Loss of GRB2 associated binding protein 1 in arteriosclerosis obliterans promotes host autophagy

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Abstract

Background: Arteriosclerosis obliterans (ASO) is a major cause of adult limb loss worldwide. Autophagy of vascular endothelial cell (VEC) contributes to the ASO progression. However, the molecular mechanism that controls VEC autophagy remains unclear. In this study, we aimed to explore the role of the GRB2 associated binding protein 1 (GAB1) in regulating VEC autophagy.

Methods: *In vivo* and *in vitro* studies were applied to determine the loss of adapt protein GAB1 in association with ASO progression. Histological GAB1 expression was measured in sclerotic vascular intima and normal vascular intima. Gain- and loss-of-function of GAB1 were applied in VEC to determine the effect and potential downstream signaling of GAB1.

Results: The autophagy repressor p62 was significantly downregulated in ASO intima as compared to that in healthy donor (0.80 *vs.* 0.20, $t = 6.43$, $P < 0.05$). The expression level of *GAB1* mRNA (1.00 *vs.* 0.24, $t = 7.41$, $P < 0.05$) and protein (0.72 *vs.* 0.21, $t = 5.97$, $P < 0.05$) was significantly decreased in ASO group as compared with the control group. Loss of GAB1 led to a remarkable decrease in LC3II (1.19 *vs.* 0.68, $t = 5.99$, $P < 0.05$), whereas overexpression of GAB1 significantly led to a decrease in LC3II level (0.41 *vs.* 0.93, $t = 7.12$, $P < 0.05$). Phosphorylation levels of JNK and p38 were significantly associated with gain- and loss-of-function of GAB1 protein.

Conclusion: Loss of GAB1 promotes VEC autophagy which is associated with ASO. GAB1 and its downstream signaling might be potential therapeutic targets for ASO treatment.

Keywords: Arteriosclerosis obliterans; Autophagy; GRB2 associated binding protein 1; JNK pathway; p38 kinase pathway

Introduction

Arteriosclerosis obliterans (ASO) is one of the most prevalent peripheral arteriosclerotic vascular diseases.[1] The etiology of ASO has not been fully understood. Several individual risk factors have been reported to contribute to the pathogenesis of ASO including smoking, hypertension, hyperlipidemia, diabetes mellitus, and hyperhomocysteinemia.[2] Current therapeutic approaches include by-pass surgery and endovascular intervention. Even though initially effective, high restenosis rates post-treatment remains an obstacle for long-term recovery.[3]

Autophagy is a vital catabolic process that degrades cytoplasmic components within the lysosome, which has been observed in the majority types of human cells including vascular endothelial cells (VEC).[4] In addition to its role as a stress response pathway, autophagy also acts as a quality control system that promotes the basal turnover of long-lived proteins and organelles as well as selectively degrading damaged cellular components.[5] Therefore, dysregulation of autophagy is associated with various human diseases, such as neurodegenerative disease, cancer, and cardiovascular disease.[6-11]

The GRB2 associated binding protein 1 (GAB1) belongs to the insulin receptor substrate (IRS1)-like docking adapter protein family.[2,3] Like other IRS1 adapter proteins, GAB1 has a PH domain, that is, involved in assembling complexes downstream of cell-surface receptor tyrosine kinase (RTK). Activated RTK signaling controls a variety of critical cellular processes including cell-cycle progression, differentiation, metabolism, survival, adhesion, motility, and migration. In this study, we found that *GAB1* which plays a critical role in the regulation of VEC autophagy was downregulated in ASO patients. *In vitro* experiments demonstrated that *GAB1* inhibited VEC autophagy through JNK and p38 pathway. The association between a loss of *GAB1* and excessive autophagy may provide a new direction for further drug development.

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Methods

Ethical approval

The investigations were conducted according to the *Declaration of Helsinki* principles. The study was approved by the ethical committee of Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China (No. RA-2020-071). The written informed consents were obtained from everyone who participates in this study.

Patients and healthy controls

We enrolled 11 patients diagnosed with ASO. The diagnosis of ASO of the lower extremity was made by history and physical examination. Ankle/brachial index and pulse wave velocity are widely used as complementary parameters to diagnose ASO. All ASO patients had the characteristic complaints of chronic limb ischemia, intermittent claudication, rest pain, or non-healing ischemic ulcers (Fontaine III, $n = 2$; Fontaine IV, $n = 9$) as confirmed by angiography. Individuals in our health examination center without any of the above symptoms were recruited as healthy controls. Sclerotic intima was collected, and the normal intima from amputation patients was used as control. The intima samples were stored at −80°C.

