Original Article

Angiotensin II Upregulates Acyl-CoA:Cholesterol Acyltransferase-1 *via* the Angiotensin II Type 1 Receptor in Human Monocyte-Macrophages

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Angiotensin II (Ang II) is known to accelerate the progression of macrophage-driven atherosclerotic lesions. Acyl-CoA:cholesterol acyltransferase-1 (ACAT1) converts intracellular free cholesterol into cholesterol ester (CE) for storage in lipid droplets, and promotes foam cell formation in atherosclerotic lesions. The present study explored the effect of Ang II on ACAT1 expression as a molecular mechanism of foam cell formation in primary cultured human monocyte-macrophages. Ang II significantly increased ACAT1 protein expression in a time- or concentration-dependent manner. Application of an Ang II type 1 (AT₁) receptor agonist (L162313), but not an Ang II type 2 (AT₂) receptor agonist (CGP42112A), mimicked the effects of Ang II treatment in inducing ACAT1 protein expression. ACAT activity and ACAT1 mRNA levels were also significantly increased by Ang II. Two-fold increases in ACAT1 protein expression and ACAT activity with Ang II treatment were completely inhibited by AT₁ receptor antagonists (candesartan, [Sar¹,Ile⁸]-Ang II), but not by an AT₂ receptor antagonist (PD123319). Treatment with a G-protein inactivator (GDP-β-S), a c-Src tyrosine kinase inhibitor (PP2), a protein kinase C (PKC) inhibitor (roscovitine), or a mitogen activated protein kinase (MAPK) kinase inhibitor (PD98059) significantly reduced Ang II-induced ACAT1 protein expression. Macrophage foam cell formation assessed using acetylated low-density lipoprotein (LDL)-induced CE accumulation was significantly enhanced by Ang II, which was completely inhibited by treatment with candesartan. These results suggested that Ang II enhances foam cell formation by upregulating ACAT1 expression predominantly through the actions of AT₁ receptor *via* the G protein/c-Src/PKC/MAPK pathway in human monocyte-macrophages. (*Hypertens Res* 2008; 31: 1801–1810)

Key Words: acyl-CoA:cholesterol acyltransferase-1, angiotensin II, angiotensin II receptors, atherosclerosis, macrophage

Introduction

Accumulation of macrophage foam cells is a hallmark of atherosclerosis. The intracellular free cholesterol level is increased by the uptake of oxidized low-density lipoprotein (oxLDL) *via* scavenger receptors, and is decreased by the

efflux of free cholesterol mediated by ATP-binding cassette transporter A1 (ABCA1) (1). As excessive accumulation of free cholesterol is toxic to cells, free cholesterol must be either removed through efflux to extracellular acceptors, such as apolipoprotein A1 and high-density lipoprotein (HDL), or esterified to cholesterol ester (CE) by the microsomal enzyme acyl-CoA:cholesterol acyltransferase-1 (ACAT1) (2). How-

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ever, massive accumulation of CE lipid droplets by ACAT1 in macrophages (foam cell formation) is a key event in the development of atherosclerosis (2). ACAT1 has been regarded as a target molecule of pharmacological intervention for preventing atherosclerosis and related complications (3).

Angiotensin II (Ang II), an octapeptide vasoconstrictor produced by the renin-angiotensin system, is implicated in hypertension and atherosclerosis (4), and has been identified in human macrophages in coronary atherosclerotic lesions (5). Infusion of Ang II into atherogenic apoE or LDL receptor deficient mice significantly increases macrophage-driven atherosclerotic lesions (6, 7). Ang II stimulates monocyte migration, macrophage-mediated LDL oxidation, macrophage cholesterol biosynthesis, and oxLDL uptake, and decreases HDL-induced cholesterol efflux from macrophages (7–9).

Ang II type 1 (AT₁) and type 2 (AT₂) receptors, members of the G-protein-coupled receptor (GPCR) superfamily, are expressed in human monocyte-macrophages (10) as well as in vascular smooth muscle cells (VSMCs), endothelial cells, and fibroblasts. Most of the proatherogenic effects of Ang II are mediated *via* the AT₁ receptor (11), whereas the monocyte matrix metalloproteinase-1 is upregulated *via* the AT₂ receptor (12). However, little is known about the effect of Ang II on ACAT1 expression, as a molecular and cellular mechanism of foam cell formation, particularly in primary human monocyte-macrophages.

