Angiotensin-(1-7) stimulates cholesterol efflux from angiotensin II-treated cholesterol-loaded THP-1 macrophages through the suppression of p38 and c-Jun N-terminal kinase signaling

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Abstract. Angiotensin II (Ang II) and Ang-(1-7) are key effector peptides of the renin-angiotensin system. The present study aimed to investigate the effects of Ang-(1-7) on Ang II-stimulated cholesterol efflux and the associated molecular mechanisms. Differentiated THP-1 macrophages were treated with Ang II (1 μM) and/or Ang-(1-7) (10 and 100 nM) for 24 h and the cholesterol efflux and gene expression levels were assessed. Pharmacological inhibition of peroxisome proliferator-activated receptor (PPAR)γ and mitogen-activated protein kinases (MAPKs) were performed to identify the signaling pathways involved. The results demonstrated that Ang II significantly inhibited the cholesterol efflux from cholesterol-loaded THP-1 macrophages. Treatment with Ang-(1-7) led to a dose-dependent restoration of cholesterol efflux in the Ang II-treated cells. The co-treatment with Ang-(1-7) and Ang II significantly increased the expression levels of adenosine triphosphate (ATP)-binding cassette (ABC)A1 and ABCG1 compared with treatment with Ang II alone. This was coupled with increased expression levels of PPARγ and liver X receptor (LXR)α. The pharmacological inhibition of PPARγ significantly ($P<0.05$) eliminated the Ang-(1-7)-mediated induction of ABCA1 and ABCG1 mRNA expression. Treatment with Ang-(1-7) caused the inactivation of c-Jun N-terminal kinases (JNK) and p38 MAPK signaling in the Ang II-treated THP-1 macrophages. In addition, the inhibition of JNK or p38 MAPK signaling using specific pharmacological inhibitors mimicked the Ang-(1-7)-induced expression of PPARγ and LXRα. In conclusion, the data demonstrated that treatment with Ang-(1-7) promoted cholesterol efflux in Ang II-treated THP-1 macrophages, partly through inactivation of p38 and JNK signaling and by inducing the expression of PPARγ and LXRα. Ang (1-7) may, therefore, have therapeutic benefits for the treatment of atherosclerosis.

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Introduction

Atherosclerosis is a progressive chronic inflammatory disease, characterized by the development of plaques in the artery, which ultimately lead to cardiovascular disease (1). One of the early events in atherogenesis is the formation of foam cells, which are macrophages containing ingested oxidized low-density lipoprotein (LDL) (2). It has been suggested that macrophages become foam cells as a consequence of the loss of the normal balance between cholesterol uptake from lipoproteins and cholesterol efflux. This has led to investigations to elucidate the regulation of cholesterol efflux from macrophages (3).

The adenosine triphosphate (ATP)-binding cassette (ABC) transporters, ABCA1 and ABCG1, are important in the removal of cholesterol from lipid-laden macrophages as a primary anti-atherogenic mechanism (4). ABCA1 promotes cholesterol efflux from foam cells to lipid-free apolipoprotein A-1 (apoA-1), but not to mature high-density lipoprotein (HDL) particles, whereas ABCG1 mediates cholesterol efflux to HDL and not to the lipid-poor apoA-1 (5,6). Biochemical studies have identified ABCA1 and ABCG1 as direct transcriptional targets of liver X receptor/retinoid X receptor (LXR/RXR) (7,8). Peroxisome proliferator-activated receptor gamma (PPARγ) is also involved in the transcriptional activation of the ABC transporters in an LXRα-dependent manner (9). Chawla *et al* (9) demonstrated that LXRα activation-induced expression levels of ABCA1 and ABCG1 are markedly reduced in PPARγ-deficient macrophages, suggesting the involvement of PPARγ in the LXRα-mediated upregulation of ABC proteins. PPARγ and LXRα have been demonstrated to exert anti-atherogenic effects by facilitating the removal of cholesterol from foam cells (10).