Cell culture and transfection

Human umbilical vein endothelial cells (HUVEC) were cultured within Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) as previously described.[12] For complementary DNA (cDNA) or small interfering RNA (siRNA) transfection, HUVEC cells were transiently transfected with plasmids or siRNAs using Lipofectamine® 2000 (Life Technologies) as previously described (Clonetics, San Diego, CA, USA).[4] The siRNA against GAB1 was purchased from Dharmacon (Lafayette, CO, USA).

Quantitative polymerase chain reaction

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using the SuperScript III First-Strand Synthesis kit following the manufacturer's protocol (TaKaRa, Shiga, Japan). Quantitative polymerase chain reaction (PCR) targeting *GAB1* was performed in a 20 μL reaction containing 1 μg of cDNA template, 1 μL of SYBR Green probe (TaKaRa). The PCR reactions were performed on a ViiA 7 Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA) with the following condition: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C, 15 s and 60°C, 1 min. Samples were run in triplicate concurrently with a 10× serial dilution of sample cDNA as a standard.

Western blotting analysis

The vascular tissues were ground under liquid nitrogen and then suspended in lysis buffer. Western blotting was performed as previously described.[13] Anti-β-actin and anti-GAPDH antibodies were obtained from Cell signaling Technology (#4970,#5174, CST, USA). The anti-GAB1 antibody was purchased from Cell signaling Technology (#3232, CST, USA). Anti-p-ERK1/2, the antibody was acquired from Cell signaling Technology (#4370, CST, USA).

Immunofluorescence (IF)

Tissues were fixed with 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 and blocked with 5% bovine serum album plus Tween 20. Coverslips were then incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at room temperature for about 1 h. After washing with PBS, the coverslips were counterstained with 4′,6-Diamidino-2-phenylindole (#H1200; Vector Laboratories, Burlington, ON, Canada). Images were visualized by using a Leica SP2 AOBS inverted confocal laser scanning microscope (Leica, Wetzlar, Germany).

Statistical analysis

Statistical analyses were performed using SPSS 23.0 (International Business Machines Corporation, IBM, USA). Normally distributed continuous data were expressed as mean ± standard deviation. *T*-test or Mann-Whitney *U*-test was used to test the differences between the two groups. All results presented are representative of at least three independent experiments. Statistical analysis was performed with an unpaired Student's *t* test to test the differences between the two groups. A *P* value of <0.05 was considered as statistically significant.

Results

Patient demographics

From 2013 to 2016, a total of 11 patients with an average age of 50.5 (35–76) years were enrolled in the clinical study [Table 1]. The occluded segments of each patient were located at different places including superficial artery, popliteal artery, or both. The risk factors are smoking, hypercoagulable states, diabetes mellitus, high cholesterol levels, and atrial fibrillation.

Loss of GAB1 is associated with VEC autophagy and ASO progression

It has been well-documented that *GAB1* serves as a critical regulatory component of VEC migration and capillary formation, and loss of GAB1 accelerates vascular inflammation.[14] To determine whether GAB1 also plays a regulatory role in ASO progression, we compared the GAB1 protein level in the vascular endothelial lesion of ASO patients with control vascular endothelium using immunoblotting. The expression level of GAB1 was almost three fold lower in ASO groups as compared with the control group (0.72 *vs.* 0.21, $t = 5.97$, $P < 0.05$) [Figure 1A]. Furthermore, real time-qPCR results showed that the *GAB1* mRNA level in the ASO group was

Table 1: Clinical information of patients with arteriosclerosis obliterans.

No.	Age (years)	Fontaine classification	Symptoms	Previous operation	Occluded level	Risk factors
1	76	IV	Toe gangrene and rest pain	PTA + STENT	SFA, ATA	Hypertension
2	72	IV	Toe ulcer and rest pain	PTA + STENT	PA	Hypertension, diabetes mellitus
3	64	IV	Toe ulcer and rest pain	PTA	PA, PTA	Hypertension, diabetes mellitus
4	71	IV	Foot bottom ulcer and rest pain	PTA + STENT	SFA, ATA	High cholesterol level, smoking
5	75	IV	Forefoot gangrene and rest pain	PTA + STENT	SFA, PA	Hypertension, smoking
6	55	III	Rest pain	None	SFA	Hypertension
7	54	III	Rest pain	None	ATA, PTA	Diabetes mellitus
8	58	IV	Toe ulcer and rest pain	PTA + STENT	SFA, PA	Hypertension, diabetes mellitus, smoking
9	73	IV	Toe ulcer and rest pain	PTA + STENT	EIA, SFA	Diabetes mellitus
10	63	IV	Toe gangrene and rest pain	PTA + STENT	EIA, ATA, PTA	Hypertension, diabetes mellitus, smoking
11	61	IV	Toe gangrene and rest pain	PTA + STENT	SFA, ATA	Diabetes mellitus

ATA: Anterior tibial artery; EIA: External iliac artery; PAP: Popliteal artery; PTA: Percutaneous balloon angioplasty; PTA: posterior tibial artery; SFA: Superficial femoral artery.