Therefore, in the present study, we assessed the effects of Ang II on foam cell formation and ACAT1 expression and the role of AT₁ and AT₂ receptors in Ang II-induced ACAT1 expression in primary human monocyte-macrophages. We investigated the post-receptor intracellular signal transduction pathways of Ang II-induced ACAT1 expression in human monocyte-macrophages.

Methods

This study was approved by the Ethics Committee of Showa University. Blood was collected from male healthy volunteers who were free from hypertension, diabetes, dyslipidemia, or ischemic heart disease, and were taking no medications.

Chemicals

Human Ang II, L162313, CGP42112A, [Sar¹,Ile⁸]-Ang II, PD123319, GDP-β-S, PP2, rottlerin, PD98059, AG490, and anti-β-actin antibodies were purchased from Sigma (St. Louis, USA). Anti-SR-A and anti-ABCA1 antibodies were obtained from R&D Systems (Minneapolis, USA) and the anti-CD68 antibody was from Santa Cruz Biotechnology (Santa Cruz, USA). Candesartan was a gift from Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). Anti-human ACAT1 antibody (DM10) was donated by the Department of Biochemistry, Dartmouth Medical School (Hanover, USA).

Cell Culture

Human peripheral mononuclear cells were isolated from the blood by Ficoll density gradient centrifugation (13–15). Purified monocytes were suspended in RPMI1640 medium and seeded onto 6-cm dishes (4×10^6 cells/dish) for immunoblot analysis, 10-cm dishes (2×10^7 cells/dish) for an ACAT activity assay and Northern blot analysis, and 3.5-cm dishes (1×10^6 cells/dish) for CE assay. After 1 h of incubation for adherence, the medium was replaced with RPMI1640 medium supplemented with 10% pooled human sera.

Adherent monocytes were incubated at 37°C in 5% CO₂ for 7 d to induce differentiation into macrophages in the presence or absence of the indicated concentrations of Ang II, an AT₁ receptor agonist (L162313), or an AT₂ receptor agonist (CGP42112A). The medium was replaced every 3 d (on day 3 and day 6) with fresh medium and medium replacement was followed by the addition of Ang II, the AT₁ agonist, or the AT₂ agonist. For antagonist experiments, the AT₁ receptor antagonist (candesartan or [Sar¹,Ile⁸]-Ang II) or the AT₂ receptor antagonist (PD123319) was added 1 h before the addition of Ang II. To evaluate the post-receptor intracellular signal transduction pathways of Ang II-induced ACAT1 expression, inhibitors of G-proteins (GDP-β-S), c-Src tyrosine kinase (PP2), protein kinase C (PKC) (rottlerin), mitogen activated protein kinase (MAPK) kinase (PD98059), or Jak2 (AG490) were added along with Ang II.

To ensure that the blocking activities of the antagonists or inhibitors were not due to their cytotoxic effects, dose-response studies were performed with only the antagonists or inhibitors to identify a concentration that is neither cytotoxic nor disturbs monocytic differentiation into macrophages.

Immunoblot Analysis

Cell extracts (25 μg of protein) were separated by 10% SDS-polyacrylamide gel electrophoresis and subjected to Western blotting with antibodies against human ACAT1, ABCA1, SR-A, or CD68 (13–15).

ACAT Enzyme Activity

Cell extracts (80 μg) were incubated for 30 min at 37°C with 20 μL of 250 μmol/L [¹⁴C]oleoyl-CoA followed by determination of cholesterol [¹⁴C]oleate (13–15).

Northern Blot Analysis

Total RNA was extracted from cultured cells and electrophoresed on 1% agarose gels containing 2.2 mol/L formaldehyde followed by Northern blotting for ACAT1 mRNA (13, 15).

Assays for Intracellular CE Accumulation

Human LDL ($d=1.019\text{--}1.063$) and acetylated LDL (acLDL) were prepared as described previously (13). Using mature macrophages that had been incubated for 7 d with the indicated concentrations of Ang II, cellular CE accumulation was analyzed by two different assays. First, cells were incubated for 16 h with 5 $\mu\text{g/mL}$ acLDL in the presence of 0.1 mmol/L [^3H]oleate conjugated with bovine serum albumin, followed by lipid extraction and determination of cellular cholesterol [^3H]oleate (13, 14). Second, cells were incubated for 16 h with 5 $\mu\text{g/mL}$ acLDL pre-labeled with 74 kBq/mL of [^3H]cholesterol in RPMI-1640 medium containing 10% human serum, followed by lipid extraction and determination of cellular free and esterified [^3H]cholesterol (16).

