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Lipoxin A4 restores oxidative stress-induced vascular endothelial cell injury and thrombosis-related factor expression by its receptor-mediated activation of Nrf2-HO-1 axis

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ABSTRACT

Venous thromboembolism (VTE) constitutes a common cause of hospital-related morbidity and mortality, with the proverbial clinical feature of deep venous thrombosis (DVT). Endothelial cell injury and dysfunction comprise the critical contributor for the development of DVT. Lipoxin A4 (LXA4) fulfills pleiotropic roles in injury repair. However, its role in DVT remains poorly elucidated. In the present study, LXA4 supplementation dampened H_2O_2-evoked cytotoxic injury in human umbilical vein endothelial cells (HUVECs) by increasing cell viability, suppressing cell apoptosis and caspase-3 activity. Moreover, treatment with LXA4 afforded cytoprotective effects against oxidative stress damage in response to H_2O_2 by abrogating ROS, lactate dehydrogenase (LDH) and MDA leakage, and elevating anti-oxidant SOD levels. Notably, LXA4 administration attenuated provasoconstriction factor endothelin-1(ET-1) expression in HUVECs exposed to H_2O_2, but enhanced the productions of vasodilatation factor NO and prostacyclin (PGI2). Simultaneously, H_2O_2-induced high expression of prothrombotic Von Willebrand Factor (vWF) was also inhibited by LXA4. Mechanism analysis substantiated that LXA4 further augmented activation of the Nrf2-HO-1 pathway. Nevertheless, blocking this signaling via si-Nrf2 transfection or HO-1 antagonist ZnPP both reversed LXA4-mediated effects against oxidative stress injury and thrombotic potential. Cessation of the LXA4 receptor pathway by its inhibitor Boc2 not only counteracted LXA4-evoked activation of the Nrf2-HO-1, but also reversed LXA4-mediated anti-oxidative stress and thrombosis-related factor expression. Accordingly, this study suggests that LXA4 may ameliorate vascular endothelial cell oxidative stress injury and subsequent thrombotic response via LXA4 receptor-dependent activation of the Nrf2-HO-1 signaling, implying a promising strategy for DVT and its complication.

1. Introduction

Thrombotic disease constitutes the great threaten for health worldwide, including arterial thrombosis and venous thromboembolic disease. Venous thromboembolism (VTE) is a major cause of hospital-related morbidity and mortality, with a common occurrence in patients with traumatic injury, surgery, pregnancy and active cancer [1,2]. VTE is clinically characterized with deep venous thrombosis (DVT) and it-evoked fateful pulmonary embolism (PE). Recently, a high prevalence of VTE has been observed in children [3]. Despite advances in anti-thrombotic disorder drugs, the unsatisfactory and recurrent thrombosis limit the current therapeutic strategy [4,5]. Therefore, it is urgently needed to elucidate the mechanism underlying venous thrombosis and explore a novel therapy approach against VTE.

Vascular dysfunction is a major cause of diseases like thrombosis and atherosclerotic complications [6,7]. During this process, vascular endothelial cells are the pivotal participator that form a monolayer lying in the inner surface of vessels. Under physiological conditions, endothelial cells produce abundant mediators to maintain local fibrinolysis state, such as the thrombomodulin and pro-vasorelaxation factors nitric Oxide (NO) and prostacyclin (PGI2). However, various adverse environments can injure endothelial cells. Oxidative stress, for example, is a major and initial cause of cardiovascular diseases including DVT [8]. Once injury, dysfunctional endothelial cells will evoke a pro-thrombosis response by regulating vasoconstriction, vasodilatation, platelet aggregation and disrupting the balance of coagulation/

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anti-coagulation and fibrinolysis/anti-fibrinolysis [7,9]. Previous research confirmed high oxidative stress in patients with DVT, indicating a critical role of oxidative stress in the pathology of venous thrombosis [8]. Moreover, antioxidative action protects against endothelial cell injury and ultimately ameliorates the pathologic progression of venous thrombosis [10].

Lipoxin A4 (LXA4) is a prominent family member of lipoxins (LXs) that are first identified as endogenous “breaking signals” of inflammatory process. Accumulating evidence has confirmed the critical potential of LXA4 in anti-inflammation and oxidant stress-evoked diseases [11,12]. For example, replenishing LXA4 attenuates pancreatitis-induced lung injury by suppressing endothelial inflammatory response [11]. Moreover, LXA4 deficiency causes preeclampsia that may be ascribed to a reduction in inflammation response and oxidative stress [12]. Recently, increasing study has corroborated the protective roles of LXA4 against injury progression. Administration with LXA4 ameliorates cerebral ischemia/reperfusion (I/R) injury by activating the nuclear factor erythroid 2-related factor (Nrf2) signaling [13]. Furthermore, LXA4 treatment attenuates intestinal I/R injury by acting as an antioxidant mediator to antagonize intestinal epithelium cell oxidative stress and apoptosis [14]. However, its roles in oxidative stress-triggered VTE and DVT remain poorly elucidated.

In this study, we aimed to explore the effects of LXA4 on oxidative stress-induced human umbilical vein endothelial cells (HUVECs) injury and thrombosis-related factor expression. Additionally, the signaling of Nrf2-HO-1 axis was also investigated.

2. Materials and methods

2.1. Cell culture and treatment

HUVECs were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). For culture, cells were incubated with Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml). For stimulation, cells were treated with exogenous LXA4 (Cayman Chemical Company, Ann Arbor, MI, USA) ranged from 10 to 200 nmol/l for 6 h before exposure to H_2O_2 (0.5 mM) for 12 h. Additionally, N-tert-butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe (Boc2) (100 μM), the LXA4 receptor LXA4R (also named as FPR2) antagonist, was added 30 min prior to the above treatments. All cells were housed in a humidified atmosphere containing 5% CO2 and cultured at 37 °C.

2.2. Knock down of Nrf2 expression by its specific siRNA

The siRNA targeting Nrf2 were applied to knock down expression of Nrf2. The scramble siRNA (si-NC) and si-Nrf2 were referenced [15] and obtained by Invitrogen (Carlsbad, CA, USA). After seeding into 6-well plates, HUVECs were transfected with 50 nM of si-NC or si-Nrf2 using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, the efficacy was evaluated by western blotting.

