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Regulatory Effects of AT_1R-TRAF6-MAPKs Signaling on Proliferation of Intermittent Hypoxia-induced Human Umbilical Vein Endothelial Cells*

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Summary: Endothelial dysfunction induced by intermittent hypoxia (IH) participates in obstructive sleep apnea syndrome (OSAS)-associated cardiovascular disorders. Myeloid differentiation primary response 88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6) regulate numerous downstream adaptors like mitogen-activated protein kinases (MAPKs) and the subsequent oxidative stress and inflammatory responses. This study aimed to characterize the role of MyD88/TRAF6 in IH-treated cell function and its associated signaling. Human umbilical vein endothelial cells (HUVECs) were randomly exposed to IH or normoxia for 0, 2, 4 and 6 h. Western blotting was used to detect the expression pattern of target gene proteins [angiotensin 1 receptor (AT_1R), p-ERK1/2, p-p38MAPK, MyD88 and TRAF6], and the relationships among these target genes down-regulated by the corresponding inhibitors were studied. Finally, the influence of these target genes on proliferation of HUVECs was also assessed by EdU analysis. Protein levels of AT_1R, TRAF6 and p-ERK1/2 were increased after IH exposure, with a slight rise in MyD88 and a dynamic change in p-p38MAPK. The down-regulation of TRAF6 by siRNA reduced ERK1/2 phosphorylation during IH without any effects on AT_1R. Blockade of AT_1R with valsartan decreased TRAF6 and p-ERK1/2 protein expression after IH exposure. ERK1/2 inhibition with PD98059 suppressed only AT_1R expression. IH promoted HUVECs proliferation, which was significantly suppressed by the inhibition of TRAF6, AT_1R and ERK1/2. The findings demonstrate that TRAF6 regulates the proliferation of HUVECs exposed to short-term IH by modulating cell signaling involving ERK1/2 downstream of AT_1R. Targeting the AT_1R-TRAF6-p-ERK1/2 signaling pathway might be helpful in restoring endothelial function.
Key words: intermittent hyopxia; angiotensin 1 receptor; myeloid differentiation primary response 88; tumor necrosis factor receptor-associated factor 6; mitogen-activated protein kinases; cells proliferation

Obstructive sleep apnea syndrome (OSAS) is a highly prevalent respiratory disorder characterized by the presence of repeated apneas and hypopneas during sleep, which results in episodes of intermittent hypoxia (IH). Epidemiological studies have identified OSAS as an independent risk factor for cardiovascular diseases such as hypertension, chronic heart failure, strokes and atherosclerosis[1]. Chronic intermittent hypoxia (CIH) can increase reactive oxygen species (ROS) production, leading to up-regulation of various transcription factors, e.g. nuclear factor kappa B (NF-κB), the most crucial factor, and thereby initiating inflammation. Numerous pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 8 (IL-8) activated by ROS and NF-κB can further initiate inflammatory transcription and inflammatory responses. Thus oxidative stress and inflammation contribute jointly to the development of endothelial dysfunction, which precedes the occurrence of cardiovascular disorders[2–4]. However the mechanisms underlying the association between OSAS and endothelial dysfunction are unclear.

Myeloid differentiation primary response 88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6) are identified as essential adaptors downstream of a wide variety of receptors, including the TNF receptor (TNFR), Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Activation of TRAF6 extensively participates in the regulation of intracellular signaling pathways involving NF-κB, mitogen-activated protein kinases (MAPKs) [extracellular regulated protein kinase (ERK1/2), p38 mitogen activated protein kinase (p38MAPK), c-Jun NH2-terminal kinase (JNK)] or interferon-regulatory factors (IRFs) to control diverse cellular processes, namely cell proliferation, survival, differentiation and cytokine production. Cumulative evidence shows that TRAF6 is implicated in the pathogenesis of various human disorders including immunity, stress response, atherosclerosis and inflammation[5]. Oxidative stress and inflammation are proved to be the basic components initiating endothelial dysfunction. Additionally, researchers have demonstrated a regulatory role of MyD88 and TRAF6 in hypoxia-reoxygenation injury, which resembles the nature of IH[4, 6]. We have

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previously demonstrated the involvement of ERK1/2 signaling in the angiotensin 1 receptor (AT_1R) over-expression under IH condition[7]. In the present work, we tried to elucidate the possible roles of MyD88/TRAF6 in the IH-stimulated AT_1R-MAPKs signaling pathway and endothelial cell function.