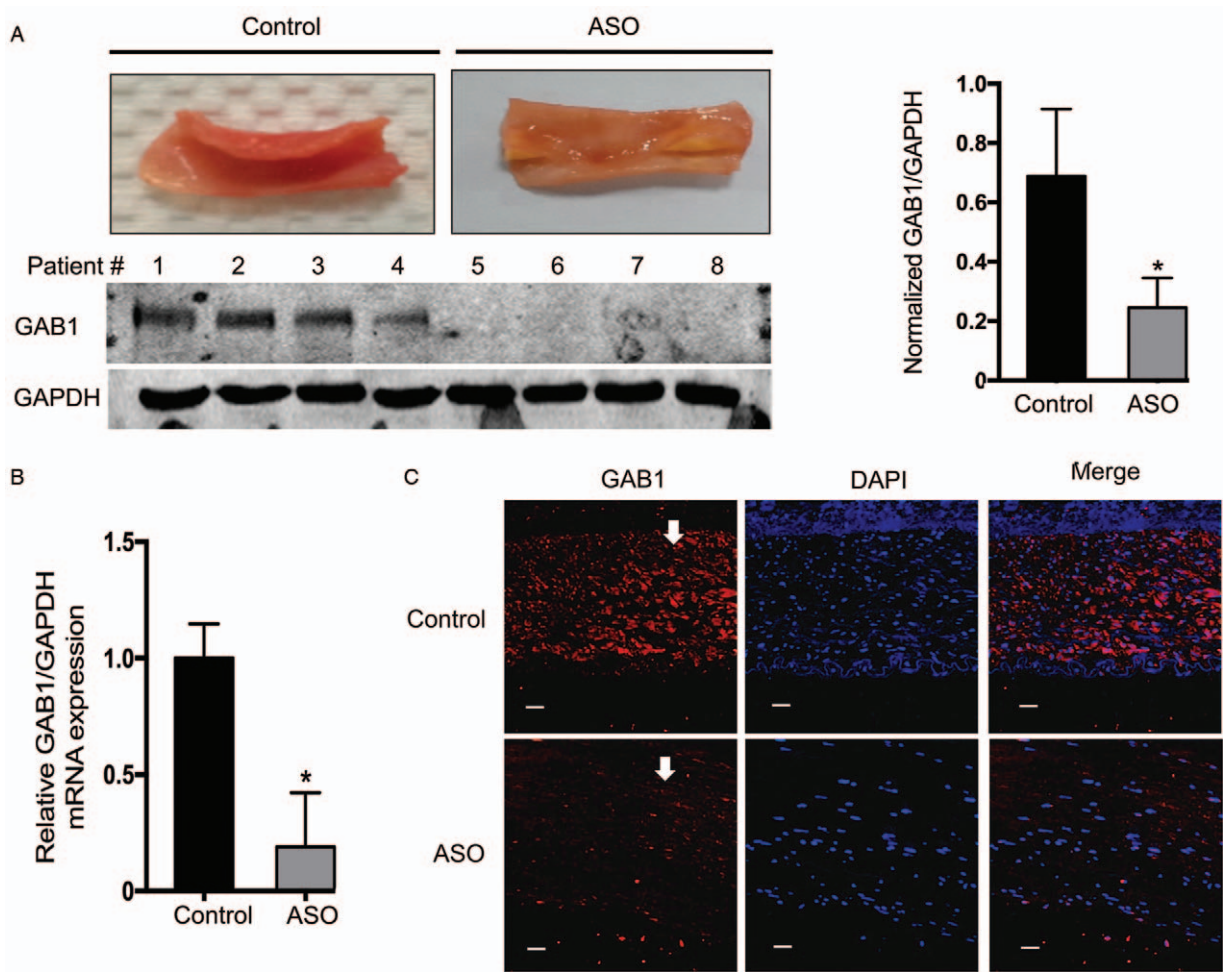

Figure 1: Expression of GAB1 in ASO patients. (A) Sclerotic intima was collected from four patients clinically diagnosed with ASO and the normal intima obtained from amputation patients was used as a control. The tissues were homogenized in RIPA buffer with a complete protease inhibitor. The lysates were processed for Western blotting for detecting the protein expression level of GAB1. The protein expression level of GAPDH was used as a loading control. (B) Protein levels of GAB1 were quantitated by means of densitometric analysis using Image J normalized to GAPDH and presented as fold changes compared with the control group (mean ± standard deviation, $n = 4$). $^{*}P < 0.05$. (C) Immunocytochemical staining was conducted using anti-GAB1 (Red). Nuclei were counterstained by DAPI (Blue). Scale bars: 10 μm. ASO: Arteriosclerosis obliterans; DAPI: 4′,6-Diamidino-2-phenylindole; GAB1: GRB2 associated binding protein 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RIPAA: Radioimmunoprecipitation assay.