The renin-angiotensin system (RAS) is considered to be involved in the pathogenesis of atherosclerosis (11,12). Angiotensin II (Ang II), the most important component of the RAS, has been found to promote the development of atherosclerosis in apolipoprotein-E (ApoE)-deficient mice (13).

Ang-converting enzyme (ACE) 2 is a homolog of ACE, which is responsible for generating Ang II from Ang I. ACE2 efficiently degrades Ang II to form Ang-(1-7), a peptide exerting actions opposite to those of Ang II (14). Our previous study demonstrated that Ang-(1-7) ameliorates Ang-II-induced apoptosis in human umbilical vein endothelial cells (15). A previous study demonstrated that Ang-(1-7) dose-dependently inhibits early atherosclerotic lesions and increases plaque stability by targeting vascular cells in $ApoE^{(-/-)}$ mice (16). Takata *et al* (17) reported that Ang II alters macrophage cholesterol homeostasis by repressing the expression of ABCA1, therefore, promoting foam cell formation. However, the effects of Ang-(1-7) on macrophage cholesterol metabolism remain to be elucidated. The present study investigated the effects of Ang-(1-7) on Ang II-stimulated cholesterol efflux and the associated molecular mechanisms.

Materials and methods

Reagents and antibodies. Ang II, Ang-(1-7), phorbol 12-myristate 13-acetate (PMA), apoA-1, SB203580 (a p38 MAPK inhibitor), SP600125 (a JNK inhibitor) and GW9662 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary mouse anti-human monoclonal antibodies (1:300) against ABCA1 (sc-53482), ABCG1 (sc-20795), PPARγ (sc-390740) and LXRα (sc-271064) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary mouse or rabbit anti-human monoclonal antibodies (1:300) against total p38 (#9212), phosphorylated (p-)p38 (#9216), total extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (#9102), p-ERK1/2 (#9108), total c-Jun NH2-terminal kinase (JNK) (#9252), p-JNK (#9255) and β-actin (#4967) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:2,000 dilution) were purchased from Santa Cruz Biotechnology, Inc.

Culture and differentiation of THP-1 cells. Human THP-1 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium, containing 10% fetal bovine serum (FBS), 10 μg/ml streptomycin and 100 U/ml penicillin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37˚C with 5% CO_2. To differentiate these cells into macrophages, the monocytes were seeded into 12-well plates at a density of $1x10^6$ cells/well in complete RPMI-1640 medium containing 160 nM PMA and incubated for 24 h at 37˚C.

Drug treatment. The differentiated THP-1 macrophages were washed three times in phosphate-buffered saline (PBS; Invitrogen Life Technologies) and labeled by incubation for 48 h at 37˚C in the presence of (3H)cholesterol ($0.37x10^9$ Bq/l; GE Healthcare, Piscataway, NJ, USA) in RPMI-1640 medium, supplemented with 10% FBS. Following washing three times in PBS, the labeled cells were treated with Ang II (1 μM) and/or 10 or 100 nM Ang-(1-7) (18) for 24 h at 37˚C. Unless stated otherwise, 100 nM Ang-(1-7) was used. To investigate the role of PPARγ in the Ang-(1-7)-mediated induction of ABCA1 and ABCG1 expression, the THP-1 macrophages were treated with GW9662 (1 μM) for 1 h (19), prior to incubation for 24 h with 1 μM Ang II with or without 100 nM Ang-(1-7). To investigate the role of mitogen-activated protein kinases (MAPKs) in the regulation of PPARγ and LXRα expression, SB203580 (10 μM) and/or SP600125 (10 μM) (20) were added to the cell culture and incubated for 1 h at 37˚C prior to the addition of 1 μM Ang II.

