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Regulation of Apelin and Its Receptor Expression in Adipose Tissues of Obesity Rats with Hypertension and Cultured 3T3-L1 Adipocytes

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Abstract: The apelin/APJ system has been implicated in obesity-related hypertension. We investigated the mechanism responsible for the pathogenesis of obesity-related hypertension with a special focus on the crosstalk between AngII/its type 1 receptor (AT1R) signaling and apelin/APJ expression. Sprague-Dawley rats fed a high-fat (obesity-related hypertension, OH) or normal-fat diet (NF) for 15 weeks were randomly assigned to one of two groups and administered vehicle or perindopril for 4 weeks. Compared to the NF rats, the OH rats showed lower levels of plasma apelin and apelin/APJ mRNAs of perirenal adipose tissues, and these changes were restored by perindopril. Administration of the AT1R antagonist olmesartan resulted in the restoration of the reduction of apelin and APJ expressions induced by AngII for 48 h in 3T3-L1 adipocytes. Among several inhibitors for extracellular signal-regulated kinases 1/2 (ERK1/2) PD98059, p38 mitogen-activated protein kinase (p38MAPK) SB203580 and phosphatidylinositol 3-kinase (PI3K) LY294002, the latter showed an additive effect on AngII-mediated inhibitory effects. In addition, the levels of p-Akt, p-ERK and p38MAPK proteins were decreased by long-term treatment with AngII (120 min), and these changes were restored by Olmesartan. Apelin/APJ appears to be impaired in obesity-related hypertension. The AngII inhibition-mediated beneficial effects are likely attributable, at least in part, to restoration of p38/ERK-dependent apelin/APJ expression in diet-induced obesity-related hypertension.

Key words: angiotensin II, apelin, hypertension, obesity, 3T3-L1 adipocytes

Introduction

The incidence and prevalence of obesity and overweight, soaring at alarming rates in industrialized countries and progressively increasing in the developing world, are pressing global health issues [1, 8]. Animal and clinical studies have shown that obesity is strongly associated with the development and aggravation of hypertension [4, 11, 28, 34], known as obesity-related hypertension (OH). Accumulated experimental evidence points to visceral obesity as the most important risk fac-

tor for hypertension and cardiovascular disease [2, 18, 25, 27]. Visceral adipose tissue is critical in the production and secretion of pathologic adipocytokines that are thought to mediate OH, such as renin, angiotensin, aldosterone, endothelin and nonesterified fatty acids (NEFAs) [2]. Changes in the types and levels of these adipocytokines contribute to the development of hypertension [2].

Apelin is a bioactive peptide originally isolated in 1998 from bovine stomach extracts [30]. Apelin was found to be an adipocytokine [3], and it has cardioprotec-

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tive properties [17, 20]. Apelin exerts a potent vasodilation effect and lowers blood pressure (BP) via a nitric oxide (NO)-dependent mechanism [16, 17, 31], and it inhibits angiotensin II (AngII)-mediated vasoconstriction through binding to its receptor, APJ [12]. The APJ receptor is a G-protein-coupled receptor that was identified in humans in 1993 [23]. APJ shares a close identity to the AngII type 1 receptor (AT1R) and named as APJ (putative receptor protein related to AT1), but does not bind AngII. The Apelin/APJ system has been shown to exert a potent hypotensive effect *in vivo* and to play a counter-regulatory role against the pressor action of AngII [14]. Lower levels of plasma apelin or levels of apelin/APJ mRNAs and protein have been observed in cardiovascular tissues in spontaneous hypertensive rats [39, 40], and essential hypertensive patients [24, 29] compared to normotensive controls, indicating the involvement of apelin in the pathophysiology of hypertension. Apelin was also implicated in the pathogenesis of OH [35].

The renin-angiotensin system (RAS) is important in BP control, and the results of an animal study suggest the activation of the systemic and adipose RAS with hypertension [4]. Diet-mediated regulation of AngII production-related gene expression (including angiotensinogen and AngII-converting enzyme) in adipose tissue has been demonstrated, with elevations in obesity and reductions in starvation [4, 10, 33]. One previous study demonstrated that the cardiac apelin mRNA is decreased in AngII-infused rats, and its restoration was achieved by treatment with angiotensin type 1 receptor blockers (ARBs) [15], suggesting that the AngII/AT1R signaling pathway is involved in the down-regulation of apelin/APJ gene expressions in the cardiovascular system.

Here, with *in vivo* and *in vitro* investigations, we examined the mechanism responsible for the pathogenesis of diet-induced obesity-related hypertension, with a special focus on the relationship between the AngII/AT1R signaling pathway and apelin/APJ expression.

Materials and Methods

Reagents

AngII was purchased from Sigma-Aldrich Corporation (St. Louis, MO). Olmesartan was donated by Daiichi Sankyo (Tokyo, Japan). Perindopril was purchased from Servier Pharmaceutical Co. (Tianjing, China). Phosphatidylinositol 3-kinase (PI3K) LY294002, extracellular

signal-regulated kinases 1/2 (ERK1/2) PD98059, and p38 mitogen-activated protein kinase (MAPK) SB203580 were obtained from Calbiochem, EMD Chemicals (San Diego, CA). Primary antibodies for phospho-Akt (Ser473), Akt, phospho-ERK1/2, ERK1/2, phospho-p38 MAPK, p38 MAPK and β -actin were purchased from Cell Signaling Technology (Beverly, MA).

