Mechanism of the inhibitory effect of atorvastatin on leptin expression induced by angiotensin II in cultured human coronary artery smooth muscle cells

Kou-Gi SHYU†, Shih-Chung CHEN‡§, Bao-Wei WANG*, Wen-Pin CHENG* and Huei-Fong HUNG*

*Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, †Graduate Institute of Clinical Medicine, Taipei Medical University, Taipei, Taiwan, ‡Department of Cardiology, New Taipei City Hospital, Taipei, Taiwan, and §Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan

ABSTRACT

Leptin contributes to the pathogenesis of atherosclerosis. Ang II (angiotensin II), a proatherogenic cytokine, increases leptin synthesis in cultured adipocytes. Statin suppresses leptin expression in adipocytes and human coronary artery endothelial cells. However, the effect of Ang II and statin on leptin expression in VSMCs (vascular smooth muscle cells), the major cell types in atheroma, is poorly understood. Thus the aim of the present study was to investigate the molecular mechanism of atorvastatin for reducing leptin expression after Ang II stimulation in VSMCs. VSMCs from human coronary artery were cultured. Ang II stimulation increased leptin protein and mRNA and phospho-JNK (c-Jun N-terminal kinase) expression. Exogenous addition of Dp44mT (2,2′-dipyridyl-N,N-dimethylsemicarbazone) and mevalonate increased leptin protein expression similarly to Ang II. Atorvastatin, SP600125, JNK siRNA (small interfering RNA) and NAC (N-acetylcysteine) completely attenuated the leptin and phospho-JNK protein expression induced by Ang II. Ang II significantly increased ROS (reactive oxygen species) formation in human VSMCs. Addition of atorvastatin and NAC significantly attenuated the formation of ROS induced by Ang II. Addition of atorvastatin and SP600125 inhibited the phosphorylation of Rac1 induced by Ang II. The gel shift and promoter activity assay showed that Ang II increased AP-1 (activator protein-1)-binding activity and leptin promoter activity, while SP600125, NAC and atorvastatin inhibited the AP-1-binding activity and leptin promoter activity induced by Ang II. Ang II significantly increased the migration and proliferation of cultured VSMCs, while addition of atorvastatin, SP600125, NAC and leptin siRNA before Ang II stimulation significantly inhibited the migration and proliferation of VSMCs induced by Ang II. Ang II significantly increased secretion of leptin from human VSMCs, and addition of SP600125, atorvastatin and NAC before Ang II stimulation almost completely inhibited the leptin secretion induced by Ang II. In conclusion, Ang II induces leptin expression in human VSMCs, and atorvastatin could inhibit the leptin expression induced by Ang II. The inhibitory effect of atorvastatin on Ang II-induced leptin expression was mediated by Rac, ROS and JNK pathways.

Key words: angiotensin II, atherosclerosis, leptin, smooth muscle cell, statin.

Abbreviations: Ang II, angiotensin II; AP-1, activator protein-1; CAD, coronary artery disease; CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; Dp44mT, 2,2′-dipyridyl-N,N-dimethylsemicarbazone; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; JNK, c-Jun N-terminal kinase; EMSA, electrophoretic mobility-shift assay; ERK, extracellular-signal-regulated kinase; HCASMC, human coronary artery smooth muscle cell; MAPK, mitogen-activated protein kinase; NAC, N-acetylcysteine; ROS, reactive oxygen species; siRNA, small interfering RNA; SMC, smooth muscle cell; VSMC, vascular smooth muscle cell.

Correspondence: Dr Huei-Fong Hung (email shyukg@tmu.edu.tw).

© The Authors Journal compilation © 2012 Biochemical Society
INTRODUCTION

Leptin, the product of the obesity gene, is a 16 kDa plasma protein mainly secreted by adipocytes and is involved in the regulation of appetite and energy metabolism [1,2]. It has peripheral actions to stimulate vascular inflammation, oxidative stress and VSMC (vascular smooth muscle cell) hypertrophy, and contributes to atherosclerosis [3,4]. Several studies have shown an independent interaction between high leptin and atherosclerosis, myocardial infarction, stroke and coronary artery intima-media thickness, suggesting that high levels of leptin may increase cardiovascular risk [5–7]. Recently, Bodary et al. [8] have shown that direct administration of leptin in apolipoprotein E-deficient mice results in increased atherosclerosis. Recent reports have shown leptin production by non-adipose cells, such as vascular endothelial cells and SMCs (smooth muscle cells) [9,10], indicating that leptin may be an inducible, locally produced, proatherogenic mediator.

