is the last residue near the C-terminus of the oxygenase domain in the dimer interface and is located between acidic (glutamine) and basic (arginine) amino acids of eNOS [32]. Collectively, these three cysteine sites play a crucial role in maintaining the normal function and activation of eNOS. To examine the correlation between OxLDL-induced eNOS S-nitrosylation and endothelial dysfunction, Cys94, Cys99 and Cys441 mutants were used in our experiments. Our findings demonstrated that the Cys94 and Cys99 sites participated in OxLDL-induced eNOS S-nitrosylation, whereas Cys441 rarely influenced OxLDL-induced eNOS S-nitrosylation (Fig. 2). The S-nitrosylation of eNOS enhanced cell migration and adhesion molecule expression in endothelial cells after treatment with OxLDL, but this effect was abolished by mutation of Cys94 and Cys99 in eNOS (Fig. 3). These results provide the first evidence that eNOS S-nitrosylation at Cys94 and Cys99 is involved in OxLDL-induced endothelial dysfunction.

In the vasculature, the interaction of VE-cadherin and β-catenin at endothelial cell-cell junctions controls vascular integrity [33]. However, OxLDL induces the activation of β-catenin in human aortic smooth muscle cells, and the active β-catenin associates with TCF4 and translocates into the nucleus, thus playing an important role in atherosclerosis [12]. Despite research on the interaction between eNOS and β-catenin in endothelial cells, the role of eNOS S-nitrosylation in regulating the eNOS/β-catenin complex and the subsequent signaling pathway in the context of endothelial dysfunction is not clearly defined [16]. Hence, we examined the involvement of eNOS S-nitrosylation in β-catenin signaling using a NOS inhibitor and mutations of eNOS. L-NAME, a dual NOS inhibitor, not only diminished eNOS S-nitrosylation (Fig. 3) but also inhibited the binding of β-catenin to eNOS induced by OxLDL in endothelial cells (Fig. 4). Furthermore, the interaction of β-catenin and eNOS, nuclear translocation and transcriptional activity of β-catenin were decreased in eNOS mutation (C94S and C99S)-transfected endothelial cells (Fig. 4). Together, our study suggested for the first time that the increased interaction of eNOS and β-catenin induced by OxLDL is dependent on the S-nitrosylation of eNOS.

OxLDL down-regulates eNOS and up-regulates iNOS, thereby augmenting the formation of NO and protein S-nitrosylation in human endothelial cells [26]. Importantly, iNOS-mediated S-nitrosylation plays an increasingly significant role in cardiovascular diseases [34]. For example, iNOS-mediated IRE1α S-nitrosylation links obesity-associated inflammation to endoplasmic reticulum dysfunction [34]. Additionally, S-nitrosylation of TSC2 by iNOS-derived NO is associated with impaired TSC2/TSC1 dimerization, mTOR pathway activation, and proliferation of human melanoma [35]. Recent studies have discovered that innate immunity is necessary for the transdifferentiation of fibroblasts to endothelial cells. Innate immune activation increases the iNOS generation of NO to S-nitrosylate RING1A, thus releasing epigenetic repression to achieve effective transdifferentiation [36]. We also confirmed that OxLDL significantly increased iNOS expression and NO release in endothelial cells (Fig. 5 and Suppl. Fig. 3), but without change in GSNOR and Trx expression (Suppl. Fig. 4). To explore whether eNOS S-nitrosylation induced by OxLDL was ascribed to iNOS-derived NO, 1400 W, which is a specific inhibitor of iNOS, was used in our study. As our results demonstrated, the iNOS inhibitor suppressed OxLDL-induced eNOS S-nitrosylation, cell migration and adhesion molecule expression (Fig. 5). Mechanistically, the iNOS inhibitor also reduced the association and nuclear translocation of eNOS and β-catenin in OxLDL-treated endothelial cells (Fig. 6). These results suggested that iNOS activation contributes to OxLDL-induced eNOS S-nitrosylation and endothelial dysfunction.

In conclusion, this study provides evidence that OxLDL increases iNOS-mediated S-nitrosylation of eNOS at Cys94 and Cys99 to regulate the interaction of eNOS and β-catenin to induce endothelial dysfunction (Fig. 7). These data highlight a novel insight into the mechanism of atherosclerosis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.02.009.

Conflict of interest

The authors declare that there is no conflict of interest.

Transparency document

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