Cholesterol efflux assay. Following the treatment described above, the labeled cells were washed three times in PBS and incubated in serum-free RPMI-1640 medium containing 10 mg/l apoA-1 as a cholesterol receptor for 12 h at 37˚C. The medium was then collected and centrifuged at 1,000 x g for 10 min at room temperature to remove cellular debris. Radioactivity in the medium and cells was analyzed by liquid scintillation counting (ZX34-LS6500; Beckman Coulter, Inc., Fullerton, CA, USA). The percentage of cholesterol efflux was calculated as: Counts/min (cpm) in the medium / (cpm in the cell + cpm in the medium) x 100.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted using TRIzol reagent, according to the manufacturer's instructions (Invitrogen Life Technologies). RT was performed using an AMV First Strand cDNA Synthesis kit (Bio Basic, Inc., Amhurst, NY, USA). qPCR amplification was performed using an Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using a SYBR green PCR master mix (Applied Biosystems). The primers used for qPCR are listed in Table I. Each PCR reaction (20 μl) contained 10 ng cDNA and ther thermal cyclin conditions were as follows: 95˚C for 10 min, followed by 35 cycles of 95˚C for 15 sec and 60˚C for 50 sec. As an internal quantitative control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified in a parallel reaction. All assays were performed in triplicate and the threshold cycle (Ct) value was determined as the cycle number at which the fluorescence signal reached the exponential phase. The relative mRNA expression levels were normalized against GAPDH and were determined using the $2^{-\Delta\Delta Ct}$ method (21).

Western blot analysis. Following treatment, the cells were lysed in lysis buffer (Santa Cruz Biotechnology, Inc.), containing 50 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1% NP-40 and 0.1% sodium dodecyl sulphate (SDS; Sigma-Aldrich), supplemented with protease and phosphatase inhibitors, including pepstatin, leupeptin, aprotinin and phenylmethylsulfonyl fluoride, which were purchased from Sigma-Aldrich. The protein samples were separated on polyacrylamide gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing 0.1% SDS, and then transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). Following blocking for 4 h in a Tris-buffered solution, containing 5% non-fat dried milk and 0.5% Tween-20 (Sigma-Aldrich), the membrane was incubated with individual primary antibodies (1:300; as described above) overnight at 4˚C. The membrane was then washed three times in PBS and incubated for 1 h with secondary antibodies at room temperature. The signals were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, Little Chalfont, UK) and developed on X-ray film (GE Healthcare). The band density was measured using the

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequence (5'-3')	Primer size (bp)
ABCA1		
Forward	GCTTTCAATCATCCCCTGAA	20
Reverse	TGACAGGCTTCACTCCACTG	20
ABCG1		
Forward	CTCCGGCTTCCTCTTCTTCT	20
Reverse	TACACGATGCTGCAGTAGGC	20
PPARγ		
Forward	GAGCCCAAGTTTGAGTTTGC	20
Reverse	CTGTGAGGACTCAGGGTGGT	20
LXRα		
Forward	ACGGTGATGCTTCTGGAGAC	20
Reverse	AGCAATGAGCAAGGCAAACT	20
GAPDH		
Forward	CGACCACTTTGTCAAGCTCA	20
Reverse	AGGGGAGATTCAGTGTGGTG	20

ABCA1, adenosine triphosphate-binding cassette, sub-family A member 1; ABCG1, ATP-binding cassette sub-family G member 1; PPARγ, peroxisome proliferator-activated receptor γ; LXRα, liver X receptor α.

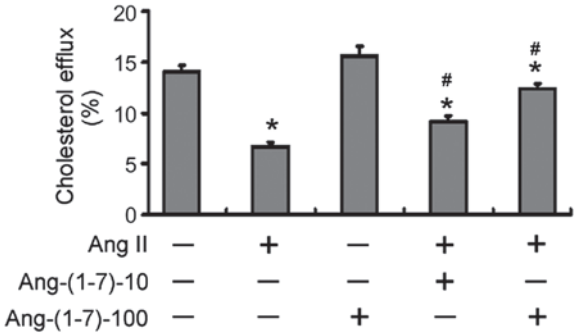

Figure 1. Effects of Ang-(1-7) on cholesterol efflux from Ang II-treated THP-1 macrophages. Differentiated THP-1 macrophages were labeled by incubation for 48 h in the presence of (3H)cholesterol and treated with Ang II and/or Ang-(1-7) for a further 24 h. Following treatment, the efflux of cholesterol to apoA-1 was measured. The data are expressed as the mean ± standard deviation of three independent experiments (*P<0.05, vs. untreated cells; #P<0.05, vs. Ang II-only cells). Ang-(1-7)-10 and Ang-(1-7)-100 indicate treatment with 10 or 100 nM Ang-(1-7), respectively. Ang, angiotensin.