1 MATERIALS AND METHODS

1.1 Antibodies, Small Interfering RNAs (siRNAs) and Reagents

The AT_1R blocker valsartan was purchased from Sigma-Aldrich (USA). The ERK1/2 inhibitor PD98059 (BML-EI360) was from Enzo (USA). The primers of human TRAF6 were synthesized by Invitrogen (USA) with the sequence 5'-AGGGACCCAGCTTTCTTTGT-3' (F), and 5'-GCCAAGTGATTCCTCTGC AT-3' (R); and human GAPDH [5'-TGAAGGTCGGAGTCAACGGA-TTTGGT-3' (F), and 5'-CATGTGGGCCATGAGGTC-CACCAC-3' (R)]. The antibodies were purchased from the following suppliers: ERK1/2 antibody (C0185) and p38MAPK antibody (B0798) from AnboBio (China), p-ERK1/2 antibody (9101) from Cell Signaling (USA), p-p38MAPK antibody (1229) and TRAF6 antibody (1660) from Epitomics (USA), MyD88 antibody (AF1701a) from Abgent (USA), AT_1R antibody (AP10119b) from Abgent (USA), GAPDH antibody (sc-365062) and Tublin (sc-53646) antibody from Santa Cruz (USA). Three different small interfering RNAs (siRNAs) specific for TRAF6 were designed by RiboBio Co., Ltd. (China): siTRAF6-001 (siB0863115523) with the sequence 5'-GGAGAAACCUGUUGUGAUUdT-dT-3' and 3'-dTdTCCUCUUUGGACAACACUAA-5', siTRAF6-002 (siB0863115537) with the sequence 5'-G-GUGAAAUGUCCAAAUGAAdTdT-3' and 3'-dTdTC-CACUUUACAGGUUUACUU-5', and siTRAF6-003 (siB0863115554) with the sequence 5'-CAUUAAGGA-UGACACAUUAdTdT-3' and 3'-dTdTGUAAUUCC-UACUGUGUAAU-5'. Lipofectamine™ 2000 transfection reagent was purchased from Invitrogen (USA). Cell Light™ 5-ethynyl-2-deoxyuridine (EdU) DNA Cell Proliferation Kit was from RiboBio Co., Ltd. (China).

1.2 Exposure to IH and Drug Treatments

Human umbilical vein endothelial cells (HUVECs) of ECV304 line were grown in M199 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (GIBCO, USA) under a humidified atmosphere of 95% air and 5% CO_2 at 37°C. According to the method described by Ryan *et al* with some modifications[8], cells at passage 4 to 7 were used and synchronized for 24 h before exposing to normoxia (N) or IH for 0, 2, 4 or 6 h. For IH treatment, cells were exposed to alternating cycles consisting of 5% O_2 for 5 min followed by 21% O_2 for 10 min at 37°C in ProOx C21 cell incubator (Biospherix, USA). HUVECs incubated under normoxic conditions (21% O_2, 5% CO_2, 37°C) served as controls. For experiments involving pre-treatments with AT_1R and ERK1/2 inhibitors, cells were pre-incubated for 30 min with vehicle (DMSO 5 μL, 0.5% and DMSO 1 μL, 0.1%) or indicated concentrations of inhibitors (valsartan 10 μmol/L: containing 5 μL DMSO; PD98059 1 μmol/L: containing 1 μL DMSO).

1.3 siRNAs Transfection and Knockdown of TRAF6

For transient knockdown experiments, HUVECs growing in six-well plates were transfected with negative control (NC) siRNA or specific siTRAF6. Briefly, serum-free medium and Lipofectamine™ 2000 transfection reagent were used to achieve a final concentration of 50 nmol/L for NC-siRNA and TRAF6-siRNA. Cells were incubated with the mixture containing siRNA in serum- and antibiotic-free medium for 48 h at 37°C. After washing twice with PBS, cells were incubated in M199 medium for another 24-h starvation treatment, then in M199 medium containing 10% fetal bovine serum and exposed to normoxic or IH treatments for 4 h as mentioned above.

1.4 Real-time Quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) and reversely transcribed into cDNA using PrimeScript RT Master Mix (Takara, Japan). Real-time quantitative PCR was performed in triplex tubes by One-step SYBR PrimeScript™ RT-PCR Kit (Takara, Japan) according to the manufacturer's instructions. Reverse transcription was performed in a condition of 15 min at 37°C and 10 s at 85°C, while PCR reaction was carried out for 40 cycles of 5 s at 95°C and 34 s at 60°C. GAPDH was used as an endogenous control to normalize the amounts of target genes.