Leptin increases oxidative stress in vascular cells [11] and promotes VSMC migration and proliferation [12], decreases arterial distensibility [13] and contributes to obesity-associated hypertension. Ang II (angiotensin II), a proatherogenic cytokine and local mediator of inflammation, increases leptin synthesis in cultured adipocytes and in rats in vivo [14,15]. However, the effect of Ang II on leptin expression in VSMCs, the major cell types in atheroma, is poorly understood.

Statin, a potent inhibitor of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase, has been shown to reduce blood leptin levels in hypercholesterolaemic rabbits and in patients with CAD (coronary artery disease) [16,17]. Statin also suppresses leptin expression in adipocytes and human coronary artery endothelial cells [18,19]. The effect of statin on leptin expression in VSMCs is poorly understood. Ang II has been shown to increase leptin expression in fat cells via the MAPK (mitogen-activated protein) kinase and ERK1/2 (extracellular-signal-regulated kinase 1/2)-dependent pathway [14]. The pleiotropic effect of statin is mainly via the Rac1 pathway. Ang II plays a crucial role in atherosclerosis. We hypothesize that Ang II may induce leptin expression in VSMCs and statin may suppress leptin expression induced by Ang II via MAPK and Rac1 pathway. Thus the aim of present study was to investigate the molecular mechanism of leptin expression in VSMCs under atorvastatin treatment with Ang II stimulation.

MATERIALS AND METHODS

Drugs

Atorvastatin, a calcium salt of a penta-substituted pyrrole, was supplied by Pfizer. A 10 mmol/l stock solution was made in 100 % DMSO. Ang II was purchased from Bachem, Torrance, CA, U.S.A. Recombinant leptin protein was purchased from PeproTech, Rocky Hill, NJ, U.S.A. Mevalonate was purchased from Sigma, St Louis, MO, U.S.A.; PD98059 (a specific and potent inhibitor of ERK kinase), SB203580 (a highly specific, cell-permeable inhibitor of p38 kinase), SP600125 (a potent, cell-permeable, selective and reversible inhibitor of JNK (c-Jun N-terminal kinase)), NAC (N-acetylcysteine; a free radical scavenger) and NSC23766 (Rac1 inhibitor; a cell-permeable pyrimidine compound that specifically inhibits Rac1) were purchased from Calbiochem®, San Diego, CA, U.S.A. These inhibitors were added 30 min before Ang II stimulation. Dp44mT (2,2′-dipyridyl- N,N-dimethylsemicarbazone), an iron chelator to generate ROS (reactive oxygen species), and anisomycin, a MAPK activator, were purchased from Calbiochem®.

VSMC culture

HCASMCs (human coronary artery smooth muscle cells) were originally obtained from PromoCell GmbH (Heidelberg, Germany). The cells were cultured in SMC growth medium supplemented with 10 % fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5 % CO₂ in air. Cells were grown to 80–90 % confluence in 10 cm² culture dishes and were sub-cultured at a ratio of 1:2. When confluent, SMCs monolayers were passaged every 6–7 days after trypsinization and were used for experiments from the third to sixth passages. Cells of these third to sixth passages were incubated for an additional 2 days to render them quiescent before the initiation of each experiment.

Western blot analysis

Cells were homogenized in modified RIPA buffer [50 mM Tris/HCl (pH 7.4), 1% Nonidet P40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin and pepstatin, 1 mM sodium orthovanadate and 1 mM sodium fluoride] and Western blot analysis was performed as previously described [20,21]. All Western blots were quantified using densitometry. Monoclonal anti-α-tubulin antibody (Sigma), polyclonal anti-leptin and monoclonal anti-phospho-JNK antibodies (Santa Cruz Biotechnology), polyclonal anti-JNK and polyclonal anti-Rac antibodies (Cell Signaling, Beverly, MA, U.S.A.) were used.