2.3. Cell viability detection by MTT assay

Cell viability was measured using the MTT assay. Briefly, cells under LXA4 condition were preconditioning with HO-1 inhibitor zinc protoporphyrin IX (ZnPP, 10 μM), si-Nrf2. Afterwards, cells were exposed to H_2O_2 for 12 h. Then, 100 μl of MTT reagent (5 mg/mL, Sigma, St. Louis, MO, USA) was added for 4 h incubation, and the subsequently formed formazan precipitate was solubilized using dimethyl sulfoxide (100 μl). Fifteen minutes later, cell viability was determined by measuring the absorbance at 570 nm, and presented as the percentage of surviving cells relative to the control group.

2.4. Cell apoptosis evaluation by Annexin V-PI staining

To evaluate cell apoptotic ratio, the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) was introduced. Following treatment with the indicated conditions, cells were trypsinized and harvested. Then, cells were resuspended in 195 μl of binding buffer. Subsequently, Annexin V-FITC (5 μl) and 200 μl of Annexin V binding buffer with PI (10 μl) were supplemented, and cells were further incubated for 20 min in darkness. All specimens were ultimately counted using a flow cytometer (BD Biosciences, San Jose, CA, USA). All protocols were performed as previously described [16].

2.5. Caspase-3 activity assay

Cells under various stimulations were trypsinized, and then treated with cell lysis buffer (100 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% TritonX-100, 1 mM EDTA, 10 mM b-glycerophosphate, 2 mM sodium vanadate and protease inhibitor) for 15 min at 4 °C. After centrifugation for 15 min, 10 μl of substrate Ac-DEVD-pNA (2 mM) was added to the obtained supernatants. The activity of caspase-3 was then detected using a commercial Caspase 3 Activity Assay Kit (Beyotime) according to the instruction of manufacturer.

2.6. Measure of reactive oxygen species (ROS) contents

For ROS measurement, the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma) was used. Briefly, cells were treated under various conditions. After that, after washing three times with phosphate buffered saline (PBS), cells were stained with 20 μM of DCFH-DA for 30 min. The fluorescence intensity of samples was captured by microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using excitation at 480 nm and emission at 530 nm.

2.7. Determination of lactate dehydrogenase (LDH) release

HUVECs were seeded into 96 well-plates at 1.5×10^4 cells per well. Cells under LXA4 condition (100 nM) were pretreated with ZnPP, Boc2 or si-Nrf2 transfection, prior to expose to H_2O_2. The LDH release into supernatants was detected according to the directions of a commercial LDH detection kit (Beyotime). OD490 nm was captured to analyze LDH content.

2.8. Measurement of malondialdehyde (MDA) content and nitric oxide (NO) production

Following experimental treatment, the contents of MDA and NO in supernatants were measured using the commercial MDA detection kit and NO detection kit (both from Nanjing Jiancheng Bioengineering Institute, Nanjing, China). For MDA measurement, the reaction was performed based on the measurement of red color produced during the reaction to thiobarbituric acid (TBA) with MDA, and the absorbance of 532 nm was determined. For NO detection, cells were incubated with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) for 10 min, the nitrite was spectrophotometrically captured at 550 nm to evaluate NO production.

2.9. Evaluation of superoxide dismutase (SOD) levels

The content of SOD was detected to assess the antioxidant status in accordance with the recommendation of a commercial SOD assay kit (Randox, Crumlin, UK). During this process, the collected supernatants were incubated with 50 mM of xanthine and xanthine oxidase (XOD) to generate superoxide radicals. Then, 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride was added to induce the formation of a red formazan dye that was spectrophotometrically captured at 505 nm and 37 °C. SOD activity was then measured by evaluating

