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p120 inhibits LPS/TNFα-induced endothelial Ang2 synthesis and release in an NF-κB independent fashion

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ABSTRACT

Adherens junction protein p120 is thought to be crucial for maintaining vascular integrity, which is important in many pathologies and diseases including atherosclerosis, vascular malformations, hemorrhagic stroke, sepsis and others. However, the mechanisms responsible for this is not completely understood. In this study, using an unbiased proteomics approach, followed by other experimental techniques, we identified that in HUVECs p120 overexpression inhibits LPS/TNFα-induced angiopoietin-2 (Ang2) expression, a key switch of endothelial destabilization. Interestingly, p120 overexpression did not inhibit LPS/TNFα-induced expression of adhesion molecules/cytokines including VCAM-1, ICAM-1, E-selectin, MCP-1, IL-8 and IL-6 in our experimental system. Furthermore, this p120-mediated repression of Ang2 is in an NF-κB independent manner, possibly via transcription factor Ets1. Our results demonstrate that p120 influences vascular integrity by secreted signals, providing new insights into the mechanisms of p120-mediated vascular stability.

1. Introduction

Endothelial cell-to-cell adherens junction plays an essential role in maintaining vascular integrity by enhancing endothelial barrier function and promoting endothelial cell quiescence. Disruption of adherens junction is implicated in many pathologies and diseases including atherosclerosis, vascular malformations, hemorrhagic stroke, sepsis and others [1]. Adherens junction is mainly mediated by vascular endothelial (VE)-cadherin, linking inside the cells to a large number of intracellular partners including β-catenin, plakoglobin and p120 [2]. Among the intracellular binding partners, p120 is thought to be crucial for vascular integrity by inhibiting VE-cadherin endocytosis [3–5]. Mice lacking endothelial p120 died embryonically and vasculature of mutant animals were disorganized, accompanied by reduced pericyte recruitment and hemorrhaging, revealing a fundamental role for p120 in regulating vascular development and vessel integrity [6]. Despite its critical role in vascular stability, the mechanisms responsible for this is not completely understood. For example, besides its role in stabilizing VE-cadherin level on cell surface, p120 can modulate the activities Rho GTPases [7], although some study suggest that p120 regulates endothelial permeability independently of its NH2 terminus and Rho GTPase binding [8]. There is also evidence that p120 has a transcription repression function through regulation of transcription factor [9]. Furthermore, most recent study shows that p120, its partner PLEKHA7 and the core microprocessor components DROSHA and DGCR8 form a microprocessor complex and possess primary microRNAs processing activity, indicating its complex role in regulating cellular behavior [10]. Finally, p120 influences the migratory behavior of neighboring cells by secreted signals [11].

Angiogenesis is a complex multistep process controlled by a wide range of positive and negative regulatory factors. Disassembly of an intact endothelial cell junction is necessary for the angiogenesis process [12]. The angiogenic factors such as vascular endothelial growth factor (VEGF) and the angiopoietins play pivotal roles in both angiogenesis and microvascular permeability. When proangiogenic signals are upregulated by hypoxic, inflammatory or tumour cell, normal quiescent endothelial cells quickly switch to an angiogenic phenotype, becoming motile and invasive and protruding filopodia, and therefore loosen their junctions [12]. However, whether and if so, which angiogenic factors are involved in p120′s vascular stabilizing effects is not clear.

In this study, using an unbiased proteomics approach, followed by other experimental techniques, we identified that p120 inhibits LPS/TNFα-induced Ang2 expression, a switch of endothelial destablization, in an NF-κB independent manner, possibly via transcription factor Ets1.

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2. Material and methods

2.1. Cell cultures and reagents

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and grown in endothelial cell growth medium (EGM-2, Lonza) containing 5% fetal bovine serum (FBS). Cells were cultured in a humidified incubator at 37 °C and 5% CO_2. The following reagents and antibodies were used: Lipopolysaccharide (LPS) (E. coli O111:B4, Sigma), TNFα (Peprotech), anti-p120 antibody (BD Bioscience), anti-VE-cadherin antibody (Santa Cruz), anti-VCAM-1 antibody (Santa Cruz), anti-IκB antibody (Milipore), anti-NF-κB antibody (p65, Santa Cruz), anti-Ets1 antibody (Proteintech), Alexa Fluor 568-conjugated secondary antibody (Thermo Scientific), Human Angiogenesis Array Kit (R&D), Human Ang2 Elisa Kit (Abcam) Following preparation, cells were treated with LPS (1 μg/ml) or TNFα (20 ng/ml) for the indicated time periods.

2.2. Adenoviral constructs and adenoviral infections

pEGFP-p120 vectors were from Dr. Andrew P. Kowalczyk (Emory University, Atlanta). Adenoviruses carrying the mouse p120 constructs was produced using the pHBAd-MCMV-GFP overexpression vector from Hanbio (Shanghai, China). HUVECs at 80–90% confluence in 6 or 24-well plates were incubated for 6 h with the 50 multiplicities of infection of the indicated adenovirus. Adenoviruses were then removed and cells were incubated with fresh medium for an additional 42 h. For most experiments, infection rates of 90% were used as monitored by GFP expression.

2.3. Transfection of siRNA

Ets1 siRNA and negative control (scrambled siRNA) were purchased from Ambion (Austin, TX, USA). Cells were transfected with indicated siRNAs (100 nM) using HiPerFect reagent (Qiagen) for 48 h, cells were then treated with LPS/TNFα for further experiments.

2.4. Measurement of angiogenic factors in culture supernatant by antibody array

After 48 h of adenovirus transfection, the cells were changed to serum-free endothelial basal medium (EBM). After LPS treatment for another 24 h, the culture medium was collected. For angiogenic factors measurement, supernatants were analyzed by Human Angiogenesis Array kit in accordance to manufacturer's instructions.

2.5. Western blotting

After indicated time of treatment, cells were washed and lysed by RIPA buffer (Pierce) in the presence of mixture protease inhibitor. Equal amounts of proteins were loaded on gels, separated by SDS-PAGE, and transferred to a PVDF membrane, incubated with blocking solution (0.2% Tween 20 in phosphate- buffered saline), and reacted with primary and HRP-linked secondary antibodies, specific bindings was detected by a chemiluminescence system.