Real-time PCR

Total RNA from cultured HCASMCs (human coronary artery SMCs) was extracted using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. Real-time reverse transcription-PCR was performed as previously described [21]. The human leptin primers were 5′-GTGGCCTTCCAGTAGATCTCTT-3′ and 5′-AGCCACAAGATCCGCACAGG-3′. A Lightcycler (Roche Diagnostics, Mannheim, Germany) was used for real-time PCR as
previously described [21]. Amplification, fluorescence detection and post-processing calculation were performed using the Lightcycler apparatus. Individual PCR products were analysed for DNA sequence to confirm the purity of the product.

**Measurement of leptin concentration**

Conditioned media from Ang II-treated cells and those from control (non-Ang II-treated) were collected for leptin measurement. The level of leptin was measured by a quantitative sandwich enzyme immunoassay technique (R&D Systems, Minneapolis, MN, U.S.A.). The lower limit of detection of leptin was 15.6 pg/ml.

**ROS**

Intracellular ROS was assessed by the ROS-specific probe CM-H2DCFDA [5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester]. CM-H2DCFDA (20 μM) was pretreated in the cultured cells for 30 min and incubated at 37°C. After Ang II stimulation, cells were collected by trypsinization and resuspended in PBS medium. ROS assay was performed according to the manufacturer’s instruction (Invitrogen, Eugene, OR, U.S.A.). Fluorescence signals were obtained with a fluorescence conversion microscope (Nikon DIAPHOT 300) and assayed by its image processing and analysis software.

**RNA interference**

Cells were transfected with 800 ng of JNK annealed siRNA (small interfering RNA) or leptin siRNA oligonucleotide (Dharmacon, Lafayette, CO, U.S.A.). JNK or leptin siRNA is a target-specific 20–25 nt siRNA designed to knock down gene expression. The JNK sense of siRNA sequences was 5′-CGUGGAUUUAUGGUCUGA-3′. As a negative control, a non-targeting siRNA (scrambled siRNA) purchased from Dharmacon was used. VSMCs were transfected with siRNA oligonucleotides using Effectene Transfection Reagent as suggested by the manufacturer (Qiagen, Valencia, CA, U.S.A.).

**EMSA (electrophoretic mobility-shift assay)**

Nuclear protein concentrations from HCASMCs were determined by Bio-Rad protein assay. Consensus and control oligonucleotides (Santa Cruz Biotechnology) were labelled by polynucleotides kinase incorporation of [γ-32P]ATP. After the oligonucleotide was radiolabelled, the nuclear extracts (4 μg of protein in 2 μl of nuclear extract) were mixed with 20 pmol of the appropriate [γ-32P]ATP-labelled consensus or mutant oligonucleotide in a total volume of 20 μl for 30 min at room temperature (22°C). The samples were then resolved on a 4% polyacrylamide gel. Gels were dried and imaged by autoradiography. Controls were performed in each case with mutant oligonucleotides or unlabelled oligonucleotides to compete with labelled sequences.

**Promoter activity assay**

Leptin gene was amplified with specific forward and reverse primers. The amplified product was digested with MluI and BglII restriction enzymes and ligated into pGL3-basic luciferase plasmid vector (Promega) digested with the same enzymes. Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into cells using a low-pressure-accelerated gene gun (Bioware Technologies, Taipei, Taiwan), essentially following the protocol from the manufacturer. Test plasmid at 2 μg and control plasmid (pGL4-Renilla luciferase), 0.02 μg, were co-transfected with a gene gun in each well, and then were replaced by the normal culture medium. Following a time course of Ang II treatment, cell extracts were prepared using Dual-Luciferase Reporter Assay System (Promega) and were measured for dual luciferase activity by a luminometer (Turner Designs, Sunnyvale, CA, U.S.A.).

**Migration assay**

The migration activity of HCASMCs was determined using the growth-factor-reduced Matrigel invasion system (Becton Dickinson) following the protocol provided by the manufacturer. Migration assay was performed as previously described [22]. 5 × 10^4 SMCs were seeded on top of an ECMatrix gel (Chemicon International, Temecula, CA, U.S.A.) prepared as described previously [23]. Cells were then incubated at 37°C for 18 h. Three different phase-contrast microscopic high-power fields per well were photographed. The migratory VSMCs with positive stain were counted and the observer was blind to the experiment.

