



Apelin receptor upregulation in spontaneously hypertensive rat contributes to the enhanced vascular smooth muscle cell proliferation by activating autophagy

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Background: Proliferation of vascular smooth muscle cells (VSMCs) plays a vital role in the progression of vascular remodeling and hypertension. Apelin-13 promotes VSMC proliferation of normal rats. This study was designed to investigate the roles of apelin receptor (APJ) and apelin-13 in VSMC proliferation of hypertension rats and underlying mechanisms.

Methods: Primary VSMCs were obtained from aorta of Wistar-Kyoto rat (WKY) and spontaneously hypertensive rat (SHR). The expressions of apelin and APJ were detected by Western blot and PCR, as well as immunohistochemistry. VSMC proliferation was evaluated with CCK-8 kit, PCNA protein expression and percentage of EdU-positive cells. Autophagy was determined by the ratio of LC3BII to LC3BI, ATG5 and p62 protein expressions, as well as LC3B immunofluorescence.

Results: APJ expression was increased while apelin expression was reduced in aorta and VSMCs of SHR compared with those of WKY. Exogenous apelin-13 promoted VSMC proliferation and autophagy of both WKY and SHR, which were prevented by APJ antagonist F13A. Blockade of APJ had no significant effects on VSMC proliferation and autophagy of WKY, but attenuated VSMC proliferation and autophagy of SHR. Administration of autophagy inhibitor 3-methyladenine (3-MA) not only attenuated VSMC proliferation of SHR, but prevented apelin-13-induced VSMC proliferation of both WKY and SHR.

Conclusions: Apelin-13 stimulates VSMC proliferation via APJ-mediated enhancement in autophagy. APJ upregulation in SHR contributes to the enhanced VSMC proliferation.

Keywords: Hypertension; apelin receptor (APJ); vascular smooth muscle cells (VSMCs); proliferation; autophagy

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Introduction

Vascular remodeling greatly contributes to the pathogenesis of hypertension, atherosclerosis, diabetic angiopathy, and vascular injury (1-3). Vascular smooth muscle cells (VSMCs) are the primary cell components in the media of arteries. VSMC proliferation plays a vital role in vascular remodeling in hypertension and atherosclerosis (4-6). Intervention of excessive VSMC proliferation serves as a therapeutic target in vascular remodeling and hypertension (6,7).

Apelin receptor (APJ) is a member of the G protein-coupled receptor gene family (8). Although APJ is similar to angiotensin II type 1 receptor (AT₁R) in structure, but it displays no affinity for angiotensin II (9). Apelin, a peptide with 77 amino, is identified as an endogenous ligand for the APJ (8). Apelin is processed to produce bioactive peptides consisting of 12, 13, 17 and 36 amino acids, which is called apelin-12, apelin-13, apelin-17 and apelin-36, respectively (10,11). Apelin-13 shows the highest biological activity among those forms (12). Apelin/APJ system plays important roles in the cardiovascular system under both normal and pathophysiological conditions (13-15). It is known that apelin promotes VSMC proliferation in normal or atherosclerotic animals (15-18). Apelin-13 and APJ in the paraventricular nucleus are involved in vasopressin release and sympathetic activation in spontaneously hypertensive rat (SHR) (19). However, the roles of peripheral vascular apelin/APJ system in VSMC proliferation in hypertension remain unknown.

Autophagy is a dynamic process that participates in cellular homeostasis by regulating cell survival and death (20). Intracellular organelles or proteins are delivered to lysosomes, which regulate cell survival through lysosome-mediated degradation (21). Hypertension and several vascular diseases are closely linked to autophagy dysfunction (22,23). Autophagy at a low level maintains homeostasis and provides energy and nutrients for cells through degradation of damaged organelles, while excessive autophagy damages cells and even causes cell death (24). Vascular autophagy is involved in physiological processes and some diseases relate to vascular structure and function (25). It has been found that AMPK activation prevents pulmonary arterial hypertension induced by monocrotaline via inhibiting NF- κ B-mediated autophagy (26). TP53-induced glycolysis and apoptosis regulator attenuates VSMC autophagy of pulmonary artery to prevent pulmonary hypertension (27). However, the role of autophagy in VSMC proliferation and its relation to apelin/APJ system in hypertension are still not elucidated. The present study aims at determining

the roles of apelin/APJ in autophagy and proliferation of VSMCs in Wistar-Kyoto rat (WKY) and SHR.

We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-6891>).

Methods

Experimental animals

Experiments were designed according to the standards of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, 8th edition, 2011). The surgical procedures and experimental protocol were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University (No.: IACUC-1811017). SHR is widely used as a model of hypertension, and WKY, derived from the same ancestral Wistar rat as the SHR, is normotensive and used as the closest genetic control for the SHR. Male WKY (n=12) and SHR (n=12) aged at 9 weeks weighing 200–250 g were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Systolic blood pressure (SBP) for SHR was higher than 150 mm Hg in the present study. Rats were exposed to a temperature-controlled room for a 12 h light/12 h dark cycle. The rats were randomly fed with a standard chow and tap water. Rat was euthanized at the end of experiments with intravenous injection of pentobarbital sodium (200 mg/kg).