2.6. Measurement of Ang2 in culture supernatant by ELISA

After reaching confluence, cells were then treated with the indicated concentrations of pharmacological agents or infected with adenoviruses. After the indicated time of treatment with LPS or TNFα, supernatants were collected. Ang2 levels were then assessed using an Ang2 sandwich ELISA kit in accordance with manufacturer's instructions.

2.7. Immunofluorescence staining

Cells were seeded onto 8-well chamber slides. After the indicated treatment, the cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X100 and incubated with 1% BSA. After that, the cells were incubated with rabbit primary antibody against VE-cadherin or NF-κB for 2 h at room temperature. Secondary corresponding IgG-Alexa Fluor 568 antibody was incubated for 1 h at room temperature. Localization of VE-cadherin or NF-κB was recorded under an inverted fluorescence microscope (Olympus).

2.8. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from the cells using the Trizol reagent in accordance with the manufacturer's instructions. One microgram of each total RNA was reverse transcribed to complementary DNA, using the Reverse Transcription System (Promega). Quantitative real-time polymerase chain reaction analysis was conducted using SYBR Green PCR Master Mix and gene-specific primers on an ABI Prism Sequence Detection System ABI 7900HT (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalization control. The following human primer pairs (with the exception of mouse primer for p120) were used:

p120 : forward, 5′-CCAGGTTCGAGTAGGTGGAA-3′; reverse, 5′-CAGACATCATGCCGTAATCG-3′,

Ang2: forward, 5′-GTGGTTTGATGCATGTGGTC-3′; reverse, 5′-CCT TGAGCGAATAGCCTGAG-3′,

VCAM-1: forward, 5′-ACCACATCTACGCTGACAATGAATCC-3′; reverse, 5′-AACACTTGACTGTGATCGGCTTCC-3′,

ICAM-1: forward, 5′-GTCACCTATGGCAACGACTCCTTC-3′; reverse, 5′-AGTGTCTCCTGGCTCTGGTTCC-3′,

E-selectin: forward, 5′-GAAGAGGTTCCTTCCTGCCAAGTG-3′; reverse, 5′-CAGAGCCATTGAGCGTCCATCC-3′,

IL-6 : forward, 5′-GCAAAGAGGCACTGGCAGAA-3′; reverse, 5′-TGC ACAGCTCTGGCTTGTTC-3′,

IL-8 : forward, 5′-TGCAGCTCTGTGTGAAGGTG-3′; reverse, 5′-TTC TGTGTTGGCGCAGTGT-3′,

Ets1 : forward, 5′-AGCTTCGACTCAGAGGACTATCCG-3′; reverse, 5′-GGCAGCAGCAGGAATGACAGG-3′.

2.9. Endothelial barrier measurement

HUVECs monolayer permeability was determined by measuring changes in trans-endothelial electrical resistance (TEER) using electrical cell-substrate impedance sensor (ECIS, Applied BioPhysics) [13]. Briefly, HUVECs were seeded onto ECIS 10E cultureware (10 small gold electrodes per well plus 1 large counter electrode, 0.8 cm^2/well) precoated with 0.1% gelatin at confluent density and allowed to form monolayers. 48 h after adenovirus infection, HUVECs monolayer were stimulated with LPS or TNFα and impedance was monitored (4000-Hz frequency) for another 24 h. Resistance values of discrete time points were calculated and plotted versus time. Each condition's end point resistance was divided by its starting resistance to give the normalized TEER.

2.10. Data analysis and statistics

Unless otherwise indicated, data are represented as the mean ± SD of two or three independent experiments. Statistical comparisons between groups were made using one-way ANOVA. Statistical differences were considered significant when $p < 0.05$.

