ARL15 overexpression attenuates high glucose-induced impairment of insulin signaling and oxidative stress in human umbilical vein endothelial cells

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ABSTRACT

Aims: Endothelial dysfunction (ED) plays a pivotal role in the development and progression of cardiovascular disease. Recently, genomic studies have found that ARL15, and some of its common genetic variants are associated with type 2 diabetes and coronary atherosclerosis. Since, the function of ARL15 is unclear we aimed at investigating the role of ARL15 in ED induced by high glucose (HG) in human umbilical vein endothelial cells (HUVECs).
Main methods: Quantitative real-time PCR was used to access the mRNA expression of ARL15. After exposure to different glucose media, nitric oxide (NO) production and the levels of superoxide dismutase (SOD), malondialdehyde (MDA), and reactive oxygen species (ROS) were studied. The underlying signaling pathway was also examined by western blot.
Key findings: Up-regulation of ARL15 attenuates HG-induced impairment in HUVECs. With insulin-stimulation, NO production and the active phosphorylation of the IR/IRS1/AKT/eNOS pathway were significantly increased. ARL15 overexpression was found to decrease the ROS and MDA production and increase SOD level. It could also reduce ERK1/2-Thr^{183}-Tyr^{185} phosphorylation, NOX2 and NOX4 expression in HG medium.
Significance: These results suggest that ARL15 could significantly alleviate the dysfunction of HUVECs induced by HG. Our findings help to identify new potential protective effects of ARL15 in HG-induced endothelial impairment.

1. Introduction

Type 2 diabetes mellitus (T2DM) has become a worldwide epidemic. T2DM patients, characterized by high blood glucose levels, usually have more than one complication [1,2]. Cardiovascular complications are the main reasons of morbidity and mortality [3]. Endothelial dysfunction (ED), resulting from high glucose levels, contributes to cardiovascular diseases, which are also induced by insulin resistance [4]. One of the metabolic actions of insulin is to stimulate production of NO as an important vasodilator. In addition, hyperglycemia increases ROS production and inflammation, which can also lead to ED. It is thus evident that high glucose levels result in phenotypic changes that cause ED [4].

Over the past decades, genome-wide association studies (GWAS) have identified many gene variants, which is useful to reveal the possible mechanisms underlying T2DM and its complicated development [5]. ADP-ribosylation factor-like 15 (ARL15), a small GTP-binding protein, the function of which is not very clear, has been associated with many metabolic disorders in different studies. GWAS had firstly found that genetic variations located in the ARL15 locus were implicated in HDL cholesterol and adiponectin concentration differences among patients with coronary heart disease and insulin resistance [6]. Additionally, other studies confirmed that ARL15 gene variations are associated with HDL cholesterol and triglyceride concentrations [7]. Due to the similarities in metabolic traits, SNPs in ARL15 are also associated with childhood obesity and T2DM, body shape [8–10]. A previous study has shown that ARL15 acts as an insulin-sensitizing

effector, which can activate PDPK1 and upregulate the IR/IRS1/ PDPK1/AKT insulin pathway in C2C12 myotubes [11]. Furthermore, ARL15 can also affect adipocyte differentiation and adiponectin secretion in 3T3-L1 cells, while the knockdown of ARL15 in β cells decreases insulin secretion [12,13]. Hence, we find that ARL15 plays an important role in insulin-dependent metabolism in different cell lines.

However, whether ARL15 affects the function of endothelial cells remains unknown. Thus, our aim was to investigate whether ARL15 plays a role in ED induced by high glucose, using conditional ARL15 overexpression in the HUVECs. The findings may accelerate our understanding of ARL15 in vascular homeostasis.

2. Materials and methods

2.1. Cell culture

HUVEC were purchased from American Type Culture Collection. The cells were cultured in Medium 199 (Invitrogen, USA) supplemented with 10% fetal bovine serum. Penicillin (100 U/ml), streptomycin (100 U/ml), and L-glutamine (2 mM) were added in the medium. Cells were maintained in an incubator with 5% CO_2 at 37 °C. HUVECs were cultured in a medium containing 5.5 mM or 35 mM glucose for at least 24 h. Cell treated with 500 nM insulin for 45 min and culture supernatants were used to measure the stable metabolite of NO. Cell lysate was used to analyze protein expression levels by western blot under different conditions.

2.2. Transfection of ARL15

The empty vector plasmid pcDNA 3.1 [pcDNA 3.1(−)] and pcDNA3.1-ARL15 [ARL15(+)] containing full-length human ARL15 were synthesized and purified by Vigene Bioscience (Shandong, China). Both the empty plasmid pcDNA3.1 and pcDNA-ARL15 fragment sequences were confirmed by DNA sequencing. The siRNA targeted ARL15 (si-ARL15) and siRNA negative control (si-NC) were synthesized and purified by Gene-Pharma (Shanghai, China). When cells reached 60% confluence, pcDNA3.1 (1 μg/ml), pcDNA-ARL15 (1 μg/ml), si-ARL15 (30 nM) or si-NC (30 nM) were transfected into the HUVECs by mixing with Lipofectamine™ 3000 reagent (Invitrogen, USA) for 24 h following the manufacturer's instructions. The following primers were used:

si-ARL15-1 (5′-3′):	CCAGAAUGCCAUCUUGAAUUTT
	AUUCAAGAUGGCAUUCUGGTT
si-ARL15-2 (5′-3′):	GCUUCUCUCAGCUGAUUAAUT
	UUAAUCAGCUGAGAGAAGCUU
si-NC (5′-3′):	UUCUCCGAACGUGUCACGUTT
	ACGUGACACGUUCGGAGAAUU