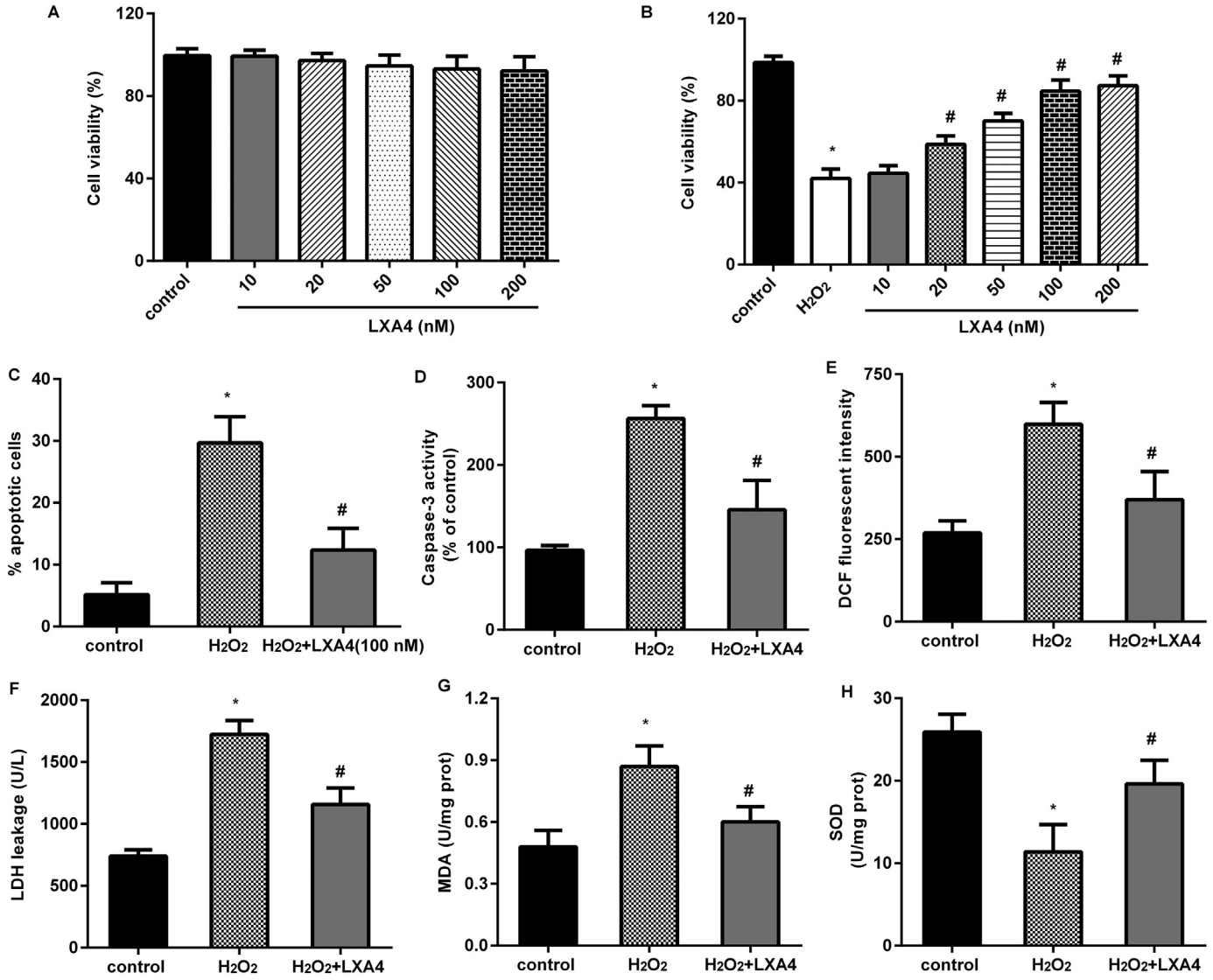

Fig. 1. LXA4 protected against H_2O_2-triggered endothelial cell oxidative stress toxic injury. (A) HUVECs were stimulated with various doses of LXA4 for 6 h, cytotoxic analysis was evaluated by MTT assay. $N = 6$. (B) Before exposure to H_2O_2 for 12 h, cells were pre-treated with 100 nM of LXA4. Cell viability was then detected by MTT analysis. $N = 6$. (C) Cells were preconditioned with LXA4 (100 nM) for 6 h, prior to H_2O_2 exposure. After staining with Annexin V-FITC/PI, cell apoptotic rate was determined by flow cytometer. $N = 5$. (D) The activity of caspase-3 was then analyzed. (E) ROS contents were measured by staining with DCFH-DA. (F–H) The generation of LDH (F), MDA (G) and SOD (H) were detected. $N = 6$. $^{*}P < .05$ vs. control group. $^{\#}P < .05$ vs. H_2O_2-treated group.

inhibitory degree of above reaction and presented as units per μg protein.

2.10. RNA extraction and qRT-PCR assay

Total RNA from HUVECs under various conditions were extracted using the TRIzol reagents (Sigma), followed by reverse transcription to synthesize cDNA using the One Step PrimeScript® miRNA cDNA Synthesis Kit (Takara, Dalian, China). Then, real time PCR was conducted to evaluate the transcriptional levels of Nrf2, HO-1, Endothelin-1(ET-1) and Von Willebrand Factor (vWF) using the SYBR Premix Ex TaqTM II Kit (TaKaRa). All procedures were conducted according to the manufacturer's instructions. Primer sequences for these genes were used as follows: Nrf2 (sense, 5′-GTTGCCCACATTCCCAAATC-3′; antisense, 5′-CGTAGCCGAAGAAACCTCAT-3′), HO-1 (sense, 5′-TCTTGGCTGGCTTC CTTAC-3′; antisense, 5′-CATAGGCTCCTTCCTCCTTTC-3′), ET-1 (sense, 5′- CAGAGGGAGAAATGGAGAGATG-3′; antisense, 5′-GGAGAAGAGAA GAGAGAGAGAGA-3′) and vWF (sense, 5′-TGTGGGAGATGTTTGCC TAC-3′; antisense, 5′-TGACCTTGCAGAAGTGAGTATC-3′). β-actin was introduced to normalize gene transcripts by the $2^{-\Delta\Delta CT}$ formula.

2.11. ELISA assay

After treatment with the indicated conditions, cells were lysed with the lysis buffer. Then, the concentration of Endothelin-1(ET-1) (Abcam, Cambridge, MA, USA) and Von Willebrand Factor (vWF) (Biomatik Corp., Wilmington, DE, USA) in supernatants were detected using the Quantative ELISA kits. Prostaglandin F1α (PGF1α) is a stable metabolite of prostacyclin (PGI2) and is typically applied to measure the levels of PGI2. Then, the 6-keto-PGF1α ELISA Kit (Abcam) was used to evaluate the concentration of PGI2 in supernatants. All protocols were performed according to the instructions provided by the manufacturers. All experiments were performed six times.

2.12. Western blotting

Cells were lysed with a RIPA lysis buffer to isolate proteins. Nuclear proteins were extracted using a Nuclear Protein Extraction Kit

(Beyotime). The BCA protein assay kit (Beyotime) was used to quantify the protein contents. Then, protein specimens (40 mg per lane) were separated using 12% SDS-PAGE, and subsequently electroblotted onto a PVDF membrane (Millipore, Billerica, MA, USA). For immunoblotting, the membrane was incubated with 5% non-fat milk for 1 h. After that, the primary antibodies against human Nrf2, Lamin B and HO-1 (Santa Cruz, CA, USA) were added. Following incubation with TBST buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Triton X-100) three times for 10 min, the membranes were probed with horseradish peroxidase-conjugated secondary antibody for 1 h. Then, the ECL reagent (Amersham Pharmacia, Piscataway, NJ, USA) was introduced to visualize the binding signals. Band intensity was quantified by the Image J software with the internal control of Lamin B for Nrf2 and β-actin for HO-1 expression.

2.13. Statistical analysis

All tests were performed at least three times and analyzed using SPSS 19.0. Data are shown as means ± SD. For statistical analysis, Student *t*-test was used for two groups, and ANOVA was introduced for three or more groups, followed by the Student-Newman-Keuls (SNK) post hoc tests. A value of $P < .05$ was deemed statistically significant.