Animals and treatment

Thirty-three male Sprague-Dawley rats (3 weeks of age, 37–51 g body weight (BW); SLACCAS, Shanghai, China) were housed in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) with a 12-h light–dark cycle. All experimental procedures were performed under protocols approved by the Shanghai Jiao Tong University Ethics Committee for the use of laboratory animals, according to the National and Institutional Guidelines for Animal Care and Use. Rats were firstly randomly separated into two groups: the normal-fat diet group (NF group, NF diet, containing [weight%]: 5.28% fat, 22.1% protein, 52% carbohydrates; SLACCAS, n=16) and the high-fat diet group (OH group, HF diet, containing [weight%]: 23% fat, 21.7% protein, 39.8% carbohydrates; SLACCAS, n=17). The high-fat diet per 100 gram is made of 57.3 gram of normal-fat rat diet, and supplemented with 20 gram lard, 10 gram sucrose, 9 gram casein, and 2 gram maltodextrin. All animals had access to chow and water *ad libitum*.

Obesity-related hypertensive (OH) rats were established by raising HF-group rats to 18 weeks of age. Then, 18-week-old NF group and OH group rats were randomly assigned to one of four groups as follows: NC (normal-fat diet group rats, n=8), NC-P (NC rats treated with an angiotensin-converting enzyme inhibitor, ACEI, perindopril, n=8), OH (obesity-related hypertensive rats, n=7) and OH-P (OH rats treated with perindopril, n=7). The NC and OH groups were administered water, whereas the OH-P and NC-P groups were administered perindopril at 1 mg/kg of BW per day, in water.

Systolic BP (SBP) was determined by using a tail-cuff pressure analysis system (BP-98A, Softron Beijing Inc., Beijing, China). Blood pressure was measured under conscious conditions, and the average of 3 pressure readings was recorded for each measurement. After 4 weeks' treatment (at the age of 22 weeks), all rats were fasted overnight and then euthanized with an intraperitoneal injection of pentobarbital (50 mg/kg). Blood was collected from the abdominal aorta for biological analysis.

Table 1. Primers sequences used in quantitative real-time RT-PCR (M: mouse; R: rat)

Gene	GenBank No.	Forward primer	Reverse primer	Amplification (bp)
Apelin (M)	NM_013912.3	CGAGTTGCAGCATGAATCTGAG	TGTTCCATCTGGAGGCAACATC	107
APJ (M)	NM_011784.3	GCATTATCGTGGTGTCTGTAGTGA	GCAAAGTCCCAGCATGTAGA	87
18sRNA (M)	NM_011296	TTCTGGCCAACGGTCTAGACAAC	CCAGTGGTCTTGGTGTGCTGA	127
Apelin (R)	NM_031612.2	CAGGCCTATCCCAGGCTCA	CAAGATCAAGGGCCCAGTCAA	116
APJ (R)	NM_031349.2	CCTGGCTTGATGCAGTTGGA	TCTGGCCTGAGACATGCAGAG	126
18sRNA (R)	NM_213557	AAGTTTCAGCACATCCTGCCAGTA	TTGGTGAGGTCAATGTCTGCTTTC	140

The visceral fat (perirenal, epididymal, and mesenteric fat pads) was excised, weighed, and stored in liquid N₂ for biological analysis.

Cell culture

Mouse 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 10% fetal bovine serum (FBS), and 1% (v/v) antibiotics in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Differentiated 3T3-L1 adipocytes were generated according to the method of Fernandez-Galilea *et al.* [9] with a minor modification. Briefly, two days post-confluence (day 0), preadipocytes were induced to differentiate into adipocytes by culturing them for 72 h in DMEM containing 4.5 g/l glucose, 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin, 1 µmol/l dexamethasone, 0.5 mmol/l isobutylmethylxanthine (IBMX) and 10 µg/ml insulin (Sigma-Aldrich). At day 3, the cells were cultured in DMEM containing 10% FBS and insulin for another 48 h. Then at day 5, the media were replaced with 10% FBS and antibiotics, without insulin, and changed every 2 days up to day 9 when the cells were considered to be differentiated into mature adipocytes. After being cultured in serum-free DMEM for 24 h, the mature 3T3-L1 adipocytes were subjected to different treatments and analyses.

Quantitative real-time PCR

Total RNA was extracted from perirenal fat deposits and 3T3-L1 cells according to the Trizol Reagent procedure (Invitrogen Life Technologies, Carlsbad, CA) and was subjected to reverse transcription with a SuperScript RT-PCR system (Invitrogen). The resulting cDNA was subjected to quantitative real-time PCR analysis with primers specific for apelin and APJ (Table 1) and with the use of a Bio-Rad CFX96™ Real-Time PCR Detec-

tion System and Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) as described [6]. Changes in gene expression were normalized to 18sRNA and calculated by the 2^{-ΔΔCt} method. All samples were analyzed in triplicate.