significantly decreased compared to the control group (1.00 *vs.* 0.24, $t = 7.41$, $P < 0.05$), suggesting downregulation of *GAB1* in the arteries was due to transcriptional regulation [Figure 1B]. Moreover, immunofluorescence (IF) analysis confirmed that the loss of GAB1 predominantly happened within the vascular intima in ASO arteries [Figure 1C]. Taken into consideration, these results indicated that loss of *GAB1* within the vascular intima was highly associated with ASO progression.

Next, we questioned whether VEC autophagy altered within ASO intima. Surprisingly, we found that the autophagy repressor p62, also known as Sequestosome-1, was significantly downregulated in ASO intima as compared to control (0.80 *vs.* 0.20, $t = 6.43$, $P < 0.05$) [Figure 2A]. Furthermore, IF analysis demonstrated that LC3II, a component of autophagosome was significantly upregulated in the endothelium of ASO intima [Figure 2B]. These results suggesting that autophagy is significantly increased in VEC of ASO intima. Taken all together, in ASO intima, loss of *GAB1* is associated with VEC autophagy and ASO progression.

GAB1 negatively regulates autophagy in VEC

Autophagy dysregulation has been found to promote atherosclerosis in part through inflammasome hyper-

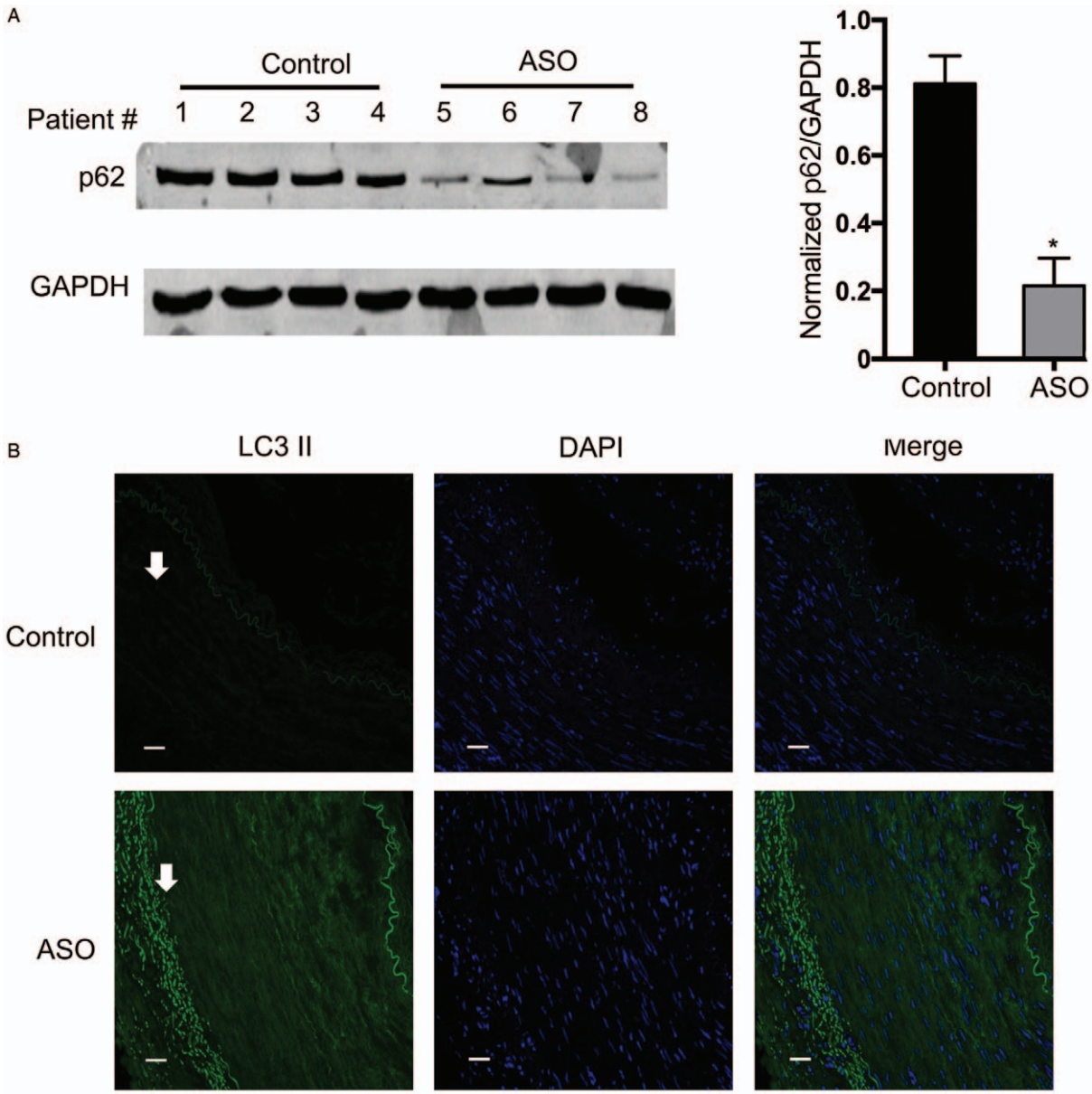

Figure 2: Autophagy is upregulated in ASO patients. (A) The same cell lysates were used as described in Figure 1 and processed for Western blotting for detecting the protein expression level of p62. The protein expression level of GAPDH was used as a loading control. Protein levels of p62 were quantitated by densitometric analysis using Image J normalized to GAPDH and presented as fold changes compared with the control group (mean ± standard deviation, $n = 4$). $^{*}P < 0.05$. (B) Immunocytochemical staining was conducted using anti-LC3 II (Green). Nuclei were counterstained by DAPI (Blue). Scale bars: 10 μm. ASO: Arteriosclerosis obliterans; DAPI: 4′,6-Diamidino-2-phenylindole; GAB1: GRB2 associated binding protein 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

