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<td>Than, Aung; Leow, Melvin Khee-Shing; Chen, Peng</td>
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Control of Adipogenesis by the Autocrine Interplays between Angiotensin (1-7) / Mas receptor and Angiotensin II / AT₁ receptor Signaling Pathways

Aung Than¹, Melvin Khee-Shing Leow²³⁴, Peng Chen¹

¹Division of Bioengineering, Nanyang Technological University, 70 Nanyang Drive, Singapore 637457
²Singapore Institute for Clinical Sciences, Brenner Center for Molecular Medicine, 30 Medical Drive, Singapore 117609
³Endocrine and Diabetes, Tan Tock Seng Hospital, 11 Jalan Tan Tock Seng, Singapore 308433
⁴Duke-NUS Graduate Medical School, 8 College Road, Singapore 169857

*Running title: Ang(1-7) and AngII regulations on Adipogenesis

To whom correspondence should be addressed: Dr. Peng Chen, Division of Bioengineering, Nanyang Technological University, Singapore 637457; Tel: (+65) 6514 1086; E-mail: chenpeng@ntu.edu.sg.

Keywords: Ang(1-7), Mas receptor, AngII, AT₁ receptor, ACE2, Adipogenesis.

Background: The autocrine regulation of Ang(1-7) on adipogenesis is unknown.

Results: The autocrine counteractive interplays between Ang(1-7)-Mas and AngII-AT₁ signaling on adipogenesis are revealed.

Conclusion: The Ang(1-7)-Mas activation stimulates adipogenesis and antagonizes the anti-adipogenic effect of AngII-AT₁ activation.

Significance: Angiotensin system in adipose tissue may serve as a potential therapeutic target for obesity and related metabolic disorders.

SUMMARY

Angiotensin II (AngII), a peptide hormone released by adipocytes, can be catabolized by adipose angiotensin-converting enzyme 2 (ACE2) to form Ang(1-7). Co-expression of AngII receptors (AT₁ and AT₂) and Ang(1-7) receptors (Mas) in adipocytes implies the autocrine regulation of local angiotensin system on adipocyte functions, through yet unknown interactive mechanisms. In the present study, we reveal the adipogenic effects of Ang(1-7) through activation of Mas receptor and its subtle interplays with the anti-adipogenic AngII-AT₁ signaling pathways. Specifically, in human and 3T3-L1 pre-adipocytes, Ang(1-7)-Mas signaling promotes adipogenesis via activation of PI3 kinase/Akt and inhibition of MAPK kinase/ERK pathways. And Ang(1-7)-Mas antagonizes the anti-adipogenic effect of AngII-AT₁ by inhibiting AngII-AT₁ triggered MAPK kinase/ERK pathway. The autocrine regulation of AngII/AT₁ - ACE2 - Ang(1-7)/Mas axis on adipogenesis has also been revealed. This study suggests the importance of the local regulations of the delicately-balanced angiotensin system on adipogenesis, and its potential as a novel therapeutic target for obesity and related metabolic disorders.

INTRODUCTION

The renin-angiotensin system (RAS) critically regulates homeostasis in various body systems (1, 2). Angiotensin II (AngII), the major bioactive component of RAS, is converted from the precursor molecule angiotensinogen (AGT) through two-step hydrolysis by renin and angiotensin-converting enzyme (ACE) (2). It acts through AngII type 1 (AT₁) and AngII type 2 (AT₂) receptors (3). AngII can be further hydrolyzed by ectoenzyme ACE2 into Ang(1-7) (4). Mediated by its interaction with the G-protein-coupled receptor Mas (Mas receptor) (5), Ang(1-7) usually antagonizes the AngII actions (6).

RAS is also a crucial regulator of energy metabolism, implicated in metabolic disorders such as obesity and insulin resistance (7-9). Adipose tissue, a highly active metabolic and
endocrine organ, is a source of components of the RAS (10, 11). On the other hand, AngII receptors and Mas receptors are co-expressed on adipocytes, implying the involvement of the local RAS system in regulating adipocyte functions (12, 13). Several lines of evidences have supported this emerging hypothesis. In particular, recent investigations start to reveal that ACE-AngII-AT₁ signaling and ACE2-Ang(1-7)-Mas signaling regulate various functions of adipocytes in an intriguing counteractive manner (6). For example, it has been demonstrated that AngII-AT₁ inhibits lipolysis and insulin-induced glucose uptake in adipocytes (14, 15) while Ang(1-7)-Mas does the opposite (16, 17). Also AngII-AT₁ induces oxidative stress in adipocytes (18) whereas Ang(1-7)-Mas interaction suppresses it (17).

Previous studies have established the inhibitory effects of AngII-AT₁ on adipogenesis (19-21). We hypothesize that ACE2-Ang(1-7)-Mas acts as an autocrine balancer (feedback route) to promote adipogenesis. We aim, for the first time, to reveal the molecular mechanisms underlying Ang(1-7)-Mas regulation on adipogenesis and its cross-talk with AngII-AT₁ pathways.

EXPERIMENTAL PROCEDURES

Cell culture and differentiation

Human subcutaneous pre-adipocytes (SP-F-2; Zen-Bio Inc., USA), collected from non-diabetic male subjects (with body-mass-index 25 - 29.9), were grown till confluence (5% CO2 and 37 °C) in pre-adipocyte growth medium (DMEM/Nutrient mixture F-12 medium (1:1, v/v) containing 15 mM HEPES, 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin). Adipocyte differentiation was then induced (defined as day 0) similarly as the previously reported (22, 23). Briefly, the cells were treated with adipocyte growth medium (serum-free DMEM/Ham’s F-12 medium containing 15 mM HEPES, 2 mM L-glutamine, 33 µM biotin, 17 µM pantothenate, 10 µg/ml transferrin, 0.2 nM triiodothyronine, 100 U/ml penicillin, and 100 mg/ml streptomycin) supplemented with 0.5 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 10 µM rosiglitazone. After 48 hr (on day 2), the medium was replaced with adipocyte growth medium containing insulin and dexamethasone, and thereafter, the medium was refreshed every 2 days in the following 8 - 10 days.