GelDoc 2000 system equipped with Quantity One 4.6 software (Bio-Rad Laboratories, Inc.) and normalized against β-actin.

Statistical analysis. The data are expressed as the mean ± standard deviation. All statistical analyses were performed using SPSS 19.0 software (IBM, Armonk, NY, USA). Statistical differences among multiple groups were calculated using a one-way analysis of variance followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

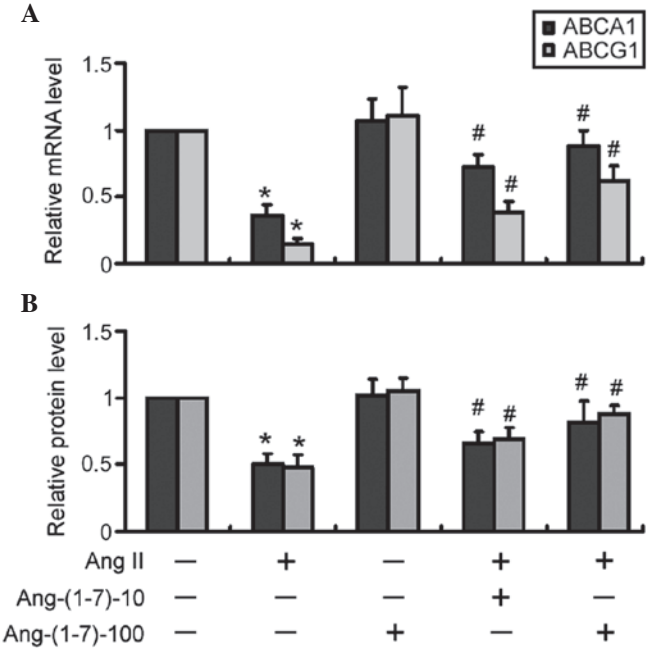

Figure 2. Effects of Ang-(1-7) on the expression levels of ABCA1 and ABCG1 in Ang II-treated THP-1 macrophages. Differentiated THP-1 macrophages were treated with Ang II and/or Ang-(1-7) for 24 h and the (A) mRNA and (B) protein expression levels of ABCA1 and ABCG1 were determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. The data are expressed as the mean ± standard deviation of three independent experiments (*P<0.05, vs. untreated cells; #P<0.05, vs. Ang II-only cells). Ang-(1-7)-10 and Ang-(1-7)-100 indicate treatment with 10 or 100 nM Ang-(1-7), respectively. Ang, angiotensin; ABC, adenosine triphosphate-binding cassette transporters.

Results

Ang-(1-7) antagonizes the Ang II-mediated repression of cholesterol efflux. Compared with the untreated control THP-1 macrophages, treatment with Ang II (1 μM) for 48 h significantly reduced the rate of cholesterol efflux to apoA-1 by ~50% (7.69±0.48, vs. 14.06±0.56%; P<0.05; Fig. 1),. Notably, treatment with Ang-(1-7) led to a dose-dependent restoration in cholesterol efflux to apoA-1. When the cells were treated with 100 nM Ang-(1-7), the cholesterol efflux rate (12.39±0.50) increased to a level similar to that observed in the control group.

Ang-(1-7) relieves the Ang II-mediated suppression of the gene expression levels of ABCA1 and ABCG1. The mRNA expression levels of ABCA1 and ABCG1 were significantly (P<0.05) reduced in the Ang II-treated THP-1 macrophages compared with the control cells (Fig. 2A). The co-treatment with Ang-(1-7) and Ang II significantly increased the mRNA expression levels of ABCA1 and ABCG1 in a dose-dependent manner (P<0.05), compared with Ang II only (Fig. 2A). In addition, western blot analysis revealed that treatment with Ang-(1-7) significantly (P<0.05) attenuated the Ang II-mediated reduction in the protein expression levels of ABCA1 and ABCG1 in a dose-dependent manner (Fig. 2B).