**Proliferation assay**

The proliferation of HCASMCs was determined using [3H]thymidine incorporation. SMCs were seeded on ViewPlate (Packard Instrument, Meriden, CT, U.S.A.) at a density of 5 × 10^4 cells/well in serum-free medium. Thymidine uptake was studied by an addition of 500 nCi/ml [3H]thymidine (PerkinElmer, Boston, MA, U.S.A.) for 6 and 18 h. Cells were washed twice with PBS. Non-specific uptake was studied in the presence of 10 μM cytochalasin B and subtracted from the measured value. MicroScint-20 (50 μl) was added, and the plate was read with TopCount (Packard Instrument).

**Statistical analysis**

The results are expressed as means ± S.D. Statistical significance was performed with ANOVA (GraphPad Software, San Diego, CA, U.S.A.). The Dunnett’s test was used to compare multiple groups to a single control group. The Tukey–Kramer comparison test was used for pairwise comparisons between multiple groups after the
**RESULTS**

**Ang II increases leptin expression in cultured VSMCs**

Western blot was used first to investigate the effect of Ang II on leptin expression on HCASMCs. Leptin protein expression was significantly induced at 4 h after Ang II stimulation, maximally induced at 18 h, and remained elevated for 24 h (Figures 1A and 1B). The leptin protein expression was confirmed with another antibody that reacts to the N-terminus of leptin of human origin purchased from Santa Cruz Biotechnology. The expression of leptin mRNA was significantly induced by Ang II from 4 to 24 h of stimulation (Figure 1C).

**Atorvastatin inhibits the effect of Ang II on leptin expression via JNK and Rac1 pathways**

To investigate the atorvastatin inhibitory mechanism on induction of leptin by Ang II, JNK and Rac pathways were studied. PD98059 (50 μM), a potent inhibitor of p42/p44 MAPK and SB203580 (3 μM), a potent inhibitor of p38 MAPK had partial inhibition effect on leptin protein expression induced by Ang II (Figures 2A and 2B). SP600125 (20 μM), a potent inhibitor of JNK and JNK siRNA completely blocked the leptin protein expression induced by Ang II. Exogenous addition of Dp44mT increased leptin protein expression similarly to Ang II and NAC (500 μM), an antioxidant scavenger, completely blocked the leptin expression induced by Ang II. These findings indicate that the JNK and ROS pathways are the main signalling pathways mediating the induction of leptin protein expression by Ang II. To test whether atorvastatin can inhibit the effect of Ang II on leptin expression in HCASMCs, different doses of atorvastatin were added before Ang II stimulation. As shown in Figures 2(C) and 2(D), atorvastatin inhibited the leptin protein expression induced by Ang II in a dose-dependent manner. Atorvastatin (10 μM) almost completely inhibited the leptin protein expression induced by Ang II. The addition of mevalonate at 200 μM for 18 h had an effect similar to Ang II stimulation in leptin protein expression (Figures 2E and 2F). However, atorvastatin did not have any effect on leptin protein expression induced by mevalonate. Atorvastatin alone had a neutral effect on leptin expression similar to control cells. This finding suggested that mevalonate has proinflammatory effect on leptin expression in HCASMCs and atorvastatin inhibited the Ang II-induced leptin expression via the HMG-CoA reductase pathway.

Rac1 inhibitor (50 μM) almost completely blocked the effect of Ang II on leptin induction. Addition of Rac1 inhibitor did not affect the leptin expression induced by Dp44mT (Figures 2E and 2F). Anisomycin (20 μM) significantly increased leptin protein expression similarly to Ang II (Figures 2G and 2H). Under anisomycin stimulation, addition of atorvastatin and NAC did not have inhibitory effect on leptin protein expression. Under Dp44mT stimulation, atorvastatin did not have an inhibitory effect on leptin expression, while the addition of SP600125 completely attenuated the leptin protein expression induced by Dp44mT (Figures 2G and 2H). The phospho-JNK protein expression was significantly induced by Ang II from 2 to 24 h (Figures 3A and 3B). Atorvastatin, SP600125, JNK siRNA and NAC significantly attenuated the phospho-JNK protein expression induced by Ang II. As shown in Figures 3(C) and 3(D), Ang II induced phosphorylation of Rac1 in a time-dependent manner. Ang II did not have any effect
Atorvastatin inhibits leptin expression in VSMCs