The 3T3-L1 mouse pre-adipose cell line (American Type Culture Collection, USA) was cultured in DMEM supplemented with 10% (v/v) bovine calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, in a 5% CO₂ humidified atmosphere at 37 °C. Adipocyte differentiation of 3T3-L1 cells follows the previously established protocol (24, 25). Specifically, two days after the cells became confluence, adipocyte differentiation was induced (defined as day 0) by incubation with DMEM with 10% FBS, 5 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. On day 2 of differentiation, DMEM containing 10% FBS and 5 µg/ml insulin was used as the culture medium. On day 4, the medium was then changed to DMEM with 10% FBS only, and thereafter, the medium was refreshed every 2 days in the following 2 - 4 days.

In some experiments, 10 µM PD98059, 50 µM PD98059, 100 mM PMA, 10 µM A779, 10 µM PD123319, 10 µM ZD7155, 1 µM AngII, or various concentrations of Ang(1-7) was added to the culture medium. In some experiments, 30 min pre-treatment of LY294002 or 60 min pre-treatment of PD98059 was performed prior to the induction of differentiation. Ang(1-7), AngII, ZD7155, and PD123319 were purchased from Tocris Bioscience (USA), and A779 from GenWay Biotech Inc. (USA). All cell culture media, supplements and sera are purchased from Life Technologies Corp. (Gibco; USA). All other chemicals and reagents were purchased from Sigma-Aldrich (USA).

siRNA silencing

Gene silencing of Mas receptor (Mas1), ACE2 (Ace2), AT₁ (Agtr1), AT₂ (Agtr2), ERK1 (Mapk3), ERK2 (Mapk1), or Akt (Akt1 and Akt2) in 3T3-L1 cells was achieved using a mouse MAS1 siRNA (sc62601, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), a mouse ACE2 siRNA (sc-41401, Santa Cruz Biotechnology, Inc.), a mouse AT₁ siRNA (sc29751, Santa Cruz Biotechnology, Inc.), a mouse AT₂ siRNA (sc29753, Santa Cruz Biotechnology, Inc.), mouse ERK1 and ERK2 siRNAs (sc-29308 and sc-35336, Santa Cruz Biotechnology, Inc.), and a mouse Akt siRNA (sc-43610, Santa Cruz Biotechnology, Inc.), respectively. Transfection was done as described previously (26, 27). Specifically, one day after being split, the cells were incubated with OPTI-MEM (Invitrogen) containing a complex of Lipofectamine-RNAiMAX transfection reagent (0.5% (v/v); Invitrogen) with siRNAs (20 nM)
for 4 hr, followed by addition of equal amount of DMEM containing 20% bovine calf serum and incubation for another 8-12 hr. The cells were then maintained in DMEM containing 10% bovine calf serum until confluence, and adipocyte differentiation was initiated as described above.

**Confocal fluorescence microscopy**

Cells grown on the glass cover-slips were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature (RT), and then washed with PBS for 3 times. After being blocked with 1% bovine albumin (BSA) in PBST (PBS with 0.1% tween20) at room temperature for 1 hr, the cells were incubated (at 4 °C) overnight with goat anti-Mas IgG in PBST with 1% BSA. After washing with PBS 3 times, the cells were incubated in FITC-labeled anti-goat IgG for 1 hr at room temperature in dark, followed by washing with PBS 3 times. Fluorescence signals were visualized on a confocal laser scanning microscope (LSM 510 Meta, Carl Zeizz GMBH, Germany).

**Western blot analysis**

The cells were washed twice with ice-cold phosphate-buffered saline (PBS), and then scraped in radioimmune precipitation assay lysis buffer (sc-24948; SantaCruz Biotechnology, Inc.) containing freshly added protease/phosphatase inhibitor mixture (Cell Signaling Technology, USA). After centrifugation at 4 °C, the supernatant of the sample was collected, and its protein content was determined using a protein assay kit (Bio-Rad, USA). Each cell lysate with the equal amount of protein content (as the loading control) was separated on SDS-PAGE by electrophoresis, followed by transfer onto a nitrocellulose membrane. After blocking the membrane for 2 - 3 hr in TBST (Tris-buffered saline-Tween; 10 mM Tris, 150 mM NaCl and 0.05% Tween-20, pH7.4) containing 5% bovine serum albumin (BSA, Sigma), the membrane was incubated with primary antibodies (1:200-400 dilution) in TBST with 1% BSA overnight. After washing three times with TBST buffer, the membrane was subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000-3000 dilution) for 6 - 8 hr. The protein bands were detected in a G:BOX Chemi XT4 imaging system (Syngene, USA) by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA).

Antibodies against peroxisome proliferator-activated receptor γ (PPARγ) (sc7196), phospho-PPARx (sc28001), C/EBPα (sc61), aP2 (A-FABP; sc18661), FAS (sc20140), AT1 (sc31181), AT2 (sc9040), MAS1 (sc54848), ACE2 (sc20998), Akt (sc8312), and phospho-Akt (Ser473) (sc135651) were purchased from Santa Cruz Biotechnology, Inc. Antibody against ACC (3662) was purchased from Cell Signaling Technology. Antibodies against phospho-ERK1/2 (3441-100) and ERK1/2 (3085-100) were purchased from Biovision Inc. (USA).

**Oil Red O staining**

Lipid droplets in adipocytes were stained with Oil Red O (Sigma-Aldrich) as described previously (28). Briefly, after being fixed in 10% formalin for 60 min, the cells were washed twice with PBS and stained with filtered Oil Red O solution (1.8 mg/ml Oil Red O and 60% (v/v) isopropanol in distilled water) for 60 min. The cells were then thoroughly washed with distilled water before being photographed under an optical microscope.