Western blot analysis

Total extracted protein was separated with 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Nonspecific binding was blocked with 5% skim milk/Tris-buffered saline with Tween for 1 h at room temperature and then incubated with primary antibodies (diluted 1:1,000) at 4°C overnight. β-actin was loaded as control. The band intensity was analyzed by densitometry using Image J software.

Apelin and angiotensin II assay

Cell-cultured supernatants and plasma levels of apelin were quantified with apelin-12 EIA Kits (Phoenix Pharmaceuticals, Burlingame, CA) following the manufacturer's instructions. Plasma AngII was measured with a commercially available radioimmunoassay kit (BNIBT, Beijing, China).

Statistical analysis

Data are expressed as mean ± S.D. Student's *t*-test (for comparisons of two groups) or one-way ANOVA (for comparisons of ≥3 groups) followed by Bonferroni post hoc tests were used for the statistical analyses. For the animal study, an ANOVA of repeated measurement data was used for the comparison of the two means of BW and BP between the NF and HF groups. Pearson's correlation analysis was performed to screen potential associations among variables. All statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL). A *P*-value < 0.05 was considered significant.

Table 2. Characteristics of the NC, NC-P, OH, and OH-P rats after 4 weeks of perindopril treatment

	NC	NC-P	OH	OH-P
Body weight (g)	627.4 ± 20.7	612.5 ± 15.5	731.9 ± 30.3**	690.0 ± 17.6##
SBP (mmHg)	137.0 ± 4.2	106.6 ± 8.9**	160.6 ± 7.2**	98.2 ± 6.8##
Heart rate (bpm)	386 ± 38	375 ± 40	393 ± 31	371 ± 46
Perirenal fat (g)	9.0 ± 0.3	8.9 ± 0.2	12.9 ± 1.7**	11.0 ± 1.2
Epididymal fat (g)	6.8 ± 0.4	6.8 ± 0.4	30.3 ± 3.2**	27.1 ± 1.6
Mesenteric fat (g)	4.8 ± 0.1	4.8 ± 0.1	8.9 ± 1.2**	7.7 ± 0.6
Total visceral fat (g)	20.7 ± 0.6	20.6 ± 0.6	52.1 ± 3.9**	45.9 ± 2.4#
Visceral fat/BW	0.033 ± 0.001	0.034 ± 0.002	0.071 ± 0.005**	0.067 ± 0.002

NC: normal control, n=8; NC-P: normal control rats treated with perindopril, n=8; OH: obesity-related hypertensive rats, n=7; OH-P: obesity-related hypertensive rats treated with perindopril, n=7. Data are mean ± S.D. ** $P < 0.01$ vs. NC; # $P < 0.05$, ## $P < 0.01$ vs. OH.

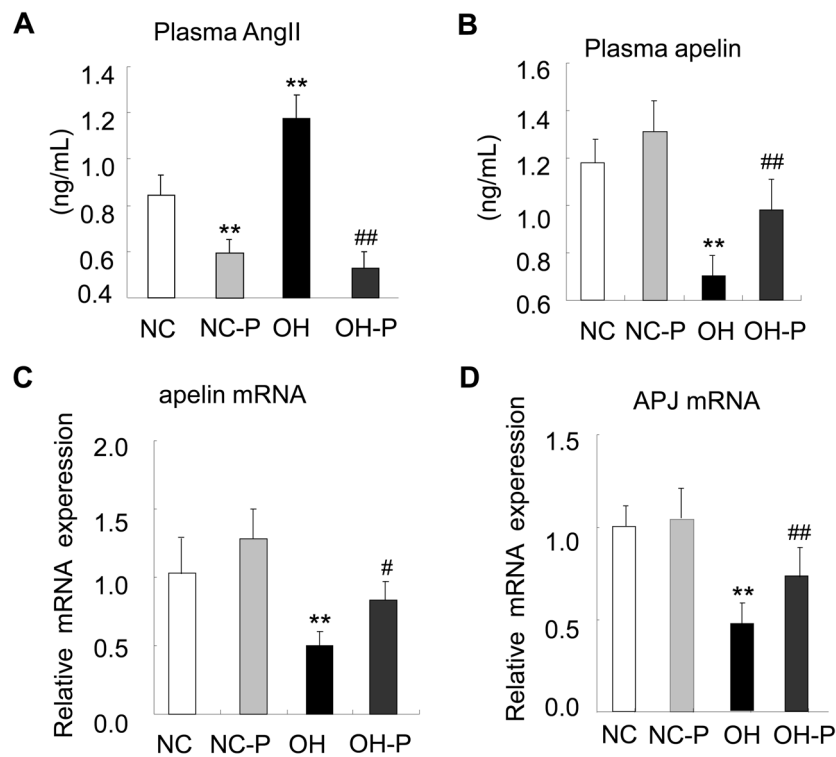


Fig. 1. Effects of perindopril on the levels of plasma AngII (A) and plasma apelin (B), and apelin (C) and APJ (D) mRNAs expression in adipose tissue of OH rats. The plasma AngII levels were measured by RIA and plasma apelin by ELISA analysis. C and D, quantitative real-time PCR was used to detect apelin/APJ mRNAs of perirenal adipose tissue. NC: normal control rats, n=8; NC-P: normal control rats treated with perindopril, n=8; OH: obesity-related hypertensive rats, n=7; OH-P: obesity-related hypertensive rats treated with perindopril, n=7. Data are mean ± S.D. of two replicates. ** $P < 0.01$ vs. NC; # $P < 0.05$, ## $P < 0.01$ vs. OH.