Figure 2 Induction of leptin protein expression by Ang II is mediated by the JNK and Rac1 pathways and atorvastatin attenuates the leptin protein expression induced by Ang II (A, C, E and G) Representative Western blots for leptin in HCASMCs treated with Ang II in the presence or absence of various inhibitors, atorvastatin or mevalonate (200 μM). (B, D, F and H) Quantitative analysis of leptin protein levels. The values from treated VSMCs have been normalized to matched α-tubulin measurement and then expressed as a ratio of normalized values to protein expression in control cells. (n = 4 per group). (B) *P < 0.05 compared with 18 h. **P < 0.001 compared with 18 h. + P < 0.001 compared with control. (D) **P < 0.001 compared with control. + P < 0.001 compared with Ang II (lane 2). ++ P < 0.001 compared with Ang II (lane 2). (F) **P < 0.001 compared with control (lane 1), + P < 0.001 compared with Ang II (lane 2). (H) *P < 0.001 compared with Dp44mT alone (lane 5). **P < 0.001 compared with Ang II (lane 2).

on total Rac1. The addition of atorvastatin and SP600125 inhibited the phosphorylation of Rac1 induced by Ang II. Rac 1 inhibitor completed inhibited the phosphorylation of Rac1.

Ang II significantly increased ROS formation in HCASMCs (Figures 4A and 4B). Exogenous addition of Dp44mT (30 nM) increased ROS formation similarly to Ang II. The addition of atorvastatin and NAC (500 μM) significantly attenuated the formation of ROS induced by Ang II. These results indicate that the Rac1 pathway mediates the inhibitory effect of atorvastatin on leptin expression induced by Ang II. Rac1 was activated by Ang II before ROS formation, and the JNK pathway was activated after ROS formation.
Ang II induces phosphorylation of JNK and Rac protein expression in HCASMCs

Figure 3  Ang II induces phosphorylation of JNK and Rac protein expression in HCASMCs

(A, C) Representative Western blots for phospho-JNK, total JNK, phospho-Rac and total Rac in HCASMCs treated with Ang II in the presence or absence of atorvastatin and different inhibitors. (B, D) Quantitative analysis of phospho-JNK and phospho-Rac protein. The values from treated HCASMCs have been normalized to matched α-tubulin measurement and then expressed as a ratio of normalized values to protein expression in control cells. (n = 4 per group). **P < 0.001 compared with control. *P < 0.01 compared with control.

Ang II increases AP-1 (activator protein-1)-binding activity and leptin promoter activity

The EMSA assay showed that Ang II increased AP-1 DNA-protein binding activity (Figure 5). An excess of unlabelled AP-1 oligonucleotide competed with the probe for binding AP-1 protein, whereas an oligonucleotide containing a 2 bp substitution in the AP-1-binding site did not compete for binding. The addition of SP600125, NAC and atorvastatin 30 min before Ang II stimulation abolished DNA–protein binding activity induced by Ang II. Exogenous addition of Dp44mT without Ang II stimulation also increased DNA–protein binding activity. To study whether the leptin expression induced by Ang II is regulated at the transcriptional level, we cloned the promoter region of human leptin (−866~+35) and constructed a luciferase reporter plasmid (pGL3-Luc). The leptin promoter construct contains AP-1, GATA, HIF (hypoxia-inducible factor)-1α, Myc-Max and NF-κB (nuclear factor κB) binding sites. As shown in Figures 5(B) and 5(C), a transient transfection experiment with HCASMCs using this reporter gene revealed that Ang II stimulation for 2 h significantly caused leptin promoter activation. This result indicates that leptin expression is induced at the transcriptional level by Ang II. When the AP-1 binding sites were mutated, the increased promoter activity induced by Ang II was abolished. Moreover, addition of SP600125, NAC and atorvastatin 30 min before Ang II stimulation caused an inhibition of transcription. Dp44mT, like Ang II, also significantly increased the leptin promoter activity. These results suggested that the AP-1-binding site in the leptin promoter is essential for the transcriptional regulation by Ang II and that Ang II regulates the leptin promoter via the JNK and ROS pathways.