Ang-(1-7) restores the expression levels of PPARγ and LXRα in Ang II-treated cells. Treatment with Ang II alone

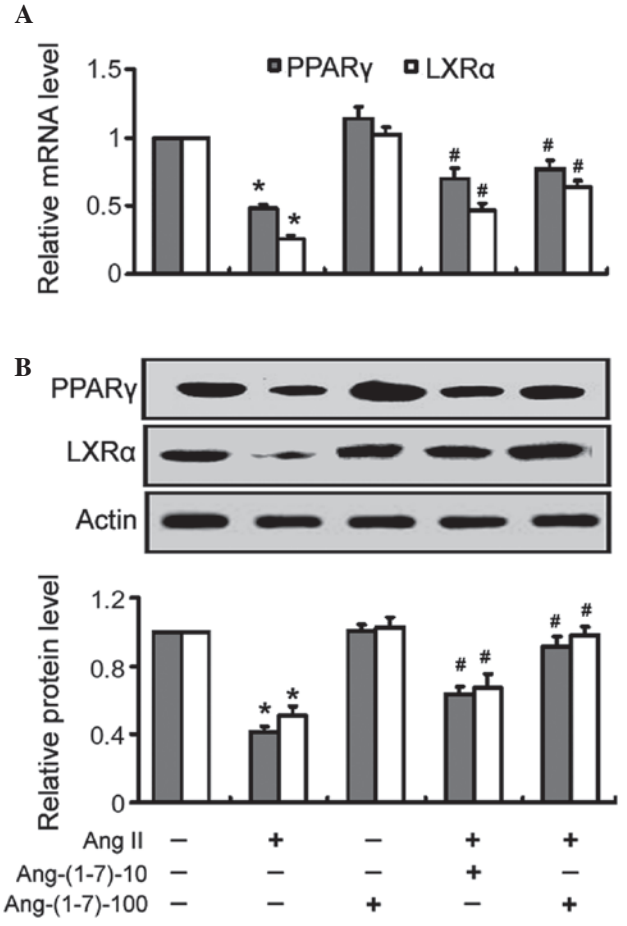

Figure 3. Ang-(1-7) stimulates the expression levels of PPARγ and LXRα in Ang II-treated THP-1 macrophages. The THP-1 macrophages were treated with Ang II and/or Ang-(1-7) for 24 h. (A) Reverse transcription quantitative polymerase chain reaction analysis of the mRNA expression levels of PPARγ and LXRα and (B) western blot analysis of the protein expression levels of PPARγ and LXRα. were performed. Representative blots of three independent experiments and densitometric quantification of the blots are shown (*P<0.05, vs. untreated cells; #P<0.05, vs. Ang II-only cells). Ang-(1-7)-10 and Ang-(1-7)-100 indicate treatment with 10 or 100 nM Ang-(1-7), respectively. PPAR, peroxisome proliferator-activated receptor; LXR, liver X receptor; Ang, angiotensin.

