Atg7 Mediates Metformin Protected Abdominal Aortic Aneurysm by Inducing Autophagy

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Research

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Abstract

Background

Abdominal aortic aneurysm (AAA) is pathologic dilation of the abdominal aorta and is often asymptomatic but has high susceptibility to rupture. Our previous study showed that metformin protected the pathophysiology of AAA by reducing the activation of PI3K/AKT/mTOR pathway.

Methods

Angiotensin II (Ang-II) was used to construct the AAA model in vascular smooth muscle cells (VSMCs). The expression of Atg7 and Atg4 was determined by qRT-PCR and Western blot. Atg7 expression was regulated by overexpressed plasmid or siRNA to examine the cell proliferation, cell migration, cell apoptosis and autophagy caused by Ang-II.

Results

Ang-II induced the expression of Atg7 and metformin reversed this effect. Suppression of Atg7 inhibited cell proliferation and cell migration, reduced cell apoptosis and autophagy, while overexpression of Atg7 enhanced cell proliferation and cell migration, induced cell apoptosis and autophagy. Moreover, the expression of autophagy related protein was regulated by Atg7 in Ang-II treated VSMCs. We further showed that the Atg7 mediated-autophagy was attenuated by metformin.

Conclusion

Metformin-reduced autophagy in AAA was mediated by Atg7, suggesting that Atg7 was a potential downstream effector of metformin in protecting the pathophysiology of AAA.

Introduction

Abdominal aortic aneurysm (AAA), a degenerative vascular disease, is a pathological dilation that predisposes to the potentially fatal consequence of aortic rupture [1]. Recently, the incidence of AAA declines [2], however, the mortality of AAA has not decreased globally [3]. Aneurysms are characterized by depletion of medial smooth muscle cells (SMCs) in the aortic wall [1]. An increasing number of studies have reported that the development of AAA is related to the decrease in the structural integrity of the vessel wall, causing by the cell apoptosis and senescence of SMCs [4, 5]. Even though the histopathological features of AAA are well documented, cellular and molecular mechanisms underlying AAA pathogenesis are not fully understood and no available pharmacological treatment has been explored to prevent the development of AAA. Therefore, searching for novel therapeutic approach remains an challenging problem.

Autophagy, a highly regulated process, delivers damaged organelles or cytoplasmic constituents to lysosome for clearance [6]. Autophagy-related genes (Atg) are the major regulatory factors in autophagy

[7]. Atg7 is accordingly well positioned as a central regulator for autophagy and serves as an E1 like enzyme for the ubiquitin like protein (UBL) Atg12 and Atg8 [8]. Mice on an *ApoE*^{-/-} background with SMC-specific Atg7 deficiency present with larger plaque sizes and exaggerated SMC senescence in the fibrous cap [9]. SMC-specific deletion of Atg7 showed reduced serum-induced cell growth, increased cell death, and decreased cell proliferation rate [10]. Moreover, loss of Atg7 in SMCs exacerbates Ang-II associated aortic remodeling [11]. Disruption of autophagic flux by VSMC-specific deletion of Atg7 is linked to defective mitophagy and exacerbate VSMC apoptosis and in turn plaque vulnerability [12]. We and other studies have reported the critical roles of autophagy in the pathogenesis of AAA and found that metformin inhibited Ang-II induced AAA progression by decreasing the activation of NF-κB and STAT3 signaling pathway [13-15]. The levels of the autophagy factors, Beclin, and LC3 were elevated in human and mouse AAA tissue [16]. As the major cellular constituent of the aorta, loss of vascular smooth muscle cells (VSMCs) through apoptosis or necroptosis is a major defining feature of AAA [17]. Moreover, recent study show that autophagy play important roles in regulating VSMCs death and aortic wall homeostasis and repair [18].

In the present study, we showed that Ang-II induced the expression of Atg7 and metformin reversed this effect. The expression of Atg7 regulated cell proliferation, cell migration, cell apoptosis and autophagy. Additionally, the expression of autophagy related protein was mediated by Atg7 in Ang-II treated VSMCs. We further showed that the Atg7 induced- autophagy was decreased by metformin. Conclusively, our data indicated that metformin reduced-autophagy in AAA was mediated by an autophagy central regulator Atg7, suggesting that Atg7 was a potential downstream effector of metformin in protecting the pathophysiology of AAA.

Materials And Methods

Cell culture and treatment

Rat VSMCs were isolated from the normal arteries of healthy donors as described previously [19] and cultured in DMEM medium supplemented with 10% FBS at 37 °C with 5% CO₂. VSMCs were treated with Ang-II (1 μM), metformin (10 mM) or combination of siRNA or plasmid with Ang-II.