Cell culture

VSMCs were isolated from thoracic aorta of WKY and SHR and cultured as we reported previously (5). Simply, the cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 IU·mL⁻¹ penicillin and 100 mg·mL⁻¹ streptomycin in humidified containing 5% CO₂ at 37 °C. VSMCs were identified with negative PECAM-1 (marker of endothelial cells) and vimentin (marker of fibroblasts), and positive α -SMA (marker of VSMCs). VSMCs from the 3rd to the 5th passages were used in the present study. The VSMCs were incubated 24 h after PBS or apelin-13 (1 μ M, MedChem Express, Monmouth Junction, NJ, USA) treatments.

Evaluation of cell proliferation

Cell proliferation was evaluated with cell counting kit-8

(CCK-8) kit, expression of proliferating cell nuclear antigen (PCNA) protein and percentage of 5-ethynyl-2'-deoxyuridine (EdU)-positive cells as we previously reported (5). Absorbance was measured at 450 nm with a microplate reader (ELX800, BioTek, Vermont, USA). PCNA protein expression was determined with Western blotting. DNA synthesis was examined with EdU incorporation assay to evaluate the VSMC proliferation with a Cell-Light™ EdU Apollo®567 *In Vitro* Imaging Kit (RiboBio, Guangzhou, China). Images were taken with a fluorescence microscopy (DP70, Olympus Optical, Tokyo, Japan). The number of EdU-positive cells was normalized by total number of cells.

Immunohistochemistry

Immunohistochemistry was used to detect APJ and apelin expressions in aorta of WKY and SHR. The aorta was fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into 10 µm sections. The deparaffinized and rehydrated sections were incubated with primary anti-APJ antibody (1:100) or anti-apelin antibody (1:100) overnight at 4 °C, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody.

Immunofluorescent analysis for LC3B

VSMCs were grown on glass coverslips in 6-well plates and fixed with 4% paraformaldehyde for 20 min. The cells were rinsed with 1×PBS for three times and permeabilized with 0.1% Triton X-100 for 5 min. After blocking in 1% BSA at room temperature for 1 h, cells were incubated with microtubule-associated protein-1 light chain-3B (LC3B) antibody (1:100) and α -SMA antibody (1:100) overnight. The cells were rinsed with 1×PBS and incubated with the secondary fluorescein anti-rabbit IgG (1:1,000) and anti-mouse IgG (1:1,000) for 1 h. The nuclei were stained with DAPI (Southern Biotech, Birmingham, AL, USA), a blue-fluorescent DNA stain.

RNA extraction and qRT-PCR

Total RNA was extracted from thoracic aorta and VSMCs with a Trizol reagent (Life Technologies, Gaithersburg, MD, USA). Then, 1 µg total RNA was reversed to cDNA using the PrimeScript® RT reagent Kits (Takara, Otsu, Shiga, Japan) and StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The

quantitative reactions were made with SYBR Green (Takara Biotechnology Co., Ltd., Tokyo, Japan) by StepOnePlus™ system. GAPDH was used as the internal control. All primers used for qRT-PCR are listed in a table (Table S1).

Western blot analysis

Total protein was extracted from VSMCs and thoracic aorta respectively. Then, 30 µg total proteins were separated with SDS-PAGE and transferred to PVDF membrane. The membranes were blocked with 5% defatted milk in TBST, and then incubated with primary antibody overnight at 4 °C. The Enhanced Chemiluminescence Detection Kit (Thermo Fisher Scientific, USA) was used to visualize protein bands. All the antibodies used for immunofluorescent and Western blot analysis are listed in a table (Table S2).

Statistical analysis

Data are expressed as mean \pm SE. Student's unpaired *t*-test was used to compare the difference between two groups. One-way or two-way ANOVA followed by post hoc Bonferroni test was employed for multiple comparisons. Statistical significance was considered as $P < 0.05$.

Results

APJ and apelin expressions in the aorta and VSMCs

APJ mRNA level was increased but the apelin mRNA level was not significantly altered in aorta of SHR compared with those of WKY (Figure 1A). APJ protein was upregulated (+73.8%) while apelin was downregulated (-28.1%) in the aorta of SHR compared with those of WKY (Figure 1B). Immunohistochemistry analysis further confirmed that the APJ expression was increased (Figure 1C) while apelin expression was reduced in the aorta of SHR (Figure 1D). Consistently, APJ protein expression was increased (+63.7%), but apelin protein expression was reduced (-23.0%) in VSMCs of SHR compared with those of WKY (Figure 2A). These findings suggest that apelin/APJ may play an important role in regulating VSMCs.

Effects of apelin-13 and F13A on VSMC proliferation

VSMC proliferation was evaluated with CCK-8 kit, PCNA protein expression and percentage of EdU-positive cells. Apelin-13 promoted VSMC proliferation of both WKY