1.5 Western Blotting

Equal amounts of cell lysates (25–50 μg) were separated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinyl difluoride (PVDF) membranes (Millipore, USA). Membranes were blocked with 5% nonfat dry milk at room temperature for 2 h and probed with primary antibodies specific for AT_1R (1:250), MyD88 (1:1000), TRAF6 (1:1000), ERK1/2 (1:1000), p38MAPK (1:1000), p-ERK1/2 (1:1000), p-p38MAPK (1:1000), GAPDH (1:3000) or Tublin (1:3000) overnight at 4°C. Then the membranes were washed and incubated with alkaline phosphatase (AP)-conjugated anti-rabbit or anti-mouse IgG (1:3000) for 1 h at room temperature. Immunoreactivity was detected by BCIP/NBT AP Color Development Kit (Beyotime Institute of Biotechnology, China). Quantification of protein expression was analyzed using Image-J software.

1.6 Cell Proliferation Assays

Cells were divided into 8 groups: normoxia (N) group, IH group, IH+$DMSO_{5\ \mu L}$ group, IH+valsartan (10 μmol/L: containing 5 μL DMSO) group, IH+NC (negative control) group, IH+siTRAF6 group, IH+$DMSO_{1\ \mu L}$ group, and IH+PD98059 (1 μmol/L: containing 1 μL DMSO) group. Cell proliferation was determined by EdU labeling according to the manufacturer's instructions. Cells were seeded at an initial density of 1×10^3 cells/well in 96-well plates and allowed to grow for 24 h before the transfections with siTRAF6 or NC-TRAF6. After 2-h exposure to normoxia or IH, the cells were incubated with 50 μmol/L EdU for another 2 h. After fixation in 4% paraformaldehyde at room temperature for 30 min, cells were permeabilized in 0.2% Triton X-100 and treated with Apollo™ 567, followed by the counterstaining with Hoeschst 33342. Five random fields were captured at 100× magnification, and the percentage of EdU-positive cells (identified by Apollo ™ 567, red) in total cells (identified by Hoeschst 33342, blue) was calculated. Three duplicate wells were set up for each group.

1.7 Statistical Analysis

All statistical analyses were performed by Graph Pad Prism 4.0 (GraphPad Software, USA). Data were expressed as $\bar{x}\pm s_{\bar{x}}$. Statistical differences were calcu-

lated by Student's *t*-test or one-way ANOVA. A *P* value less than 0.05 was considered to be statistically significant.

2 RESULTS

2.1 Expression of AT_1R, TRAF6, MyD88 and MAPKs Proteins under IH Exposure

Protein levels of AT_1R, TRAF6 and p-ERK1/2 were increased in HUVECs of IH group at 2, 4 and 6 h as compared with sham and normoxia groups. The expression of AT_1R at 6 h in IH group was significantly reduced as compared with that at 2 and 4 h (fig. 1A, 1B, 1C and 1E). There was a slight but not significant rise in the expression of MyD88 after IH exposure compared with the sham group and normoxia group (fig. 1A and 1D). The p-p38MAPK protein expression was inhibited by 2-h IH treatment, but finally overreached that of the normoxia group at 6 h (fig. 1A and 1F).