activation.[15,16] It is also reported that autophagy can be regulated by insulin growth factor 1 receptor (IGF-1R) signaling.[17] *GAB1* is a key downstream adapt protein of IGF-1R signalling; so the loss of *GAB1* may contribute to arteriosclerosis through inducing VEC autophagy. To validate this hypothesis, we knocked down *GAB1* in HUVECs and found that loss of GAB1 led to a remarkable increase of LC3II, and this effect cannot be reversed by the autophagy inducer Hank balanced salt solution (HBSS) (1.19 *vs.* 0.68, 0.46 *vs.* 0.14, and 0.54 *vs.* 0.40) [Figures 3A and 3C]. Correspondingly, overexpression of *GAB1* significantly decreased LC3II level (0.41 *vs.* 0.93, 0.17 *vs.* 0.34, and 0.21 *vs.* 0.60) [Figures 3B and 3D]. These results suggested that *GAB1* plays a negative role in regulating VEC autophagy.

GAB1 regulates autophagy through stress-activated protein kinase pathway

Autophagy is upregulated in response to extra- or intracellular stress, such as starvation, growth factor deprivation, ER stress, and pathogen infection. Several stress-activated protein kinase pathways, such as mTOR1, JNK, Akt, and Erk have been demonstrated to play a key role in regulating autophagy upon those stimulations.[17-20] As an adapt protein, GAB1 served as an upstream of those signaling. Here, we demonstrated that the phosphorylation levels of JNK and p38 were significantly increased in ASO intima compared with control (0.54 *vs.* 2.67, $t = 16.3$, $P < 0.05$) [Figure 4A]. Meanwhile, the phosphorylation levels of Akt and ERK1/2