3. Results

3.1. LXA4 dose-dependently affords cytoprotective effects on cell cytotoxic injury upon H_2O_2 exposure

As shown in Fig. 1A, we first explored the cytotoxic effects of LXA4 on HUVECs, and found that LXA4 executed little cytotoxicity to HUVECs with doses from 10 to 200 nmol/l. More importantly, exogenous supplementation with LXA4 antagonized the adverse effects of H_2O_2 on cell viability in a dose-dependent manner, but there was little difference between the 100 nmol/l-treated and 200 nmol/l-treated groups (Fig. 1B). Therefore, the concentration of LXA4 at 100 nmol/l was chosen for the subsequent experiments. Furthermore, exposure to H_2O_2 evoked cell apoptosis, which was reversed by pretreatment with 100 nmol/l of LXA4 (Fig. 1C). Simultaneously, H_2O_2-enhanced caspase-3 activity also was abrogated after LXA4 preconditioning (Fig. 1D).

3.2. Administration with LXA4 alleviates H_2O_2-triggered oxidative stress

To assess whether LXA4 protected HUVECs against oxidative stress injury, cells were stimulated with H_2O_2. As presented in Fig. 1E, LXA4 attenuated H_2O_2-induced production of ROS, an indicator of cellular oxidative state. Furthermore, LDH release, a marker for cell injury, induced by H_2O_2 exposure was also reduced following LXA4 preconditioning (Fig. 1F). Concomitantly, H_2O_2-elevated MDA leakage was inhibited in LXA4-pretreated groups (Fig. 1G). Additionally, LXA4 treatment also ameliorated the adverse effect of H_2O_2 on SOD production, a marker of enzymatic antioxidant state (Fig. 1H). These results indicate the beneficial efficacy of LXA4 against H_2O_2–triggered oxidative stress injury in HUVECs.

3.3. LXA4 preconditioning alleviates thrombosis-related factor expression in HUVECs upon H_2O_2 stimulation

Convincing evidence supports the fact that endothelial cell injury possesses the critical role in thrombosis [7,9]. We therefore further investigated the role of LXA4 in thrombosis-related factor expression in HUVECs under H_2O_2 condition. QRT-PCR assay confirmed that LXA4 inhibited H_2O_2-induced increase in transcripts of ET-1, a critical regulator for vasoconstriction (Fig. 2A). Moreover, high levels of ET-1 in supernatants from H_2O_2-stimulated HUVECs was also inhibited following LXA4 treatment (Fig. 2B). Relative to the H_2O_2 groups, the decreases in vasodilatation-related factor contents of NO (Fig. 2C) and PGF1α, a stable metabolite of PGI2 (Fig. 2D), were restored in LXA4-pretreated groups. Simultaneously, administration with LXA4 suppressed the transcript and release of prothrombotic risk factor vWF in HUVECs upon H_2O_2 exposure (Fig. 2E and F).

3.4. Exogenous supplementation with LXA4 activates the Nrf2-HO-1 signaling in H_2O_2-exposed HUVECs

Activation of the Nrf2-HO-1 signaling elicits an extraordinary effect on the response to adverse stimuli, including oxidative stress injury [13,14]. We hence explored the possibility of LXA4 on this pathway activation. As presented in Fig. 3A and B, exposure to H_2O_2 just evoked a certain degree elevation in Nrf2 mRNA (Fig. 3A) and protein expression (Fig. 3B). However, LXA4 preconditioning dramatically increased the transcript and nuclear expression of Nrf2 in HUVECs upon H_2O_2. Concomitantly, treatment with LXA4 also evoked the notable up-regulation in subsequent HO-1 mRNA (Fig. 3C) and protein (Fig. 3D), compared with the the H_2O_2 groups.

3.5. Blockage of Nrf2-HO-1 pathway affords the protective effects of LXA4 on H_2O_2-induced oxidative stress injury and thrombosis-related factor expression

To further elucidate the correlation between Nrf2-HO-1 activation and LXA4-mediated protective efficacy on HUVECs, we silenced the expression of Nrf2 by transfection with its siRNA and suppressed downstream expression of HO-1 (Fig. 4A). Functional assay corroborated that cessation of Nrf2 reversed the positive role of LXA4 on H_2O_2-inhibited cell viability (Fig. 4B), but increased cell apoptosis relative to the LXA4-pretreated groups under H_2O_2 condition (Fig. 4C). Additionally, LXA4-triggered inhibition of ROS production (Fig. 4D) and increases in SOD secretion (Fig. 4E) were also counteracted after si-Nrf2 transfection in H_2O_2-exposed HUVECs. Furthermore, the inhibitory effects of LXA4 on ET-1 generation were also abrogated following Nrf2 silencing (Fig. 4F). Adversely, LXA4-elevated pro-vasodilatation factors NO (Fig. 4G) and PGF1α (Fig. 4H) levels were attenuated when blocking the Nrf2 signaling, concomitant with the increases in prothrombosis vWF generation (Fig. 4I). Analogously, pre-treatment with HO-1 antagonist ZnPP also offset the protective effects of LXA4 against H2O2-induced oxidative stress injury and thrombosis-related factor expression.

3.6. LXA4 possesses the positive function in the activation of Nrf2-HO-1 signaling and anti-injury, anti-thrombosis potential partially through elevating its receptor FPR2

Previous research has confirmed that LXA4 often exerts its role in a LXA4 receptor LXA4R/FPR2-dependent manner [13,17]. To further clarify the mechanism how LXA4 regulates the Nrf2-HO-1 signaling, the specific antagonist Boc2 was used to block the FPR2 pathway. Intriguingly, treatment with Boc2 neutralized LXA4-evoked expression of Nrf2 and HO-1, implying that Boc2 counteracted LXA4-induced activation of the Nrf2-HO-1 signaling (Fig. 5A). Moreover, preconditioning with Boc2 attenuated LXA4-induced increase in cell viability (Fig. 5B) and LXA4-evoked decrease in cell apoptosis (Fig. 5C). Simultaneously, Boc2 treatment reversed the suppressive functions of LXA4 on H_2O_2-induced ROS generation (Fig. 5D), and attenuated LXA4-evoked antioxidant stress SOD production (Fig. 5E). In contrast to the LXA4 and H_2O_2-treated groups, Boc2 augmented generation of pro-vasoconstriction ET-1 (Fig. 5F), but diminished pro-vasodilatation NO (Fig. 5G) and PGF1α(Fig. 5H) contents in supernatants. Additionally, LXA4-inhibited vWF expression was also attenuated when blocking the LXA4 receptor FPR2 signaling (Fig. 5I).