Cellular Assay for Endocytic Uptake of [^{125}I]acLDL

Monocytes were incubated for 7 d with or without the indicated concentrations of Ang II. The cells in each dish were incubated for 18 h with 5 $\mu\text{g/mL}$ [^{125}I]acLDL (13, 14). Aliquots (0.75 mL) of the culture medium were mixed with 0.25 mL of 40% trichloroacetic acid. To this solution, 0.2 mL of 0.7 mol/L AgNO_3 was added, and the resulting solution was centrifuged at 2,500 rpm for 10 min. Trichloroacetic acid-soluble radioactivity in the supernatant and cell-associated radioactivity were determined as previously described (13, 14).

Statistical Analysis

Data expressed as mean $\pm$ SEM were compared by Student's unpaired *t*-test between two groups and by ANOVA followed by Bonferroni's post hoc test when >2 groups were examined. A *p*-value <0.05 was considered significant.

Results

Time-Dependent Effect of Ang II on ACAT1 Expression in Human Monocyte-Macrophages

The expression of ACAT1 protein during differentiation from monocytes into mature macrophages in the presence or absence of exogenous Ang II was determined by immunoblot analysis. At the same time, the levels of Ang II endogenously produced by cultured monocyte-macrophages were determined by radioimmunoassay (Ang II RIA kit; SRL, Inc., Tokyo, Japan). In the absence of exogenous Ang II, ACAT1 expression increased significantly, in a time-dependent manner, during monocytic differentiation into macrophages, and reached the maximal level on day 7 (Fig. 1A). Under these conditions, Ang II concentrations in the culture medium were 7.0 ± 3.0 pmol/L on day 0, 11.2 ± 2.2 pmol/L on day 3, and 10.2 ± 3.1 pmol/L on day 7; there were no significant differences among these concentrations. The concentrations of Ang

Fig. 1. Time- and dose-dependent effects of Ang II on ACAT1 protein expression in human monocyte-macrophages. *A:* Human monocytes were incubated for the indicated time periods in the absence or presence of Ang II (1 $\mu\text{mol/L}$). *B:* Human monocytes were incubated for 7 d with the indicated concentrations of Ang II. Bar graphs show densitometric analyses of ACAT1 immunoblotting after normalization with β -actin. Data are expressed as mean $\pm$ SEM from six independent experiments using monocytes from six different donors. * $p < 0.005$ vs. corresponding day of control. † $p < 0.05$, ‡ $p < 0.005$ vs. Ang II 0 $\mu\text{mol/L}$.

II produced endogenously by monocyte-macrophages during culture were minor in comparison to the concentration of Ang II added exogenously. During monocytic differentiation into macrophages without exogenous Ang II, treatment with candesartan (10 $\mu\text{mol/L}$), an AT_1 receptor-selective antagonist, or PD123319 (10 $\mu\text{mol/L}$), an AT_2 receptor-selective antagonist, did not affect ACAT1 protein expression (0.98 ± 0.07 and 1.00 ± 0.05 fold of the control). In the presence of exogenous

Fig. 2. Effects of AT_1 or AT_2 receptor agonist on ACAT1 protein expression in human monocyte-macrophages. Human monocytes were incubated for 7 d with the indicated concentrations of L162313, an AT_1 receptor agonist (A), or CGP42112A, an AT_2 receptor agonist (B). Bar graphs show densitometric analyses of ACAT1 immunoblots after normalization with β -actin. Data are expressed as mean $\pm$ SEM from five independent experiments using monocytes from five different donors. * $p < 0.001$, † $p < 0.05$ vs. L162313 0 μ mol/L.

Ang II, the expression of ACAT1 was significantly enhanced compared with expression in the absence of Ang II on days 5 and 7 (Fig. 1A).

Dose-Dependent Effect of Ang II on ACAT1 Expression in Human Monocyte-Macrophages

Figure 1B shows the concentration-dependent effect of Ang II

on ACAT1 protein expression in human monocyte-macrophages on day 7. Ang II significantly increased ACAT1 expression in a concentration-dependent manner. A maximal increase in ACAT1 expression of two-fold was observed at 1 μ mol/L.