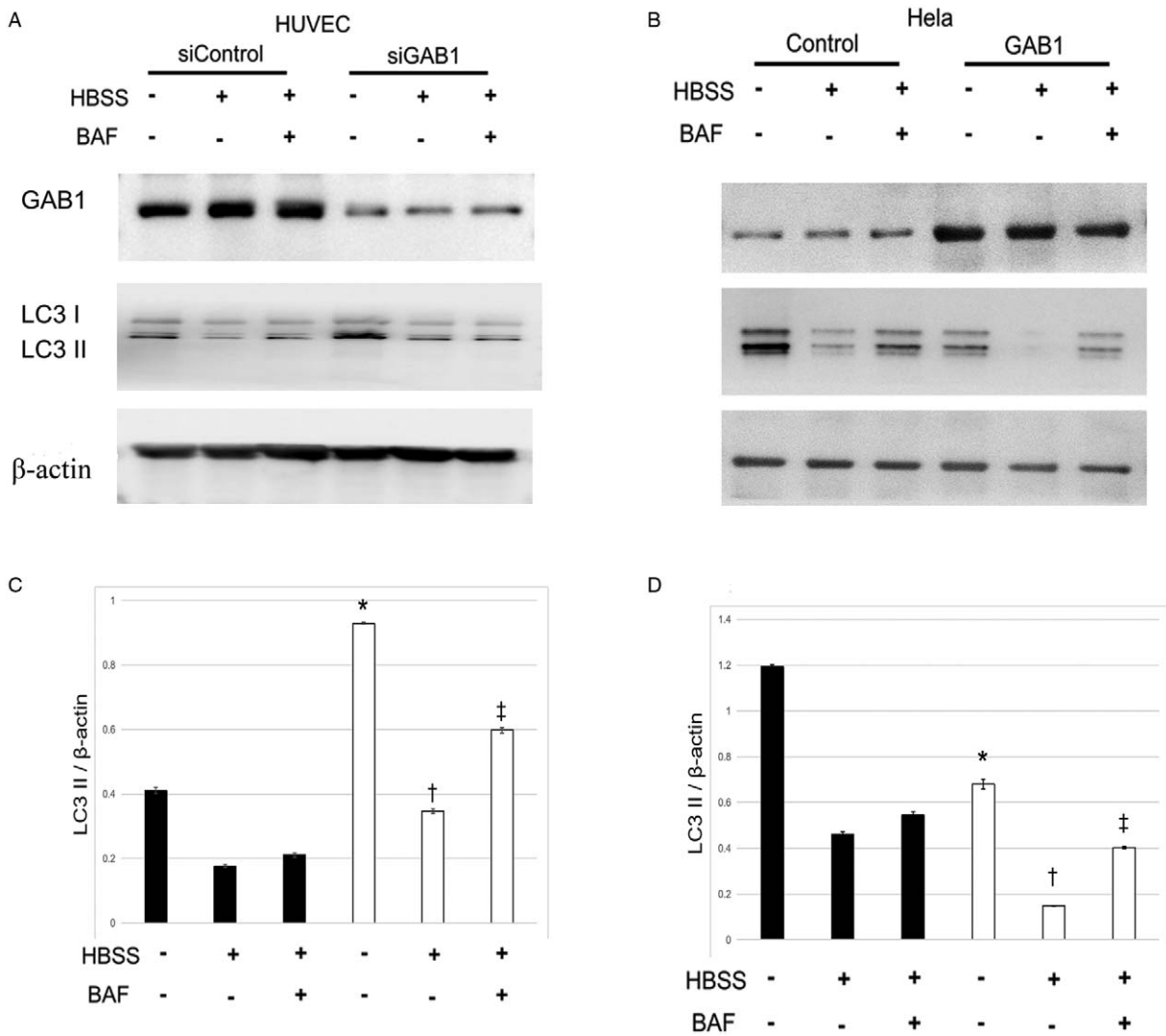

Figure 3: GAB1 natively regulates autophagy. (A) HUVECs were treated with either control siRNA (siControl) or GAB1-targeting siRNA (siGAB1) for 48 h, followed by treatment of HBSS for 6 h for the induction of autophagy presented with or without BAF. Cell lysates were harvested to examine the protein levels of GAB1 (using anti-GAB1 antibody), LC3I/II, and β-actin by Western blotting. (B) HeLa cells were transiently transfected with control or GAB1 expression vector control for 24 h, followed by the treatment as mentioned above. Western blotting was performed and analyzed as mentioned above. (C) Protein levels of LC3II were quantitated by densitometric analysis using Image J normalized to β-actin and presented as fold changes compared with the control group. *siControl *vs.* siGAB1 in HBSS− and BAF−; †siControl *vs.* siGAB1 in HBSS+ and BAF−; ‡siControl *vs.* siGAB1 in HBSS+ and BAF+. (D) Protein levels of LC3II were quantitated by densitometric analysis using Image J normalized to β-actin and presented as fold changes compared with the control group. *Control *vs.* GAB1 in HBSS− and BAF−; †Control *vs.* GAB1 in HBSS+ and BAF−; ‡Control *vs.* GAB1 in HBSS+ and BAF+. ASO: Arteriosclerosis obliterans; BAF: Bafilomycin A1; DAPI: 4′,6-Diamidino-2-phenylindole; GAB1: GRB2 associated binding protein 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HBSS: Hank balanced salt solution; HUVEC: Human umbilical vein endothelial cells.