Results

Obesity-related hypertensive (OH) rats were established

At the age of 18 weeks, the rats fed the HF diet showed much higher BW and SBP levels compared to the control rats fed the normal diet (BW: 660.8 ± 54.3 vs. 567.1 ±

14.9 g; SBP: 152.7 ± 8.7 vs. 132.5 ± 3.2 mmHg, respectively; $P < 0.01$). Any reading of SBP higher than 140 mmHg was considered hypertension. In this study, 3 of 17 rats fed the high-fat diet did not develop hypertension. These three rats were then removed from the OH group.

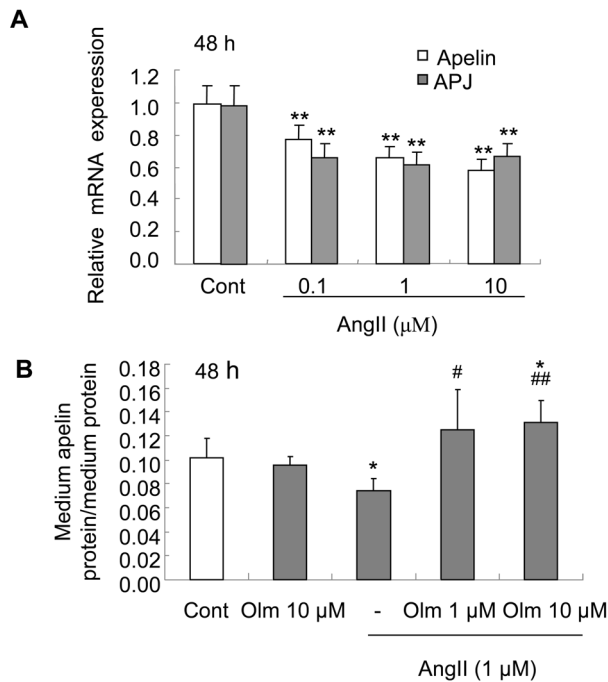


Fig. 2. Effects of olmesartan on AngII-induced apelin and APJ expressions in cultured 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were subjected to serum-free culture for 24 h before treatments. (A) Real-time PCR showed apelin/APJ mRNAs levels of the 3T3-L1 adipocytes incubated with AngII at indicated concentrations for 48 h. (B) ELISA showed the levels of apelin protein in the cultured medium of 3T3-L1 adipocytes treated with AngII (1 μmol/l) in the absence (–) or presence of olmesartan (1 or 10 μmol/l) for 48 h. Results are the means ± S.D. of three or more independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. corresponding controls. # $P < 0.05$, ## $P < 0.01$ vs. AngII (1 μmol/l). Cont: control; AngII: angiotensin II; Olm: olmesartan; μM: μmol/l.

Effects of perindopril on apelin and APJ expression in OH rats

At the age of 22 weeks, the OH rats still showed not only higher BW and SBP values, but also more visceral fat (including perirenal fat, Epididymal fat, mesenteric fat, total visceral fat weight and total visceral fat/BW) compared to the NC rats ($P < 0.01$; Table 2). Perindopril significantly reduced the BW, BP and total visceral fat in the OH rats compared to the untreated OH rats ($P < 0.05$ or $P < 0.01$). There was no significant difference in heart rate among the four experimental groups (Table 2).

The OH rats had significantly higher plasma AngII levels than the NC rats ($P < 0.01$, Fig. 1A). The levels of plasma AngII were dramatically decreased in both the NC-P and OH-P groups compared to the respective control

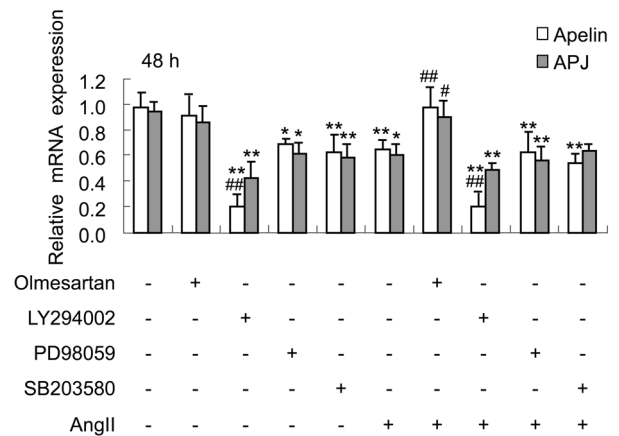


Fig. 3. Effects of several signaling pathway inhibitors on AngII-mediated apelin/APJ mRNA expression in cultured 3T3-L1 adipocytes. Following culture in serum-free for 24 h, the differentiated 3T3-L1 adipocytes were treated with AngII (1 μmol/l) in the absence (–) or presence (+) of several signaling pathway inhibitors (olmesartan to AT1R, LY294002 to PI3K, PD98059 to ERK1/2, and SB203580 to p38MAPK, each at 10 μmol/l, added 30 min before AngII treatment) for 48 h. Quantitative real-time PCR was used to detect apelin/APJ mRNAs levels. Results are the means ± S.D. of four or more independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$ vs. control. # $P < 0.05$, ## $P < 0.01$ vs. AngII (1 μmol/l).