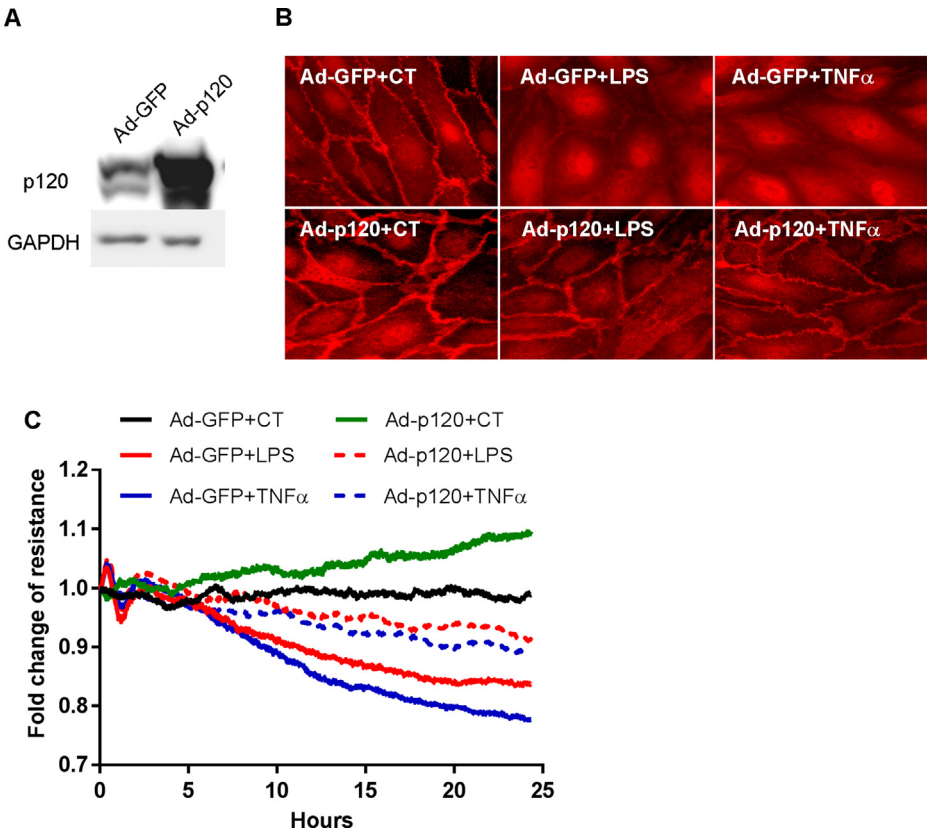


Fig. 1. p120 overexpression prevented LPS/TNFα-induced decreased junction localization of VE-cadherin at cell-cell junctions. HUVECs were infected with adenovirus containing GFP (Ad-GFP) or p120-GFP (Ad-p120) for 48 h. A. Confirmation of overexpression of p120 by Western blot. B. Cells were stimulated with LPS (1 μg/ml) or TNFα (20 ng/ml) or control vehicle (CT) for another 6 h and immunostained for VE-cadherin. C. p120 overexpression reduced the decrease in TEER induced by LPS/TNFα. After adenovirus transfection, cells were stimulated with LPS (1 μg/ml) or TNFα (20 ng/ml) or control vehicle (CT) for another 24 h and TEER was monitered. Representative image of three experiments is shown.

3. Results

3.1. p120 overexpression prevented LPS/TNFα-induced decreased junction localization of VE-cadherin

It is known that LPS/TNFα-induced breakdown of endothelial barrier functions are associated with the formation of intercellular gaps and fragmentation of VE-cadherin immunostaining at cell-cell contact. To investigate the role of p120 on LPS/TNFα-induced effects on endothelial barrier functions, we conducted adenovirus-mediated overexpression of p120 in HUVECs. As shown in Fig. 1, p120 overexpression successfully prevented the decreased localization of VE-cadherin at cell-cell contact induced by LPS/TNFα. To further test the functional role of p120 on endothelial barrier *in vitro*, we measured TEER of endothelial monolayer challenged with LPS/TNFα after p120 adenovirus or control adenovirus transfection. As shown in Fig. 1C, LPS/TNFα stimulation caused significant and sustained decrease in TEER in control adenovirus, which reflects dramatic endothelial cell barrier dysfunction induced by LPS/TNFα treatment. However, p120 overexpression significantly reduced the decrease in TEER induced by LPS/TNFα, indicating a protective role of p120 against LPS/TNFα-induced endothelial barrier disruption.

3.2. Proteome profiling of angiogenic factors in LPS-stimulated HUVECs with or without p120 overexpression

To determine whether p120 has a role in angiogenic factors induced by LPS, we used a non-biased, high-throughout approach using human angiogenesis antibody array containing 55 different angiogenesis related proteins antibodies immobilized on membrane. As shown in Fig. 2, LPS stimulation increased the levels of eight angiogenesis related proteins (over two-fold) in culture of HUVECs transfected with control adenovirus (Ang2, GM-CSF, PTX-3, Serpin E1, IGFBP-2, TSP-1, MCP-1, IL-8). p120 overexpression reduced LPS-stimulated increase of Ang2, PTX3 and GM-CSF while showing no effect on Serpin E1, IGFBP-2, TSP-1, MCP-1 and IL-8. Interestingly, p120 overexpression also reduced the basal secretion of MCP-1, PIGF and Ang2.

3.3. p120 overexpression inhibited LPS/TNFα-stimulated Ang2 synthesis and release in HUVECs

Among the 3 angiogenic factors inhibited by p120 overexpression in supernatant from LPS-stimulated HUVECs, Ang2 receives great attention in recent years, because of its critical role in vascular stability [14–16]. Thus, our subsequent experiments are focused on Ang2. To further confirm the inhibitory effects of p120 on LPS/TNFα-induced release of Ang2, we measured the concentration of Ang2 in supernatant of HUVECs by Elisa. In consistent with the results of antibody array, p120 overexpression significantly reduced the release of Ang2 in HUVECs after LPS/TNFα stimulation (Fig. 3A). To further evaluate the Ang2 change at transcriptional level, we examined Ang2 mRNA level by RT-qPCR. In line with the results at protein level, the Ang2 mRNA level was decreased by p120 overexpression both at baseline and under LPS/TNFα-stimulated conditions (Fig. 3B).

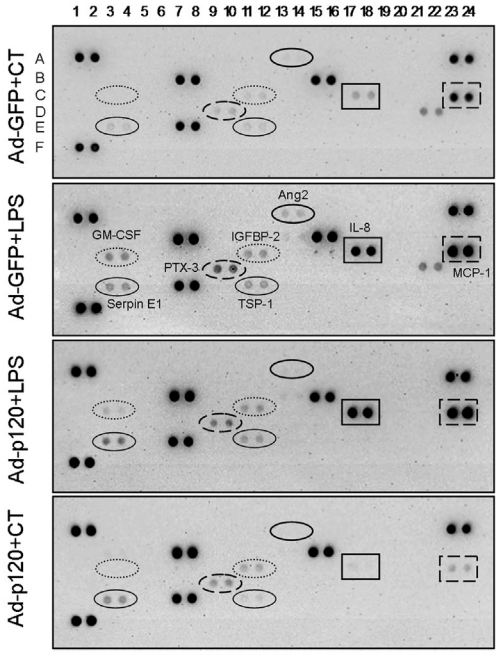
3.4. p120 overexpression did not inhibit LPS/TNFα-induced VCAM-1 protein expression and the activation of NF-κB signaling

Next, we sought to explore the mechanisms responsible for the inhibitory role of p120 on LPS/TNFα-induced Ang2 release and synthesis. Previous study has shown that NF-κB pathway has been involved in regulating Ang2 in endothelial cells [17]. However, in our antibody array results described above, we found that LPS-stimulated increase of IL-8 and MCP-1, which are typical gene product of NF-κB pathway, was not inhibited by p120 overexpression. To expand this, we examined VCAM-1 expression, another NF-κB dependent gene product. Although LPS (Fig. 4A) and TNFα (Fig. 4B) significantly stimulated VCAM-1 protein expression, p120 overexpression did not have inhibitory effects