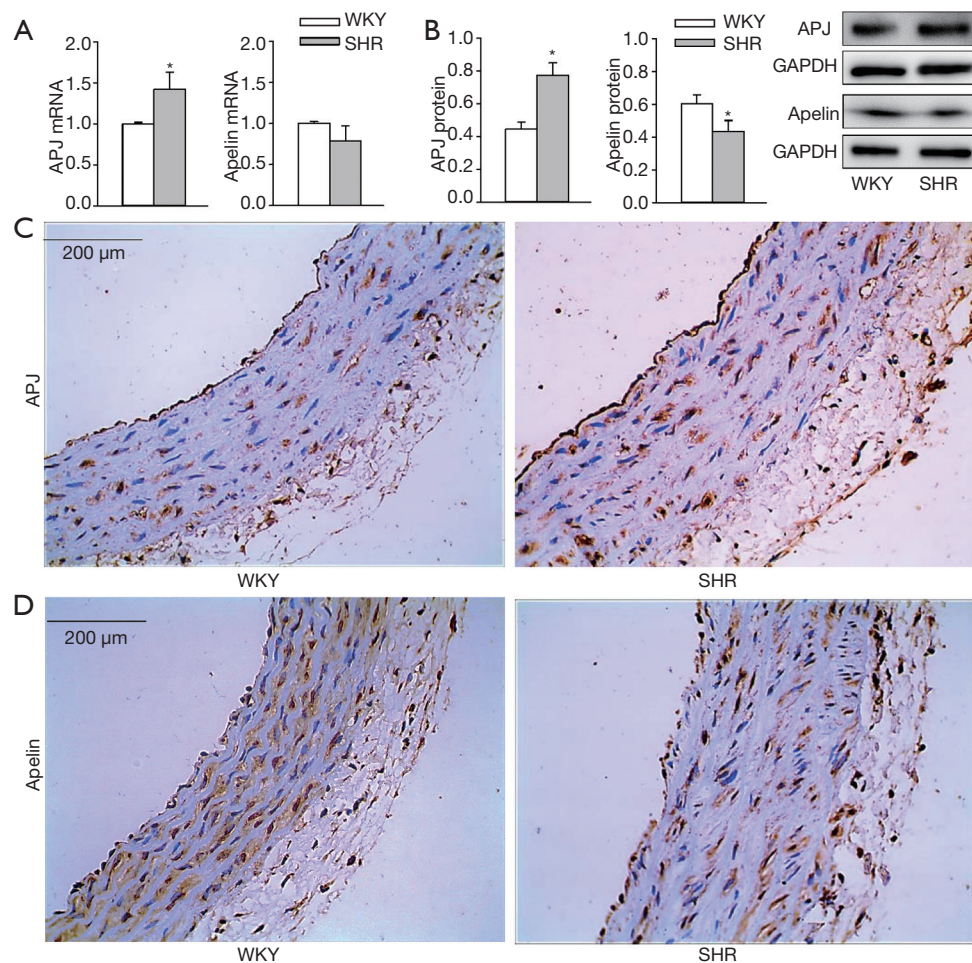


Figure 1 APJ and apelin expressions in aorta of WKY and SHR. (A) APJ and apelin mRNA expressions. (B) APJ and apelin protein expressions. (C) Immunohistochemistry of aorta sections for APJ (brown color). (D) Immunohistochemistry of aorta sections for apelin (brown color). Values are mean \pm SE. *, $P < 0.05$ vs. WKY. $n = 6$. WKY, Wistar-Kyoto rat; SHR, spontaneously hypertensive rat; APJ, apelin receptor.

and SHR. APJ antagonist F13A had no significant effects on VSMC proliferation of WKY, but inhibited VSMC proliferation of SHR. Furthermore, F13A prevented apelin-13-induced VSMC proliferation in VSMCs of both WKY and SHR (Figure 2B,C,D,E). These results indicated that apelin-13 stimulates VSMC proliferation via APJ in both WKY and SHR. The upregulation of APJ in VSMCs of SHR contributes to the enhanced VSMC proliferation in SHR.

Effects of apelin-13 and F13A on autophagy of VSMCs

Increased LC3B II production from LC3BI and ATG5

protein expression as well as reduced p62 protein expression are markers of autophagy (28-30). Apelin-13 promoted autophagy in VSMCs of both WKY and SHR. APJ antagonist F13A had no significant effects on autophagy in VSMCs of WKY, but attenuated autophagy in VSMCs of SHR. Furthermore, F13A prevented apelin-13-induced autophagy in VSMCs of both WKY and SHR (Figure 3A,B,C). The findings were further supported by the triple immunofluorescence staining for LC3B (Figure 4A,B). These results indicated that apelin-13 promotes autophagy via APJ in VSMCs of WKY and SHR. The increased APJ expression in VSMCs of SHR is involved in the enhanced autophagy in VSMCs of SHR.