groups (NC and OH rats; $P < 0.01$, Fig. 1A). Compared to the NC rats, the OH rats had lower levels of plasma apelin and apelin/APJ mRNAs of perirenal adipose tissues, and these changes were restored by perindopril treatment ($P < 0.05$ or $P < 0.01$; Fig. 1B, C, D). However, perindopril showed no effect on these parameters in the NC rats. The Pearson correlation analysis showed that the levels of plasma apelin were negatively correlated with BW, BP and plasma AngII ($r = -0.852, -0.498, -0.62$, respectively; $P < 0.01$) and were positively correlated with the apelin mRNA levels of the perirenal fat tissue ($r = 0.761, P < 0.01$).

Effects of olmesartan on AngII-induced apelin and APJ expressions in cultured 3T3-L1 adipocytes

The quantitative PCR revealed that AngII treatment for 48 h reduced the levels of apelin and APJ mRNAs of cultured 3T3-L1 adipocytes in a dose-dependent manner ($P < 0.01$, Fig. 2A). ELISA showed that the level of apelin protein was reduced in the condition medium of 3T3-L1 cells treated with AngII for 48h, and this change was restored by olmesartan treatment (1 or 10 μmol/l, $P < 0.05$ or $P < 0.01$, Fig. 2B). Next, we tested several signaling pathway inhibitors including PI3K inhibitor

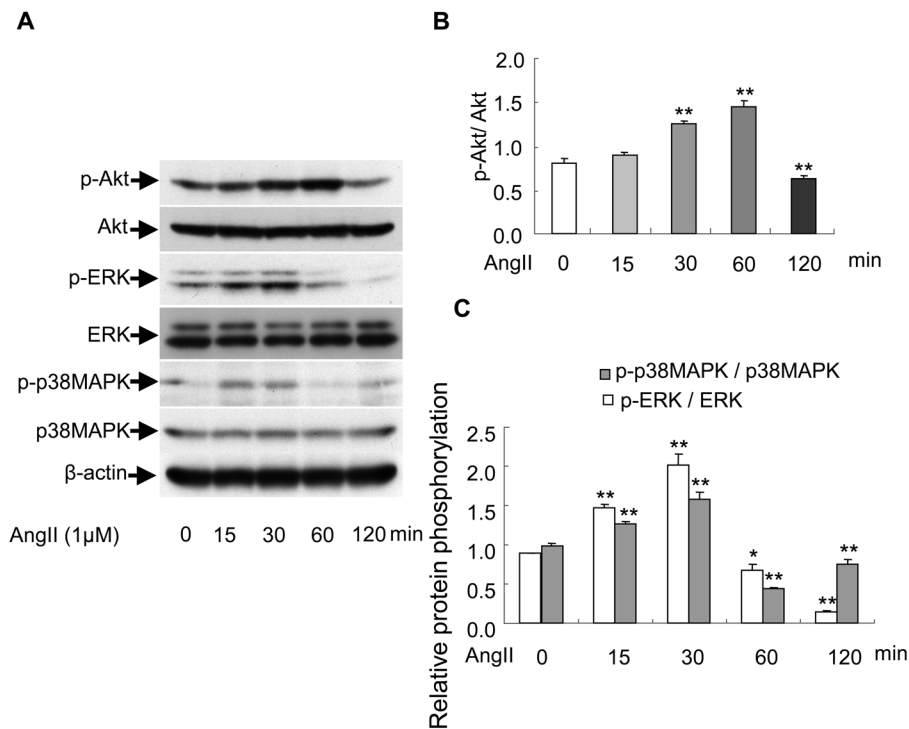


Fig. 4. Effects of AngII on the phosphorylation of Akt, ERK1/2 and p38MAPK in cultured 3T3-L1 adipocytes. A, B and C, Representative images of Western blots (A) combined with quantitative data of the levels of p-Akt (Ser-473), p-ERK1/2, and p-p38MAPK (B and C) induced by AngII (1 $\mu\text{mol/l}$) at indicated time points. Results are the means \pm S.D. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. corresponding controls.

LY294002 (10 $\mu\text{mol/l}$), ERK1/2 inhibitor PD98059 (10 $\mu\text{mol/l}$), and p38MAPK inhibitor SB203580 (10 $\mu\text{mol/l}$) in the presence and absence of AngII (1 $\mu\text{mol/l}$) for 48 h. As shown in the Figure 3, pretreatment with the AT1R antagonist olmesartan (10 $\mu\text{mol/l}$) diminished the inhibitory effect of AngII (1 $\mu\text{mol/l}$) on apelin/APJ mRNAs expressions ($P < 0.05$ or $P < 0.01$). Even though at the basal condition, SB203580 and PD98059 had a comparable inhibitory effect on both gene expressions, neither PD98059 nor SB203580 had an additive effect on the gene expressions in the presence of AngII. LY294002 almost completely suppressed the apelin mRNA expression, not only in absence but also in the presence of AngII, by 80%.