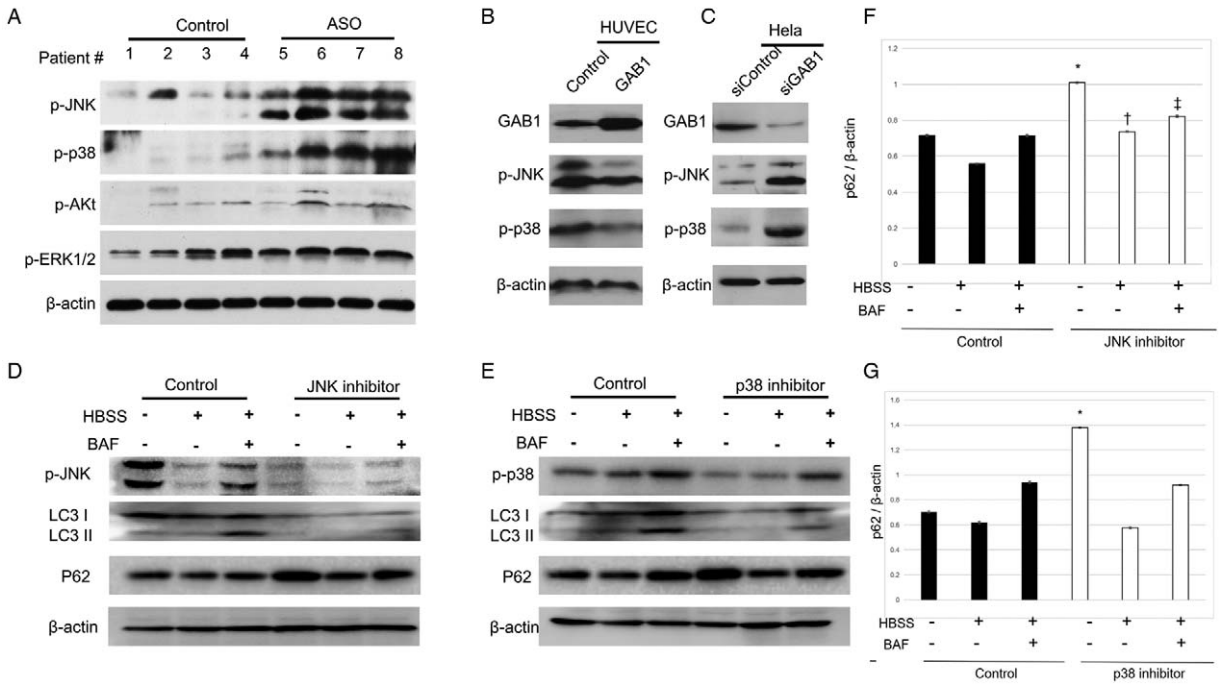

Figure 4: Stress-activated protein kinase pathway was involved in GAB1-mediated autophagy. (A) The tissue lysates were harvested as mentioned above to examine the protein levels of p-JNK, p-p38, p-Akt, p-ERK1/2, and β-actin by Western blotting. (B) HUVECs were treated with either control siRNA (siControl) or GAB1-targeting siRNA (siGAB1) for 48 h. (C) HeLa cells were transiently transfected with control or GAB1 expression vector control for 24 h, cell lysates were harvested to examine the protein levels of GAB1, p-p38, p-JNK, and β-actin by Western blotting. HUVEC with either (D) JNK inhibitor (SP600215) or (E) p38 inhibitor (SB203580) for 24 h, followed by treatment of HBSS for 6 h for the induction of autophagy presented with or without BAF. Cell lysates were harvested to examine the protein levels of GAB1, LC3I/II, and β-actin by Western blotting. (F) Protein levels of p62 were quantitated by densitometric analysis using Image J normalized to β-actin and presented as fold changes compared with the control group. *control *vs.* p38 inhibitor in HBSS− and BAF−; †control *vs.* p38 inhibitor in HBSS+ and BAF−; ‡control *vs.* p38 inhibitor in HBSS+ and BAF+. (G, F) Protein levels of p62 were quantitated by densitometric analysis using Image J normalized to β-actin and presented as fold changes compared with the control group. *control *vs.* JNK inhibitor in HBSS− and BAF−; †control *vs.* JNK inhibitor in HBSS+ and BAF−; ‡control *vs.* JNK inhibitor in HBSS+ and BAF+. ASO: Arteriosclerosis obliterans; BAF: Bafilomycin A1; DAPI: 4′,6-Diamidino-2-phenylindole; GAB1: GRB2 associated binding protein 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HBSS: Hank's balanced salt solution; HUVEC: Human umbilical vein endothelial cells.