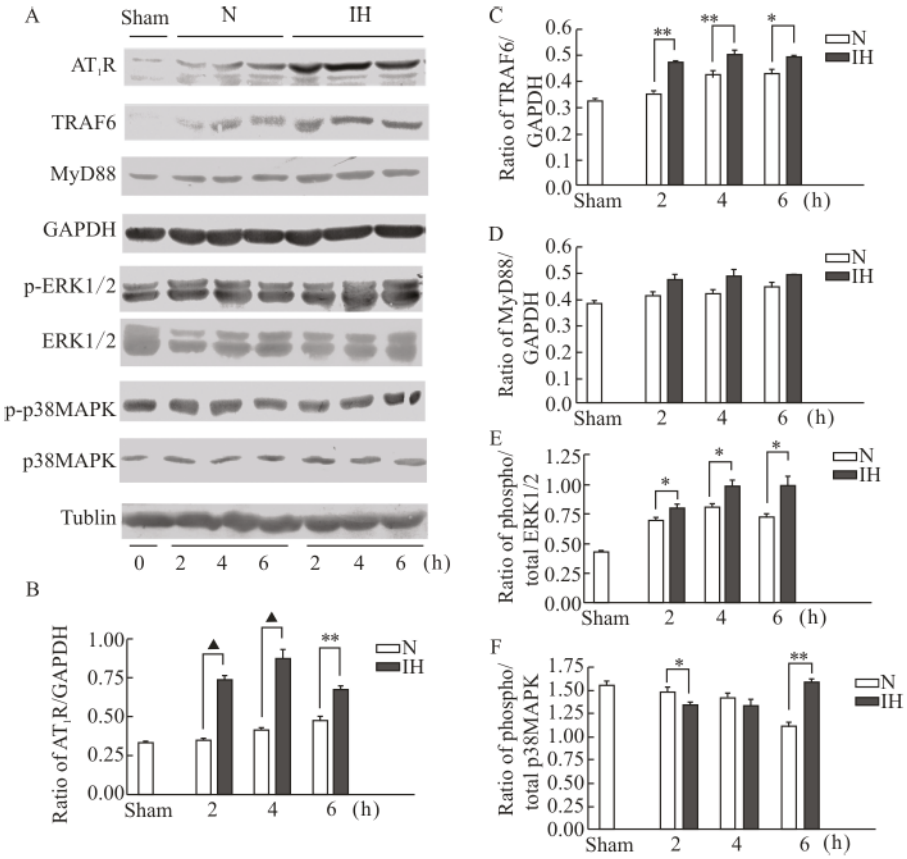

Fig. 1 Protein expression levels of AT_1R, TRAF6, MyD88 and MAPKs at baseline (sham) or under normoxia (N) and intermittent hypoxia (IH) conditions

A: representative image of Western blotting; B, C, E: Protein levels of AT_1R (B), TRAF6 (C) and p-ERK1/2 (E) were increased in HUVECs exposed to 2, 4 and 6 h of IH without a time-dependent manner as compared with sham or normoxia (N) group, which tended to decrease at the 6-h time point; D: There was a slight but not significant rise in the expression of MyD88 after IH exposure compared with the normoxia group; F: The phosphorylation of p38MAPK was inhibited by 2-h IH treatment, but overreached that of the normoxia group at 6 h. n=5 for each group. $^{*}P<0.05$, $^{**}P<0.01$, $^{▲}P<0.001$

2.2 Knockdown of TRAF6

To determine the possible role of TRAF6 in the regulation of HUVECs signaling under IH exposure, TRAF6 was knocked down using specific siRNAs. Following transfections with siRNAs and treatments with normoxia or IH for 4 h, real-time PCR and Western blotting were performed. Cells exhibited significantly reduced expression of TRAF6 at both mRNA and protein levels in normoxia and IH groups (fig. 2).

2.3 Effect of TRAF6 on IH-induced MAPKs Expression

HUVECs were treated with TRAF6-specific siRNAs under normoxia or IH conditions for 4 h and then collected for protein analysis. AT_1R protein expression remained unchanged after TRAF6 silencing under both normoxia and IH conditions (fig. 3A and 3B). The phosphorylation of ERK1/2 remained almost unchanged after TRAF6 inhibition under normoxia condition. Whereas transfection with TRAF6 greatly inhibited p-ERK1/2 expression induced by IH exposure (fig. 3A and 3C). However, there was no effect on p38-MAPK expression with TRAF6 inhibition either under normoxia or IH (fig. 3A and 3D). These data suggest that TRAF6 plays a role in regulating p-ERK1/2 protein expression in HUVECs, particularly under exposure to IH.