2.3. Assessment of mRNA levels by quantitative real-time PCR

Total RNA from HUVECs was isolated by RNAiso Plus (TaKaRa Bio, Tokyo, Japan) according to the manufacturer's protocol. Aliquots of 1000 ng RNA were reverse-transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Bio). To perform qRT-PCR analyses for the expressions of ARL15 vs. GAPDH, the SYBR Green RT-PCR kits (TaKaRa Bio, Tokyo, Japan) were used. The sequences of the primers used for amplification were:

ARL15 (5′-3′):	TGAAAGCCCCGACAACGTCG
	AGTGCATAACTGCGGATGCTA
GAPDH (5′-3′):	CTGGGC-TACACTGAGCACC
	AAGTGGTCGTTGAGGGCAATG

The transcripts were quantified using the ΔΔCt method.

2.4. Quantification of NO

The NO production in different culture medium was measured by the Griess method according to the instruction from total NO assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, after exposure to varied treatment conditions, nitrate reductase was used to reduce nitrate to nitrite. Then nitrite was measured by Griess reagent, and the absorbance was detected at 540 nm using a microplate spectrophotometer (BioTek, USA).

2.5. Detection of malondialdehyde (MDA)

As a compound yielded from lipid peroxidation, the levels of MDA were measured using Lipid Peroxidation Malondialdehyde Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). The detection process is based on a chromogenic reaction with thiobarbituric acid [14]. The optical density at 532 nm was measured by a microplate spectrophotometer (BioTek, USA).

2.6. Detection of reactive oxygen species (ROS)

ROS in HUVECs was measured by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) following the instructions from Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, after the specific treatments, cells were harvested and re-suspended in a serum-free medium containing DCFH-DA (10 μM). After incubating in the medium for 30 min at 37 °C and washing with the serum-free medium, the fluorescent intensity of HUVECs was measured using a flow cytometer at 488 nm excitation source and 525 nm emission source [15].

2.7. Measurement of intracellular superoxide dismutase (SOD) activities

The cellular SOD activity levels were determined by Superoxide Dismutase Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, cells were harvested and washed, lysed on ice, and then the supernatant was collected by centrifuging at $12000 \times g$ for 5 min at 4 °C. A modification of the xanthine/xanthine oxidase method was used to assay cellular SOD levels [16], which were then determined by microplate spectrophotometer (BioTek, USA) at 450 nm.

2.8. Western blot analyses

Cells were lysed using RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Jiangsu, China) and protease inhibitor cocktail, then protein concentration was determined using the BCA kit (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts (30 μg) of protein were separated by electrophoresis and then transferred onto a PVDF membrane (Millipore, USA). After blocking with 5% non-fat dry milk for 1 h at room temperature (25 °C) and incubated with primary antibodies and homologous secondary antibodies, the protein bands were visualized by ECL (Thermo Scientific™, USA). The targeted proteins were quantified by the Image J 1.38X software (USA).

The primary antibodies used were as follows: GAPDH (Sigma, dilution 1:3000); ERK1/2 (Wanleibio, dilution 1:500); p-ERK1/2 (Cell Signaling Technology, dilution 1:1000); ARL15 (Proteintech, dilution 1:1000); Akt (Cell Signaling Technology, dilution 1:1000); p-Akt (Cell Signaling Technology, dilution 1:1000); eNOS (Abcam, dilution 1:1000); p-eNOS (Abcam, dilution 1:500); NOX2 (Abcam, dilution 1:1000); NOX4 (Abcam, dilution 1:1000).