Effects of olmesartan on AngII-mediated phosphorylation of Akt, ERK1/2 and p38MAPK in cultured 3T3-L1 adipocytes

We first examined AngII-mediated time-dependent effects on the phosphorylation of Akt, ERK1/2 and p38MAPK. The quantitative Western blotting analysis

revealed that 1 $\mu\text{mol/l}$ of AngII increased the levels of p-Akt, p-ERK1/2 and p-p38MAPK in a time-dependent manner (Fig. 4). The level of p-Akt (Ser-473) reached a peak at 60 min after AngII treatment, and the levels of both p-ERK1/2 and p-p38MAPK reached a peak at 30 min.

To further explore the potential role of olmesartan on AngII-mediated phosphorylation of Akt, ERK1/2 and p38MAPK in adipocytes, following pretreatment with olmesartan for 30 min, we then treated the adipocytes with AngII for a long term (120 min) for analysis. As expected, we found that the levels of p-Akt, p-ERK and p38MAPK were reduced by the long-term AngII treatment, and that these levels were restored by olmesartan (10 $\mu\text{mol/l}$; $P < 0.05$ or $P < 0.01$, Fig. 5).

Effect of AngII on the apelin secretion in cultured 3T3-L1 adipocytes

Lastly, we performed apelin ELISAs to examine the effect of AngII on apelin secretion in 3T3-L1 adipocytes (Fig. 6). Interestingly, AngII (1 $\mu\text{mol/l}$) treatment for 1

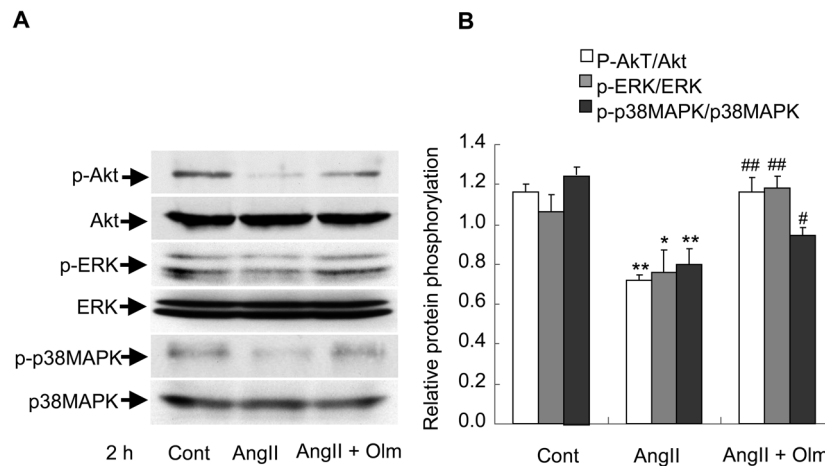


Fig. 5. Effects of olmesartan on the phosphorylation of Akt, ERK1/2 and p38MAPK in response to long-term AngII treatment in cultured 3T3-L adipocytes. After pretreatment with or without olmesartan (10 $\mu\text{mol/l}$) for 30 min, the cells were incubated with AngII (1 $\mu\text{mol/l}$) for an additional 120 min. A and B, Representative images of Western blots (A) and combined with quantitative levels of p-Akt (Ser-473), p-ERK1/2 and p-p38MAPK (B). Results are the means \pm S.D. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. corresponding controls; # $P < 0.05$, ## $P < 0.01$ vs. AngII (1 $\mu\text{mol/l}$).

h increased the levels of apelin in the cultured media compared to the control group ($P < 0.01$, Fig. 6). Brefeldin A (a Golgi inhibitor, 5 $\mu\text{g/ml}$) significantly reduced the basal secretion of apelin ($P < 0.05$), whereas LY294002 (10 $\mu\text{mol/l}$) had no significant effect on apelin secretion. In addition, pretreatment with Brefeldin A or LY294002 did not change the AngII-induced increase in apelin secretion in the media.

Discussion

Several important observations can be made on the basis of the present study's results. First, the levels of plasma apelin and apelin/APJ mRNAs of perirenal adipose tissue were reduced in obesity-related hypertensive rats fed a high-fat diet, and these changes were restored by AngII inhibition with ACE inhibitor treatment with perindopril. Second, long-term treatment with AngII down-regulates Apelin and APJ expressions through the inhibition of the p38/ERK signaling pathway in cultured 3T3-L1 cells, and this effect was also restored by AT1R antagonism with olmesartan. The supposed mechanisms underlying the chronic effect of AngII on apelin expression in adipose tissue are schematically represented in Fig. 7.