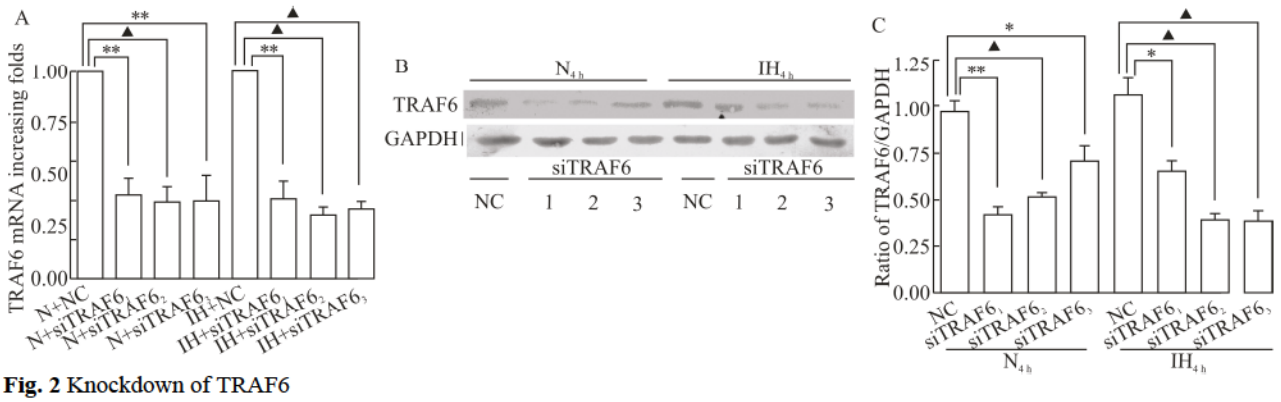

Fig. 2 Knockdown of TRAF6

A: TRAF6 mRNA expression levels were significantly down-regulated in HUVECs transfected with specific TRAF6 siRNAs in both normoxia (N) and IH groups as compared with negative control group; B and C: HUVECs exhibited significant inhibition of TRAF6 at protein levels by transfections with specific TRAF6 siRNAs in both normoxia and IH groups. n=5 for each group. $^{*}P$<0.05, $^{**}P$<0.01, $^{▲}P$<0.001

2.4 Effects of AT_1R and ERK1/2 Down-regulation on IH-induced TRAF6 Expressions

It was found that TRAF6 participated in the regulation of ERK1/2 phosphoryaltion in HUVECs exposed to 4-h IH. Phosphorylation of p38MAPK was significantly reduced by IH exposure and had no change with TRAF6 inhibition. Only the AT_1R inhibitor valsartan (10 μmol/L) and the ERK1/2 inhibitor PD98059 (1 μmol/L) were used to further delineate the possible interaction between TRAF6 and AT_1R-ERK1/2 signaling pathway. Pre-treatment of valsartan decreased TRAF6 and p-ERK1/2 protein expression in HUVECs under 4-h exposure of IH. Blockade of ERK1/2 with PD98059 suppressed AT_1R protein expression, but exerted no effect on TRAF6 protein levels (fig. 3).