2.9. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) of results derived from more than three independent experiments. Statistical comparisons were performed using Student's *t*-tests or one-
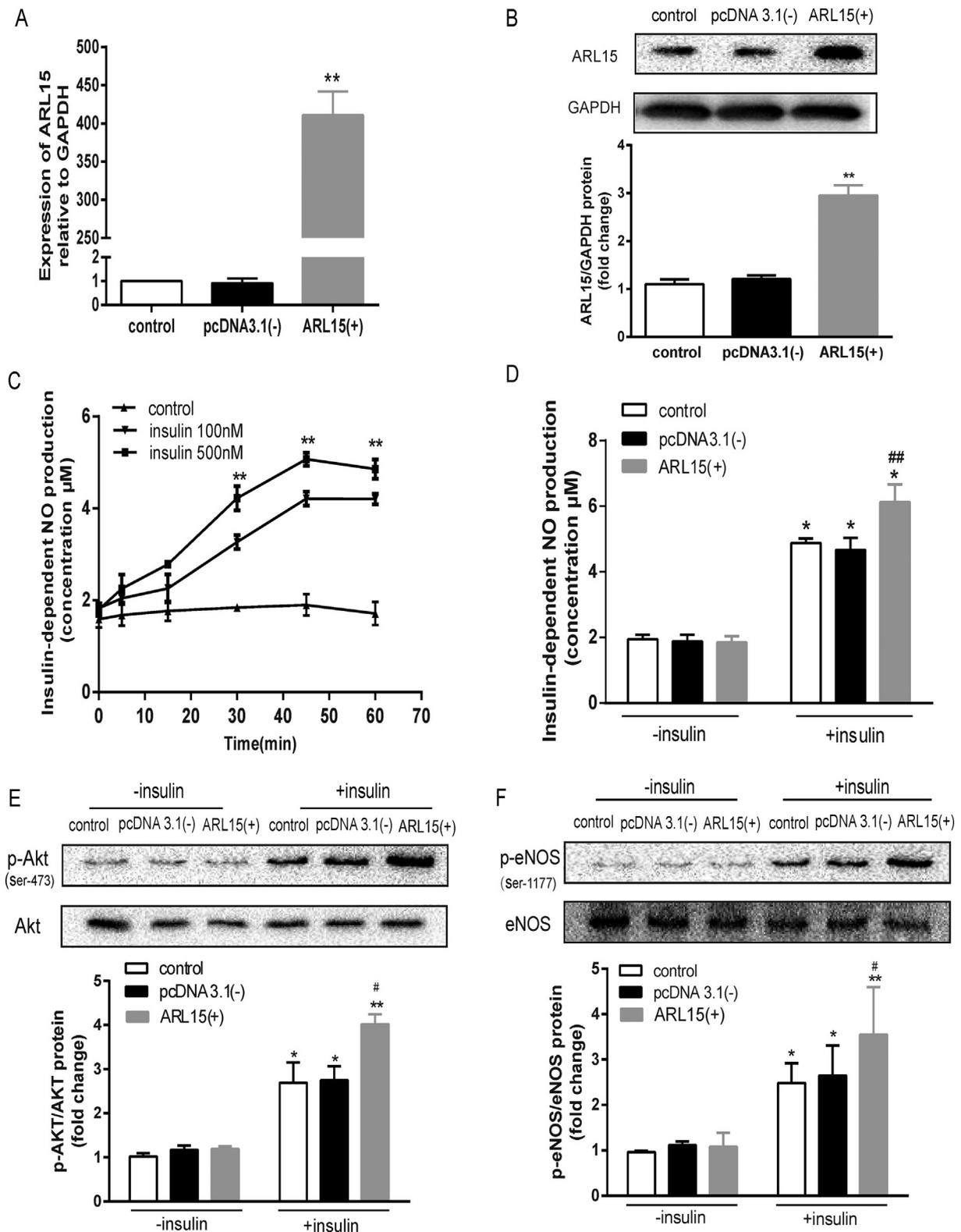
Fig. 1. ARL15 overexpression enhances NO production in HUVECs stimulated by insulin. Expression of ARL15 at the level of mRNA determined by qRT-PCR (A); and protein determined by western blot (B); in HUVECs transfected with ARL15 plasmid [ARL15 (+)] or empty vector [pcDNA 3.1(−)], the NO production under different insulin concentrations was detected by the Griess method (C); insulin-mediated (500 nM, 45 min) NO production in low glucose (LG, 5.5 mM) was measured when transfected with or without ARL15 (+) (D); the protein expression of phosphor-Akt (ser473) (E); and phosphor-eNOS (ser1177) (F); were measured by western blot under LG with ARL15(+) or pcDNA 3.1(−) transfected in LG. Results are mean ± SEM (n = 3) of densitometric values normalized to the corresponding Akt or eNOS. All data are mean ± SEM (n = 3). $*P < 0.05$, $**P < 0.01$ when compared to HUVECs transfected with pcDNA 3.1(−) and without insulin stimulation. $\#P < 0.05$, $\#\#P < 0.01$ when HUVECs transfected with pcDNA 3.1(−) and exposed to insulin (500 nM) stimulated for 45 min were used as control.

way ANOVA followed by least significant test (LSD) test. P < 0.05 was considered significant compared to the respective control.

3. Results

3.1. ARL15 overexpression increases insulin-stimulated NO production, Akt, and eNOS phosphorylation

When HUVECs were cultured in low-glucose medium (5.5 mM, LG) and transfected with ARL15 (+) for 24 h, the mRNA and protein levels of ARL15 showed obvious increase (Fig. 1A, B). When effect of various concentrations of insulation and incubation times on NO production by HUVECs was tested it was found that incubating with insulin at 500 nM for 45 min showed the largest effect compared with non-stimulated cells (Fig. 1C). With ARL15 overexpression, the insulin-stimulated NO production in HUVECs increased significantly compared with control (Fig. 1D). It is well known that PI3K/Akt-eNOS signaling pathway is important in insulin stimulated NO production in endothelial cells. Thus, we evaluated whether Akt-eNOS signaling was mediated by ARL15 in HUVECs. As shown in Fig. 1E and F, insulin induced an increase in both Akt and eNOS phosphorylation. Moreover, ARL15 overexpression induced the insulin-stimulated phosphorylation of Akt and eNOS significantly as compared to control (Fig. 1E, F).

3.2. High glucose reduces NO production stimulated by insulin

High glucose (HG) is the main risk factor for the development of ED. We examined the effects of HG on NO production in HUVECs. Incubation in HG medium (35 mM) for 24 h reduced insulin (500 in M)-dependent NO production compared with HUVECs exposed to normal glucose (Fig. 2A). Furthermore, there were significantly reduced phosphorylation of Akt and eNOS when cells incubated in HG medium were stimulated with 500 nm insulin for 45 min (Fig. 2B and C).