Effect of AT_1 or AT_2 Receptor Agonists on ACAT1 Expression in Human Monocyte-Macrophages

We assessed the concentration-dependent effect of agonists of the AT_1 receptor (L162313) and the AT_2 receptor (CGP42112A) on ACAT1 protein expression in monocyte-macrophages on day 7. L162313 increased ACAT1 protein expression in a concentration-dependent manner, with a maximal increase of 1.8-fold observed at 1 μ mol/L (Fig. 2A). In contrast, CGP42112A did not have a statistically significant effect on ACAT1 expression at any of the concentrations tested (Fig. 2B).

Dose-Dependent Effect of Ang II on ACAT Activity in Human Monocyte-Macrophages

Figure 3A shows the concentration-dependent effect of Ang II on ACAT activity in human monocyte-macrophages on day 7. Ang II significantly increased ACAT activity in a concentration-dependent manner, with a maximal increase of two-fold observed at 1 μ mol/L. The increase in ACAT activity paralleled the increase in ACAT1 protein expression.

Effects of AT_1 or AT_2 Receptor-Selective Antagonists on Ang II-Induced ACAT1 Expression and ACAT Activity in Human Monocyte-Macrophages

As shown in Fig. 3B, Ang II significantly increased ACAT1 protein expression and increased ACAT activity by two-fold. Pretreatment with the AT_1 receptor-selective antagonist (candesartan or [Sar¹,Ile⁸]-Ang II) significantly inhibited Ang II-induced increases in ACAT1 expression and ACAT activity, whereas the AT_2 receptor-selective antagonist (PD123319) did not affect those parameters.

Effect of Ang II on ACAT1 mRNA Levels in Human Monocyte-Macrophages

Densitometric scanning of Northern blots of monocyte-macrophages on day 7 revealed that Ang II significantly increased the levels of the 2.8- and 3.6-kb transcripts by 1.6-fold and 1.4-fold, but had no significant effect on the 4.3- or 7.0-kb transcripts (Fig. 4A, B).

Intracellular Signal Transduction of Ang II-Induced ACAT1 Expression in Human Monocyte-Macrophages

We assessed the effects of inhibitors of G proteins (GDP- β -

Fig. 3. *A:* Effects of Ang II on ACAT activity in human monocyte-macrophages. *B:* Effects of an AT₁ or AT₂ receptor-selective antagonist on Ang II-induced ACAT1 protein expression (top) and ACAT activity (bottom) in human monocyte-macrophages. Human monocytes were incubated for 7 d with the indicated concentrations of Ang II. Candesartan (10 μmol/L) or [Sar¹,Ile⁸]-Ang II (10 μmol/L), AT₁ receptor-selective antagonists, or PD123319 (10 μmol/L), an AT₂ receptor-selective antagonist, was added 1 h before the addition of Ang II. Bar graphs show ACAT activity expressed as mean ± SEM from seven independent experiments using monocytes from seven different donors. Control value = 43 ± 14 pmol/min/mg protein (B). *p < 0.002, †*p* < 0.001 vs. Ang II 0 μmol/L. ‡*p* < 0.0005 vs. control, §*p* < 0.0001, #*p* < 0.005 vs. vehicle (Ang II 1 μmol/L).

S), c-Src tyrosine kinase (PP2), PKC (rottlerin), MAPK kinase (PD98059), or Jak2 (AG490) on Ang II-induced ACAT1 protein expression. As shown in Fig. 4C, the increase in ACAT1 expression by Ang II was completely inhibited by GDP-β-S, PP2, rottlerin, or PD98059 and partially inhib-

ited by AG490.

Effects of Ang II on Differentiation Markers and Endocytosis of acLDL in Human Macrophages

Incubation of monocyte-macrophages with Ang II (1 μmol/L) for 7 d did not significantly alter the protein expression of ABCA1, SR-A, or CD68 as differentiation markers (Fig. 5A). Figure 5B and C show the effects of Ang II on the endocytosis of acLDL as SR-A function in human macrophages. Neither the cell association nor endocytic degradation of [¹²⁵I]acLDL was significantly affected by Ang II (1 μmol/L).

Effect of Ang II on Intracellular CE Accumulation in Human Macrophages

The effect of Ang II on cholesterol [³H]oleate accumulation in human macrophages was observed at 5 μg/mL of acLDL more clearly than at higher concentrations in our preliminary experiments (data not shown). As shown in Fig. 6A, cholesterol [³H]oleate accumulation was maximal at 1.5-fold at 1 μmol/L of Ang II. Pretreatment with an AT₁ receptor-selective antagonist (candesartan) significantly inhibited Ang II-induced cholesterol [³H]oleate accumulation (Fig. 6B). Figure 6C shows the cellular free and esterified [³H]cholesterol derived from acLDL pre-labeled with [³H]cholesterol in macrophages. Ang II treatment significantly increased [³H]cholesterol ester, but not free [³H]cholesterol.