Atorvastatin attenuates VSMC migration and proliferation induced by Ang II

To test the effect of Ang II and leptin on the function of VSMCs, the migration activity of HCASMCs was examined. As shown in Figures 6(A) and 6(B), Ang II significantly increased the SMC migration activity as compared with control. Addition of atorvastatin, SP600125, NAC and leptin siRNA significantly abolished the migration activity of SMCs induced by Ang II. Control siRNA did not affect the migration activity of SMCs. Dp44mT had a similar effect on SMC migration activity compared with Ang II. Ang II and leptin recombinant protein for 6 and 18 h stimulation also significantly increased the proliferation of cultured SMCs (Figure 6C). The addition of atorvastatin, SP600125, NAC and leptin siRNA before Ang II stimulation significantly inhibited the proliferation of SMCs induced by Ang II. Dp44mT had a similar effect on SMC proliferation compared
Atorvastatin inhibits leptin expression in VSMCs

Figure 4 Ang II increases ROS formation in HCASMCs
(A) Representative microscopic image for ROS assay with (upper panel) or without (lower panel) green fluorescence in HCASMCs subjected to Ang II stimulation for 1–2 h or control cells without Ang II in the absence or presence of atorvastatin, NAC or Dp44mT. (B) Quantitative analysis of the positive fluorescent cells (n = 4 per group). *P < 0.01 compared with control. **P < 0.001 compared with control. +P < 0.01 compared with 1 h.

with Ang II and leptin. The leptin siRNA almost completely inhibited the leptin protein expression induced by Ang II, while scrambled siRNA did not have the inhibitory effect (see Supplementary Figure S1 at http://www.clinsci.org/cs/122/cs1220033add.htm).

Ang II increases secretion of leptin from HCASMCs
Ang II stimulation for 4–24 h significantly increased the secretion of leptin from HCASMCs (see Supplementary Figure S2 at http://www.clinsci.org/cs/122/cs1220033add.htm). Addition of SP600125, atorvastatin and NAC 30 min before Ang II stimulation almost completely inhibited the leptin secretion induced by Ang II.

DISCUSSION
In the present study, we demonstrated several significant findings. First, Ang II up-regulates leptin expression in cultured HCASMCs; secondly, Ang II induces secretion of leptin from HCASMCs; thirdly, Rac1, JNK kinase, ROS and AP-1 transcription factor are involved in the signalling pathway of leptin induction; fourthly, atorvastatin inhibits leptin expression induced by Ang II; fifthly, atorvastatin inhibits migration and proliferation of HCASMCs induced by Ang II. Leptin is considered to be a specific adipocyte product. Recent reports [9,10] and our present study have shown leptin production by non-adipose cells. Sugiyama et al. [10] have reported that a combination of hypoxia and high low-density...
Figure 6  Ang II increases migration activity and proliferation of HCASMCs

(A) HCASMCs that had migrated through a filter were stained. HCASMCs were treated with Ang II for 18 h in the presence or absence of atorvastatin, siRNA or inhibitors. (B) Migration of HCASMCs was quantified by staining and counting the number of cells that migrated to the bottom of the filter in five fields under an 400× high-power field (HPF; n = 4 per group). *P < 0.001 compared with control. +P < 0.001 compared with Ang II (lane 2). (C) Quantitative analysis of proliferation of HCASMCs by [3H]thymidine incorporation assay (n = 4 per group). *P < 0.001 compared with control. +P < 0.01 Ang II.

Lipoprotein level conditions induced leptin expression in HCASMCs. Our study is the first one to investigate VSMC leptin in detail. The result in our study may change our conception of leptin from an adipose-tissue-derived circulating adipokine to an inducible, locally produced, proatherogenic mediator.

Higher plasma leptin levels are associated with hypertension, suggesting plasma leptin level as a novel adipose-tissue-derived circulating adipokine to an inducible, locally produced, proatherogenic mediator.

Higher plasma leptin levels are associated with hypertension, suggesting plasma leptin level as a novel adipose-tissue-derived circulating adipokine to an inducible, locally produced, proatherogenic mediator.