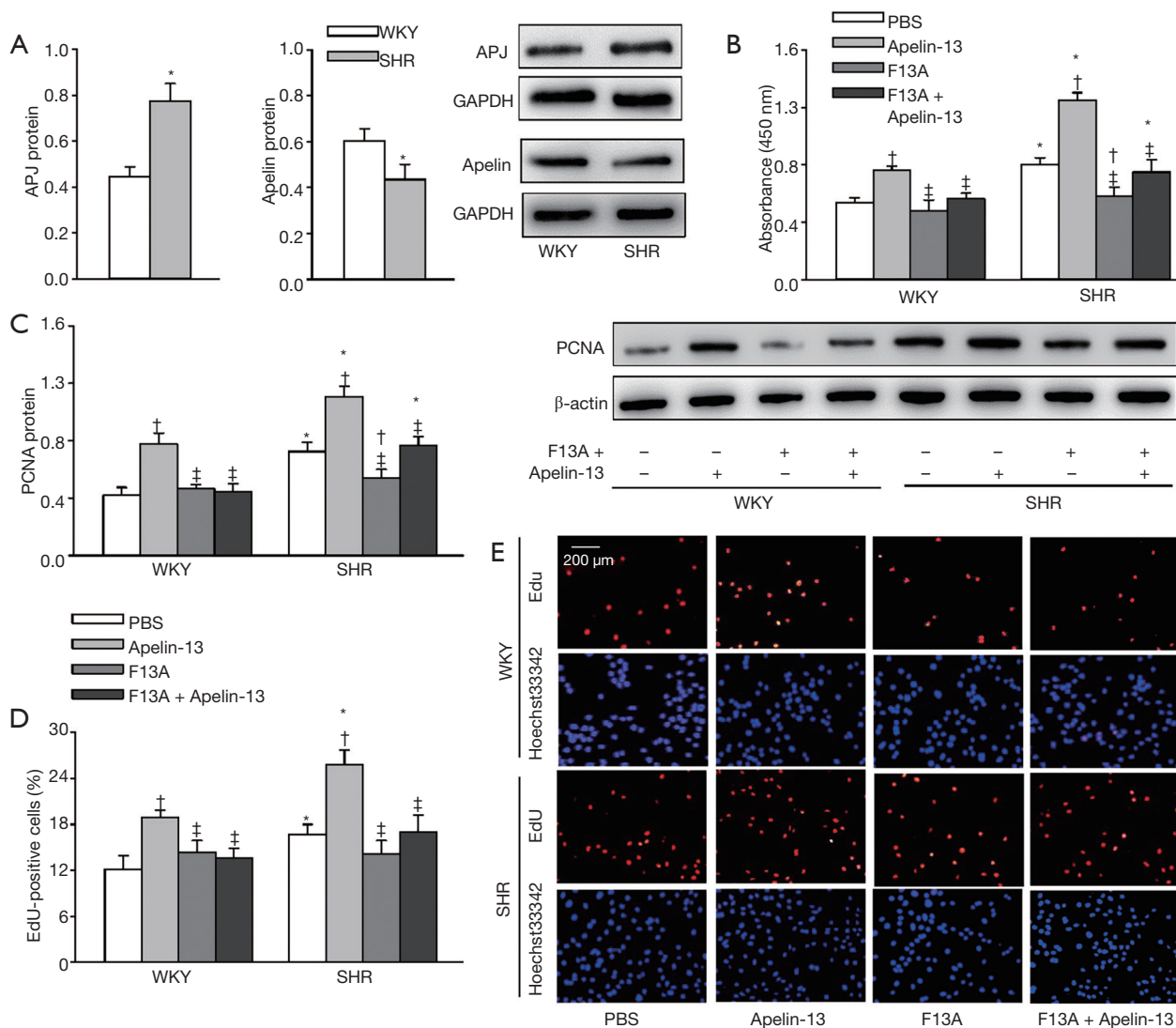


Figure 2 APJ and apelin protein expressions in VSMCs and the effects of apelin-13 and APJ receptor antagonist F13A on VSMC proliferation of WKY and SHR. (A) APJ and apelin protein expressions in VSMCs of WKY and SHR. (B) VSMC proliferation evaluated with CCK-8 kit. (C) VSMC proliferation evaluated with PCNA protein expression. (D) VSMC proliferation evaluated with the percentage of EdU-positive cells. (E) Representative images of EdU staining. Red, EdU-positive cells. Blue, Hoechst 33342 was used to label double-stranded DNA and thus to visualize nuclei. The measurements were carried out 24 h after PBS, apelin-13 (1 μ M), or F13A (1 μ M) treatment. Values are mean \pm SE. *, $P < 0.05$ vs. WKY; †, $P < 0.05$ vs. PBS; ‡, $P < 0.05$ vs. Apelin-13. $n = 6$. VSMC, vascular smooth muscle cell; F13A, APJ antagonist; CCK-8, cell counting kit-8; PCNA, proliferating cell nuclear antigen; EdU, 5-ethynyl-2'-deoxyuridine.

Time-effects of apelin-13 on VSMC autophagy and proliferation

Apelin-13 time-relatedly promoted VSMC autophagy and proliferation in WKY and SHR. The autophagy was significantly enhanced at the 8th h after application

of apelin-13 (Figure 5A,B), while the proliferation was significantly enhanced at the 16th h after application of apelin-13 (Figure 5C). The results indicate that the effects of apelin-13 on autophagy occur earlier than those on proliferation.