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	PC				Activin A		ADAMTS1		ANG		Ang1		Ang2		Angiostatin		AR		Artemin				PC	
B	Factor III		CXCL16		DPPIV		EGF		EG-VEGF		Edoglin		Endostatin		ET-1		FGF-1		FGF-2		FGF-4		FGF-7	
C	GNDF		GM-CSF		HB-EGF		HGF		IGFBP-1		IGFBP-2		IGFBP-3		IL-1β		IL-8		TGFβ1		Leptin		MCP-1	
D	MIP-1α		MMP-8		MMP-9		NRG1-β1		PTX-3		PD-ECGF		PDGF-AA		PDGF-AB		Persephin		PF4		PIGF		Prolactin	
E	Serpin B5		Serpin E1		Serpin F1		TIMP-1		TIMP-4		TSP-1		TSP-2		uPA		Vasohibin		VEGF		VEGF-C			
F	PC																						NC	

B



C

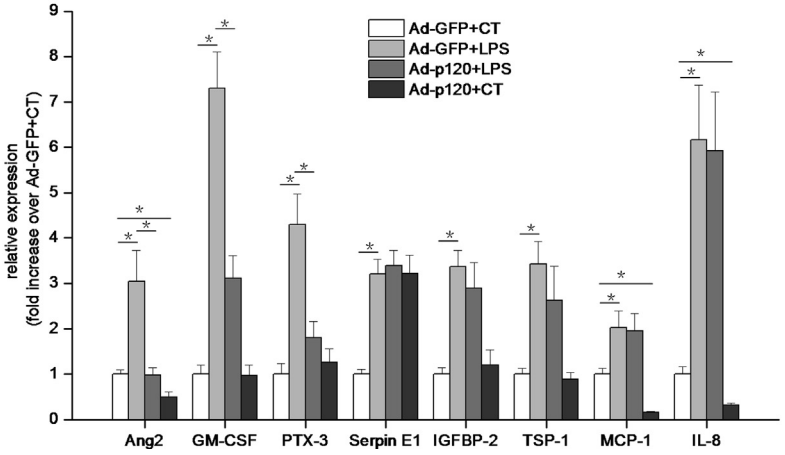


Fig. 2. Angiogenic factor profiles of supernatant from LPS-stimulated HUVECs with p120 overexpression. A. Map of the angiogenic factor antibody array. B. Angiogenic factor profiles of supernatant from CT or LPS-stimulated (24 h) HUVECs with or without p120 overexpression. Experiments were done in duplicate. C. Relative expression of 55 different angiogenesis related proteins were determined based on optical densitometry of the corresponding bands of angiogenesis related proteins that were at least 2-fold differentially regulated in supernatant from CT or LPS-stimulated HUVECs with Ad-GFP overexpression. Eight factors including Ang2, GM-CSF, PTX-3, Serpin E1, IGFBP-2, TSP-1, MCP-1 and IL-8 were at least 2-fold increased after LPS stimulation, among which Ang2, GM-CSF and PTX-3 were decreased by p120 overexpression. PC: positive control, NC: negative control. $^{*}p < 0.05$.

on VCAM-1 expression at 4 and 8 h.

It is well established that activation of NF-κB typically involves the phosphorylation and degradation of IκB, leading to the release of NF-κB into the nucleus. To further evaluate whether p120 overexpression can prevent LPS/TNFα-induced IκB degradation, we measured IκB expression by Western blot. As shown in Fig. 4A and B, LPS or TNFα stimulation for 1 h induced an obvious degradation of IκB. However, this effect was not prevented by p120 overexpression. Consistent with this, our results showed that p120 overexpression did not prevent nuclear translocation of NF-κB induced by LPS/TNFα as measured by immunofluorescence (Fig. 4C).

3.5. p120 overexpression did not inhibit LPS/TNFα-induced adhesion molecules/cytokines mRNA expression

To further explore the effects of p120 overexpression on LPS/TNFα-stimulated NF-κB pathway, we examined the mRNA expression of several NF-κB pathway targeted genes, including cell adhesion molecules/cytokines by RT-qPCR. Our results showed that p120 overexpression had no effects on LPS/TNFα-stimulated mRNA increase of VCAM-1, ICAM-1, E-selectin, IL-8 and IL-6 (Fig. 5A and B).

Taken together, the above results suggested that p120 overexpression had no effect on LPS/TNFα-stimulated NF-κB activation and that the inhibitory effects of p120 on LPS/TNFα-induced Ang2