**Triglyceride assay**

Cellular triglyceride content was determined by using an Adipogenesis Assay kit (Biovision Inc., USA) according to the manufacturer’s protocol. Briefly, after 6 - 8 days differentiation of 3T3-L1 cells, or 10-12 days differentiation of human pre-adipocytes, cells were washed twice with PBS, scraped, sonicated to homogenize the suspension, and then assayed for total triglyceride. The total protein concentration, which was used as internal control, in these cells was measured with a protein assay kit from Bio-Rad Lab (USA).

**Enzyme-linked immunosorobent assay (ELISA)**

The concentration of Ang(1-7) or AngII in the medium was determined using a mouse Ang(1-7) ELISA kit (CSB-E13763m; Cusabio Biotech, USA) or a mouse AngII ELISA kit (CSB-E04495m; Cusabio Biotech), respectively. Each sample contains the same amount of proteins (determined by Bradford method as the internal control of the total cell number).

**Statistical analysis**

Data were analyzed using Student t-test, and expressed as mean ± SEM. p < 0.05 was considered statistically significant.

**RESULTS**

Ang(1-7)-Mas receptor promotes adipogenesis and antagonizes the anti-adipogenic effect of AngII-AT1 receptor in human pre-adipocytes

Pre-adipocytes isolated from human adipose tissue are used here as the in-vitro cell model for adipogenesis (29, 30). It has been known that
AngII receptors are expressed in pre-adipocytes, and the majority of AngII receptors found in human pre-adipocytes and adipocytes are of AT1 subtype (13, 31) (Supplemental Fig. S1). Here, we report that Mas receptors are also expressed in human pre-adipocytes and adipocytes (Fig. 1A, Supplemental Fig. S1). To evaluate the roles of Ang(1-7) and AngII in adipogenesis, human pre-adipocytes were induced to differentiate in the presence of Ang(1-7) and/or AngII.

Transformation of pre-adipocytes into mature adipocytes requires the coordinated regulations of various transcriptional factors (30). Among them, peroxisome proliferator-activated receptor γ (PPARγ) is considered as the most essential one (32). Because adipogenesis is accompanied by lipogenesis, enzymes responsible for lipogenesis (e.g., fatty acid synthase - FAS and acetyl-CoA carboxylase - ACC) are also regarded as the important markers for adipogenesis (24, 33).

After 10-12 days of hormone-induced differentiation, many of the fibroblast-like spindle-shaped human pre-adipocytes were differentiated into mature adipocytes, as evidenced by their morphological change and intracellular accumulation of lipid droplets (Fig. 1B). Lipid accumulation, an indicator of the extent of adipogenesis, can be assessed visually by Oil Red O staining of lipid droplets and quantitatively by direct measurement of intracellular triglyceride (TG) content. As shown in Fig. 1C and D, exogenous application of Ang(1-7) during differentiation considerably increased Oil Red O staining and TG content. And the number of lipid-laden adipocytes was also increased by Ang(1-7) treatment (Fig. 1E; Supplemental Fig. S2A). Consistently, addition of Ang(1-7) enhanced the expression of adipogenic transcriptional factor PPARγ, lipogenesis markers (FAS and ACC), and adipocyte protein-2 (aP2, a specific late-marker of adipogenesis (24)) (Fig.1F - J). Clearly, Ang(1-7) stimulates the adipocytic differentiation of human pre-adipose cells.

It has been reported that AngII suppresses adipogenesis of human pre-adipocytes via AT1 receptor (19, 20). As expected, addition of AngII during differentiation suppressed the adipogenesis, as evidenced by reduced Oil Red O staining, TG content, number of adipocytes, and expression of adipogenesis markers: PPARγ, FAS, ACC, and aP2 (Fig. 1). Such AngII-induced suppression is mediated by AT1 receptors because it can be rectified by specific AT1 antagonist (ZD7155) (34), but not by specific inhibitor of AT2 (PD123319) (35) (Supplemental Fig. S2B).

Interestingly, co-incubation with Ang(1-7) effectively abolished the anti-adipogenic effects of AngII (Fig.1). To determine if Ang(1-7) mediates its stimulatory actions through Mas receptor, the selective Mas receptor antagonist A779 (36) was applied during differentiation. As shown in Fig. 1, addition of A779 completely eliminated the stimulatory effects of Ang(1-7) on adipogenesis and its counteraction on AngII. Taken together, we conclude that Ang(1-7), via its interaction with Mas receptor, not only stimulates adipogenesis but also counteracts the anti-adipogenic effect of AngII.

The adipogenic effect of Ang(1-7) and its counteraction against AngII-AT1 activation are confirmed in 3T3-L1 pre-adipocytes

Because human pre-adipocytes grow and differentiate slowly and cannot be easily transfected (37), we used an established mouse pre-adipocyte cell line (3T3-L1 cells) (29, 30) instead as the cell model in all the following experiments in order to investigate the cross-talks between Ang(1-7) and AngII signaling observed from human pre-adipocytes. We first confirmed that 3T3-L1 cells express Ang(1-7) receptor (Mas receptor) and AngII receptors (both AT1 and AT2 receptors) (Fig. 2A, Supplemental Fig. S1) (38, 39).

As 3T3-L1 pre-adipocytes were differentiating into adipocytes (day 0 – 6 after hormonal induction of differentiation), the expression of PPARγ, C/EBPα, FAS, ACC, and aP2 were significantly up-regulated, along with their morphological change and increased intracellular accumulation of lipid droplets (Fig. 2). Consistent with the observations from human pre-adipocytes (Fig. 1), Ang(1-7) treatment during differentiation increased the expression of adipogenic markers (PPARγ, C/EBPα, FAS, ACC, and aP2), Oil Red O staining, and TG content (Fig. 2, Supplemental Fig. S3). Ang(1-7) stimulated adipogenesis is apparently dose-dependent (Supplemental Fig. S3).