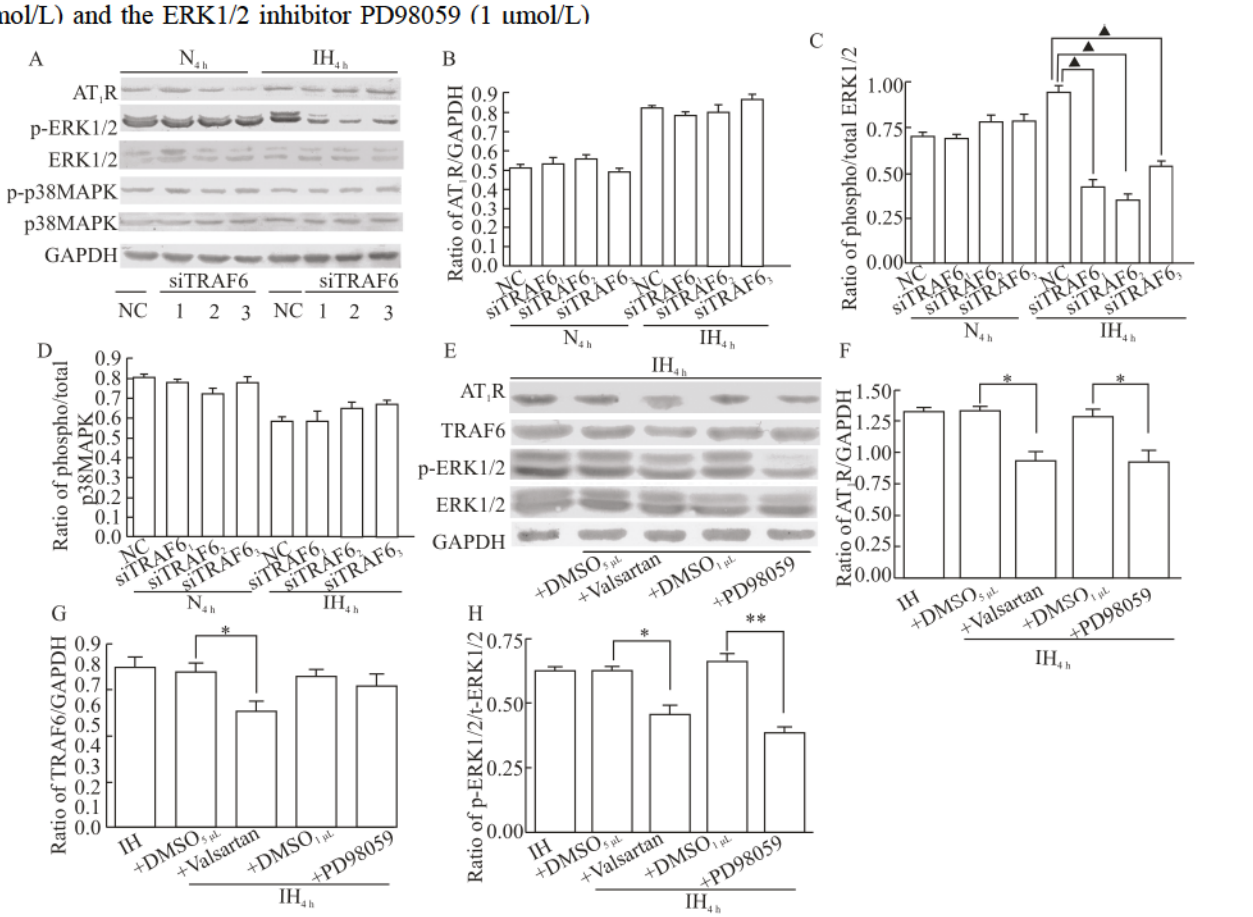

Fig. 3 Regulatory effects of TRAF6 on IH-induced MAPKs expression

A: representative image of Western blotting; B and D: The protein expression of AT_1R (B) and p38MAPK (D) remained unchanged after TRAF6 silencing under both normoxia (N) and IH conditions; C: Transfection with TRAF6 greatly inhibited p-ERK1/2 expression under 4-h IH exposure; E: representative image of Western blotting; F, G and H: Blockade of ERK1/2 with PD98059 (1 μmol/L) suppressed AT_1R protein expression (F), but exerted no effect on TRAF6 protein levels (G). Pre-treatment of 10 μmol/L valsartan decreased TRAF6 (G) and p-ERK1/2 (H) protein expression in HUVECs under 4-h IH. n=5 for each group. NC: the negative control siRNAs. $^{*}P$<0.05, $^{**}P$<0.01, $^{▲}P$<0.001

2.5 Inhibitory Effects of Blockade of TRAF6, AT_1R and ERK1/2 on Cell Proliferation

To determine whether IH can promote the proliferation of HUVECs and the possible role of TRAF6, AT_1R and ERK1/2, cells were assessed by EdU analysis (fig. 4). It was found that proliferation of S-phase cells was promoted by 4-h exposure of IH as compared with normoxia exposure (25.37%±1.63% *vs.* 17.35%±1.35%, $P<0.01$, $n=3$). After transfection of HUVECs with $siTRAF6_2$, down-regulation of AT_1R with valsartan and ERK1/2 with PD98059, the cell proliferation rate in S-phase was significantly reduced in IH groups (IH+$siTRAF6_2$ 18.63%±0.77% *vs.* IH+NC-siRNA 25.09%±1.99%, $P<0.05$; IH+valsartan 22.18%±0.92% *vs.* IH+$DMSO_{5\ \mu L}$ 26.67%±0.99%, $P<0.01$; IH+PD98059 21.43%±0.87% *vs.* IH+$DMSO_{1\ \mu L}$ 26.07%±1.10%, $P<0.01$, $n=3$).