3.3. ARL15 overexpression alleviates the HG-induced impairment of the insulin-Akt-eNOS pathway

To investigate whether ARL15 is involved in the ED induced by HG, we cultured the HUVECs transfected with ARL15 (+) in HG medium. Compared with control, ARL15 overexpression showed a benefit in NO production stimulated by insulin (Fig. 3A, B). ARL15 overexpression (Fig. 3C) did enhance NO production with insulin stimulation under HG, but it did not restore the damage caused by HG. Furthermore, phosphorylation of Akt and eNOS was increased by ARL15 (Fig. 3D, E) under both normal and high glucose condition. To further confirm the beneficial effects of ARL15 overexpression, we performed rescue experiments in HUVEC-ARL15 (+) with si-ARL15 in HG condition. In cells transfected with si-ARL15, the NO production induced by insulin stimulation as well as the phosphorylation of Akt and eNOS were decreased (Supporting information Fig. S1).

3.4. Up-regulation of ROS, MDA expression and down-regulation of SOD activity in cells exposed in high-glucose medium

High glucose concentration augments oxidative stress which plays a vital role in the progress of ED. The inflammatory and oxidative stress levels (e.g., ROS, MDA and SOD) in HUVECs were determined. Compared with cells incubated in normal glucose medium, ROS production showed a significant elevation in high-glucose cultured cells (Fig. 4A). Similarly, as a marker of lipid peroxidation, the levels of MDA also increased in HUVECs treated with high glucose (Fig. 4B) although, the SOD activity is down-regulated in HG medium (Fig. 4C). The increased expression of NADPH oxidase (NOX) has been shown to promote oxidative stress in cells. In this study, we demonstrated that under HG conditions, NOX2 and NOX4 proteins showed a higher expression than in LG conditions (Fig. 4D, E). Since, ROS has been demonstrated to

activate a lot of transcription factors including ERK1/2, it is proposed that the increased ROS production under HG conditions may result in the increased phosphorylation of ERK1/2. Western blotting revealed that there was an increase in phosphorylation of ERK1/2 in cells treated with HG (Fig. 4F).

3.5. ARL15 overexpression ameliorates the oxidative stress induced by HG

In this study, it has been shown that ARL15 overexpression can enhance NO production. Interestingly, as shown in Fig. 5A and B, HG induced-ROS and MDA increase was reduced by ARL15 overexpression, though normal expression levels were not attained. The SOD level was increased in comparison to the control under HG (Fig. 5C). With ARL15 overexpression (Fig. 5D), the expression of NOX2 and NOX4 showed a decrease (Fig. 5E, F). Moreover, ARL15 overexpression resulted in significantly lower basal ERK1/2 phosphorylation than that occurring without overexpression (Fig. 5G). With ARL15 knockdown, the ROS and MDA levels increased and SOD level decreased (Supporting information Fig. S2). The expression of all related proteins significantly changed when ARL15 was overexpressed in HUVECs (Supporting information Fig. S2).

4. Discussion

T2DM is a chronic disease that has acquired epidemic proportions and is characterized by high levels of blood glucose, which results from the inadequate production of insulin and inability to respond to insulin. Hyperglycemia, if unchecked over the long term, can cause damage to various organs and lead to the development of complications, especially macrovascular and microvascular diseases [17,18]. ED plays a fundamental role in the pathogenesis of vascular complications, and interventions aimed at tackling ED can help in controlling diabetes [19].

Vascular endothelial cells (ECs) perform some key functions to maintain homeostasis, such as maintaining blood fluid content by regulating the exchange between macromolecule and fluid [20]. Thus, when the ECs are incapable of regulating the balance between vasorelaxation and vasoconstriction ED develops [21]. High glucose is the main risk factor for the development of ED. A lot of experimental studies have indicated that a decrease in NO production and increase in oxidative stress in ECs play essential roles in the pathological progression of ED [22,23]. Therefore, NO production and oxidative stress are potential targets for the development of novel therapeutic strategies for ED.

Previous studies showed that ARL15 acts as an insulin-sensitizer which can enhance the phosphorylation of IRS1/PI3K/AKT signaling pathway in C2C12 myotubes [11]. ARL15 can also enhance insulin secretion in β cells [13]. To date, no research is available on its function in ECs. Herein, we demonstrate that the overexpression of ARL15 in HUVECs can increase NO production stimulated by insulin under basal conditions. Furthermore, overexpressing ARL15 can alleviate the high glucose-induced impairment of insulin-stimulated NO production and oxidative stress. It seems that the protective effects of ARL15 on both sides are related.