In order to evaluate the role of AngII in adipogenesis of 3T3-L1 cells, we have to differentiate the effects mediated by AT1 or AT2 receptor. This is realized by challenging the AT2-siRNA knockdown 3T3-L1 cells or AT1-siRNA knockdown cells with AngII. Knockdown of AT1 or AT2 gene (Agtr1 or Agtr2) was confirmed by western blot analyses (Supplemental Fig. S1). As shown in Fig. 3,
activation of AT₁ receptors decreased Oil Red O staining, TG content, and expression of all adipogenic markers (PPARγ, C/EBPα, FAS, ACC, and aP2) whereas AT₂ activation did the opposite. These results were corroborated in naïve 3T3-L1 cells by selective inhibition of AT₁ or AT₂ using their specific antagonists (Supplemental Fig. S4). These experiments establish the inhibitory roles of AngII-AT₁ signaling in adipogenesis, which is in agreement with the previous reports (19-21) and our observation in human pre-adipocytes (Fig. 1). Also similar to the observation from human pre-adipocyte (Fig. 1), co-incubation of Ang(1-7) essentially reversed the inhibitory effects of AT₁ receptor activation on adipogenesis. On the other hand, Ang(1-7) did not affect AT₂ stimulated adipogenesis (Fig. 3, Supplemental Fig. S4). Taken together, we show that 3T3-L1 is a good and convenient alternative model to human pre-adipocytes to study the stimulatory roles of Ang(1-7)-Mas signaling in adipogenesis and its cross-talks with AngII-AT₁ pathways.

**Autocrine interplays between Ang(1-7) and AngII**

It is known that production of AngII and its precursor peptide (angiotensinogen) increases during adipogenesis (20, 40). AngII peptides, in turn, are cleaved by a membrane-bound ectoenzyme (ACE2) to form Ang(1-7) (4). It is thus conceivable that Ang(1-7) production might increase as well during adipogenesis. Indeed, as shown in Fig. 4A and B, both Ang(1-7) and AngII released into the culture media were elevated during adipogenesis. Because the receptors for Ang(1-7) and AngII are expressed in pre-adipocytes and adipocytes (12, 39) (Fig. 1 and 2, Supplemental Fig. S1), it is likely that these locally produced Ang(1-7) and AngII may serve as the autocrine feedback signals upon adipocyte differentiation, in an interactive way.

*Ace2* gene expression in 3T3-L1 cells was blocked by specific ACE2-siRNA before the initiation of differentiation (verified by western blot analyses shown in Supplemental Fig. S1). As a result, it was found that the release of Ang(1-7) from these cells was considerably lowered throughout the differentiation whereas the AngII release was increased in the early stage (day 1 - 2) (Fig. 4A and B). It was also noted that adipogenesis was suppressed in ACE2-siRNA knockdown cells, as evidenced by the reduced number of lipid-laden adipocytes and decreased expression of adipocyte markers: aP2 and FAS (Fig. 4C – F). This is presumably due to the decrease of adipogenic factor – Ang(1-7) and increase of anti-adipogenic factor – AngII. In support of this, we show that down-regulation of adipogenesis by ACE2-knockdown can be effectively rectified by application of Ang(1-7) or blocking of AT₁ receptor. These results demonstrate that ACE2 is a critical linker to control the balance between Ang(1-7) and AngII signaling.

Fig. 4G and H show that ACE2 expression steadily increased over time during the 3T3-L1 differentiation, promoting adipogenesis by strengthening Ang(1-7) signaling. Intriguingly, exogenous application of Ang(1-7) suppressed the increase of ACE2 expression (Fig. 4G) whereas AngII-AT₁ activation further enhanced ACE expression in the early differentiation phase (day 1). These observations suggest that a delicate balance is established by the interplays between AngII, ACE2, and Ang(1-7).

It is known that the antiadipogenic effect of AngII-AT₁ involves the activation (phosphorylation) of extracellular signal-regulated kinases (ERK1/2) (19). Here, we show that Ang(1-7) neutralizes the antiadipogenic effect of AngII-AT₁ by interfering ERK1/2 activation because, in AT₂-siRNA knockdown cells, AngII-enhanced ERK1/2 phosphorylation was abolished by co-incubation of Ang(1-7) (Fig. 4I). As the summary, we provide evidence that the local autocrine regulations of Ang(1-7) and AngII play important and counteracting roles in adipogenesis, and the cross-talks between Ang(1-7)-Mas and AngII-AT₁ signaling are linked by ACE2 and ERK1/2.

**Involvement of Mas receptor**

The experiments on human pre-adipocytes (Fig. 1) demonstrate that the adipogenic and anti-AngII effects of Ang(1-7) are mediated by Mas receptor. Here, we further analyzed the involvement of Mas receptor using *Mas1*-gene knockdown 3T3-L1 cells. The knockdown of Mas receptor expression by MAS1-siRNA was verified by western blot analyses (Supplemental Fig. S1).

In the Mas-knockdown cells, the expressions of adipogenic markers (PPARγ, FAS, ACC, and aP2) were reduced (Fig. 5A and B) and as expected, Ang(1-7) failed to increase the expression of these markers, and to abolish the inhibition by AngII. Some studies have reported low-affinity interaction between Ang(1-7) with AT₁ and AT₂ receptors in other cell types (41, 42). To rule out the possible contributions from AT₁ and AT₂ receptors, we show that Ang(1-7)-
stimulated expression of the adipogenic markers was not compromised when AT1 and AT2 receptors were simultaneously blocked by their respective antagonists. These results establish that the adipogenic effects of Ang(1-7) are mediated by Mas receptors.

Furthermore, we also observed that Mas receptor expression increased over time during the 3T3-L1 differentiation, and application of exogenous Ang(1-7) elevated Mas receptor expression at the early differentiation phase (day 1 - 2) (Fig. 5C). These experiments demonstrate the positive regulation of Ang(1-7)-Mas signaling to accelerate adipogenesis, particularly, in the early stage.