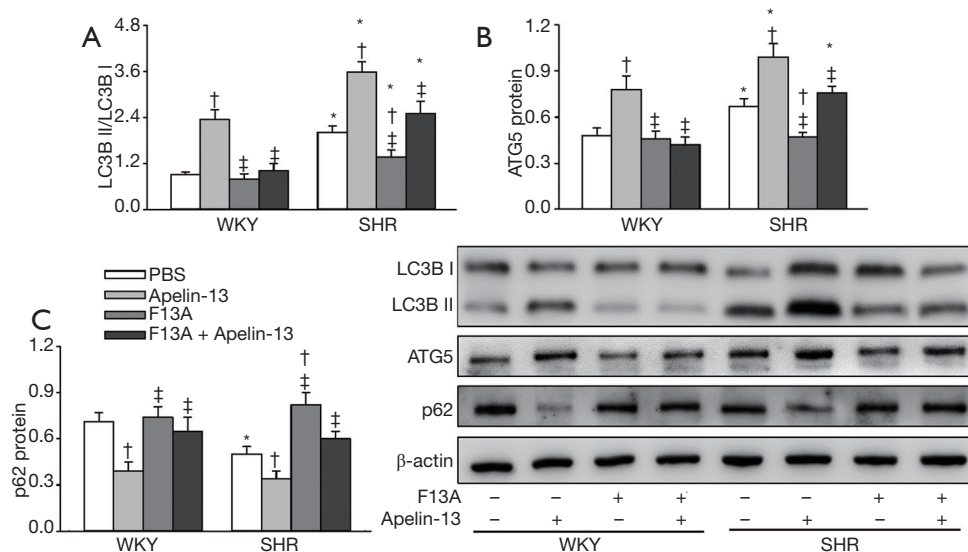


Figure 3 Effects of apelin-13 and apelin receptor antagonist F13A on VSMC autophagy of WKY and SHR. Autophagy determined by LC3B, ATG5 and p62 protein expressions. (A) Ratio of LC3B II to LC3B I. (B) ATG5 protein expression in VSMCs. (C) p62 protein expression in VSMCs. The measurements were carried out 24 h after PBS, apelin-13 (1 μ M) or F13A (1 μ M) treatment. Values are mean \pm SE. *, $P < 0.05$ vs. WKY; †, $P < 0.05$ vs. PBS; ‡, $P < 0.05$ vs. Apelin-13. $n = 6$. LC3B, microtubule-associated protein-1 light chain-3B; ATG5, autophagy related protein 5.

Effects of 3-MA on apelin-induced VSMC proliferation

3-Methyladenine (3-MA) is a widely used autophagy inhibitor (31-33). 3-MA had no significant effects on VSMC proliferation of WKY, but inhibits the enhanced VSMC proliferation of SHR. More importantly, apelin-induced VSMC proliferation was prevented by 3-MA treatment (Figure 6A,B,C,D). These data indicated that apelin promotes VSMC proliferation at least partially mediated by enhancing autophagy in VSMCs.

Discussion

VSMC proliferation is important for vascular remodeling in hypertension (34-36). Vascular remodeling may initially be adaptive in hypertension, but eventually becomes maladaptive and contributes to the development of hypertension and occurrence of its complications (37-39). The primary novel findings are that APJ upregulation in SHR greatly contributes to the VSMC proliferation, and apelin-13 promotes VSMC proliferation by APJ-mediated activation of autophagy in both WKY and SHR. Blockade of APJ attenuates VSMC autophagy and proliferation, and inhibition of autophagy suppresses VSMC proliferation in SHR and prevents apelin-13-induced VSMC proliferation

in both WKY and SHR. These findings suggest that APJ in VSMCs may be a therapeutic target for inhibiting VSMC autophagy and proliferation in hypertension.

APJ is an orphan G protein-coupled receptor and apelin is the endogenous ligand of APJ. Apelin/APJ system is widely distributed in many tissues and cells including VSMCs (15). It is known that apelin-13 promotes VSMC proliferation in VSMCs of normal animals (40). However, the roles of apelin/APJ in VSMC proliferation of hypertension remain unknown. We found that APJ expression was increased in VSMCs of SHR as well as in the aorta of SHR. APJ antagonist inhibited VSMC proliferation of SHR and prevented apelin-13-induced VSMC proliferation of both WKY and SHR. The findings indicate that the upregulation of APJ plays a crucial role in the enhanced VSMC proliferation in SHR. It is noted that apelin expression was slightly reduced in VSMCs of SHR as well as in the aorta of SHR. The downregulation of apelin in the SHR might be a compensatory protective mechanism to avoid the roles of excessive APJ activation in VSMC proliferation.

Autophagy is crucial for lysosomal degradation and recycling process in cells, which is responsible for maintaining the homeostasis of cell survival and cell death