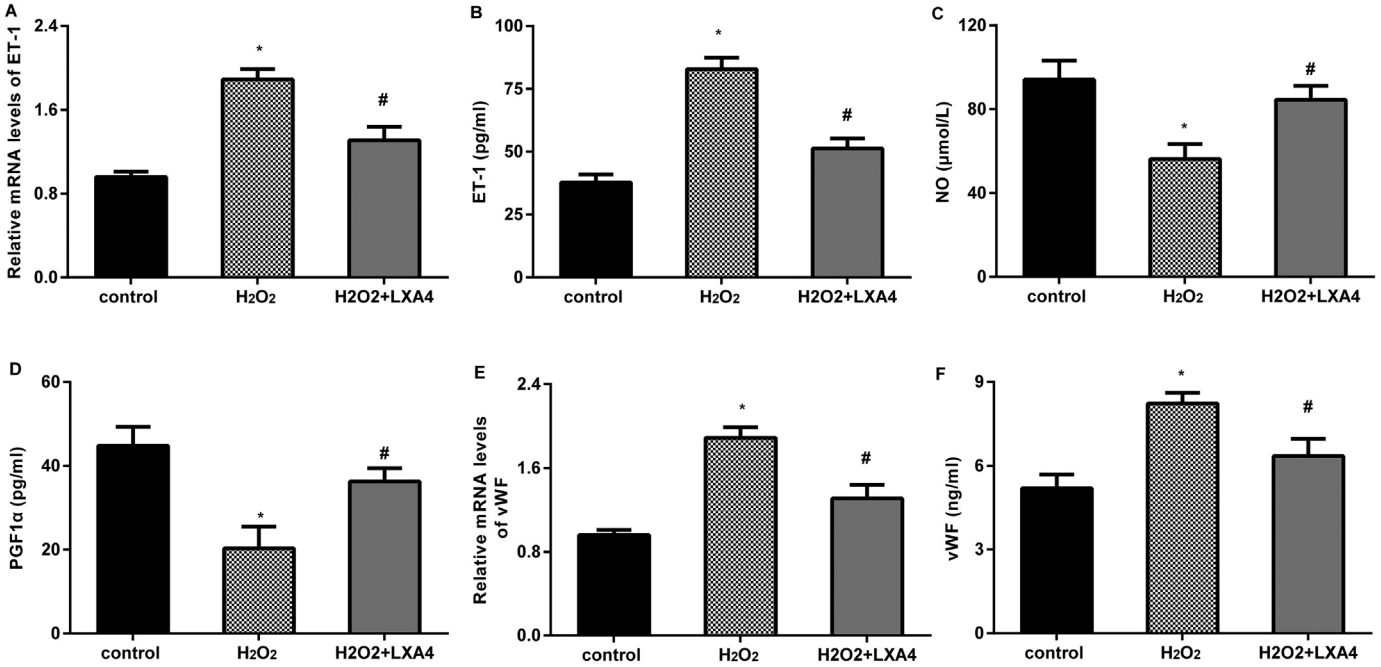

Fig. 2. Preconditioning with LXA4 suppressed thrombosis-related factor expression in HUVECs upon H_2O_2. (A) Cells were pre-treated with 100 nM of LXA4 for 6 h, prior to H_2O_2 exposure for 12 h. Then, the transcript levels of ET-1 were determined by qRT-PCR. (B) The contents of ET-1 in supernatants were measured by ELISA assay. (C) The generation of NO was evaluated by Griess reaction. (D) The effects on the production of PGF1α, a stable metabolite of PGI2, were analyzed by ELISA. (E, F) The mRNA levels and generation of vWF were also assessed. N = 6. $^{*}P < .05$ vs. control group. $^{\#}P < .05$ vs. H_2O_2-treated group.