**Stimulation of adipogenesis by Ang(1-7) is MAPK kinase/ERK and PI3 kinase/Akt dependent**

Next, we explored the signaling pathways responsible for the stimulatory effect of Ang(1-7) on adipogenesis of 3T3-L1 cells. It is known that the phosphorylation (activation) of ERK1/2 is required for mitotic clonal expansion in the early stage of adipogenesis (43, 44); however, ERK1/2 activity must quickly return to the low level to avoid inactivation (phosphorylation) of the critical adipogenic factor (PPARɤ) (45, 46). Indeed, as shown in Fig. 6A, hormonal stimulation of adipogenesis caused acute increase of ERK1/2 phosphorylation (within 15 min) which was followed by a quick decay to a low level. Interestingly, such decay was exaggerated by addition of Ang(1-7) (Fig. 6A). As discussed above, it would help to sustain the adipogenic process by preventing ERK1/2-induced inactivation (phosphorylation) of PPARɤ. This is confirmed in Fig. 6B.

PI3k/Akt signaling triggered PPARɤ expression is also well-known for its critical role in adipogenesis (47, 48). Regulation of Ang(1-7) on PI3 kinase/Akt pathway has been reported in other cell types (49, 50). As shown in Fig. 6C, phosphorylation of Akt was acutely induced (within 5 min) upon induction of adipogenic differentiation, and this acute phosphorylation was largely enhanced by the addition of Ang(1-7) although such enhancement is short-lived (within 15 min).

ERK1/2 and Akt are the substrates of MAPK kinase and PI3 kinase, respectively. Stimulation of MAPK kinase by phorbol 12-myristate 13-acetate (PMA) produced significant inhibitory effect on adipogenesis as evidenced by the reduced number of mature adipocytes and expression of adipogenic markers (aP2, FAS, ACC and PPARɤ)(Fig. 7A - G). On the contrary, inhibiting MAPK kinase by PD98059 (51) slightly enhanced adipogenesis (Fig. 7; Supplemental Fig. S5). PD98059 inhibition and PMA stimulation on adipogenesis has also been reported previously (52-54). Here, we further show that PMA was able to partially eliminate Ang(1-7)-induced adipogenesis (Fig. 7; Supplemental Fig. S5). In another line of experiments, it was found that blocking PI3 kinase by LY294002 (55) significantly inhibited adipogenesis and abolished the stimulatory effect of Ang(1-7)(Fig. 7A - G).

The ERK1/2 inhibition and Akt stimulation on adipogenesis were further confirmed by knocking down of ERK1/2 (Mapk3 and Mapk1) or Akt1/2 genes bbefore initiation of 3T3-L1 cell differentiation. The knockdown of the protein expression was verified by western blot (Supplemental Fig. S5). In the ERK1/2-knockdown cells, the expressions of adipogenic markers (aP2 and FAS) were significantly increased, whereas in the Akt-knockdown cells, the expressions of these markers were largely suppressed (Fig. 7H-J). Also as expected, Ang(1-7) failed to increase the expressions of these markers in the Akt knockdown cells (Fig. 7H – J).

Taken together, it may be concluded that Ang(1-7) stimulates adipogenesis through two distinct pathways: 1) inhibiting MAPK kinase-ERK1/2 signaling which leads to PPARɤ inactivation; 2) enhancing PI3 kinase-Akt signaling which leads to PPARɤ expression. The latter pathway appears to be more potent.

**Ang(1-7) acts in the early stage of differentiation**

The acute action of Ang(1-7) on ERK1/2 and Akt phosphorylation (shown in Fig. 6) suggests the importance of Ang(1-7) signaling in the early stage of differentiation. In support of this view, it was found, based on the number of differentiated adipocytes and expressions of adipogenic markers (aP2 and FAS), that administration of Ang(1-7) even 1 day after the hormonal initiation of differentiation failed to stimulate adipogenesis whereas incubation with Ang(1-7) only for the first day of differentiation was as potent as its presence for the whole differentiation process (6 days) (Fig. 8).

To further confirm that Ang(1-7) acts mainly on the early stage of differentiation, Mas receptor was knocked down at different stages of differentiation (at day -2/2 days before the initiation of differentiation) or at day0, day2, or
day4) (Fig. 8G - I). The Mas receptor expression was largely suppressed even one day after the transfection with MASH1-siRNA, which was verified by western blot analyses (Supplemental Fig. S1). Consistent with our data shown inFig. 5, Ang(1-7) failed to increase the expressions of adipogenic markers (aP2 and FAS) in the 3T3-L1 cells transfected with MASH1-siRNA at day -2. However, Ang(1-7) was able to significantly stimulate the expressions of these markers in the cells transfected with MASH1-siRNA at day0, day2, or day4 (Fig. 8G - I).

It is known that, in the early stage of differentiation, pre-adipocytes undergo several rounds of cell division, known as mitotic clonal expansion (MCE), before undertaking terminal differentiation through which they acquire the characteristics of maturity (33, 43). We show in Fig. 8C that induction of differentiation resulted in 2 – 3 fold increase in cell number within 2 days, but Ang(1-7) treatment had insignificant effect on cell proliferation. This observation rule out the possibility that the adipogenic effects of Ang(1-7) is due to increased mitotic clonal expansion.

**DISCUSSION**

Adipocytes, which were previously regarded as the passive storage depots for excess energy (fat), actually play perplexing and integrative roles in regulating metabolism (56). Complicated cross-talks exist between adipocytes and other cells in the metabolic networks (57). For example, the functions of adipocytes (adipogenesis, lipid and carbohydrate metabolism) are highly regulated by insulin secreted by pancreatic beta cells (58), catecholamines secreted by chromaffin cells (59), and glucocorticoid secreted by adrenal cortex (60). On the other hand, adipocytes release a variety of adipokines (e.g., leptin, apelin, AngII) to control various aspects of metabolism and influence the secretion of other metabolic factors or hormones from other cells (59, 61). Intriguingly, the receptors of some adipokines are identified in adipocytes, implying the existence of autocrine regulatory loops on adipocyte functions (39, 62, 63). For instances, adiponectin-adiponectin receptor (AdipoR1 and AdipoR2) promotes adipogenesis and insulin-induced glucose uptake, and inhibits lipolysis (64, 65); leptin-leptin receptor (ObR) inhibits adipogenesis and glucose uptake, and promotes lipolysis (54, 66); apelin-apelin receptor (APJ) inhibits adipogenesis and lipolysis (26), and promotes insulin sensitivity and glucose uptake (67).