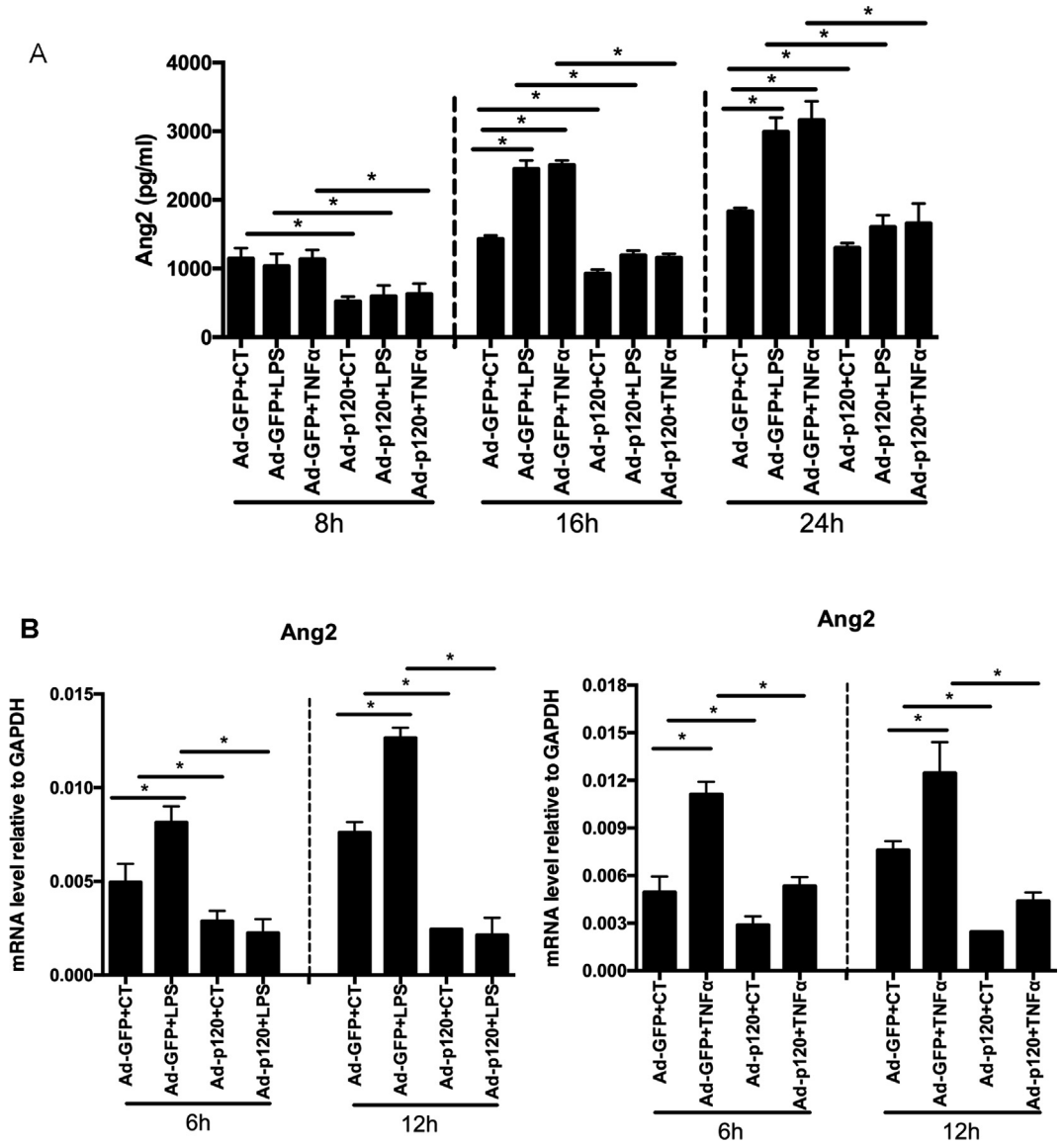


Fig. 3. p120 overexpression inhibited LPS/TNFα-stimulated Ang2 synthesis and release in HUVECs. After adenovirus transfection for 48 h, subsequent experiments were done. A. Ang2 levels from supernatant from LPS or TNFα-treated HUVECs for 8, 16, or 24 h were determined by Elisa. B. Endothelial Ang2 mRNA was determined by RT-qPCR after LPS or TNFα treatment for 6 or 12 h. $^{*}p < 0.05$. Data are expressed as the mean ± SD of three experiments.

expression may be mediated by other mechanism(s).

3.6. Transcription factor Ets1 mediated LPS/TNFα-induced upregulation of Ang2

Ets are a large family of transcription factors participating in early endothelial differentiation and angiogenesis [18]. Many members of this family are expressed very early in the developing vasculature and Ets consensus binding domains are present in essentially all endothelial cell-specific gene promoters [18]. Previous studies report that Ang2 was found to be regulated by the Ets family transcription factors Ets1 [19–21]. To test whether the transcription factor Ets1 mediates LPS/TNFα-induced Ang2 upregulation, we used siRNA to knockdown Ets1. After confirmation of Ets1 knockdown by both at mRNA and protein level by Western blotting or RT-qPCR (Fig. 6A and B), respectively, we found that the mRNA expression of Ang2 and concentrations of Ang2 in supernatant were both decreased significantly (Fig. 6B and C), demonstrating that Ets1 is involved in the upregulation of Ang2 after LPS/TNFα-induced stimulation.

3.7. p120 overexpression inhibited LPS/TNFα-stimulated increased expression of Ets1

Based on our above results showing that p120 overexpression prevented LPS/TNFα-induced endothelial-specific gene Ang2, but not other cell adhesion molecules/cytokines, we subsequently investigated whether p120 has any effects on Ets1 expression. As shown in Fig. 7A, LPS/TNFα stimulation increased Ets1 protein expression, but this increased expression of Ets1 was inhibited by p120 overexpression. Similar results were observed at mRNA level (Fig. 7B), suggesting that Ets1 may be involved in the inhibitory role of p120 in LPS/TNFα-induced expression of Ang2.

4. Discussion

Ang2, together with Ang1, have been identified as ligands of the endothelial receptor tyrosine kinase Tie2 [22,23]. Ang2 is primarily expressed in endothelial cells and stored in Weibel-Palade bodies, which can be rapidly released upon stimulation [24]. While Ang1-mediated activation of Tie2 regulates endothelial cell survival, blood