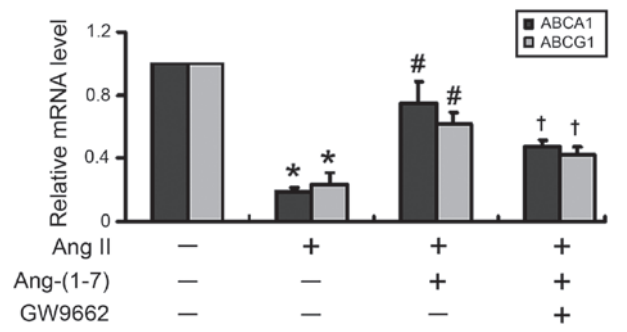

Figure 4. Pharmacological inactivation of PPARγ reverses the inductive effects of Ang-(1-7) on the expression levels of ABCA1 and ABCG1. The THP-1 macrophages were treated with Ang II with or without Ang-(1-7) with or without pretreatment with the PPARγ inhibitor, GW9662. Following incubation for 24 h, the mRNA expression≠ levels of ABCA1 and ABCG1 were determined by reverse transcription-quantitative polymerase chain reaction. The data are expressed as the mean ± standard deviation of three independent experiments (*P<0.05, vs. untreated cells; #P<0.05, vs. Ang II-only-cells; †P<0.05, vs. cells treated with Ang II and Ang-1-7). ABC, adenosine triphosphate-binding cassette transporter; Ang, angiotensin; PPAR, peroxisome proliferator-activated receptor.

significantly (P<0.05) reduced the mRNA expression levels of PPARγ and LXRα in the THP-1 macrophages, compared with the control cells (Fig. 3A). Treatment with Ang-(1-7) inhibited the Ang II-mediated suppression of PPARγ and LXRα expression in a dose-dependent manner (Fig. 3A). Similarly, the Ang II-mediated reduction in the protein expression of PPARγ and LXRα was almost completely inhibited following treatment with Ang-(1-7), as determined by western blot analysis (Fig. 3B).

Pharmacological inactivation of PPARγ attenuates the stimulatory effects of Ang-(1-7) on the mRNA expression levels of ABCA1 and ABCG1. Subsequently, the present study inhibited the activity of PPARγ to investigate whether the Ang-(1-7)-mediated stimulation of ABCA1 and ABCG1 expression was PPARγ-dependent. As shown in Fig. 4, inhibition of PPARγ with GW9662, an irreversible and selective PPARγ antagonist, significantly (P<0.05) eliminated the Ang-(1-7)-mediated induction of the mRNA expression of ABCA1 and ABCG1.

Inactivation of JNK and p38 MAPKs is associated with the Ang-(1-7)-mediated induction of PPARγ and LXRα expression. To elucidate the mechanisms underlying the Ang-(1-7)-induced expression of PPARγ and LXRα, MAPK signaling pathways were investigated by western blot analysis. It was revealed that treatment with Ang-(1-7) significantly (P<0.05) antagonized the inductive effect of Ang II on the phosphorylation of JNK and p38 MAPKs, but not ERK1/2 (Fig. 5A). The total protein expression levels of the MAPKs were not affected by treatment with Ang-(1-7). Notably, co-incubation with SB203580, a specific inhibitor of p38 MAPK, significantly (P<0.05) restored the expression levels of PPARγ and LXRα in Ang II-treated THP-1 macrophages (Fig. 5B), which mimicked the indusive effects of Ang-(1-7). Similarly, inhibition of JNK signaling using SP600125 significantly (P<0.05) attenuated the inhibition of PPARγ and LXRα expression by Ang II. Taken together, these results suggested that inactivation of p38 and JNK MAPK signaling was involved in the Ang-(1-7)-mediated induction of PPARγ and LXRα expression.

Discussion

Ang II ha been implicated in a variety of cardiovascular diseases, including atherosclerosis, hypertension, left ventricular hypertrophy, myocardial infarction and heart failure (22). It has been demonstrated that Ang II accelerates foam cell formation by upregulating the expression of acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT1), which converts intracellular free cholesterol into cholesterol ester for storage in lipid droplets (23). In addition, the upregulation of ACAT1, suppression of ABCA1 expression and subsequent cholesterol transport is known to mediate the atherosclerotic effect of Ang II (24). Consistently, the present study demonstrated that treatment with Ang II resulted in a significant reduction in cholesterol efflux from the THP-1 foam cells to apoA-1. This inhibition of cholesterol accumulation was coupled with the downregulation of ABCA1 and ABCG1. Notably, the presence of Ang-(1-7) significantly reversed the Ang II-induced repression of cholesterol efflux. Taken together, these results provided evidence that Ang-(1-7)