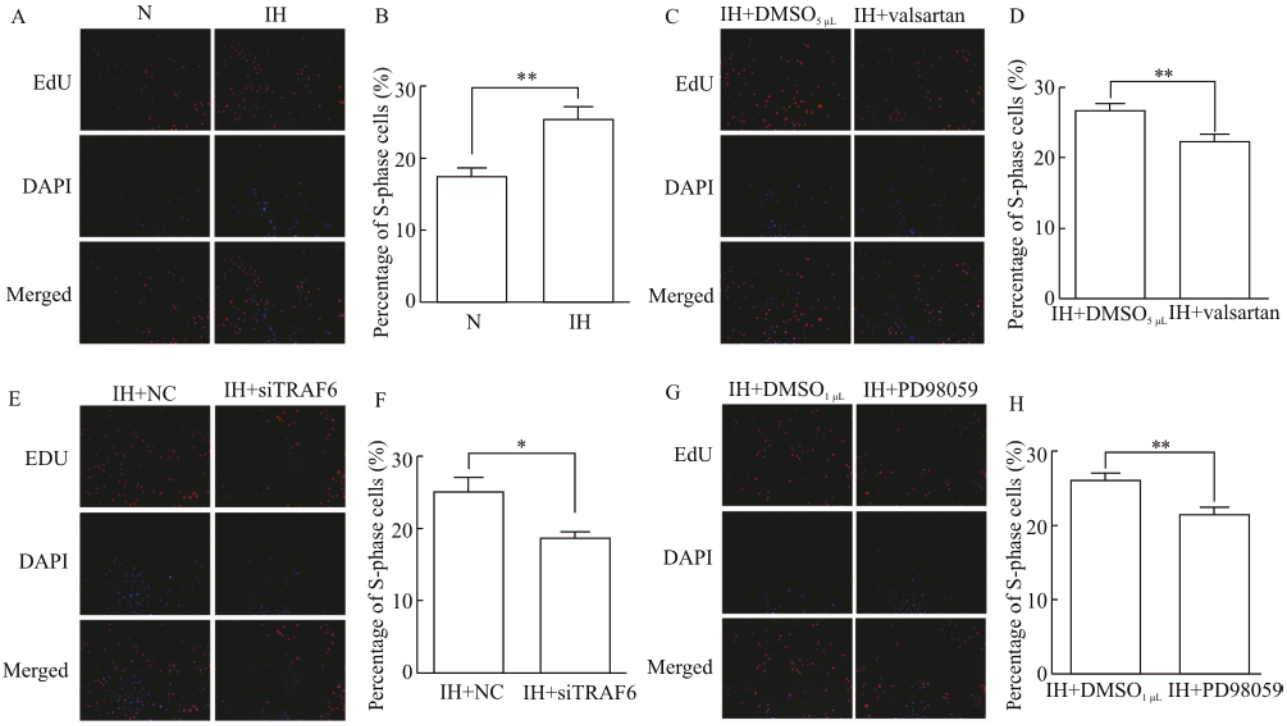

Fig. 4 Inhibitory effects of blockade of TRAF6, AT_1R and ERK1/2 on proliferation of HUVECs

EdU assays indicated that 4-h exposure of IH promoted cell proliferation as compared with normoxia exposure (A and B). After inhibition of AT_1R with valsartan (C and D), transfections of HUVECs with $siTRAF6_2$ (E and F), and inhibition of ERK1/2 with PD98059 (G and H), the cell proliferation rate was significantly suppressed in IH groups. $n=3$ for each group. NC: the negative control siRNAs. $^*P<0.05$, $^{**}P<0.01$

3 DISCUSSION

This study found that short-term IH exposure led to enhanced protein expression of AT_1R, TRAF6 and p-ERK1/2 in HUVECs, with a decrease in p-p38MAPK during the early stages of IH (2 h and 4 h), accompanied by increased cell proliferation. Inhibition of AT_1R suppressed IH-induced TRAF6 protein expression and ERK1/2 activation. Moreover, TRAF6 deficiency would down-regulate IH-triggered ERK1/2 phosphorylation without any effects on AT_1R expression. Activation of AT_1R, TRAF6 and ERK1/2 was required for HUVECs proliferation under IH conditions.

TRAF6 acts as a critical signaling transducer downstream of various cell surface receptors namely TNFR and IL-1R/TLR superfamilies to trigger the activation of NF-κB and MAPKs pathways including ERK1/2, p38MAPK and JNK[5]. It is important for survival and activation of all cells in the body, from those immune cells to epithelial cells[6]. In the setting of hypoxia-reoxygenation, TRAF6 interacts with its binding protein, namely TRAF-interacting protein with forkhead-associated domain (TIFA) constitutively to activate NF-κB and thus participates in the inflammatory stress, which is TLR4/MyD88-dependent[9]. Whereas, endogenous Cezanne may prevent Lys63 polyubiquitination TRAF6 in response to hypoxia-reoxygenation, resulting in decreased NF-κB activity and inflammation injury[10]. The cyclic nature of the intermittent hypoxia episodes during sleep in OSAS patients resembles hypoxia-reoxygenation injury and thus contributes to the ROS overproduction and oxidative stress as well as inflammatory reactions[4]. As expected, we found that the expression of TRAF6 was augmented in HUVECs by IH treatments. However, there was only a slight rise in MyD88 expression after IH exposure. In ischemic mouse kidney, both MyD88 and TRAF6 protein levels are enhanced[11]. The discrepancy may be attributed to a short-term exposure in the present observation. Or there may be an alternate upstream kinase of TRAF6 during the IH treatment.

Members of MAPKs families, namely ERK, p38MAPK and JNK, play a significant role in complex cellular processes like proliferation by regulation of cell cycle engines and cooperation with other related signal pathways[12]. Once TNFR or IL-1R/TLR receptors on the cell surface are activated, the adapter protein MyD88 is recruited to interact with interleukin-1 recep-

tor-associated kinase (IRAK), followed by the dissociation of IRAK from MyD88. Then IRAK becomes associated with TRAF and the TRAF6-IRAK complex triggers the activation of TGFβ-associated kinase 1 (TAK1), which is the activator of the MAPKs cascades[13–15]. To determine the potential functional interaction between TRAF6 and MAPKs in response to IH, HUVECs were treated with TRAF6-specific siRNA. It showed that TRAF6 inhibition decreased p-ERK1/2, but not p-p38MAPK expression induced by IH exposure. Blockade of ERK1/2 with PD98059 greatly suppressed HUVECs proliferation without any effect on TRAF6 protein levels. These data suggest that ERK1/2 may act as the downstream regulators of TRAF6 cascade in the control of cell proliferation under IH conditions.