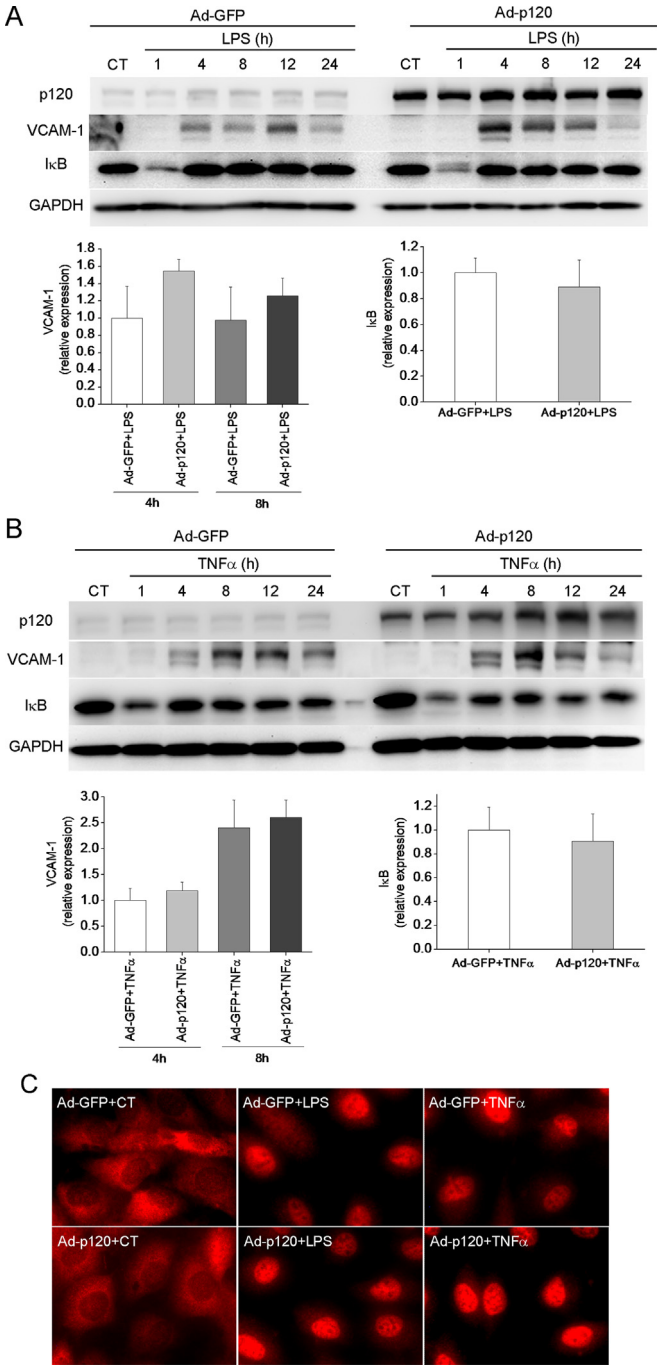


Fig. 4. p120 overexpression did not inhibit LPS/TNFα-induced VCAM-1 protein expression, IκB degradation and NF-κB nuclear translocation. After adenovirus transfection, HUVECs were stimulated with LPS (A) or TNFα (B) for the indicated time periods. A and B. VCAM-1 and IκB were determined by Western blot. Relative expression of VCAM-1 (4 h and 8 h) and IκB (1 h) were evaluated based on optical densitometry of the corresponding bands. Representative immunoblot of three experiments is shown. Data are expressed as the mean ± SD of three experiments. C. Immunofluorescence localization of NF-κB after LPS/TNFα stimulation for 1 h.

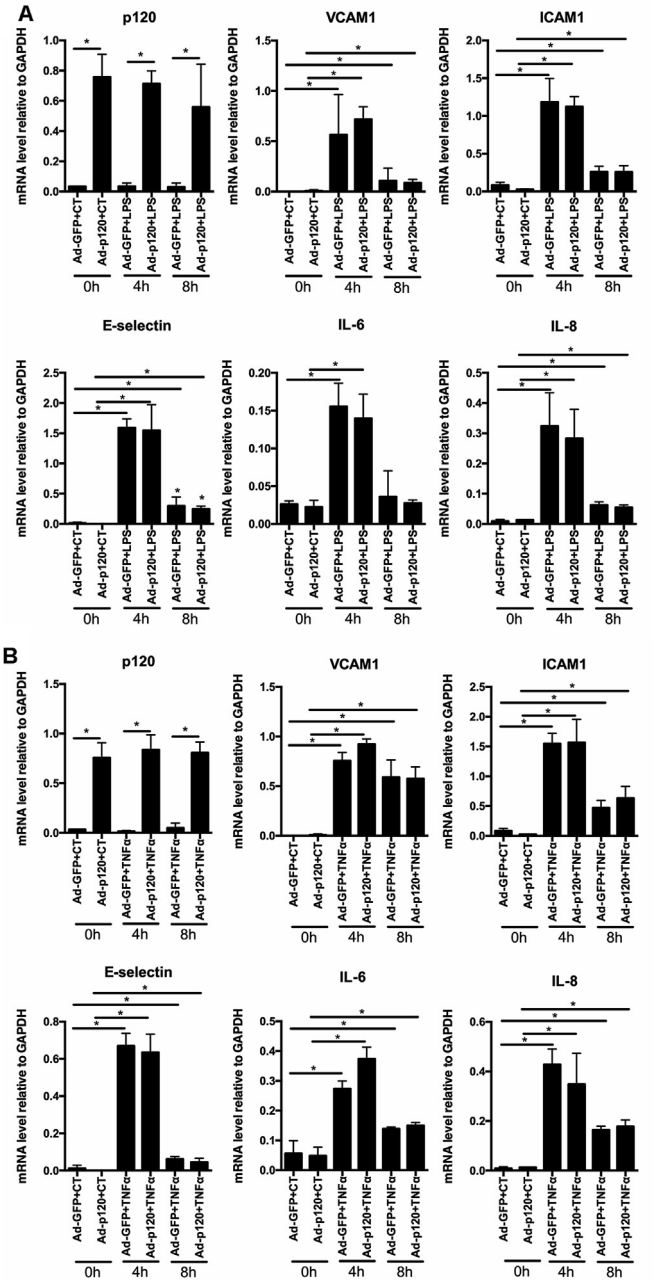


Fig. 5. p120 overexpression did not inhibit LPS/TNFα-induced mRNA expression of adhesion molecules/cytokines. After adenovirus transfection, HUVECs were stimulated with LPS (A) or TNFα (B) for 4 or 8 h. Then mRNA expression of the indicated adhesion molecules/cytokines were determined by RT-qPCR. $^{*}p < 0.05$. Data are expressed as the mean ± SD of three experiments.

vessel maturation and vessel-sealing effect, Ang2 acts in blood vessels primarily as a functional antagonist of Ang1/Tie2, acting as an autocrine regulator mediating vascular destabilization by inhibiting Ang1-mediated Tie2 activation [23,25]. The opposing effects of Ang-1 and Ang2 support a model of constitutive Ang1/Tie2 interactions controlling vascular homeostasis as a default pathway and Ang2 acting as a dynamically regulated antagonizing cytokine. Previous studies have shown that overexpression of Ang2 disrupts blood vessel formation [23]. In adult mice and humans, Ang2 is expressed only at sites of vascular remodeling [23]. Furthermore, Ang2 was up-regulated in the sites of neovascularization and some known angiogenic stimuli, such as hypoxia and vascular endothelial growth factor (VEGF), can increase Ang2 expression, implicating its role in deteriorating the integrity of preexisting vasculature [26]. Inflammatory mediators/cytokines, such as TNFα and thrombin, have also been shown to regulate Ang2 expression [17,27]. Accumulating evidence show that Ang2 is critical for cytokine-induced vascular leakage [28]. In this study, we found that p120 overexpression can decrease the synthesis and release of Ang2 in both basal and LPS/TNFα-induced inflammatory conditions, providing additional mechanism for vascular stabilizing effects of p120.