In this study, we demonstrate the local autocrine regulations by the angiotensin system. Specifically, the counteracting interplays between Ang(1-7)-Mas and AngII-AT1 signaling on adipogenesis are revealed, as illustrated in Fig. 9. Based on our experiments and the previous reports (5, 19, 45, 48), the following scenario may be proposed. During adipogenesis, the production of AngII (Fig. 4) and AT1 receptor are up-regulated (20, 40), acting as a dampening feedback (Fig. 1 and 3). Local accumulation of AngII stimulates the expression of ACE2 and production of Ang(1-7) by ACE2 (Fig. 4). Mediated by Mas receptor, Ang(1-7), in turn, enhances adipogenesis (Fig. 1 - 5) by inhibiting AngII-AT1, triggered MAP kinase ERK1/2 pathway to activate the adipogenic factor PPARγ (Fig. 4, 6 and 7) and by stimulating PI3 kinase-Akt pathway to increase PPARγ expression (Fig. 6 and 7). This action is further augmented by Ang(1-7) induced Mas receptor expression (Fig. 5). Ang(1-7), on the other hand, reduces ACE2 expression presumably to avoid over-production of Ang(1-7) (Fig. 4). As described, multilateral negative and positive feedback loops peculiarly co-exist. Presumably, such complex interplays are balanced to ensure appropriate pace and phasing in differentiation. Disruption of these subtle interplays by silencing ACE2 expression or Mas receptor expression freezes adipogenesis while addition of exogenous Ang(1-7) accelerates adipogenesis.

It is increasingly accepted that formation of new adipocytes (adipogenesis) is a physiological adaptive response to excessive calorie intake whereby to maintain an overall healthier adipose tissue because newly-differentiated adipocytes are more insulin-sensitive (thus more capable of energy storage) (68, 69). Otherwise, hypertrophic adipocytes, which are less insulin-sensitive, will result (70, 71). The adverse metabolic consequences of enlarged adipocytes (particularly in visceral fat) have been extensively documented, including insulin-resistance caused type 2 diabetes and hypertriglyceridemia (72, 73). The adipogenic potential of pre-adipocytes from different fat depots differs. Subcutaneous pre-adipocytes are more sensitive to the adipogenic stimuli (e.g., PPARγ agonist) than visceral ones (74, 75). Adipogenesis in subcutaneous adipose tissues thus assist to prevent the notorious hypertrophy.

*Ang(1-7) and AngII regulations on Adipogenesis*
of visceral adipose (76). Therefore, the adipogenic effect of Ang(1-7) signaling should, albeit counter-intuitively, be beneficial. This is supported by the previous studies on animal models. For instances, increasing the circulating level of Ang(1-7) in rats improves insulin sensitivity in adipose tissue and decreases plasma level of triglyceride and visceral fat mass (7, 77) whereas knocking-down of Mas receptors in mice results in impaired insulin sensitivity and increased visceral fat mass (78). In contrast, the anti-adipogenic AngII-AT₁ signaling increases visceral adipose tissue growth and insulin resistance (19, 79, 80), while administration of AT₁ receptor blockers reduces fat mass and improves insulin sensitivity in human and several animal models (81-83).

Through circulation, the autocrine balance between AngII and Ang(1-7) signaling can be influenced by other angiotensin-peptide producing cells (e.g., vascular endothelial and smooth muscle cells, cardiac myocytes, pancreatic β-cells, hepatocytes, etc.) (2, 6). On the other hand, adipose tissue is a major source of angiotensin peptides (10, 84) which play critical roles in regulating, for example, blood vessel contraction (85), angiogenesis (86), glucose uptake in skeletal muscles (9), and hormone secretion (39, 87). Such cross-talks may underlie the association between obesity and other metabolic disorders (e.g., diabetes and cardiovascular diseases). Hence, this study not only reveals the intriguing autocrine interplays between AngII and Ang(1-7) signaling on adipogenesis but also raise the possibility of targeting these molecular pathways to treat metabolic disorders.
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**FOOTNOTE**

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**FIGURE LEGENDS**

**FIGURE 1.** Ang(1-7)-Mas receptor enhances adipogenesis and antagonizes the anti-adipogenic effect of AngII in human pre-adipocytes. [A] Confocal images of immunostained Mas receptors in human pre-adipocyte and adipocyte. Scale bars = 10 µM. [B] Representative optical images of human pre-adipocytes and differentiated human adipocytes (day 12). Scale bars = 60 µM. [C] Representative images of Oil Red O stained adipocytes, differentiated from pre-adipocytes for 10 - 12 days without (control) or with exposure to 1 µM Ang(1-7), or Ang(1-7) plus 10 µM A779 (specific Mas receptor antagonist), or 1 µM AngII, or AngII plus Ang(1-7), or AngII plus Ang(1-7) and A779. Scale bars = 240 µM. [D] Quantification of intracellular triglyceride content (mean ± SEM, n = 4 - 5). [E] Number of adipocytes (lipid-droplet-containing cells) per field-of-view (40x). The data is presented as mean ± SEM (n = 6 - 7 images from 3 - 4 different culture batches). [F - J] Western blot analyses of the expression of PPARγ (~54 kDa), FAS (~270 kDa), ACC (~280 kDa), ap2 (~15 kDa), and actin (~43 kDa) in differentiated human adipocytes (day 10 - 12 after induction of differentiation). [F] shows the representative immunoblots. [G - J] Show the statistics (mean ± SEM, n = 4 - 5) of the blot densities normalized to actin density (as the internal control). Each sample contains same amount of total proteins (as the loading control). Student’s t-test: *p < 0.05, **p < 0.01 vs. control; #p < 0.05, ##p < 0.01 between indicated pairs.