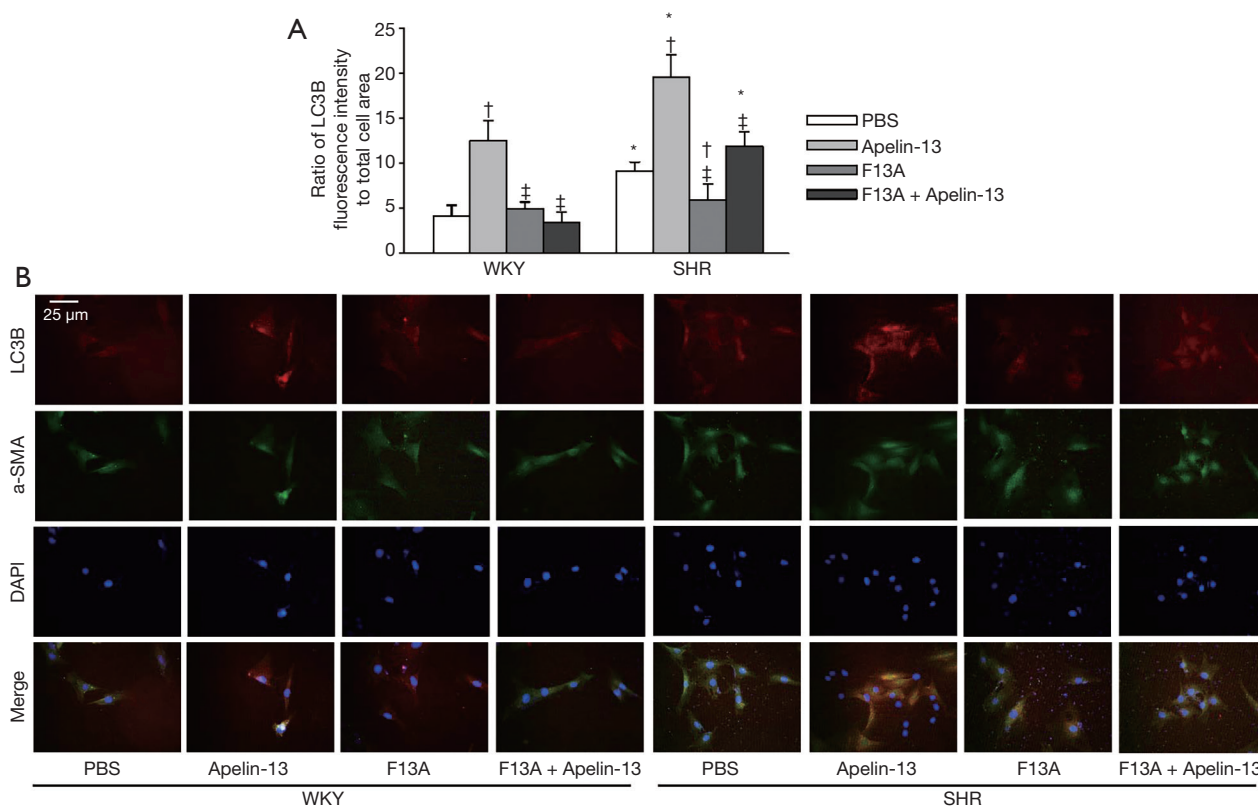


Figure 4 Effects of apelin-13 on LC3B expressions in VSMCs of WKY and SHR. LC3B expression was determined with immunofluorescence staining. The measurements were carried out 24 h after PBS, apelin-13 (1 μ M) or F13A (1 μ M) treatment. (A) Relative fluorescence intensity of LC3B normalized with cell area. (B) Representative images of triple immunofluorescence staining for LC3B (red), α -SMA (green) and DAPI (blue). The α -SMA and DAPI staining were respectively used to show VSMC cells and nuclei. Values are mean \pm SE. *, $P < 0.05$ vs. WKY; †, $P < 0.05$ vs. PBS; ‡, $P < 0.05$ vs. Apelin-13. $n = 5$. α -SMA, α -smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole.

as well as the cellular function. It is very important for waste removal and structural reconstruction in cells and is involved in the pathogenesis of hypertension (22). Generally, basal or mild autophagy level is beneficial for cellular homeostasis and cell survival, while moderate autophagy promotes cellular phenotype change, proliferation and migration, and excessive autophagy causes severe self-digestion and even cell death. Therefore, moderate or excessive autophagy is involved in vascular diseases including atherosclerosis and restenosis (41). We found that autophagy was enhanced in VSMCs of SHR, which was attenuated by blockade of APJ. The roles of apelin-13 in promoting VSMC autophagy of WKY and SHR were prevented by the blockade of APJ. More importantly, autophagy inhibitor attenuated VSMC proliferation of SHR, and prevents apelin-13-induced VSMC proliferation of both WKY and SHR. These

findings provide solid evidences that apelin/APJ activation promotes VSMC proliferation by activating VSMC autophagy in SHR, which is supported by the fact that TP53-induced glycolysis and apoptosis regulator inhibits autophagy of pulmonary artery smooth muscle cells to prevent pulmonary arterial hypertension (27).

Recent studies have shown the complicated signal pathways of apelin/APJ in VSMC proliferation of normal animals. Apelin in VSMCs activates PI3K/Akt, Egr-1, 14-3-3/Raf-1, and NOX4/ROS, which accelerates the conversion from G1 phase to S phase during mitosis, and transmits the signal from ERK to cyclin D1, and then enhances proliferation of VSMCs (15). Furthermore, PINK1/Parkin-mediated mitophagy stimulates apelin-13-induced VSMC proliferation via activation of AMPK α , and exacerbates atherosclerotic lesion (42), suggesting that the roles of