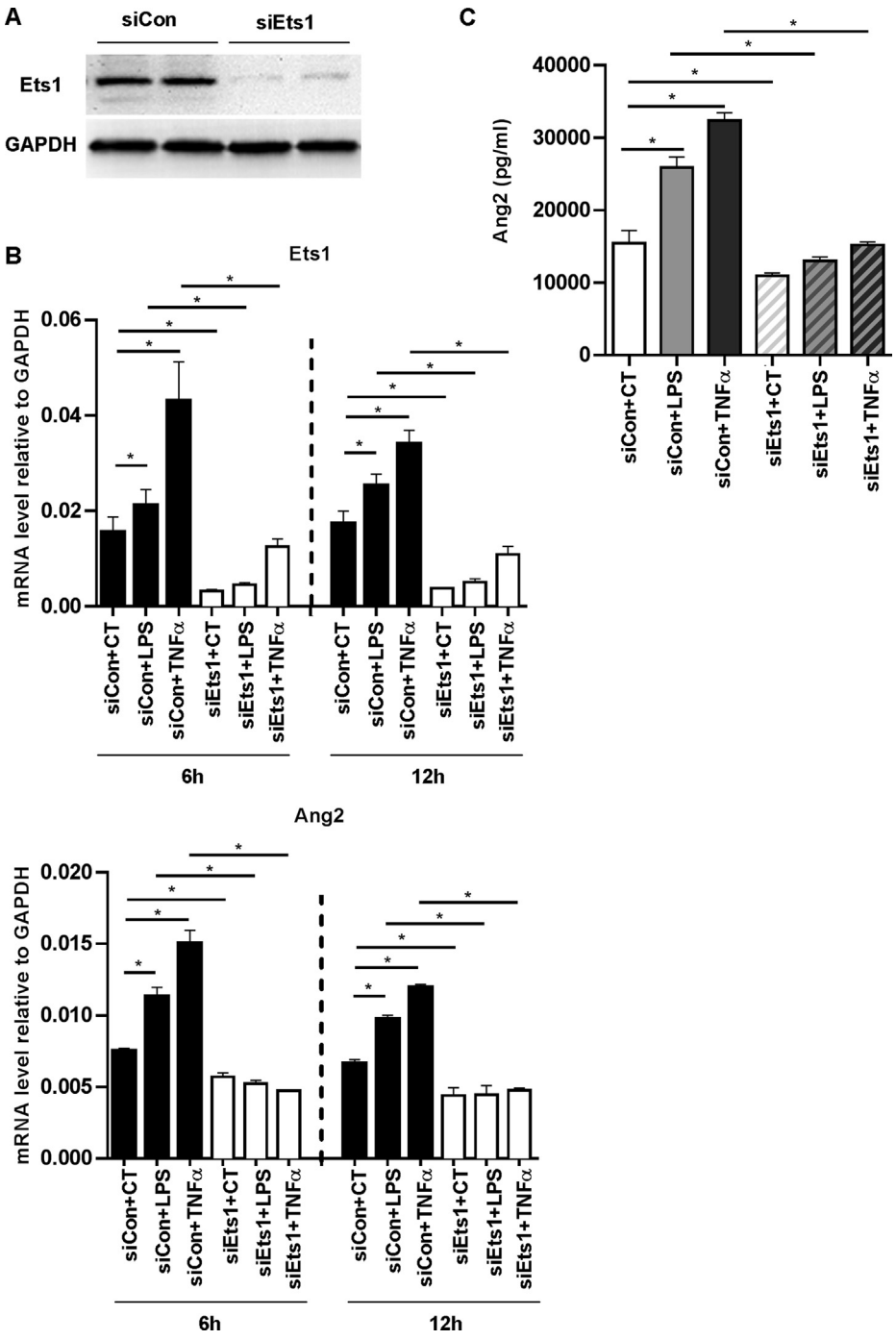


Fig. 6. Transcription factor Ets1 mediated LPS/TNFα-induced upregulation of Ang2. A. Ets1 knockdown 48 h after siRNA transfection determined by Western blot. B. Ets1 and Ang2 mRNA expression examined by RT-qPCR. 48 h after siRNA transfection, HUVECs were stimulated with LPS or TNFα for 6 h or 12 h. C. The concentration of Ang2 in supernatant of HUVECs following siRNA transfection evaluated by Elisa. After siRNA transfection for 48 h, culture medium was changed to EBM. HUVECs were then stimulated with LPS/TNFα or CT for 24 h. siCon, siRNA Control. siEts1, siRNA Ets1. $^{*}p < 0.05$. Data are expressed as the mean ± SD of three experiments.

Previous study has shown that TNFα increased Ang2 in part by NF-κB pathway [17], although analyses of human Ang2 promoter have not revealed abundant putative sites for NF-κB binding [29], indicating that NF-κB may regulate Ang2 expression indirectly through cooperative interactions with other transcription factors. However, in present study, p120 overexpression did not prevent NF-κB activation, evidenced by failure of prevention of IκB degradation and NF-κB nuclear translocation. In addition, p120 overexpression had no effects on LPS/TNFα-induced adhesion molecules/cytokines expression including VCAM-1, ICAM-1, E-selectin, MCP-1, IL-8 and IL-6. This is in contrast with a previous study showing that p120 regulates endothelial response to LPS by NF-κB pathway [30]. The reason for this discrepancy is unclear. However, one possible explanation may be endothelial heterogeneity [31]. While that study used rat lung microvascular endothelial cells, our study used HUVECs. Interestingly, Alcaide et al. also showed a normal pattern of TNFα-inducible adhesion molecules VCAM-1 and ICAM-1 in the presence of p120 overexpression when using HUVECs, which is in consistent with our findings [32].