Plasmid or siRNA transfection

The overexpression vector of pcDNA-Atg7 were constructed and confirmed by restriction enzyme digestion and DNA sequencing analysis. Negative control (NC) or Atg7 siRNA was purchased from GenePharma. The plasmid or siRNA was transfected to VMSCs by using Lipofectamine 2000 (Invitrogen, CA, USA) according to manufacturer's instructions.

Immunofluorescence

VMSCs were treated with Ang-II and transfected with NC siRNA, Atg7 siRNA, control plasmid or overexpressed Atg7 plasmid for 48 hours (h). Cells were fixed and blocked by 1% BSA for 30 minutes

(min) at room temperature. Then incubated with anti-Atg7 antibody at 4 °C overnight. Followed by washing with PBS for three times, FITC conjugated secondary antibody was used. Cellular nuclear was stained by DAPI. The images were required by Olympus CX71.

Cell apoptosis

Cells were harvested and stained with Annexin V and PI according to the manufacturer's instructions of Annexin V-FITC apoptosis detection kit (BD Biosciences). Cell samples were analyzed in a FACS Calibur instrument (BD Biosciences) with Cell Quest software.

EdU staining

Cells were permeabilized and stained with Click-iT EdU Imaging Kit (Invitrogen). Briefly, cells were fixed with 4% formaldehyde for 15 min and incubated with 0.1% Triton X-100 for 10 min. Thereafter, cells were treated with 0.5 mL Click-iT reaction cocktail for 30 min in the dark. After removal of the reaction cocktail, cells were washed with 3% BSA in PBS, and cell nuclei were stained with 1 µg/mL DAPI for 5 min in the dark. Finally, the cells were washed with PBS, and images were obtained by Olympus CX71.

Transwell assay

Cells were starved overnight and re-seeded onto the top chamber of transwell plate (1×10^5 in 100 µL medium), which was pretreated with 1% Matrigel (BD Biosciences) in PBS. Total of 500 µL cell culture media was added to the bottom chambers. After 24 h, not migrated cells were removed and migrated cells were fixed and stained with crystal violet. The images were obtained by Olympus CX71.

Western blot

Cells were lysed by ice-cold RIPA lysis buffer and quantified with a BCA protein kit. Total of 30 µg protein was separated by 12% SDS-PAGE gel. After transferring to PVDF membranes and blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with primary antibodies against Atg7, p62, Beclin1, LC3 or GAPDH overnight at 4°C. Membranes were washed by TBST for three times and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. The protein bands were visualized by enhanced chemiluminescence.

Transmission electron microscope (TEM)

Cells were fixed by 2.5% glutaraldehyde and washed by PBS for four times. One percent of osmic acid was used to fix cells again. After washing, cells were dehydrated by 50% acetone, 70% acetone, 90% acetone and 100% acetone respectively. Cells were infiltrated, embedded, sliced and stained by uranium acetate lead citrate. The cell morphology was viewed by H-7500 transmission electron microscope.

Statistical analysis

All experiments were conducted for at least three times and corresponding data were expressed as mean $\pm$ SD. Statistical differences for two groups were performed with Student's *t*-test and those for multiple groups with One-way analysis of variance (ANOVA), followed by a post hoc Tukey test. Values of *p* value less than 0.05 were considered to be statistically significant.

Results

Ang-II induces Atg7 expression

Our previous study found that metformin protected the pathophysiology of AAA by inhibiting autophagy *in vivo* and *in vitro*. To explore the mechanism under these effects, we used Ang-II to construct the AAA model in VSMCs and tested the expression of autophagy related protein, Atg4 and Atg7. Our results found that Ang-II induced the protein and RNA expression level of Atg7 (**Figure 1A**), while the expression of Atg4 was not changed. Additionally, metformin reversed the induction of Atg7 (**Figure 1A**). As Atg7 is considered as an essential factor for the induction of autophagy, the increased level of Atg7 might contribute to the induction of autophagy in AAA *in vitro* model.

Atg7 mediates cell proliferation and migration of VSMCs under Ang-II treatment

To access the functional role of Atg7 in AAA *in vitro* model (Ang-II treated VSMCs), we overexpressed or suppressed Atg7 by plasmid or siRNA and performed IF to show the transfection efficiency, as shown in **Figure 1B**, the expression of Atg7 was enhanced or reduced by transfection. Next, we detected the cell proliferation rate by Edu staining, and found that overexpression of Atg7 significantly increased cell proliferation rate. Overexpression of Atg7 occupied a mean of 82% versus 38% of control. Reduction of Atg7 by siRNA, consistently, decreased cell proliferation from 37% to 8% (**Figure 2A and C**). Moreover, the cell migration induced by Atg7 and reduced by inhibiting Atg7 (**Figure 2B and D**). These data indicated that Atg7 regulated the cell proliferation and cell migration in AAA *in vitro* cell model.