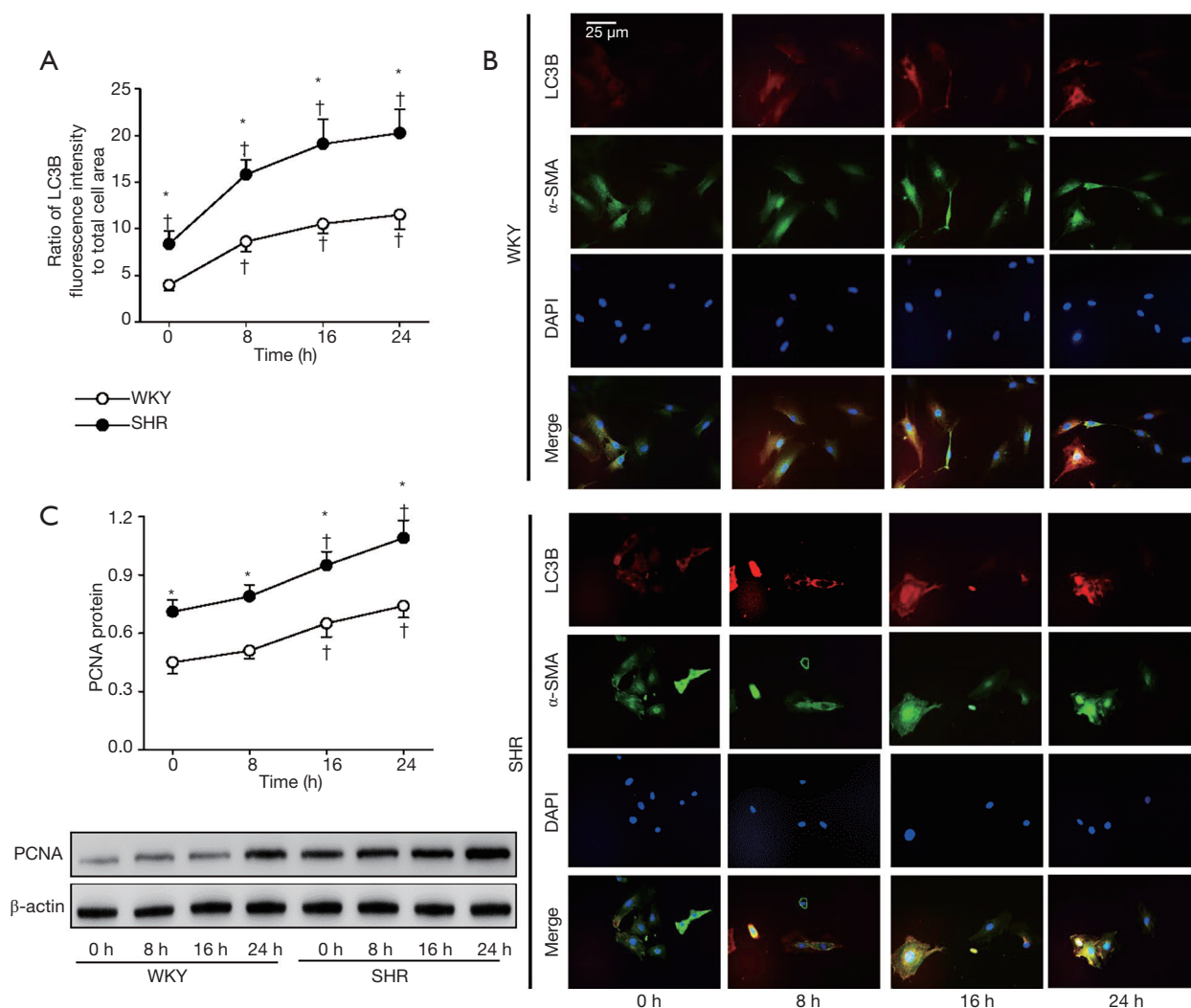


Figure 5 Time-effects of apelin-13 on LC3B and PCNA expressions in VSMCs of WKY and SHR. LC3B expression was determined with immunofluorescence staining. PCNA expression was examined with Western Blot. The measurements were respectively examined 0, 8, 16 and 24 h after apelin-13 (1 μ M) treatment. (A) Relative fluorescence intensity of LC3B normalized with cell area. (B) Representative images of triple immunofluorescence staining for LC3B (red), α -SMA (green) and DAPI (blue). The α -SMA and DAPI staining were respectively used to show VSMC cells and nuclei. (C) VSMC proliferation evaluated with PCNA protein expression. Values are mean \pm SE. *, $P < 0.05$ vs. WKY; †, $P < 0.05$ vs. 0 h. $n = 4$.

apelin in promoting VSMC proliferation is mediated by AMPK α /PINK1/parkin-autophagy signal pathway (15). However, the primary signal pathways of apelin/APJ in VSMC autophagy and proliferation in SHR remain further investigation.

Conclusions

Apelin-13 stimulates VSMC proliferation via APJ-

dependent autophagy activation in WKY and SHR. Increased APJ expression contributes to VSMC proliferation in SHR. Blockade of APJ inhibits VSMC autophagy and proliferation in SHR, and inhibition of autophagy attenuates VSMC proliferation in SHR and apelin-13-induced VSMC proliferation of both WKY and SHR. Intervention of APJ in VSMCs might be a strategy for attenuating VSMC proliferation and autophagy in hypertension.