Discussion

The present study demonstrates that Ang II accelerated foam cell formation by upregulating ACAT1 expression predominantly through the AT₁ receptor followed by the G protein/c-Src/PKC/MAPK pathway in primary human monocyte-macrophages. To our knowledge, our study provides the first evidence that treatment with an AT₁ receptor blocker (ARB) can suppress Ang II-enhanced ACAT1 expression and foam cell formation in human monocyte-macrophages, contributing to the prevention of atherosclerosis.

Human monocytes express AT₁ receptors at higher levels than AT₂ receptors (17). Expression of both receptors increases during differentiation of human monocytes into macrophages (10). AT₁ receptor expression in the vasculature is increased by oxLDL, high glucose, and inflammatory cytokines, such as interleukin-6 and C-reactive protein (18). The expression levels of the AT₁ receptor define the biological efficacy of Ang II. Deletion of the AT₁ receptor in apoE-deficient mice results in attenuated atherosclerotic lesions (19). ARB has been widely used as an antihypertensive drug with the expectation of anti-atherosclerotic effects. Previous studies have shown that ARB decreases oxidative stress and LDL oxidation in the vessel wall and reduces the development of atherosclerosis (20). In addition, ARB has been shown to reduce plaque size by attenuating macrophage infiltration and

Fig. 4. *A and B: Effects of Ang II on ACAT1 mRNA levels in human monocyte-macrophages. C: Intracellular signal transduction of Ang II-induced ACAT1 protein expression in human monocyte-macrophages. Human monocytes were incubated for 7 d with Ang II (1 $\mu\text{mol/L}$) in the presence or absence of GDP- β -S (100 $\mu\text{mol/L}$), a G-protein inactivator; PP2 (1 $\mu\text{mol/L}$), a c-Src tyrosine kinase inhibitor; rottlerin (1 $\mu\text{mol/L}$), a PKC inhibitor; PD98059 (1 $\mu\text{mol/L}$), a MAPK kinase inhibitor; or AG490 (10 $\mu\text{mol/L}$), a Jak2 inhibitor. Bar graphs show densitometric analyses of Northern or Western blotting after normalization with 18S or β -actin, respectively. Data are expressed as mean $\pm$ SEM from four independent experiments using monocytes from four different donors. * $p < 0.02$, † $p < 0.05$ vs. Ang II 0 $\mu\text{mol/L}$, ‡ $p < 0.0001$ vs. control (Ang II 0 $\mu\text{mol/L}$), ^s $p < 0.05$ vs. vehicle (Ang II 1 $\mu\text{mol/L}$).*

VSMC proliferation in the aorta of diabetic apoE-deficient mice. Johnstone *et al.* (21) showed that candesartan reduces macrophage accumulation within the plaque but increases

collagen deposition, contributing to plaque stabilization. Koga *et al.* (22) reported that inflammatory mechanisms are involved in the development of atherosclerosis based on their

Fig. 5. Effects of Ang II on ABCA1, SR-A, and CD68 protein expression (A) and SR-A function as assessed by cell association (B) and endocytic degradation (C) of [^{125}I]acLDL in human monocyte-macrophages. A: Bar graphs show densitometric analyses of ABCA1, SR-A, and CD68 immunoblotting after normalization with β -actin. A–C: Data are expressed as mean $\pm$ SEM from triplicate determinations of three independent experiments using monocytes from three different donors.

data that a blockade of the interferon- γ receptor can suppress atherosclerotic lesions in apoE-deficient mice. An ARB that acts as a peroxisome proliferators-activated receptor- γ agonist shows anti-inflammatory effects (23, 24). ARB ameliorates inflammatory changes by decreasing both monocyte chemoattractant protein-1 expression and macrophage infiltration in glomeruli in patients with hypertensive nephrosclerosis (25).