Insulin-signaling pathways regulating physiological processes reveal parallels in skeletal muscle and adipose tissue [24]. To perform the biological functions in HUVECs, insulin firstly binds with its cell surface receptor IRS1, which is a ligand-activated tyrosine kinase. After tyrosine-phosphorylation of IRSs, it specifically binds to the p58 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) and activates the catalytic p110 subunit of PI3K. The activation of PI3K leads to phosphorylation and activation of PDK-1, which then phosphorylates Akt. Finally, activated Akt directly phosphorylates the endothelial NO synthase (eNOS) at Ser^{1177}, resulting in NO production [25]. Intriguingly, we found when ARL15 was overexpressed in HUVECs, the insulin-stimulated NO production increased. Hence, this observation encouraged us to investigate if ARL15 plays a role in HUVECs impaired by
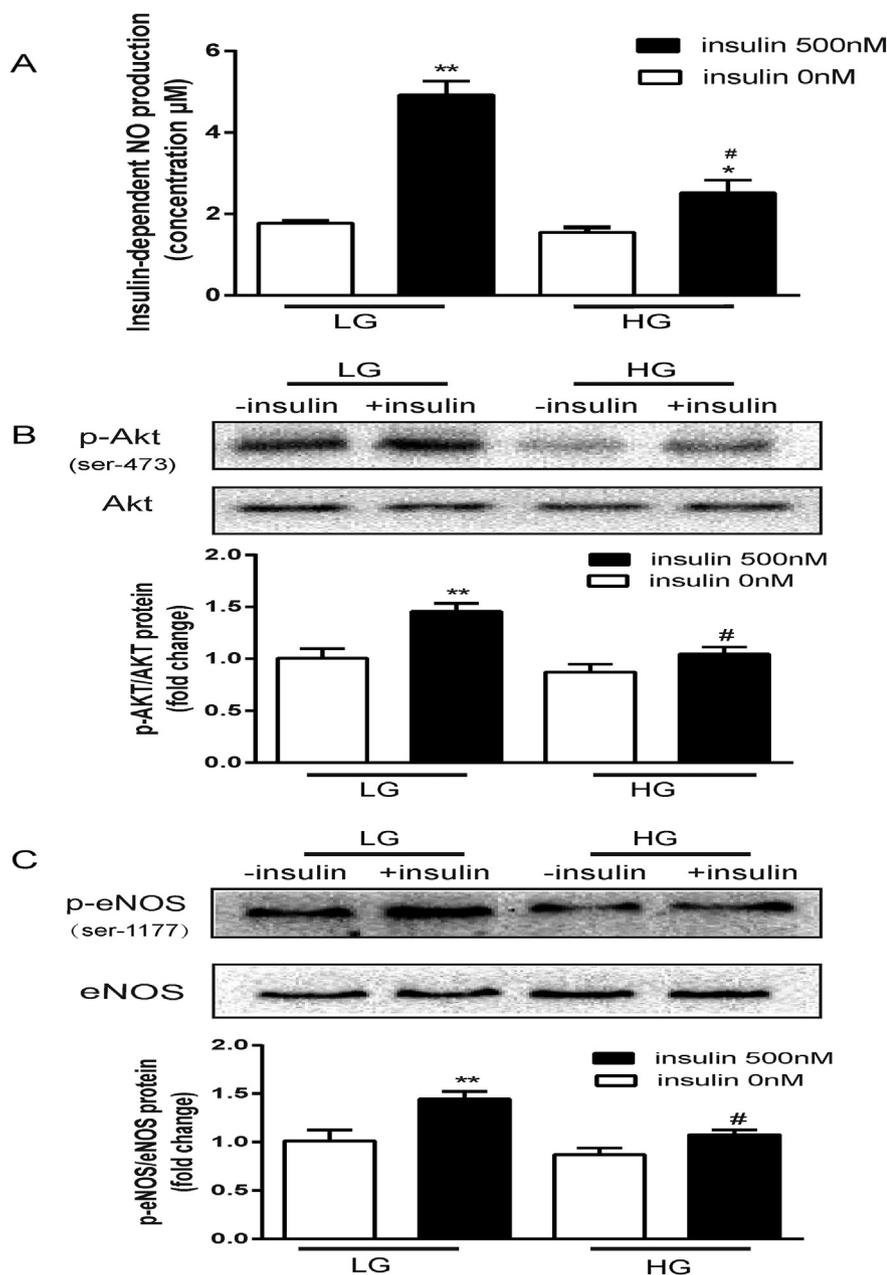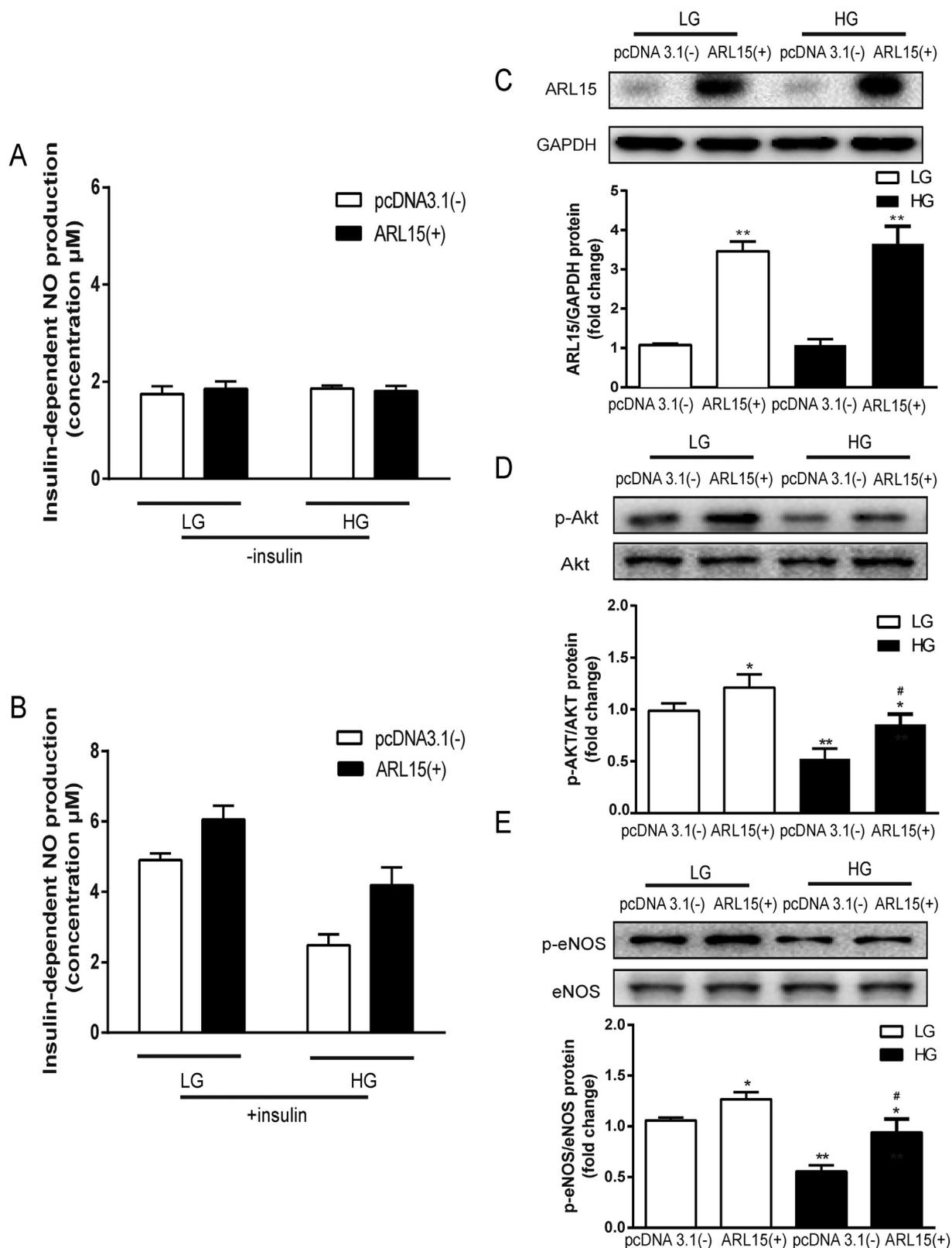
Fig. 2. Impairment of insulin signaling in HUVECs when exposed to high glucose (HG) medium. NO released from cells incubated in LG or HG for 24 h was measured by the Griess method. Cells were stimulated with insulin (500 nM) for 45 min (A) and NO was measured. Results are mean ± SEM (n = 3–5) and *P < 0.05, **P < 0.01 as compared to HUVECs exposed to LG without insulin stimulation. #P < 0.05, ##P < 0.01 as compared to cells exposed to LG with insulin (500 nM) stimulated for 45 min. The protein expression of phosphor-Akt (B) and phosphor-eNOS (C) by western blot under LG or HG medium with insulin stimulation (500 nM, 45 min). Results are mean ± SEM (n = 4) of densitometric values normalized to the corresponding Akt or eNOS.