Inhibition of Atg7 reduces cell apoptosis and autophagy in Ang-II treated VSMCs

To further determine the apoptotic effects of Atg7 in VSMCs, we performed Annexin V assay to test the early and late stages of cell apoptosis. As shown in **Figure 3A**, inhibition of Atg7 decreased cell apoptosis (22% versus 13%), while overexpression of Atg7 enhanced cell apoptosis (19% versus 30%). Moreover, overexpression of Atg7 increased autophagic vacuoles and suppression of Atg7 decreased autophagic vacuoles (**Figure 3B**). We further detected the expression of autophagy related protein and showed that the expression of LC3 II/I and Beclin1 was elevated by Atg7 overexpression, while the level of p62 was reduced by overexpressing Atg7 (**Figure 3C**). Consistently, suppression of Atg7 by siRNA inhibited LC3 II/I and Beclin1, while increased p62 expression (**Figure 3C**). These results suggested that Atg7 mediated both Ang-II induced cell apoptosis and autophagy.

Metformin reduced-autophagy in AAA is mediated by Atg7

To test whether Atg7 regulated metformin reduced autophagy in Ang-II treated VSMCs, we overexpressed Atg7 in both metformin and Ang-II treated VSMCs (**Figure 4A**), and found that autophagic vacuoles induced by Atg7 was reversed by metformin (**Figure 4A-B**). In addition, the expression of LC3 II/I and Beclin1 was increased and p62 level was decreased by Atg7 overexpression, metformin relapsed these effects (**Figure 4C**). Thus, our results suggested that metformin reduced autophagy through suppressing Atg7.

Discussion

AAA is pathologic dilation of the abdominal aorta and is often asymptomatic but has high susceptibility to rupture. Here, we found that the expression of autophagy related protein, Atg7 was increased in AAA *in vitro* cell model and metformin reversed the induced expression of Atg7. Additionally, the cell proliferation, cell migration, cell apoptosis and autophagy of *in vitro* AAA cell model were regulated by Atg7. Moreover, Atg7 induced these effects were reversed by metformin. Taken together, our data suggested that Atg7 was a potential downstream effector of metformin in protecting the pathophysiology of AAA.

Up-regulation of LC3, Atg5 and Atg7 was found in dissecting AAA tissues from Ang-II treated *ApoE*^{-/-} mice and Ang-II challenged SMC Atg7 specific knockout mice exhibits severe cardiac dysfunction and larger suprarenal aortic diameters [11]. As a major component of the vessel wall, VSMCs are responsible for the control of blood flow and arterial pressure by regulating the lumen's diameter of resistance vessels in response to Ang-II [20]. Ang-II induced autophagy by increasing Beclin-1, Vps34, Atg-12-Atg5, Atg4 and Atg7 protein levels, Beclin-1 phosphorylation, and the number of autophagic vesicles [21]. Here, we found that Atg7 was up-regulated in Ang-II treated VSMCs, as our previous study showed that metformin represses the pathophysiology of AAA by inhibiting autophagy pathway [15], in this study, we showed that the induced expression of Atg7 in AAA was reversed by metformin. Until recently, there are no reports evaluating whether metformin regulate Atg7 in Ang-II induced VSMCs. Our experiments suggested that Atg7 might be involved in metformin reduced autophagy in AAA, which was evidenced by an decrease in autophagy related protein.

AAA results from changes in the aortic wall structure, including the loss of vascular smooth muscle cells and degradation of the extracellular matrix caused thinning of the media and adventitia [22]. The proliferation and apoptosis of VSMCs are associated with the progression of AAA [18]. Besides, loss of autophagy in VSMCs promotes VSMCs death and endoplasmic reticulum stress-dependent vascular inflammation and aggravates AAA [11]. Autophagy is a multistep process, consists of Beclin 1 and Vps34 and two ubiquitin-like system, LC3 and the autophagy-related (ATG) proteins [23]. Atg7 acts as the E1 enzyme of the ubiquitin-proteasome system to mediate the formation of Atg12-Atg5-Atg16 complex, which is required for the elongation of the autophagosome [24]. Recent findings revealed that in atherosclerotic lesions of *ApoE*^{-/-} mice deleted for Atg7 in VSMCs displayed enhanced apoptosis [12]. In this study, we provided evidence that inhibition of Atg7 repressed cell proliferation and cell migration, reduced cell apoptosis and autophagy, while overexpression of Atg7 increased cell proliferation and cell

migration, enhanced cell apoptosis and autophagy. Our results indicated that Atg7 served as an important regulator of proliferation, migration, apoptosis and autophagy in Ang-II treated VSMCs.