Deletion of ACAT1 brought destabilization of cellular membrane function on cholesterol accumulation and cell death in macrophages from ACAT1-deficient mice (26). In contrast, partial ACAT inhibition *in vivo* by an ACAT1-selective inhibitor suppressed atherosclerosis without cytotoxicity to macrophages (27). Moreover, adverse effects have not been reported in recent clinical trials of ACAT inhibitors (28). Inhibition of Ang II-induced ACAT1 expression by candesartan may not produce unfavorable effects on macrophages *in vivo*.

In primary human monocyte-macrophages, ACAT1 is upregulated by urotensin II, serotonin, salusin- β , and transforming growth factor- β_1 (TGF- β_1) and is downregulated by salusin- α and adiponectin (13–15, 29, 30). Ang II significantly increases ACAT1 expression, but not ABCA1, SR-A, and CD68, which are known to increase during monocyte-macrophage differentiation (14). These findings suggest that ACAT1 upregulation by Ang II is independent of cell differentiation.

The human ACAT1 gene encodes four different mRNA species of 2.8, 3.6, 4.2, and 7.0 kb. These four transcripts contain a full-length ACAT1 open reading frame and various sizes of untranslated regions, including different promoters. It is possible that all four transcripts produce ACAT1 protein. In general, the major transcripts (2.8 and 3.6 kb) are regarded as quantitatively important players for ACAT1 transcription. However, the exact contribution of each transcript to ACAT1 translation is unknown. We demonstrated increases in two of

Fig. 6. *A: Dose-dependent effects of Ang II on cholesterol [3H]oleate accumulation induced by acLDL along with [3H]oleate in human monocyte-macrophages. B: Inhibitory effect of AT₁ receptor-selective antagonist, candesartan (10 μmol/L), on Ang II (1 μmol/L)-induced cholesterol [3H]oleate accumulation in macrophages. Candesartan was added 1 h before the addition of Ang II. C: Accumulation of intracellular free and esterified [3H]cholesterol derived from acLDL pre-labeled with [3H]cholesterol in macrophages. Data are expressed as mean ± SEM from triplicate determinations of three independent experiments using monocytes from three different donors. *p < 0.005 vs. Ang II 0 μmol/L, †*p* < 0.005 vs. control, ‡*p* < 0.005 vs. Ang II (1 μmol/L), §*p* < 0.05 vs. cholesterol [3H]oleate accumulation by Ang II 0 μmol/L.*

these transcripts (2.8 and 3.6 kb) in response to Ang II in the present study as well as other vasoactive agents and TGF-β₁ in the previous studies (13, 15, 29).

In our previous studies, the effects of Ang II on VSMC proliferation and hypertrophy were shown to be mediated by AT₁ receptors followed by various intracellular signal transduction mechanisms, such as Gq, tyrosine kinases, PKC, and MAPK. In addition to the MAPK pathway, Ang II activates the Jak2/STAT3 pathway *via* AT₁ receptors (11). In the present study, we examined the pathways of ACAT1 expression that are stimulated by Ang II in human monocyte-macrophages. Ang II-induced ACAT1 upregulation was completely abolished by G protein, c-Src, PKC, or MAPK kinase inhibitors, but partially inhibited by a Jak2 inhibitor. These observations suggested that the G protein/c-Src/PKC/MAPK pathway is mainly involved in Ang II-induced ACAT1 upregulation in human monocyte-macrophages. Similarly, this signal transduction pathway participates in ACAT1 expression enhanced by other GPCR agonists, such as urotensin II and serotonin (13, 15).

The concentration of Ang II necessary for ACAT1 stimulation was relatively high compared with physiological plasma levels of Ang II in humans. Our previous studies demonstrated that Ang II within the same range of concentrations significantly induced VSMC proliferation *via* the AT₁ receptor (11, 31). Several animal studies have shown that release of Ang II from cardiomyocytes in response to mechanical stretch reaches 100-fold over baseline *in vitro* (32), and Ang II concentrations in interstitial fluid from the heart or the kidney are 100- or 1,000-fold higher, respectively, than in plasma (33). In human atherosclerotic lesions, a large amount of Ang II is generated locally by angiotensin-converting enzyme and chymase in the autocrine/paracrine manner (34, 35). It is possible that Ang II concentrations around monocyte-macrophages within atherosclerotic lesions may reach those required for ACAT1 stimulation. This phenomenon requires further investigation *in vivo* to assess its clinical relevance. Expression of both the AT₁ and AT₂ receptors increased during differentiation of THP1 monocytes into macrophages in the absence of exogenous Ang II (10). In the presence of exogenous Ang II, AT₁ and AT₂ receptors are downregulated in THP1-derived macrophages (10). However, changes in AT₁ or AT₂ receptor expression during differentiation of primary cultured human monocytes into macrophages in the presence of exogenous Ang II have not been elucidated. Further studies are needed to clarify the role of the expression of AT₁ and AT₂ receptors in human monocyte-macrophage culture system used in the present study.