remained unchanged. Furthermore, we found that the p-p38 and p-JNK levels were significantly associated with gain- and loss-of-function of GAB1 protein [Figures 4B and 4C]. The p38 inhibitor (SB203580) attenuated host autophagy at basal condition by demonstrating the up-regulation of p62. However, the autophagic flux did not affect by the reduction of p62 in the Bafilomycin (BAF) + HBSS group, suggesting inactivation of p38 may only play a role in attenuating autophagy under normal conditions (0.70 *vs.* 1.38, 0.61 *vs.* 0.58, and 0.94 *vs.* 0.92) [Figures 4E and 4G]. On the other hand, we found that JNK inhibitor (SP600215) led to an up-regulation of p62 in normal condition, HBSS group and BAF+HBSS group (0.72 *vs.* 1.01, 0.56 *vs.* 0.73, and 0.71 *vs.* 0.82) [Figures 4D and 4F], indicating that JNK can positively activate basal autophagy and autophagic flux. Taken all together, our results suggest that the JNK pathway may play an important role in the loss of GAB1 induced VEC autophagy.

Discussion

As a docking protein, *GAB1* has been well-documented in regulating cellular processes including cell proliferation, differentiation, apoptosis, and stress responses.[21] The depletion of *GAB1* has been proved to result in embryonic lethality due to severe organ development defects.[22] As *GAB1* was involved in multiple intracellular signaling pathways, the dysregulation of *GAB1* was reported to be associated with different human diseases.[23] For instance, selective deletion of hepatic *GAB1* showed enhanced hepatic insulin sensitivity with reduced glycemic and improved glucose tolerance.[24] Endothelial *GAB1* deletion accelerates AngII-dependent vascular inflammation and atherosclerosis.[25] Atherosclerosis is a complex inflammatory process involving several signaling pathways, such as transforming growth factor-β (TGF-β) and CD40/CD40L.[26-28] In this study, we found that both JNK and p38 pathways were involved in ASO. It has been reported that the activation of JNK is involved in the induction of autophagy during oxidative stress and persistently hyper-expressed and activated SAPK/JNK in lesions plays a key role in mediating cell differentiation and apoptosis during the development of atherosclerosis.[17] The p38 pathway was previously reported to play a critical role in the tight control of the early development of atherosclerosis.[29] In this study, we found loss of *GAB1* repressed JNK and p38 signaling which contributes to VEC autophagy o and eventually promotes ASO progression.

Previous studies showed that blocking autophagy promotes plaque necrosis in the atherosclerosis mouse model.[30] Moreover, autophagy has been reported to be involved in the regulation of atherosclerosis plaque stability.[31] In this study, we found that loss of *GAB1* induced VEC autophagy and this can be attenuated by blockage of JNK and p38 signaling. It has been reported

JNK engaged activator protein-1 bind to the p62 promoter. Meanwhile, activation of p38 can promote autophagy in an mTOR-dependent manner. These may explain how *GAB1* regulated autophagy by JNK. Even though the detailed mechanism by which *GAB1* controls JNK and p38 remains unclear, our results suggest targeting downstream signaling of *GAB1* can significantly repress the VEC autophagy.

The mechanisms that control *GAB1* transcription in VEC remain unknown. No methylation in the GAB1 promoter region of these ASO patients was found (data not shown). However, we cannot exclude other genomic alterations that may indirectly induce *GAB1* repression. A recent study reported that microRNA is associated with *GAB1* expression in colorectal cancer.[32] Whether such alterations regulate *GAB1* expression warrants further investigation. Autophagy is a complex process in a variety of physiological activities. The regulation of autophagy under different physiological activities may vary from each other. The arteries consist of three different structures including intima, tunica media, and adventitia. In this study, we focused on the intima autophagy, which represents the initiation of the arteriosclerosis. Further study will focus on the mechanism by which *GAB1* regulates host autophagy in mice models.

In this study, we did not profile all signaling upon *GAB1* alteration. As an adapt protein, while it cannot be excluded that *GAB1* may also exert its autophagy regulating function through other pathways, we demonstrated that the p38 and JNK inhibitor significantly antagonized HUVEC autophagy, suggesting that the p38 and JNK may not be essential for the loss of *GAB1* induced cell autophagy. We also cannot rule out the possibility that *GAB1* may be associated with diabetes as 7 of 11 patients included in this study have diabetes. However, this does not impede the conclusion that *GAB1* is associated with VEC autophagy in ASO patients.

However, taken all together, our study reported that loss of *GAB1* in ASO patients. Moreover, we found that GAB1 plays a negative role in promoting autophagy in HUVECs. This finding suggests a new mechanism to explain the development of the pathogenesis of ASO, which provides a new target for future drug development.

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Conflicts of interest

None.

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