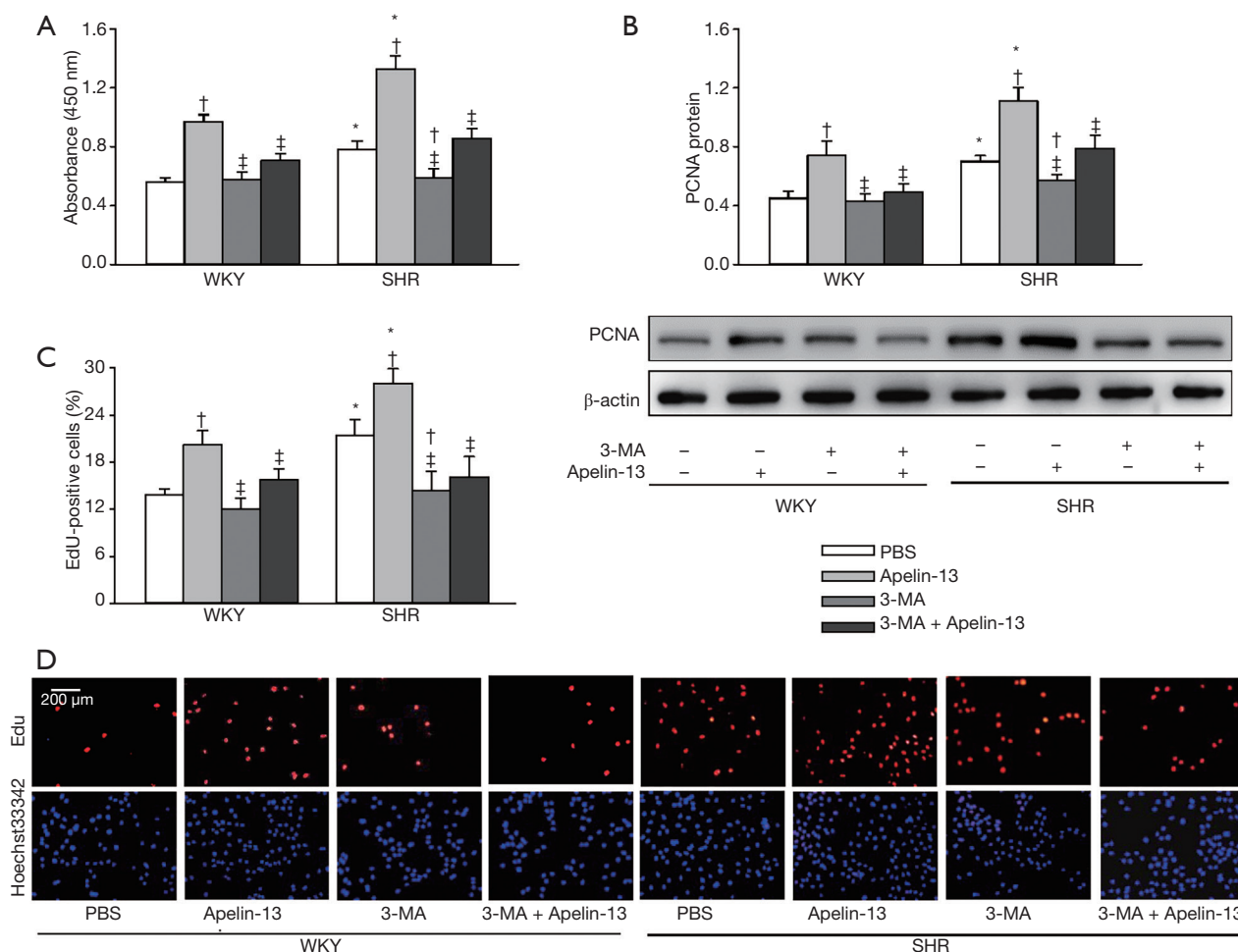


Figure 6 Effects of autophagy inhibitor 3-MA on apelin-13-induced VSMC proliferation of WKY and SHR. (A) VSMC proliferation evaluated with CCK-8 kit. (B) VSMC proliferation evaluated with PCNA protein expression. (C) VSMC proliferation evaluated with the percentage of EdU-positive cells. (D) Representative images of EdU staining. Red, EdU-positive cells. Blue, Hoechst 33342 was used to label double-stranded DNA and thus to visualize nuclei. The measurements were carried out 24 h after PBS, apelin-13 (1 μ M), or 3-MA (5 mM) treatment. Values are mean \pm SE. *, $P < 0.05$ vs. WKY; \dagger , $P < 0.05$ vs. PBS; \ddagger , $P < 0.05$ vs. Apelin-13. $n = 6$. 3-MA, 3-methyladenine.

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Footnote

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Data Sharing Statement: Available at <http://dx.doi.org/10.21037/atm-20-6891>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm-20-6891>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were

performed under a project license (No.: IACUC-1811017) granted by the Experimental Animal Care and Use Committee of Nanjing Medical University, in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, 8th edition, 2011).

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Table S1 Primers for real-time quantitative PCR analysis

	Primer	Sequences
Apelin	Forward	5'-TCTGGCTCTCCTTGACTG-3'
	Reverse	5'-AGGCATGGGTCCCTTATG-3'
APJ	Forward	5'-GGAAGATGATGGTTACAACACTAC-3'
	Reverse	5'-AGGCTGGCAATGAAGATG-3'
GAPDH	Forward	5'-AGGTCGGTGTGAACGGATTTG-3'
	Reverse	5'-TGTAGACCATGTAGTTGAGGTCA-3'

APJ, apelin receptor.

Table S2 Antibodies for Western blot or immunohistochemistry

	No.	Source
Anti-apelin	DF13350	Affinity Biosciences, Cincinnati, OH, USA
Anti-APJ	20341-1-AP	Protein Tech Group Inc, Chicago, IL, USA
Anti-PCNA	10205-2-AP	Protein Tech Group Inc, Chicago, IL, USA
Anti-GAPDH	60004-1-Ig	Protein Tech Group Inc, Chicago, IL, USA
Anti- β -actin	4970	Cell Signaling Technology, Beverly, MA, USA
Anti-LC3B	3868	Cell Signaling Technology, Beverly, MA, USA
Anti- α -SMA	48938	Cell Signaling Technology, Beverly, MA, USA
Anti-ATG5	12994	Cell Signaling Technology, Beverly, MA, USA
Anti-p62	5114	Cell Signaling Technology, Beverly, MA, USA

APJ, apelin receptor; PCNA, proliferating cell nuclear antigen; LC3B, microtubule-associated protein-1 light chain-3B; α -SMA, α -smooth muscle actin; ATG5, autophagy related protein 5.