In conclusion, Ang II enhances foam cell formation by upregulating ACAT1 expression predominantly *via* the AT₁ receptor acting through the G protein/c-Src/PKC/MAPK pathway in human monocyte-macrophages. These findings provide an understanding of potential molecular mechanisms by which hypertension promotes the development of atherosclerosis. Our study suggests that ARB against monocyte-

macrophages as well as VSMCs may become promising therapeutic strategies for the prevention of atherosclerotic complications in hypertension.

References

- Pennings M, Meurs I, Ye D, et al: Regulation of cholesterol homeostasis in macrophages and consequences for atherosclerotic lesion development. *FEBS Lett* 2006; **580**: 5588–5596.
- Miyazaki A, Sakashita N, Lee O, et al: Expression of ACAT-1 protein in human atherosclerotic lesions and cultured human monocytes-macrophages. *Arterioscler Thromb Vasc Biol* 1998; **18**: 1568–1574.
- Miyazaki A, Kanome T, Watanabe T: Inhibitors of acyl-coenzyme A:cholesterol acyltransferase. *Curr Drug Targets Cardiovasc Haematol Disord* 2005; **5**: 463–469.
- Daugherty A, Manning MW, Cassis LA: Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest* 2000; **105**: 1605–1612.
- Potter DD, Sobey CG, Tompkins PK, Rossen JD, Heistad DD: Evidence that macrophages in atherosclerotic lesions contain Ang-II. *Circulation* 1998; **98**: 800–807.
- Ayabe N, Suganuma E, Babaev VR, et al: Angiotensin II amplifies macrophage-driven atherosclerosis. *Arterioscler Thromb Vasc Biol* 2004; **24**: 2143–2148.
- Keidar S, Heinrich R, Kaplan M, Hayek T, Aviram M: Angiotensin II administration to atherosclerotic mice increases macrophage uptake of oxidized LDL: a possible role for interleukin-6. *Arterioscler Thromb Vasc Biol* 2001; **21**: 1464–1469.
- Keidar S, Attias J: Angiotensin II injection into mice increases the uptake of oxidized LDL by their macrophages via a proteoglycan-mediated pathway. *Biochem Biophys Res Commun* 1997; **239**: 63–67.
- Kaplan M, Aviram M, Knopf C, Keidar S: Angiotensin II reduces macrophage cholesterol efflux: a role for the AT-1 receptor but not for the ABC1 transporter. *Biochem Biophys Res Commun* 2002; **290**: 1529–1534.
- Okamura A, Rakugi H, Ohishi M, et al: Upregulation of renin-angiotensin system during differentiation of monocytes to macrophages. *J Hypertens* 1999; **17**: 537–545.
- Watanabe T, Pakala R, Katagiri T, Benedict CR: Serotonin potentiates angiotensin II-induced vascular smooth muscle cell proliferation. *Atherosclerosis* 2001; **159**: 269–279.
- Kim MP, Zhou M, Wahl LM: Angiotensin II increases human monocyte matrix metalloproteinase-1 through the AT₂ receptor and prostaglandin E₂: implications for atherosclerotic plaque rupture. *J Leukoc Biol* 2005; **78**: 195–201.
- Suguro T, Suguro T, Kanome T, et al: Human urotensin II accelerates foam cell formation in human monocyte-derived macrophages. *Hypertension* 2005; **46**: 738–744.
- Watanabe T, Nishio K, Kanome T, et al: Impact of salusin- α and - β on human macrophage foam cell formation and coronary atherosclerosis. *Circulation* 2008; **117**: 638–648.
- Suguro T, Watanabe T, Kanome T, et al: Serotonin acts as an up-regulator of acyl-coenzyme A:cholesterol acyltransferase-1 in human monocyte-macrophages. *Atherosclerosis* 2006; **186**: 275–281.
- Dove DE, Su YR, Zhang W, et al: ACAT1 deficiency disrupts cholesterol efflux and alters cellular morphology in macrophages. *Arterioscler Thromb Vasc Biol* 2005; **25**: 128–134.
- Nahmod KA, Vermeulen ME, Radien S, et al: Control of dendritic cell differentiation by angiotensin II. *FASEB J* 2003; **17**: 491–493.
- Wassmann S, Nickenig G: Pathophysiological regulation of the AT₁-receptor and implications for vascular disease. *J Hypertens* 2006; **24**: S15–S21.
- Wassmann S, Czech T, van Eickels M, Fleming I, Bohm M, Nickenig G: Inhibition of diet-induced atherosclerosis and endothelial dysfunction in apolipoprotein E/angiotensin II type 1A receptor double-knockout mice. *Circulation* 2004; **110**: 3062–3067.
- Rueckschloss U, Quinn MT, Holtz J, Morawietz H: Dose-dependent regulation of NAD(P)H oxidase expression by angiotensin II in human endothelial cells: protective effect of angiotensin II type 1 receptor blockade in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1845–1851.
- Johnstone MT, Perez AS, Nasser I, et al: Angiotensin receptor blockade with candesartan attenuates atherosclerosis, plaque disruption, and macrophage accumulation within the plaque in a rabbit model. *Circulation* 2004; **110**: 2060–2065.
- Koga M, Kai H, Yasukawa H, et al: Postnatal blocking of interferon- γ function prevented atherosclerotic plaque formation in apolipoprotein E-knockout mice. *Hypertens Res* 2007; **30**: 259–267.
- Cianchetti S, Fiorentino AD, Colognato R, Stefano RD, Franzoni F, Pedrinelli R: Anti-inflammatory and anti-oxidant properties of telmisartan in cultured human umbilical vein endothelial cells. *Atherosclerosis* 2008; **198**: 22–28.
- Yano Y, Hoshida S, Ishikawa J, et al: The differential effects of angiotensin II type 1 receptor blockers on microalbuminuria in relation to low-grade inflammation in metabolic hypertensive patients. *Am J Hypertens* 2007; **20**: 565–572.
- Imakiire T, Kikuchi Y, Yamada M, et al: Effects of renin-angiotensin system blockade on macrophage infiltration in patients with hypertensive nephrosclerosis. *Hypertens Res* 2007; **30**: 635–642.
- Fazio S, Major AS, Swift LL, et al: Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *J Clin Invest* 2001; **107**: 163–171.
- Ikenoya M, Yoshinaka Y, Kobayashi H, et al: A selective ACAT-1 inhibitor, K-604, suppresses fatty streak lesions in fat-fed hamsters without affecting plasma cholesterol levels. *Atherosclerosis* 2007; **19**: 290–297.
- Nissen SE, Tuzcu EM, Brewer HB, et al: ACAT Intravascular Atherosclerosis Treatment Evaluation (ACTIVATE) Investigators: Effect of ACAT inhibition on the progression of coronary atherosclerosis. *N Engl J Med* 2006; **354**: 1253–1263.
- Hori M, Miyazaki A, Tamagawa H, et al: Up-regulation of acyl-coenzyme A:cholesterol acyltransferase-1 by transforming growth factor- β 1 during differentiation of human monocytes into macrophages. *Biochem Biophys Res Commun*