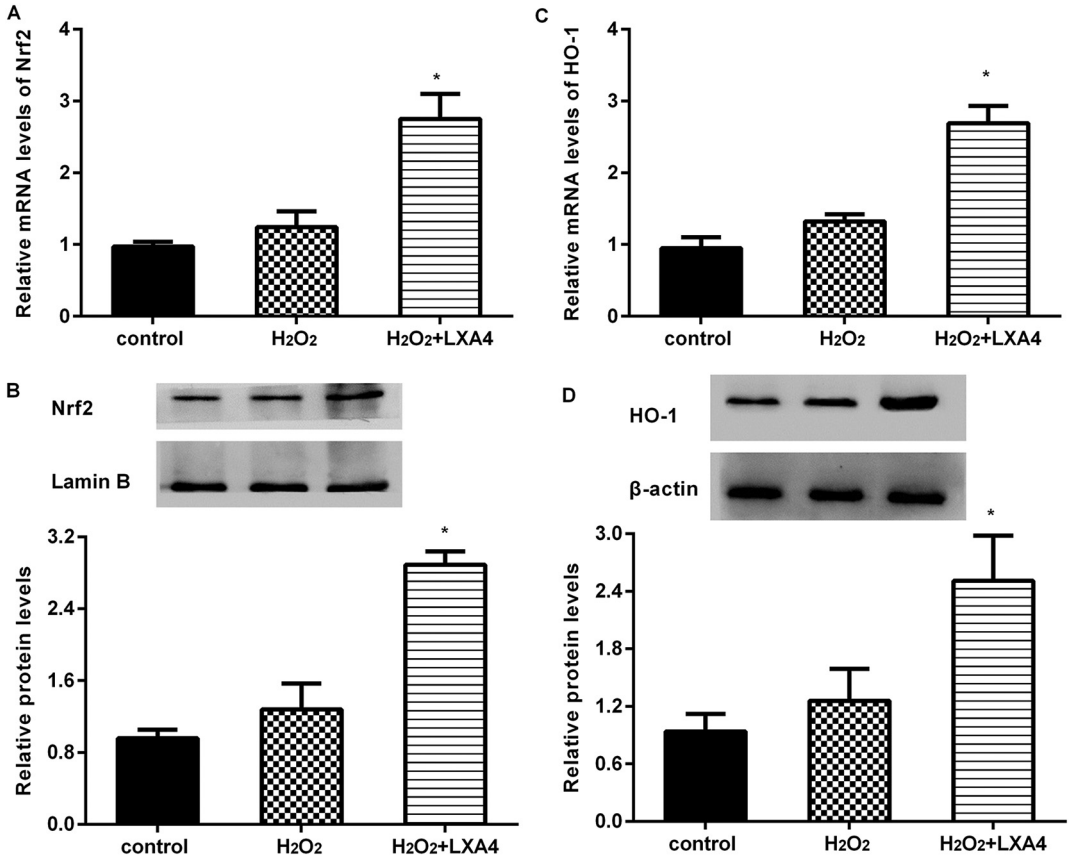

Fig. 3. LXA4 addition enhanced the activation of the Nrf2-HO-1 pathway in H_2O_2-exposed HUVECs. (A) Cells were pre-administrated with 100 nM of LXA4 for 6 h, and then cultured under H_2O_2 conditions. The mRNA levels of Nrf2 were then detected by qRT-PCR. N = 6. (B) The protein expression of Nrf2 in nuclei was quantified by western blotting. *N* = 3. (C-D) The corresponding effects on the mRNA (C) and protein (D) levels of HO-1 were also measured. $^{*}P < .05$ vs. control group. $^{\#}P < .05$ vs. H_2O_2-treated group.

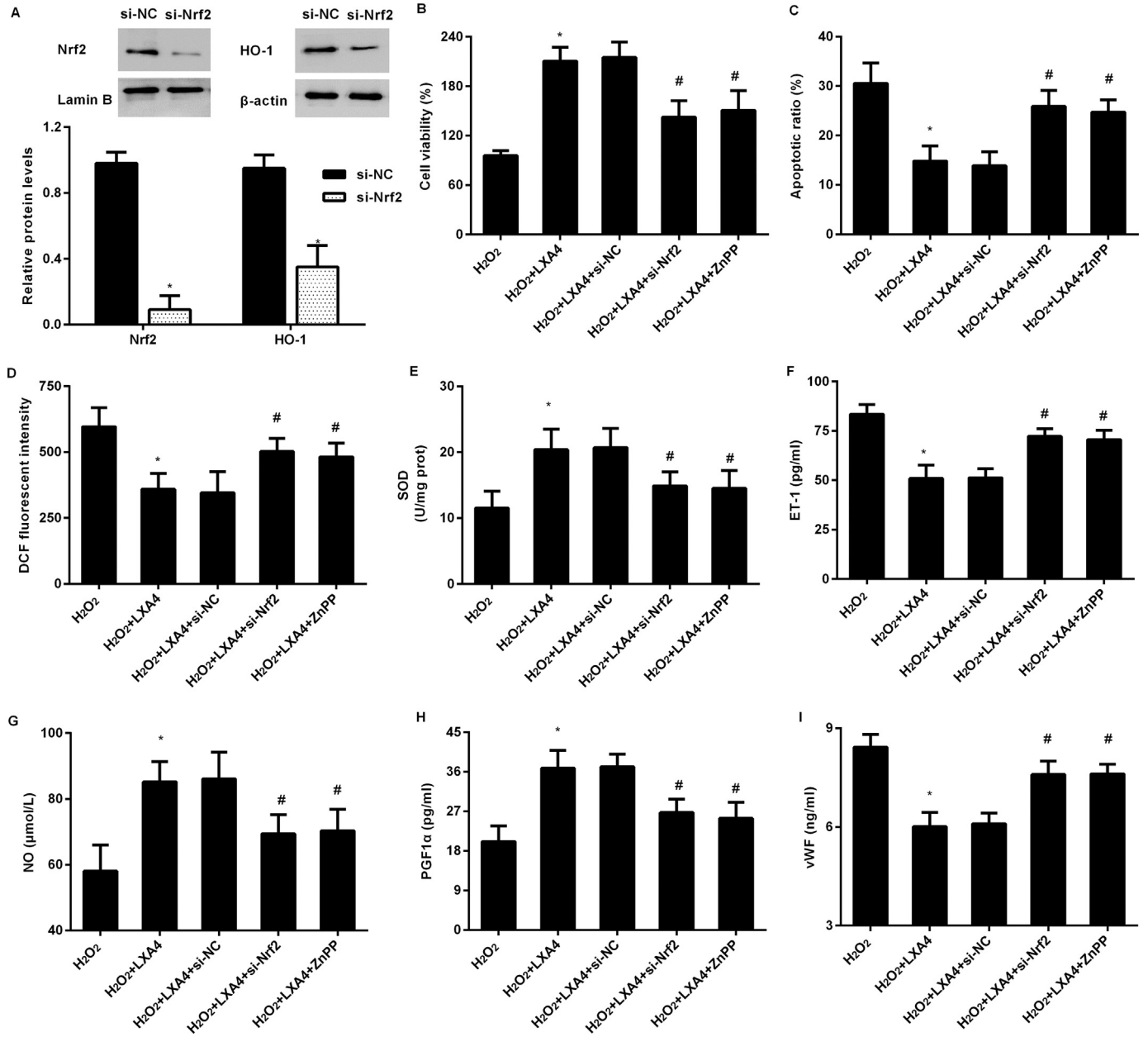

Fig. 4. Cessation of the Nrf2-HO-1 pathway afforded the protective effects of LXA4 on H_2O_2-induced oxidative stress injury and thrombosis-related factor expression. (A) After transfection with si-Nrf2 or si-NC, the protein expression of Nrf2 and HO-1 was assessed by western blotting. N = 3. (B) Following pretreatment with si-Nrf2 or ZnPP, cells incubated with LXA4 were exposed to H_2O_2 for 12 h. Then, cell viability was determined. N = 6. (C) Flow cytometer was performed to detect cell apoptosis. N = 5. (D, E) The levels of ROS (D) and SOD (E) were measured. (F–I) The subsequent effects on the generations of ET-1 (F), NO (G), PGF1α (H) and vWF (I) were also evaluated. N = 6. $^{*}P < .05$ vs. H_2O_2-treated group; $^{\#}P < .05$ vs. H_2O_2 + LXA4 group.