HG. It had already been clarified that when exposed to HG, the insulin-signaling pathway and NO production in HUVECs are impaired [26]. These findings were confirmed in our experiments. In addition, we found that ARL15 exerts a positive effect on ameliorating HUVECs impairment under HG exposures.

Hitherto, many studies have demonstrated a reciprocal relationship between NO production and oxidative stress that deteriorates ED. ECs are especially vulnerable to the HG-induced toxic effects. When exposed to a HG environment, ECs fail to produce enough NO and generate more ROS [27]. Previous studies have identified that NO is a major target for ROS. When O_2^- interacts with NO, peroxynitrite is produced and the endothelium-dependent vascular relaxation is contained [28]. Peroxynitrite can oxidize tetrahydrobiopterin (BH4) and promote eNOS uncoupling. Uncoupled eNOS in the endothelium can also lead to oxidative stress since it begins to produce O_2^- and NO· simultaneously. In addition, the radicals tend to attack other cellular targets because of diminished production of NO [29]. All of these circumstances can increase oxidant production, oxidative damage and cause further deterioration of the ED.

ROS includes cellular molecular oxygen and its derivatives [30]. Both mitochondrial electron transport chain and enzymatic sources, such as NOX, nitric oxide synthase (NOS), and dual oxidases (DUOX), can produce ROS. It has been demonstrated that increased activity of NOX contributes to the pathogenesis of vascular disease [31,32]. In addition, NOX2 and NOX4 are expressed abundantly in HUVEC [33]. In our study, overexpressing ARL15 can attenuate NOX2 and NOX4 protein expression in HUVECs under HG medium. Increased ROS, by surpassing endogenous antioxidant defense mechanism, can oxidize many macromolecules, such as DNA and protein [34]. This condition has commonly been defined as oxidative stress. It is also known that MDA, an indicator to estimate ROS-induced cell injury, increases under HG treatment [35]. However, SOD can reduce ROS signaling and control oxidative damage in cells. In addition, it is well known that oxidative stress can induce the activation of ERK in many cell types [36,37]. We found that ARL15 was involved in HG-induced oxidative stress in HUVECs. We also demonstrated that overexpressing ARL15 could alleviate the oxidative stress in HUVECs induced by HG, which is evident from the changes in the ROS, MDA and SOD levels.