Previous studies indicate that visceral adipose tissue

is critical in the production of pathologic adipocytokines, and changes in the types and levels of adipocytokines lead to the development of hypertension [2]. The ability of diet-induced obesity to decrease plasma or/and adipose tissue apelin/APJ levels is likely to contribute to the development of hypertension under experimental conditions. Here, we observed that the levels of both plasma apelin and apelin/APJ mRNAs of perirenal adipose tissue were significantly reduced in the OH rats. Our observations reveal that the levels of plasma apelin were negatively correlated with BW and SBP, and positively correlated with the apelin mRNA levels of perirenal fat tissue. It was reported that circulating apelin levels were decreased in patients with essential hypertension [24, 29]. Because the apelin/APJ system exerts potent vasodilation, we propose that diet-induced OH may be due to the impairment of systemic and adipose tissue apelin/APJ-mediated hypotensive action. It should be noted that the data from a few clinical studies showed an increase in circulating apelin in obese patients compared to normal-weight controls [5, 13]. This discrepancy between previous clinical studies and the present animal study might be attributable to differences in obesity stages or to obesity with complications such as hypertension and heart failure. This notion was supported by a 2011 finding from the Przewlocka-Kosmala

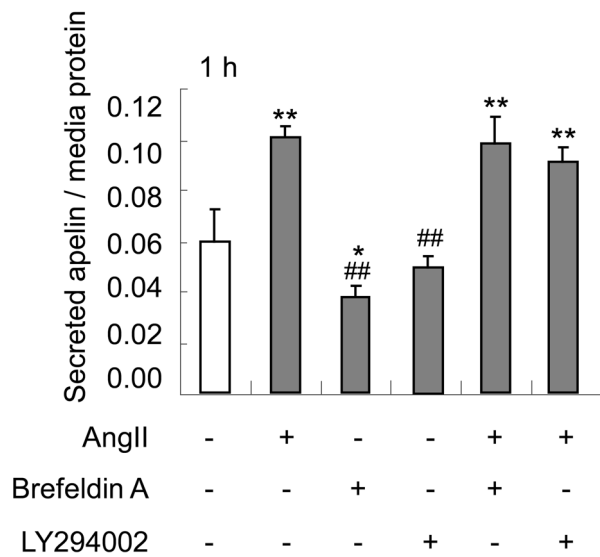


Fig. 6. Treatment with AngII for 1 h stimulated apelin secretion via a vesicle-releasing pathway. 3T3-L1 adipocytes were cultured serum-free for 24 h, and then treated with AngII (1 $\mu\text{mol/l}$) for 1 h with (+) or without (-) pretreatment with the Golgi inhibitor Brefeldin A (5 $\mu\text{g/ml}$), or LY294002 (10 $\mu\text{mol/l}$) for 30 min. The apelin secretion in cultured media was detected by ELISA. Results are the means \pm S.D. of three or four independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control. ## $P < 0.01$ vs. the AngII group.

group that obese patients with hypertension and heart failure had lower levels of plasma apelin compared to control patients [24]. Further studies are necessary to investigate this issue.

Accumulating evidence shows an activation of the systemic and local adipose RAS in animals with obesity and hypertension [4]. Animal and human adipose tissues have been shown to possess all of the components necessary for the production of AngII, including angiotensinogen, renin-like activity, and ACE [4]. In the present study, obesity increased the plasma AngII concentration, and the ability of obesity to induce hypertension was abrogated by ACE inhibitor treatment. AngII inhibition also showed an improvement of decreased plasma apelin levels and the apelin-APJ gene expressions in local adipose tissue. We further demonstrated that AT1R antagonism restored the AngII-induced down-regulation of apelin-APJ gene expressions in cultured 3T3-L1 cells. Thus, diet-induced obesity appears to impair the apelin/APJ-mediated vascular beneficial effect through its ability to activate systemic and local adipose tissue RAS. It was reported that AngII-infusion decreased apelin mRNA expression in the cardiac tissue, and this change was

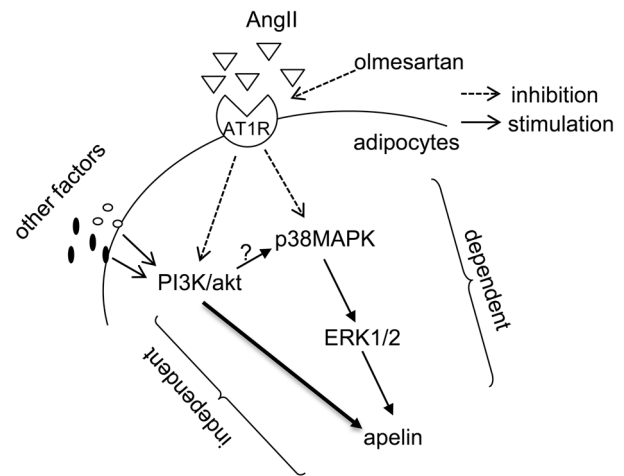


Fig. 7. Proposed mechanism of chronic inhibitory effect of AngII on apelin expression in adipose tissue.

restored by olmesartan [15]. Taken together, these findings suggest that AngII inhibition-mediated beneficial effects are likely attributable, at least in part, to improvement of the impaired apelin/APJ system associated with an activation of systemic or/and adipose tissue RAS in diet-induced OH.