Ets is one large family of transcription factors. Ets consensus binding domains are present in essentially all endothelial cell-specific gene promoters [18]. Ets1 is a central factor in endothelial specific Ang2 expression [19,20,33]. Consistent with previous studies, our results showed that in unstimulated endothelial cells, Ets1 was expressed at low level. However, LPS/TNFα induced Ets1 expression significantly. We also demonstrated that Ets1 is involved in the upregulation of Ang2 after LPS/TNFα stimulation. To note, the concentration of Ang2 in supernatant in siRNA experiments was significantly higher than that in adenovirus transfection experiments. A possible explanation for this

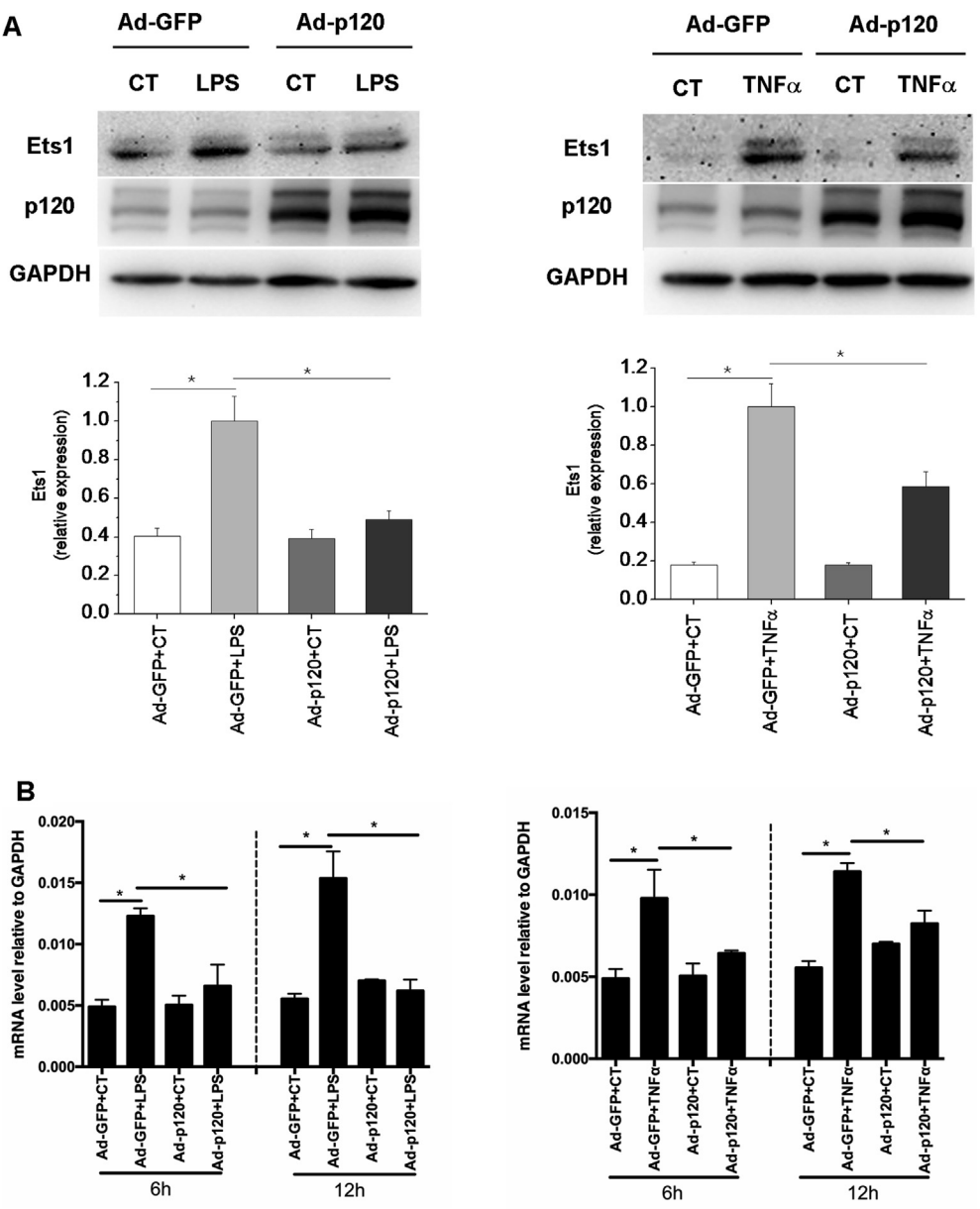


Fig. 7. p120 overexpression prevented LPS/TNFα-induced Ets1 expression. A. Ets1 expression was determined by Western blot. After adenovirus transfection, HUVECs were stimulated with LPS or TNFα for 8 h. Representative immunoblot of three experiments is shown. B. Ets1 mRNA was determined by RT-qPCR. After adenovirus transfection, HUVECs were stimulated with LPS or TNFα for 6 h or 12 h. $^{*}p < 0.05$. Data are expressed as the mean ± SD of three experiments.

may be that virus infection can induce expression and release of a significant amount of Ang2 in endothelial cells [34]. Therefore, in our experiments following adenovirus transfection, endothelial cells may be less responsive to LPS/TNFα stimulation with regard to the regulation of Ang2. Furthermore, p120 overexpression prevented LPS/TNFα induced Ets1 expression. Thus, our results demonstrated that p120 may inhibit LPS/TNFα-induced Ang2 expression via repression of Ets1 but independent of NF-κB. Our findings provided new insights into the mechanisms of p120-mediated vascular stability by demonstrating that p120 influences vascular integrity by secreted signals.

Acknowledgements

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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