Oxidative stress seems to play a pivotal role in the leptin effect [4]. Statin has been shown to inhibit the sympathetic nervous system through inhibition of ROS in the rostral ventrolateral medulla [29]. The ROS are mainly produced by the AT1 receptor (angiotensin II type I receptor). In the present study, Ang II increased ROS formation, and exogenous addition of Dp44mT had a similar effect to Ang II. The increased ROS could be significantly attenuated by atorvastatin and NAC, a free radical scavenger. NAC also inhibited the leptin secretion from HCASMCs induced by Ang II. The inhibition of ROS by atorvastatin in VSMCs may explain the clinical effect of atorvastatin to reduce blood pressure [30].

The effect of leptin on proliferation of SMCs is controversial. A few studies have reported that leptin inhibits cell growth of VSMCs and inhibits independent of its lipid-lowering action [17]. Statins have been shown to reduce lipid-lowering effects as well as pleiotropic properties. Statins have been shown to reduce leptin expression in human coronary artery endothelial cells by reducing C-reactive protein production [19] and in adipocytes by inhibition of protein prenylation [18]. These results implicate that statins may control inflammatory responses by inhibiting leptin expression. In the present study, Ang II was able to increase leptin secretion from VSMCs and atorvastatin significantly inhibited the leptin secretion induced by Ang II.

Ang II has been shown to promote leptin production in fat cells in a dose- and time-dependent fashion [28]. In the present study, we have demonstrated that Ang II-induced leptin protein and mRNA expression in HCASMCs and atorvastatin decreased Ang II-induced leptin expression in a dose-dependent manner. The induction of leptin protein by Ang II was largely mediated by the JNK kinase pathway because the specific and potent inhibitors of an upstream JNK kinase, SP600125 and JNK siRNA, inhibited the induction of leptin protein. Atorvastatin also inhibited the phosphorylation of Rac induced by Ang II. The promoter activity of wild-type leptin promoter after Ang II stimulation was significantly higher than that of AP-1 mutant leptin promoter. This finding indicates that Ang II regulates leptin in rat VSMCs at the transcriptional level and that an AP-1-binding site in the leptin promoter is essential for transcriptional regulation. Taken together, our results indicate that Ang II may increase the AP-1 transcriptional activity in HCASMCs. Leptin induced by Ang II was largely through JNK, Rac and leptin promoter pathways, and atorvastatin could inhibit leptin expression through inhibition of Rac phosphorylation, reduced AP-1 binding activity and leptin promoter activity.

Statin has been shown to reduce lipid-lowering effects as well as pleiotropic properties. Statins have been shown to reduce leptin expression in human coronary artery endothelial cells by reducing C-reactive protein production [19] and in adipocytes by inhibition of protein prenylation [18]. These results implicate that statins may control inflammatory responses by inhibiting leptin expression. In the present study, Ang II was able to increase leptin secretion from VSMCs and atorvastatin significantly inhibited the leptin secretion induced by Ang II.

Ang II has been shown to promote leptin production in fat cells in a dose- and time-dependent fashion [28]. In the present study, we have demonstrated that Ang II-induced leptin protein and mRNA expression in HCASMCs and atorvastatin decreased Ang II-induced leptin expression in a dose-dependent manner. The induction of leptin protein by Ang II was largely mediated by the JNK kinase pathway because the specific and potent inhibitors of an upstream JNK kinase, SP600125 and JNK siRNA, inhibited the induction of leptin protein. Atorvastatin also inhibited the phosphorylation of Rac induced by Ang II. The promoter activity of wild-type leptin promoter after Ang II stimulation was significantly higher than that of AP-1 mutant leptin promoter. This finding indicates that Ang II regulates leptin in rat VSMCs at the transcriptional level and that an AP-1-binding site in the leptin promoter is essential for transcriptional regulation. Taken together, our results indicate that Ang II may increase the AP-1 transcriptional activity in HCASMCs. Leptin induced by Ang II was largely through JNK, Rac and leptin promoter pathways, and atorvastatin could inhibit leptin expression through inhibition of Rac phosphorylation, reduced AP-1 binding activity and leptin promoter activity.