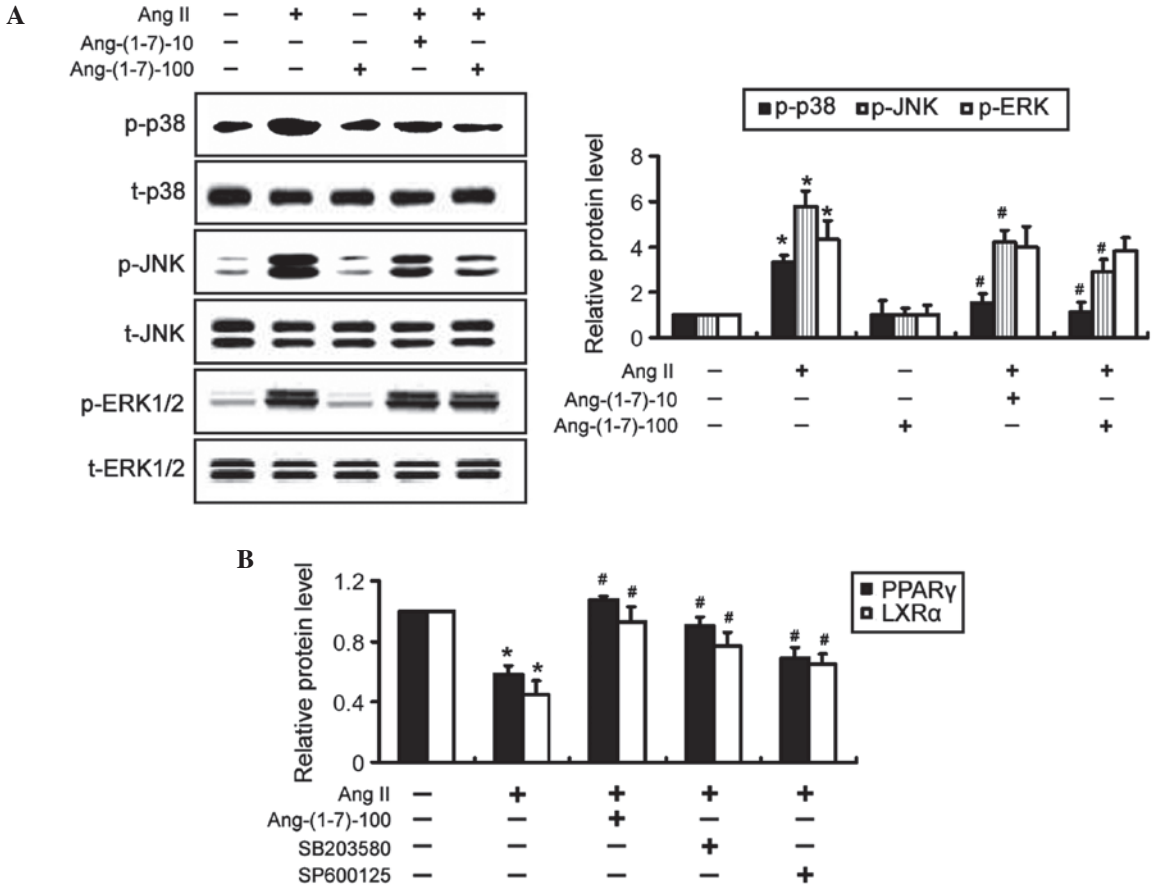

Figure 5. Ang-(1-7)-mediated induction of PPARγ and LXRα is associated with the suppression of JNK and p38 mitogen-activated protein kinase activation. (A) THP-1 macrophages were exposed to Ang II and/or Ang-(1-7) for 24 h and the phosphorylation levels of MAPKs were examined by western blot analysis. Representative blots of three independent experiments and densitometric quantification of the blots are shown. (B) THP-1 macrophages were treated with 10 μM SB203580 or SP600125 for 1 h prior to the addition of 1 μM Ang II. Following treatment, the protein expression levels of PPARγ and LXRα were determined by western blot analysis. The data are expressed as the mean ± standard deviation of three independent experiments (*P<0.05, vs. untreated cells; $^{\#}$P<0.05 vs. Ang II-only cells). Ang-(1-7)-10 and Ang-(1-7)-100 indicate treatment with 10 or 100 nM Ang-(1-7), respectively. Ang, angiotensin; p-, phosphorylated; t-, total; JNK, c-Jun NH2-terminal kianse; ERK, extracellular signal-regulated kinase; PPAR, peroxisome proliferator-activated receptor; LXRα, liver X receptor α.