4. Discussion

Deep venous thrombosis (DVT) and it-evoked PE are the major manifestations of VTE, the proverbial and common life-threatening cardiovascular diseases in United States [18]. High prevalence of VTE has been validated in elderly, and is predicated to increase due to the ageing populations in many countries [19]. The annual costs of VTE-related healthcare are estimated to $2–10 billion [18]. Though advances in traditional strategy for DVT treatment, the outcomes (e.g., thrombocytopenia) in some cases is disappointing [5]. Endothelial cell injury under oxidative stress and inflammation stimulation is the key regulator in the development of DVT by evoking pro-thrombosis response [7]. The present study confirmed that LXA4 treatment attenuated H_2O_2-induced vascular endothelial cell oxidative stress injury. Simultaneously, administration with LXA4 also suppressed pro-vasoconstriction ET-1 and vWF expression, but increased pro-vasodilatation factors NO and PGI2 stable metabolite PGF1α releases in H_2O_2-stimulated HUVECs. Moreover, activation of the Nrf2-HO-1 pathway accounted for the protective efficacy of LXA4 in this process, which was particularly regulated by the LXA4 receptor. Accordingly, this study may support a potential approach against DVT and its complications.

The integrity of vascular endothelium is essential for blood vessel function and maintenance of non-thrombotic state. Once injury occurs under various pathological conditions including oxidative stress and inflammation, vascular endothelial cell apoptosis is a core event in the progression of thrombotic diseases including DVT [20,21]. In contrast to healthy volunteers, patients with DVT had increased oxidative stress levels [8]. Recent study has confirmed that disruption of endothelial

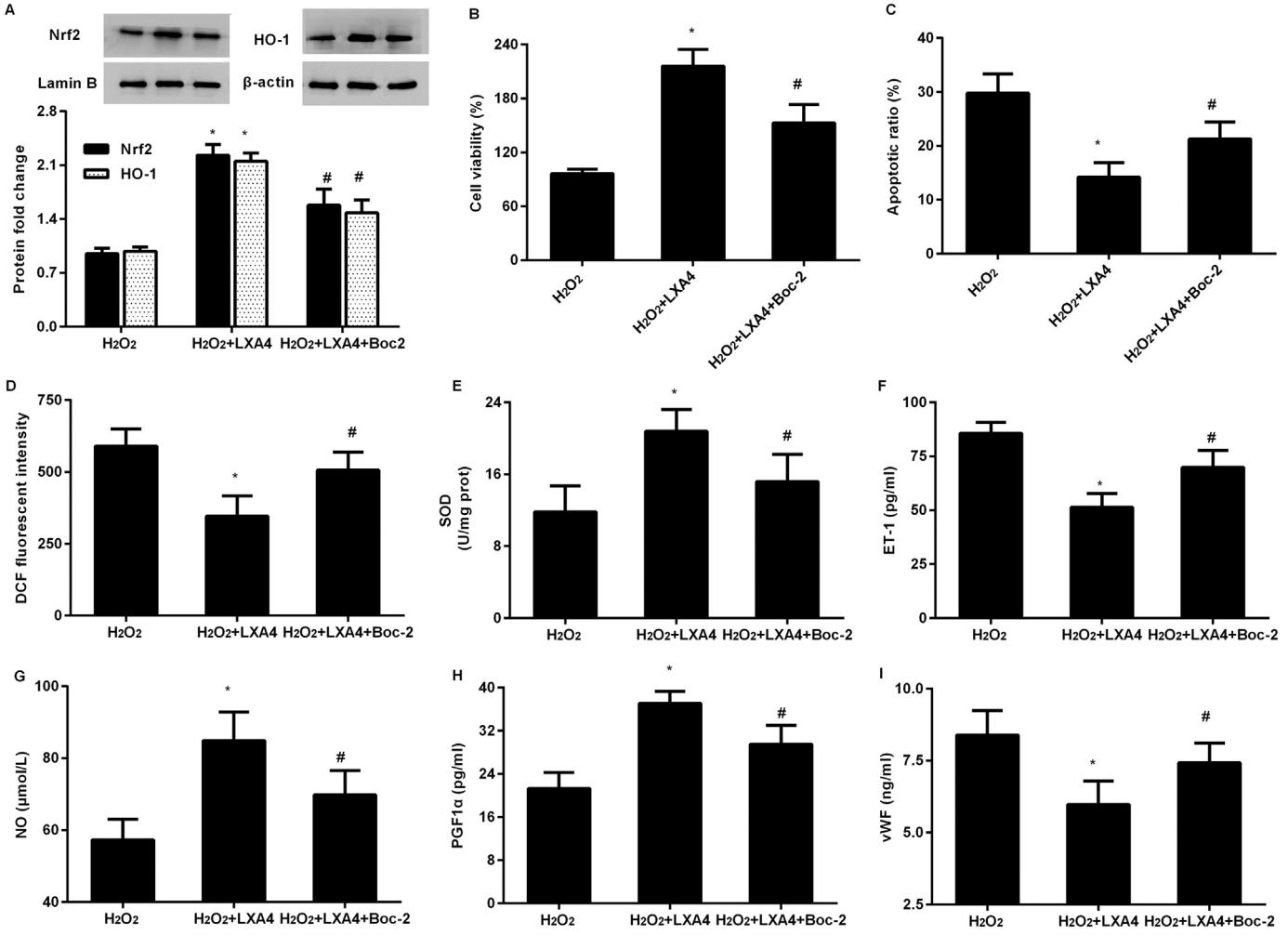

Fig. 5. The LXA4 receptor signaling was involved in LXA4-mediated anti-oxidative stress injury and thrombotic potential by activating the Nrf2-HO-1 pathway. (A) Cells under LXA4 incubation was pre-treated with LXA4 receptor antagonist Boc2 (100 μM), followed by the exposure to H_2O_2. Then, the expression of Nrf2 and HO-1 was analyzed. N = 3. (B) Cell viability was detected by MTT assay. N = 6. (C) After staining with Annexin V/PI, flow cytometer was conducted to analyze cell apoptosis. N = 5. (D-I) The contents of ROS (D), SOD (E), ET-1 (F), NO (G), PGF1α (H) and vWF (I) were measured subsequently. N = 6. $^{*}P < .05$ vs. H_2O_2-treated group; $^{\#}P < .05$ vs. H_2O_2 + LXA4 group.