Oxidative stress seems to play a pivotal role in the leptin effect [4]. Statin has been shown to inhibit the sympathetic nervous system through inhibition of ROS in the rostral ventrolateral medulla [29]. The ROS are mainly produced by the AT1 receptor (angiotensin II type I receptor). In the present study, Ang II increased ROS formation, and exogenous addition of Dp44mT had a similar effect to Ang II. The increased ROS could be significantly attenuated by atorvastatin and NAC, a free radical scavenger. NAC also inhibited the leptin secretion from HCASMCs induced by Ang II. The inhibition of ROS by atorvastatin in VSMCs may explain the clinical effect of atorvastatin to reduce blood pressure [30].

The effect of leptin on proliferation of SMCs is controversial. A few studies have reported that leptin inhibits cell growth of VSMCs and inhibits
proliferation of SMCs induced by Ang II [31–33]. However, more studies have demonstrated that leptin induces proliferation and hypertrophy of VSMCs in cell culture and animal studies [12,34–37]. These contradictory results raise some doubts as regards the potential involvement of leptin in vascular remodelling. Different cell types and cultured conditions may have different results on leptin effect on VSMCs. However, results from clinical studies indicate that leptin increases cardiovascular risk, and plasma leptin levels may become a biomarker for hypertension. Our result also demonstrated that recombinant leptin enhanced migration and proliferation of HCASMCs, both of which could be attenuated by atorvastatin. These results indicate that leptin still seems to be a proatherogenic cytokine in vascular pathophysiology.

Ang II increased leptin secretion from HCASMCs and increased migration and proliferation of HCASMCs. Leptin siRNA inhibited leptin secretion and migration and proliferation of HCASMCs induced by Ang II. This finding indicates that there is an autoregulatory loop of leptin expression. The autoregulatory loop of leptin expression is mediated by JNK and ROS signalling pathways, since both JNK inhibitor and NAC inhibited leptin secretion and migration and proliferation of HCASMCs induced by Ang II.

The pathway that controls induction of leptin expression under Ang II stimulation in HCASMCs and the inhibitory effect of atorvastatin on leptin expression is shown in Figure 7. Our findings provide further evidence for the pleiotropic effect of statins. A better understanding of the detailed mechanisms of statin will provide us with new insight into the prevention and therapeutic development for leptin-induced atherosclerosis that is frequently encountered in patients suffering from CAD.

**AUTHOR CONTRIBUTION**

Kou-Gi Shyu participated in the design of the study and drafted the paper. Shih-Chung Chen made substantial contributions to conception and design, and analysis and interpretation, of data. Bao-Wei Wang made substantial contributions to conception and design, and acquisition, of data or analysis and interpretation of data. Wen-Pin Cheng made substantial contributions to acquisition of data or analysis and interpretation of data. Huei-Pong Hung participated in the design of the study and final approval of the paper prior to submission.

**FUNDING**

This work was supported by Pfizer, Taiwan (research grant to H.F.H.) and Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan [grant number SKH-8302-99-DR-02].

**REFERENCES**

Supplementary Online Data

Mechanism of the inhibitory effect of atorvastatin on leptin expression induced by angiotensin II in cultured human coronary artery smooth muscle cells

Kou-Gi SHYU†, Shih-Chung CHEN‡§, Bao-Wei WANG*, Wen-Pin CHENG* and Huei-Fong HUNG*

*Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, †Graduate Institute of Clinical Medicine, Taipei Medical University, Taipei, Taiwan, ‡Department of Cardiology, New Taipei City Hospital, Taipei, Taiwan, and §Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan

Figure S1 Effect of leptin siRNA on leptin protein expression

(A) Representative Western blottings for leptin in rat VSMCs treated with Ang II (10 nM) for 18 h in the presence or absence of siRNA. (B) Quantitative analysis of leptin protein levels. The values from treated VSMCs have been normalized to values in control cells (n = 4 per group).

Figure S2 Ang II increases leptin secretion

Leptin collected from cultured medium was measured by the ELISA method.

*P < 0.01 compared with control. **P < 0.001 compared with control.

+P < 0.001 compared with 18 h (n = 4 per group).

Correspondence: Dr Huei-Fong Hung (email shyukg@tmu.edu.tw).