**FIGURE 2.** Ang(1-7) enhances adipogenesis of 3T3-L1 pre-adipocytes. [A] Confocal images of immunostained Mas receptor in 3T3-L1 pre-adipocyte and differentiated 3T3-L1 adipocyte (day 8). Scale bars = 10 µM. [B] Representative images of 3T3-L1 cells at different stages of differentiation (day 2, 4 and 6), without (control) or with exposure to Ang(1-7). Scale bars = 60 µm. [C - H] Western blot analyses of PPARγ, C/EBPα (~42 kDa), FAS, ACC, ap2, and actin expression in 3T3-L1 cells at different stages of differentiation (day 1, 2, 4 and 6), without (-) (control) or with (+) 1 µM Ang(1-7). Representative immunoblots were shown in [C], and the statistics (mean ± SEM, n = 4 - 5) of the optical density ratio to actin blot (internal control) were shown in [D - H]. Each sample contains the same amount of total protein (loading control). Student’s t-test: *p < 0.05, **p < 0.01 vs. control.

**FIGURE 3.** Ang(1-7) stimulates adipogenesis and neutralizes the anti-adipogenic effect of AngII-AT1 receptor activation in 3T3-L1 pre-adipocytes. The cells were transfected with control siRNA or AT1 receptor siRNA or AT2 receptor siRNA, and cultured till confluence. This was followed by induction of differentiation for 7 - 8 days, without (-) (control) or with (+) 1 µM Ang(1-7) and/or 1 µM AngII. [A] Representative images of Oil Red O stained 3T3-L1 adipocytes differentiated under different conditions (day 8). [B - G] Western blot analyses of the expression of PPARγ, C/EBPα, FAS, ACC, ap2, and actin in 3T3-L1 adipocytes (day 7 - 8). The representative immunoblots were shown in [B]. The statistics (mean ± SEM, n = 5 - 6) of the optical densities of the blots normalized to that of actin were shown in [C - G]. [H] Intracellular triglyceride content (mean ± SEM, n = 4 - 5) in 3T3-L1 adipocytes (day 7 - 8). Student’s t-test: *p < 0.05, **p < 0.01 vs. control; #p < 0.05, ##p < 0.01 between indicated pairs.

**FIGURE 4.** Autocrine interplays between Ang(1-7) and AngII in the differentiation of 3T3-L1 pre-adipocytes. The cells were transfected with control siRNA or ACE2 siRNA, and cultured till confluence. This was followed by 7 - 8 days of differentiation, with (+) or without (-) 1 µM Ang(1-7) or 10 µM ZD7155 (selective AT1 receptor antagonist) or 1 µM AngII plus PD123319 (selective AT2 receptor antagonist). [A and B] The concentrations of released Ang(1-7) and AngII in the culture
media (24 hr incubation) at different differentiation stages (day0 – day 6). The concentrations at day 0 from the control-siRNA transfected cells were regarded as the control). [C] Representative images of differently cultured 3T3-L1 cells (day 7 - 8). Scale bars = 60 μm. [D] Number of adipocytes (lipid-droplet laden cells) per field-of-view (40x). The data is presented as mean ± SEM (n = 6 – 7 images from 3 – 4 different culture batches). [E - F] Representative immunoblots of αP2, FAS, and actin (day7 - 8); and the statistics (mean ± SEM, n = 4 - 5) showing the optical densities of the blots normalized to actin density. [G and H] Western blot analyses of ACE2 (~90 kDa) and actin expression in untreated 3T3-L1 cells at different differentiation stages (day 0 – 6) (expression level at day 0 as control). The top panel shows the representative immunoblots and the lower panel shows the statistics (mean ± SEM, n = 4 - 5) of the optical density of the ACE2 normalized to that of actin. [I] Western blot analyses of the phosphorylated and total ERK1/2 (pERK1/2 and ERK1/2, ~ 42 – 44 kDa) in AT2 siRNA-transfected cells (1 or 4 hr after induction of differentiation). The top panels show the representative immunoblots, and the lower panel shows the statistics (mean ± SEM, n = 4 – 5) of the optical density ratio between pERK1/2 and ERK1/2. Student’s t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs. control; #p < 0.05, ##p < 0.01 between indicated pairs.

**FIGURE 5.** Involvement of Mas receptor in 3T3-L1 adipogenesis. The cells were transfected with control siRNA or MAS1- siRNA, and cultured till confluence. Differentiation was then induced for 7 – 8 days, without (-) (control) or with (+) 1 µM Ang(1-7), or 1 µM AngII, or 10 µM ZD7155, or 10 µM PD123319. [A and B] Western blot analyses of PPARx, FAS, ACC, αP2, and actin (day 7 - 8). The representative immunoblots were shown in [A] and the statistics (mean ± SEM, n = 4 - 5) of the optical densities of the blots normalized to actin density were shown in [B]. [C] Western blot analyses of Mas receptor (~ 37 kDa) and actin in the untreated cells at different stages of differentiation (12 hr – day 6). Protein level at 12 hr without Ang(1-7) treatment was regarded as the control. The top panel shows the representative immunoblots, and the lower panel shows the statistics (mean ± SEM, n = 4 - 5) of the optical density ratio between the blot of Mas receptor and that of actin. Student’s t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs. control; #p < 0.05, ##p < 0.01 between indicated pairs.