functional integrity impairs endothelial permeability in vivo and facilitates thrombosis [22]. In the present study, LXA4 pretreatment attenuated H_2O_2-inhibited cell viability and cell apoptosis. Endothelial cell apoptosis after DVT also has been observed in rats [20]. Furthermore, administration with LXA4 antagonized H_2O_2-triggered oxidative stress injury by lowering ROS accumulation, MDA and LDH release, but increasing antioxidant enzymatic SOD levels. Similarly, LXA4 also suppressed intestinal epithelium cell oxidative stress and apoptosis, and ultimately ameliorated intestinal I/R injury [14]. Intriguingly, several reports have confirmed that attenuating oxidative stress damage and apoptosis in vascular endothelial cells inhibits the development of DVT [9,10,20].

In normal, vascular endothelial cells elicits critical roles in regulating the balance between fibrinolysis and coagulation processes. However, endothelial cells evoke pro-thrombosis response after injury by inducing the productions of vasoconstrictors and pro-platelet aggregation factor [9]. Here, treatment with LXA4 inhibited ET-1 generation, a common vasoconstrictor, in HUVECs upon H_2O_2 stimulation. Conversely, LXA4 preconditioning restored H_2O_2-restrained release of NO and PGI2. When generated due to endothelial injury or dysfunction, NO and PGI2 act as proverbial endothelial vaso-relaxing peptides that suppress platelet activation and adhesion [23]. vWF often serves as risk marker of thrombotic disease by acting as a critical participator for platelet adhesion, aggregation and subsequent thrombosis [24]. Notably, vWF-mediated platelet adhesion is essential for DVT formation [25]. We further observed a fact that H_2O_2-induced production of vWF in vascular endothelial cells were counteracted following LXA4 treatment. Therefore, these data suggest that LXA4 may ameliorate thrombosis by attenuating pro-thrombotic factor expression that is triggered after endothelial cell injury.

Nrf2, a redox-sensitive transcriptional factor, can translocate to the nucleus to induce expression of antioxidant stress enzyme HO-1. Activation of the Nrf2-HO-1 signaling affords protective efficacy against injury-related pathogenic progressions, such as cerebral I/R injury, spinal cord injury and ischemic stroke [26,27]. We next elucidated the mechanism lying beneath LXA4-mediated anti-endothelial cell injury and anti-thrombosis potential, and confirmed that LXA4 further enhanced activation of the Nrf2-HO-1 pathway, though little increase in Nrf2 and HO-1 expression after H_2O_2 exposure. Previous research corroborated that activating the Nrf2-HO-1 pathway attenuated oxidative damage and facilitated neurological recovery after ischemic stroke [27]. Intriguingly, blockage of Nrf2 or HO-1 expression muted the beneficial role of LXA4 against endothelial cell oxidative stress injury. Notably, LXA4 also protects against spinal cord injury by regulating the AKT/Nrf2/JO-1 signaling [26]. More intriguingly, cessation of Nrf2-HO-1 pathway increased ET-1 and vWF expression, but decreased NO

and PGI2 levels in LXA4-treated HUVECs upon H_2O_2 exposure. Analogously, former study also corroborated that activating the Nrf2-HO-1 inhibited hypoxia-induced ET-1 secretion [28]. Additionally, enhancement of HO-1 expression not only increased thrombomodulin expression [29], but also dampened thrombus formation in sepsis [30]. Hence, activation of the Nrf2-HO-1 pathway may account for LXA4-mediated anti-oxidative stress injury and anti-thrombotic potential.

LXA4 fulfills pleiotropic efficacy by binding to its receptor FPR2 [13,17]. For instance, LXA4 effectively reduced cerebral infarct volumes and improved neurological function in a FPR2-dependent manner [13]. Intriguingly, in this research, blocking the FPR2 pathway by its antagonist Boc2 not only muted LXA4-induced activation of Nrf2-HO-1 signaling, but also counteracted the beneficial effects of LXA4 on ameliorating cell oxidative stress injury and pro-thrombotic potential. However, Boc2 treatment did not fully neutralized the positive effects of LXA4. Previous study confirmed that LXA4 preconditioning attenuated intestinal I/R injury in a FPR2-independent manner [14]. In an experimental model of stroke, LXA4 also caused neuroprotective effects in a Peroxisome proliferator-activated receptors gamma (PPAR γ)-dependent manner [31]. Whether PPAR γ is involved in LXA4-mediated protection against injury and thrombotic ability of endothelial cells. BOC2 often acts as an FPR1 and FPR2 antagonist. Previous reports confirmed that LXA4 exerted pleiotropic efficacy by binding to its receptor LXA4R/FPR2 [13,17]. In this study, FPR subtype has not been identified. Recent research has confirmed the key roles of FPR1 in ventricular remodeling after ischemia/reperfusion injury [32]. However, does FPR1 participate in LXA4-mediated function of HUVECs? Do other pathways account for above progression? These hypotheses will be explored in our next study.

In conclusion, the present study corroborated that supplementation with LXA4 antagonized oxidative stress-induced HUVEC injury and thrombosis-related factor expression via receptor-mediated activation of the Nrf2-HO-1 pathway. Therefore, these findings highlight that LXA4 may attenuate the pathogenic progression of DVT by ameliorating vascular endothelial cell injury and subsequent pro-thrombotic response, implying a promising strategy for DVT and its complications.

Compliance with ethical standards

This research did not involve any Human Participants and/or Animals experiment.

Declaration of interest

There is no conflicts of interest concerning this article.

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