Metformin, a well-known drug, acts on both metabolism and inflammatory response, which leads it as a potential therapeutic target in several cardiovascular diseases [25]. Several studies have found that metformin significantly slows the growth of AAA [13, 14, 26] and inhibits the cell growth and proliferation, finally leading to cell apoptosis [27]. More importantly, metformin prescription status may be associated with a decreased risk of AAA [28]. Recently, we demonstrated that metformin protected Ang-II induced AAA by activating PI3K/Akt/mTOR/autophagy pathway [15], however, it remained unclear the molecular mechanism under this pathway. In this study, by using *in vitro* AAA model, we provide evidence that autophagy induced by Atg7 was reversed by metformin, suggesting that metformin reduced autophagy through suppressing Atg7.

One of the main limitations of our study is that, although key findings were obtained from *in vitro* AAA model, a *in vivo* AAA model was used to further confirm our findings. In conclusion, the main findings of this study, metformin reduced-autophagy in AAA was mediated by Atg7, suggesting that Atg7 was a potential downstream effector of metformin in repressing the pathophysiology of AAA (**Figure 5**).

Abbreviations

AAA: abdominal aortic aneurysm, Ang-II: angiotensin II, Atg: autophagy-related genes, EdU: 5-ethynyl-2'-deoxyuridine, Met: metformin, mTOR: mammalian/mechanistic target of rapamycin, PI3K: phosphoinositide 3-kinase, qRT-PCR: quantitative real-time PCR, SMCs: smooth muscle cells, TEM: transmission electron microscope, VSMCs: vascular smooth muscle cells, UBL: ubiquitin like protein

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article.

Competing interests

None.

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None.

Authors' contributions

Jingjing Guo, Zhu Wang, Ming Xue and Xinqiang Han: Conception and design, financial support, manuscript writing.

Lei Mi, Mengpeng Zhao, Chao Ma and Jian Wu: Administrative support, Collection and/or assembly of data, Data analysis and interpretation.

All authors read and approved the final manuscript.

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Figures

Figure 1

Ang-II induces Atg7 expression. (A). VSMCs were treated with Ang-II alone or the combination of Ang-II (1 μ M) and metformin (Met, 10 mM) for 48 h. The cells were collected and lysed for Western blot and qRT-PCR. GAPDH was used as a loading control. (B). VMSCs were treated with Ang-II (1 μ M) and transfected with NC siRNA, Atg7 siRNA, control plasmid or overexpressed Atg7 plasmid for 48 h. Cells were fixed and stained by Atg7 antibody. $**p < 0.01$.

Figure 2

Atg7 mediates cell proliferation and migration of VSMCs under Ang-II treatment. VMSCs were treated with Ang-II (1 μ M) and transfected with NC siRNA, Atg7 siRNA, control plasmid or overexpressed Atg7 plasmid for 48 h. Cells were fixed and stained and performed Edu staining (A) and tranwell assay (B). The summary of Edu and transwell assays were shown (C-D). The respective images were shown. $**p < 0.01$.

Figure 3

Inhibition of Atg7 reduces cell apoptosis and autophagy in Ang-II treated VSMCs. VSMCs were treated with Ang-II (1 μ M) and transfected with NC siRNA, Atg7 siRNA, control plasmid or overexpressed Atg7 plasmid for 48 h. Ang-II Cells were performed Annexin V staining (A), TEM assay, the blue arrow represents autophagic vacuoles (B) and Western blot with indicated antibodies (C). * $p < 0.05$, ** $p < 0.01$.

Figure 4

Metformin reduced-autophagy in AAA is mediated by Atg7. VMSCs were treated with the combination of Ang-II and metformin and transfected with Atg7 overexpressed plasmid. The expression of Atg7 was tested by IF (A), the autophagic bodies and vesicles were shown by TEM assay, the blue arrow represents autophagic vacuoles (B), and the expression of autophagy related protein was determined by Western blot (C). * $p < 0.05$, ** $p < 0.01$.

Figure 5

Schematic displaying Metformin decreases Atg7 induced cell proliferation, cell migration, cell apoptosis and autophagy in in vitro AAA model.