Data from previous studies highlight the role of renin-angiotensin system (RAS) activation in the pathogenesis of cardiovascular effects associated with OSAS, perhaps through modulating sympathetic nerve activity or its capacities to trigger multiple processes like oxidative stress, which requires the activation of AT_1R[16–18]. In our study, we found AT_1R, TRAF6 and p-ERK1/2 proteins were increased by time. We further found that pre-treatment of valsartan decreased both TRAF6 and p-ERK1/2 protein expression in HUVECs under 4-h exposure of IH. And ERK inhibition exerted no effect on TRAF6 expression, whereas TRAF6 silencing downregulated ERK1/2 activity. Together, the results indicate that TRAF6 lies downstream of AT_1R to activate ERK1/2, which has been proven by previous work[19]. Interestingly, we found that blockade of ERK1/2 rather than TRAF6 could suppress AT_1R protein levels in turn. Therefore, the findings suggest a feedback relation between AT_1R and ERK1/2 under IH conditions, which might be mediated by other signaling pathways independent of TRAF6.

Previous studies have confirmed an active role of TRAF6 in modulating cell proliferation under various pathological conditions ranging from tumorigenesis, stress and inflammation responses[20, 21]. Knockdown of TRAF6 could inhibit glioma cells viability, suppress cell proliferation, invasion and migration and increase cell apoptosis, potentially via abrogation of NF-κB activity[20]. On oxidative stress stimulation, TRAF6 is activated by interaction with Atg9, a transmembrane protein involved mainly in autophagy control, leading to JNK activation and the subsequent intestinal stem cell proliferation or apoptotic cell death in mammals[21]. Differentiated osteoclasts treated with lipopolysaccharide (LPS) promoted protein expression of TRAF6 and cell proliferation via MAPKs pathways involving augmented phosphorylation of JNK and ERK1/2[22]. We consistently discovered that short duration of IH improved HUVECs proliferation, which was mediated by the activation of TRAF6 and ERK1/2. However, the mechanisms underlying IH-stimulated cell proliferation are not clear. Activated Notch1 family[23], regenerating gene (Reg) family, hepatocyte growth factor (HGF)[24], epidermal growth factor (EGF) family and erbB family receptor[25] mediated signaling pathways have also been implicated in regulating the cell proliferation processes. However, we have previously found that 8-h IH induced aortic endothelial apoptosis owing to endoplasmic reticular stress[26]. Other researches also discover that IH exposure accelerates cell apoptosis due to enhanced oxidative stress and inflammatory responses via NF-κB and Nrf2/HO-1 pathways or caspase-3 dependent/independent pathways[27]. We suppose that, depending on the duration and magnitude of exposure, IH might result in the promotion of either cell proliferation or apoptosis. The compensatory mechanisms of cell proliferation mediated by AT_1R-TRAF6-ERK1/2 pathway could help maintain endothelial cell function to some extent, and long-term exposure will unavoidably lead to the development of endothelial dysfunction[2, 4].

Our study has several limitations. Firstly, like all *in vitro* models, pathological system of OSAS can't be mimicked completely. Apart from IH, sleep fragmentation contributes jointly to its multiple pathological processes. Secondly, the IH models in this study can't be on behalf of chronic intermittent hypoxia and IH profiles various among different researches. The time points were set at 2, 4, and 6 h. Much longer exposure should be conducted to examine the dynamic changes in p38MAPK activity. Thirdly, MyD88 protein level remained nearly unchanged by IH. Further investigations should elucidate its potential role in controlling AT_1R-TRAF6-ERK1/2 signaling. Finally, HUVECs have been chosen instead of primary arterial endothelial cells due to its rapid growth in culture for carrying out the different assays at the same time. Studies on primary arterial endothelial cells will be necessary to verify the current results in the future.

Our findings together demonstrate a key role of TRAF6 in regulating HUVECs proliferation on exposure to short-term IH by modulating cell signalings involving ERK1/2 downstream of AT_1R. Modulating the AT_1R-TRAF6-ERK1/2 signaling pathway might be helpful in restoring endothelial function and treating the subsequent cardiovascular disorders.

Conflict of Interest Statement

The authors declared no conflict of interest.

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