Fig. 3. ARL15 overexpression alleviates the impaired insulin signaling in HUVECs under HG condition. Insulin (500 nM, 45 min)-stimulated NO production in HUVECs incubated in LG or HG for 24 h co-incubated with or without the ARL15 (+) plasmid (A); effect of ARL15 (+) transfection on production of NO by HUVECs exposed to LG or HG in the absence of insulin stimulation (B); expression of ARL15 measured by western blot in HUVECs transfected with ARL15 (+) or pcDNA 3.1 (−), GAPDH was used as a loading control (C); the phosphorylation of Akt (D) and eNOS (E) was determined in HUVECs exposed to HG or LG medium for 24 h co-incubated with or without ARL15 (+) and then with insulin stimulation for 45 min. Data were presented as densitometric values protein band normalized to the corresponding Akt or eNOS. All data are mean ± SEM (n = 3–4). *P < 0.05, **P < 0.01 compared to pcDNA 3.1 (−) transfection incubated in LG condition. #P < 0.05, ##P < 0.01 compared to pcDNA 3.1 (−) transfection incubated in HG condition.
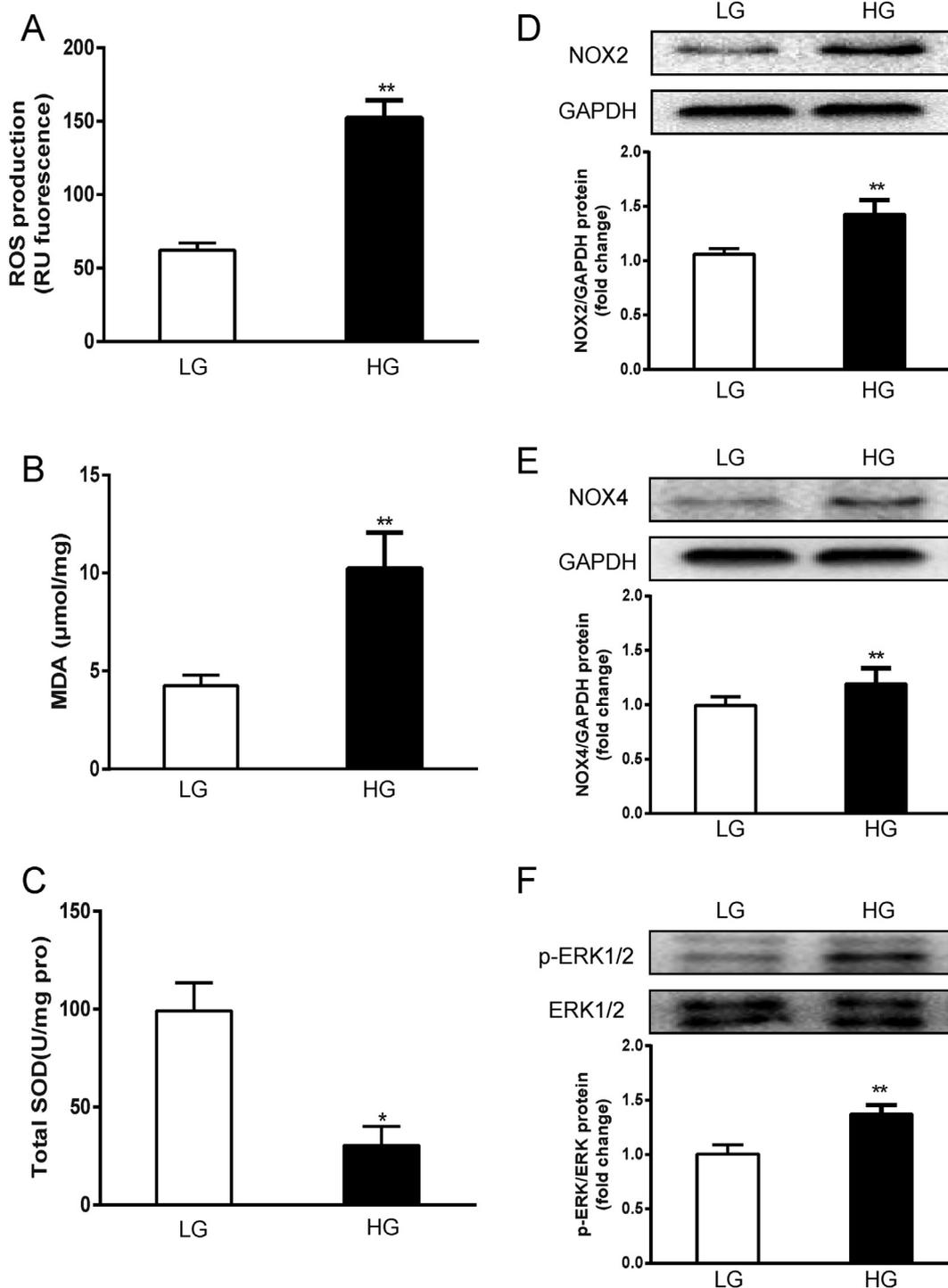
Fig. 4. HUVECs cultured in HG medium increases oxidative stress. HUVECs were incubated in a HG medium and ROS levels detected by DCFH-DA fluorescence probe method (A), MDA levels were detected by thiobarbituric acid reaction (B); and SOD activities were detected by WST-8 assay (C); protein expression levels of NOX2 (D); NOX4 (E); and p-ERK1/2 (F) were measured. All data are mean ± SEM (n = 3). *P < 0.05, **P < 0.01 compared to cells incubated in LG medium.

A more effective therapeutic intervention of vascular complications in diabetes requires complete understanding of hyperglycemia-induced ED. In this study, we demonstrated that ARL15 can affect both oxidative stress and NO production processes under HG condition and reduce the effects of ED. However, we performed all of our studies in cells now and the in vivo experiments are needed in the future. This research, by elucidating the role of ARL15 in ED, may aid in making ARL15 into a novel therapeutic target for ED.