**FIGURE 6.** Effects of Ang(1-7) on the phosphorylation of ERK1/2, PPARx and Akt in 3T3-L1 cells during the early stage of differentiation. Western blot analyses of the phosphorylation and total protein levels of [A] ERK1/2, [B] PPARx, and [C] Akt (~60 kDa) in 3T3-L1 cells at different times of differentiation (0 min, 5 min, 15 min, 1 hr, 4 hr, 24 hr, 48 hr), with (+) or without (-) 1 µM Ang(1-7) treatment. The top panels show the representative immunoblots. The lower panels show the statistics (mean ± SEM, n = 5 – 6) of the optical density ratio between pERK1/2 and ERK1/2 blots, or between pPPARx and PPARx blots, or between pAkt and Akt blots. Student’s t-test: *p < 0.05, **p < 0.01 between indicated pairs.

**FIGURE 7.** Effect of PI3 kinase/Akt and MAPK kinase/ERK signaling pathways on Ang(1-7) induced adipogenesis of 3T3-L1 cells. The cells were induced to differentiate for 7 – 8 days without (control) or in the presence of 1 µM Ang(1-7), or 10 µM LY294002 (selective inhibitor of PI3 kinase), or Ang(1-7) plus LY29400, or 50 µM PD98059 (selective inhibitor of MAPK kinase), or Ang(1-7) plus PD98059, or 100 nM PMA, or Ang(1-7) plus PMA. [A] Representative images of differently cultured cells (day 7 - 8). Scale bars = 60 μm. [B] Number of adipocytes (lipid-droplet containing cells) per field-of-view (40x) was counted on day 7, and presented as mean ± SEM (n = 6 - 7 images from 3 to 4 different culture batches). [C – G] Western blot analyses of αP2, FAS, PPARx, ACC, and actin expression in 3T3-L1 cells (day 7 - 8). Representative immunoblots were shown in [C]; and the statistics (mean ± SEM, n = 5 - 6) of the optical density ratio normalized to actin blot was shown in [D - G]. [H – J] 3T3-L1 cells were transfected with control siRNA or Akt siRNA or ERK1/2 siRNA, and cultured till confluence. Differentiation was then induced for 7 – 8 days, without (-) (control) or with (+) 1 µM Ang(1-7). Representative immunoblots of αP2, FAS, and actin expression in 3T3-L1 cells (day 7 - 8) were shown in [H]; and the statistics (mean ± SEM, n = 4 - 5) of the optical density ratio normalized to actin blot was shown in [I and J]. Student’s t-test: *p < 0.05, **p < 0.01 vs. control; #p < 0.05, ##p < 0.01, ###p < 0.001 between indicated pairs.

**FIGURE 8.** Ang(1-7) exerts its stimulatory effect on adipogenesis in the early stage of differentiation. 3T3-L1 cells were induced to differentiate without (control) or with exposure to 1 µM Ang(1-7) for
different periods of time: from day 0 - 6 (d0-d6), or from day 1 - 6 (d1-d6), or from day 2 - 6 (d2-d6), or from day 4 - 6 (d4-d6), or from day 0 - 1 (d0-d1). [A] Representative images of differently cultured 3T3-L1 cells (day 6). Scale bars = 60 µM. [B] Number of adipocytes (lipid-droplet containing cells) per field of view (40x) was counted on day 6, and presented as mean ± SEM (n = 6 - 8 images from 3 to 4 different culture batches). [C] Number of 3T3-L1 cells per 12-wells was counted at day 0, 1, and 2 (mean ± SEM, n = 4; the cell number at day 0 was regarded as the control). [D – F] Western blot analyses of aP2, FAS, and actin expression in differently cultured cells (day 6). Representative immunoblots were shown in [D] and the statistics (mean ± SEM, n = 4 - 5) of the optical density ratio to actin was shown in [E and F]. [G – I] 3T3-L1 cells were transfected with control siRNA or Mas receptor siRNA at different time: transfection of Mas receptor siRNA 2-days before the initiation of differentiation denoted as d(-2), or at day0 (d0), or day2 (d2), or day4 (d4). Differentiation was induced at day0 without (-) (control) or with (+) 1 µM Ang(1-7) for 7 – 8 days. Representative immunoblots of aP2, FAS, and actin expression in 3T3-L1 cells (day 7 - 8) were shown in [G]; and the statistics (mean ± SEM, n = 4 - 5) of the optical density ratio normalized to actin blot was shown in [H and I]. Student’s t-test: * p < 0.05, ** p < 0.01 vs. control; #p < 0.05, ##p < 0.01 between indicated pairs.

FIGURE 9. Illustration of the interplays between Ang(1-7)-Mas and AngII-AT\(_1\) signaling in adipogenesis.
FIGURE 1

Ang(1-7) and AngII regulations on Adipogenesis
FIGURE 2

Ang(1-7) and AngII regulations on Adipogenesis

A

Mas receptor

3T3-L1 pre-adipocyte

3T3-L1 adipocyte

B

day 2

Control

Ang(1-7)

day 4

Control

Ang(1-7)

day 6

Control

Ang(1-7)

C

PPAR-γ

C/EBP-α

FAS

ACC

aP2

Actin

Ang(1-7) - + - - + +

D

Relative Density

PPAR-γ

day 1

day 2

day 4

day 6

E

Relative Density

CiEBP-α

day 1

day 2

day 4

day 6

F

Relative Density

FAS

day 1

day 2

day 4

day 6

G

Relative Density

ACC

day 1

day 2

day 4

day 6

H

Relative Density

aP2

day 1

day 2

day 4

day 6
FIGURE 3

Ang(1-7) and AngII regulations on Adipogenesis
FIGURE 4

A

B

C

D

E

F

G

H

I

Ang(1-7) and AngII regulations on Adipogenesis
Ang(1-7) and AngII regulations on Adipogenesis

FIGURE 5
FIGURE 6
FIGURE 7

A

B

C

D

E

F

G

H

I

J

Ang(1-7) and AngII regulations on Adipogenesis
FIGURE 8
Ang(1-7) and AngII regulations on Adipogenesis

FIGURE 9