and Ang II exhibited counter-regulatory roles in macrophage cholesterol homeostasis.

The present study revealed that treatment with Ang II caused a significant decrease in the mRNA expression levels of ABCA1 and ABCG1, suggesting its importance in transcriptional regulation. Notably, co-treatment with Ang-(1-7) significantly restored the expression levels of ABCA1 and ABCG1 in the Ang II-treated THP-1 macrophages. Since PPARγ and LXRα are the 'master' transcription factors, cooperatively regulate the expression levels of ABCA1 and ABCG1 (8,9), the present study assessed the effects of Ang-(1-7) on the expression levels of PPARγ and LXRα. It was found that the Ang II-mediated suppression of t PPARγ and LXRα expression was significantly reversed following treatment with Ang-(1-7). The Ang-(1-7)-mediated induction of PPARγ expression has also been demonstrated in other biological contexts (25,26). Dhaunsi *et al* (26) reported that treatment with Ang-(1-7) prevents the inhibition of PPARγ expression in diabetes and/or hypertension. The present study further demonstrated that the pharmacological inhibition of PPARγ with GW9662 significantly inhibited the Ang-(1-7)-mediated induction of ABCA1 and ABCG1 mRNA expression. These results suggested that treatment with Ang-(1-7) facilitated the PPARγ-dependent transcriptional activation of the ABC genes, which provided a possible explanation for its promotion of cholesterol efflux from foam cells.

There is evidence that MAPK signaling is involved in foam cell formation (27-29). Ren *et al* (27) demonstrated that oxidized HDL accelerates foam cell formation via the p38 MAPK-dependent upregulation of PPARγ and downregulation of cluster of differentiation 36. Mei *et al* (29) demonstrated that activation of p38 MAPK promotes cholesterol ester accumulation in macrophages. Pharmacological inhibition of p38 MAPK signaling, including treatment with curcumin, has also been revealed to hinder oxidized LDL-induced foam cell formation (30). In addition to p38 MAPK, activation of the JNK MAPK is involved in foam cell formation from macrophages, which are exposed to oxidized LDL (31). In agreement with previous studies, the present study revealed that the Ang-(1-7)-mediated promotion of cholesterol efflux in Ang II-treated THP-1 macrophages is coupled with the inactivation of JNK and p38 MAPK signaling. Additionally, the inhibition of MAPK signaling using specific pharmacological inhibitors mimicked the induction of PPARγ and LXRα expression by Ang-(1-7). These results suggested that the Ang-(1-7)-mediated

restoration of PPARγ and LXRα expression was associated, in part, with the suppression of MAPK signaling. The inhibition of MAPK signaling by Ang-(1-7) has also been demonstrated in other biological settings. Santos *et al* (32) reported that oral treatment with Ang-(1-7) prevents obesity and hepatic inflammation in high-fat fed rats, which is associated with the suppression of the MAPK signaling pathway. Moon *et al* (33) revealed that Ang II-induced MAPK signaling activation is attenuated by Ang-(1-7) in mesangial cells.

In conclusion, the present study provided the first evidence, to the best of our knowledge, that Ang-(1-7) facilitated cholesterol efflux in Ang II-treated THP-1 macrophages, which, in part, involved the suppression of p38 and JNK signaling and the induction of PPARγ and LXRα expression. These findings suggested that Ang-(1-7) may offer therapeutic benefits in preventing the development of atherosclerosis.

Acknowledgements

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