The PI3K/Akt signaling pathway was reported to be involved in apelin synthesis and/or secretion in 3T3-L1 adipocytes [21]. The direct stimulation of apelin by insulin is clearly associated with the activation of PI3K/Akt and MAPK pathways [3]. The signaling pathways of inflammatory cytokine for the induction of apelin were dependent on PI3K, JNK and MAPK but not protein kinase C activation in 3T3F442A adipocytes [7]. Iwanaga *et al.* reported that the apelin/APJ gene levels were decreased by AngII-infusion in hypertrophied and failing hearts of rats, and the changes were improved by AT1R antagonism [15]. Here we observed that olmesartan prevented the AngII-mediated down-regulation of apelin/APJ expressions in cultured 3T3-L1 adipocytes. Furthermore, olmesartan improved the long-term AngII intervention (120 min)-mediated reduction of the p-ERK1/2 and p-p38MAPK levels in adipocytes (Fig. 5). These findings suggest that the signaling pathways of AngII for the down-regulation of apelin/APJ gene expressions may be dependent on down-regulated p38/ERK1/2 signaling pathways in adipose tissue and adipocytes. This conception was further supported by observation that both p38MAPK inhibitor SB203580 and ERK1/2 inhibitor PD98059 had no additive effects on apelin/APJ gene

expressions suppressed by AngII treatment alone. It should be noted that olmesartan also improved the long-term AngII treatment (120 min)-mediated reduction of the p-Akt levels in adipocytes. Taken together with the observation that AngII-mediated inhibitory effect on apelin-APJ expression was significantly higher in the presence of the PI3K inhibitor than in the presence of the p38 or ERK1/2 inhibitor, these findings suggest that there are p38/ERK1/2-dependent and -independent signaling pathways in apelin/APJ expression in adipocytes.

Some adipocytokines have been shown to be synthesized and stored in secretory vesicles in adipocytes, including adiponectin, leptin and resistin [37, 38]. Our present study revealed that short-term treatment with AngII for 1 h increased the levels of apelin in the cultured media, raising the possibility that apelin is secreted from a pre-existing pool present in mature adipocytes in the acute response to AngII (Fig. 6), although we had no related direct evidence. This notion was further supported by the recent findings of Than *et al.* that AngII (1 $\mu\text{mol/l}$ or 10 $\mu\text{mol/l}$) stimulated acute apelin secretion at 30 min and 2 h [32]. There are two secretory pathways for proteins secreted from the cytosol to the extracellular space, including a constitutive secretory pathway and a regulated secretory pathway [19]. The Golgi inhibitor Brefeldin A decreased the basal secretion of apelin, indicating that the Golgi system might be involved in the constitutive secretion of apelin in adipocytes. There is a storage form of apelin in cardiomyocytes [26]. Based on these findings, we favor the hypothesis that short-term treatment with AngII may trigger apelin secretion via a regulated vesicle-releasing pathway. This was further supported by our observation that pretreatment with Brefeldin A had no effect on the AngII-induced secretion of apelin in adipocytes. Furthermore, we found that pretreatment with LY294002 did not affect the apelin secretion in response to the short-term AngII treatment. Recently, a single study has reported that AngII stimulated acute apelin secretion through MAPK/ERK pathway [32]. Since the short-term treatment with AngII promoted the phosphorylation of p38MAPK and ERK1/2 in cultured 3T3-L1 cells (Fig. 4), it is possible to speculate that the signaling pathway of the AngII-mediated acute action for the induction of apelin secretion might be dependent on an ERK1/2 signaling pathway but not a PI3K/Akt pathway.

The apelin/APJ system was demonstrated to exert a potent hypotensive effect [17, 31]. Our present findings

provide evidence that there is cross-talk between AngII/AT1R signaling and the apelin/APJ system in 3T3-L1 adipocytes. The AngII inhibition-mediated beneficial effects are likely attributable, at least in part, to restoration of the impaired apelin/APJ system in diet-induced OH. Thus, our results provide new evidence for the benefits of RAS inhibitors in the treatment of hypertension with obesity.

Limitation

Several limitations of the present study should be pointed out. First, the sample size of animals for each experimental group was small. Second, the calculation of the food intake was not designed into this study protocol. Although a treatment with ACE inhibitor for a short period of time (around 4 weeks) does not affect a food intake in rodents fed a normal-fat or high-fat diet in some studies [22, 36], the effect of perindopril on the food intake in this experiment will be needed to investigate in future. Third, different angiotensin inhibitors were applied to *in vivo* (perindopril, ACE inhibitor) and *in vitro* study (olmesartan, ARB). As known, ACE inhibitor has been shown to be not quite suitable for cell experiments. Here, we have used an AT1R antagonist olmesartan in 3T3-L1 cell experiments. Fourth, with the exception of the perirenal fat, other visceral fats including epididymal and mesenteric fat pads were not studied for targeted gene expressions. Further investigation will be needed to study this issue. Fifth, since the concentration of angiotensin II in culture experiments (1 $\mu\text{mol/l}$) was 1,000 times high compared to the *in vivo* concentration (about 1 nmol/l), results of *in vitro* experiments may merely show a possible mechanism. Further studies are required to explain the *in vivo* findings by using a lower concentration of angiotensin II *in vitro* studies.

Disclosures

The authors have no conflict of interest to declare.

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