- mun* 2004; **320**: 501–505.
30. Furukawa K, Hori M, Ouchi N, *et al*: Adiponectin down-regulates acyl-coenzyme A:cholesterol acyltransferase-1 in cultured human monocyte-derived macrophages. *Biochem Biophys Res Commun* 2004; **317**: 831–836.
31. Watanabe T, Pakala R, Katagiri T, Benedict CR: Mildly oxidized low-density lipoprotein acts synergistically with angiotensin II in inducing vascular smooth muscle cell proliferation. *J Hypertens* 2001; **19**: 1065–1073.
32. Sadoshima J, Xu Y, Slayter HS, Izumo S: Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes *in vitro*. *Cell* 1993; **75**: 977–984.
33. Schuijt MP, van Kats JP, de Zeeuw S, *et al*: Cardiac interstitial fluid levels of angiotensin I and II in the pig. *J Hypertens* 1999; **17**: 1885–1891.
34. Ohishi M, Ueda M, Rakugi H, *et al*: Enhanced expression of angiotensin-converting enzyme is associated with progression of coronary atherosclerosis in humans. *J Hypertens* 1997; **15**: 1295–1302.
35. Ihara M, Urata H, Kinoshita A, *et al*: Increased chymase-dependent angiotensin II formation in human atherosclerotic aorta. *Hypertension* 1999; **33**: 1399–1405.