5. Conclusion

In summary, ARL15 is considered as a protective effector of HG-induced impairment in HUVECs. Our research demonstrated that ARL15 overexpression can enhance NO production and alleviate oxidative stress via different signaling pathways, while knockdown of ARL15 can suppress the protective effects induced by ARL15 overexpression. Future studies need to explore the action of ARL15 on the endothelium of atherosclerotic rats.
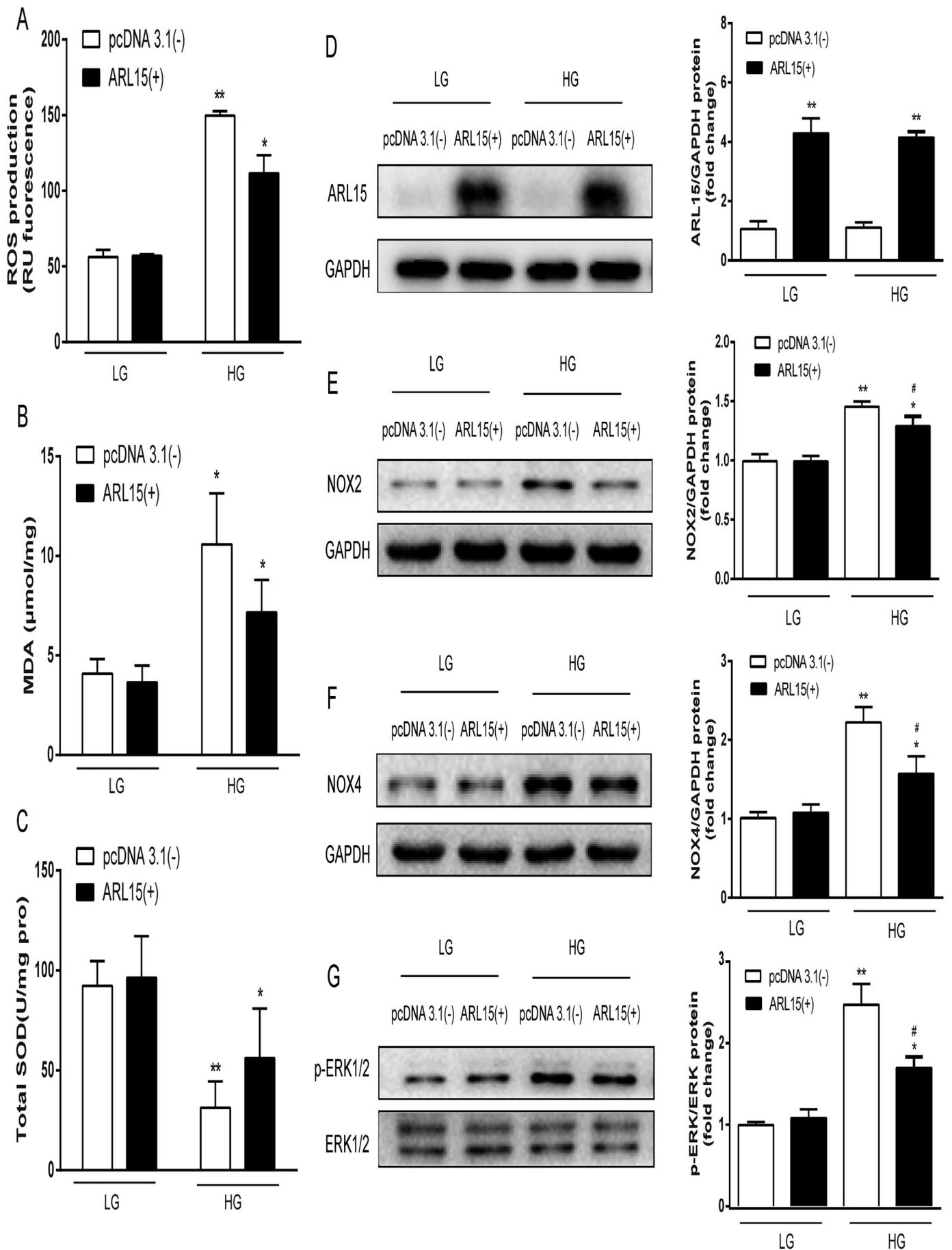
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Fig. 5. ARL15 overexpression ameliorates the oxidative stress in HUVECs incubated in HG medium. When cells transfected with pcDNA 3.1 (−) or ARL15 (+) were exposed to HG or LG, ROS levels (A); MDA levels (B); and SOD levels (C) were detected by different methods. The protein expression of ARL15 was determined by western blot with GAPDH as control (D); NOX2 (E) and NOX4 (F) expressions in HG and LG media and transfected with either ARL15 (+) or pcDNA 3.1 (−) were detected by western blot. The phosphorylation of ERK1/2 was also determined in cells exposed to HG or LG medium for 24 h co-incubated with or without ARL15 (+) (G). Data are presented as densitometric protein band and normalized to the corresponding GAPDH. Results are representative of 3 independent experiments (n = 3). *P < 0.05, **P < 0.01 compared to pcDNA 3.1 (−) transfection incubated in LG condition. #P < 0.05, ##P < 0.01 compared to pcDNA 3.1 (−) transfection incubated in HG condition.

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Conflict of interest

The authors declare that they have no competing interests and the manuscript is approved by all authors for publication.

Acknowledgements

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