Human Urotensin II Accelerates Foam Cell Formation in Human Monocyte-Derived Macrophages

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Abstract—Human urotensin II (U-II), the most potent vasoconstrictor peptide identified to date, and its receptor (UT) are involved in hypertension and atherosclerosis. Acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT-1) converts intracellular free cholesterol into cholesterol ester (CE) for storage in lipid droplets and plays an important role in the formation of macropage-derived foam cells in atherosclerotic lesions. We examined the effects of U-II on ACAT-1 expression and CE accumulation in human monocyte-derived macrophages. U-II increased ACAT activity in a concentration-dependent manner after 7 days in monocyte primary culture. Immunoblotting analysis showed that U-II at 25 nmol/L increased ACAT-1 protein expression level by 2.5-fold, which was completely abolished by anti–U-II antibody, selective UT receptor antagonists (urantide and 4-aminquinoline), a G-protein inactivator (GDP-β-S), a c-Src protein tyrosine kinase inhibitor (PP2), a protein kinase C (PKC) inhibitor (rottlerin), a mitogen-activated protein kinase kinase (MEK) inhibitor (PD98059), or a Rho kinase (ROCK) inhibitor (Y27632). Northern blotting analysis indicated that among the 4 ACAT-1 mRNA transcripts (2.8-, 3.6-, 4.3-, and 7.0-kb), the 2.8- and 3.6-kb transcript levels were selectively upregulated by ≈1.7-fold by U-II (25 nmol/L). Further, U-II (25 nmol/L) significantly increased acetylated LDL (acetyl-LDL)–induced CE accumulation in monocyte-derived macrophages but not scavenger receptor class A (SR-A) function as assessed by endocytic uptake of [125I]acetyl-LDL. Our results suggest that U-II may play a novel role in the formation of macropage-derived foam cells by upregulating ACAT-1 expression via the UT receptor/G-protein/c-Src/PKC/MEK and ROCK pathways but not by SR-A, thus contributing to the relatively rapid development of atherosclerosis in hypertension. (Hypertension. 2005;46:738-744.)

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human ACAT isozymes ACAT-1 and ACAT-2. ACAT-1 is the dominant isozyme in monocyte-macrophages. ACAT-1 is expressed at high levels by macrophage-derived foam cells in atherosclerotic lesions in vivo and upregulated during differentiation from monocytes into macrophages in vitro. Therefore, ACAT-1 plays a crucial role in the formation of macrophage-derived foam cells. However, it has not been clarified whether U-II modulates ACAT-1 and SR-A activities and foam cell formation in human monocyte-derived macrophages.

In the present study, we assessed the effects of U-II on ACAT-1 expression at protein and mRNA levels, ACAT activity, and SR-A activity in the formation of human macrophage-derived foam cells. We investigated the intracellular signal transduction pathways of U-II–induced ACAT-1 expression in human monocyte-derived macrophages.

Methods

The study was approved by the ethics committee of Showa University School of Medicine.

Chemicals

Human U-II, anti–U-II antibody, GDP-β-S, PP2, rotterlin, and PD98059 were obtained from Sigma. Wako. Urantide was purchased from Peptides International, and PD98059 were obtained from Sigma. Y27632 was purchased from Cambridge Corp. DM10 was a gift from the Department of Biochemistry, Dartmouth Medical School (Hanover, NH).

Cell Culture

Human peripheral mononuclear cells were isolated from the blood of healthy volunteers by Ficoll density gradient centrifugation as described previously. Purified monocytes were suspended in RPMI medium 1640 and seeded onto 6-cm dishes (4 × 10⁵ cells per dish) for immunoblotting analysis, cholesterol esterification assay, and endocytic uptake of [³²P]oleoyl-CoA (20 dpm/pmol), followed by incubation for 15 minutes at 37°C. Lipids were extracted and the radioactive cholesterol [³²P]oleate was determined by thin-layer chromatography.

Western Blotting Analysis

Cells were extracted with 100 μL of 10% sodium dodecyl sulfate (SDS) as described previously. In a standard experiment, 30 μg of protein was separated by 10% SDS-PAGE and subjected to Western blotting with a rabbit polyclonal antibody raised against human ACAT-1 (DM10). The densities of the bands were measured using Light-Capture and Densitograph software (AE-6962FC; CS Analyzer ver2.0; ATTO Corp.).

Results

U-II Uregulates ACAT-1 Expression in Human Monocytes/Macrophages

In the absence of U-II, the expression of ACAT-1 protein was increased during differentiation from monocytes into...
U-II Increases ACAT Activity in Human Monocyte-Derived Macrophages

We examined the effects of U-II on the ACAT activity under identical conditions. As shown in Figure 2, U-II increased ACAT activity in a concentration-dependent manner with a maximal effect at 25 nmol/L (2.3-fold increase). The increase in ACAT enzyme activity paralleled that in ACAT-1 protein expression.

Signal Transduction Pathways of U-II–Induced ACAT-1 Upregulation

To determine how U-II upregulates ACAT-1 expression, we assessed the effects of anti–U-II antibody, UT receptor antagonists (urantide and 4-aminoquinoline), or inhibitors of G-protein (GDP-β-S), c-Src tyrosine kinase (PP2), PKC (rottlerin), MEK (PD98059), or ROCK (Y27632) on U-II–induced ACAT-1 expression. As shown in Figure 3, the increase in ACAT-1 expression by U-II (25 nmol/L) was completely inhibited by anti–U-II antibody (20 μL/2 mL plate), urantide (10 nmol/L), a UT receptor antagonist peptide, 4-aminoquinoline (100 nmol/L), a UT receptor antagonist nonpeptide, GDP-β-S (100 μmol/L), a G-protein inactivator, PP2 (1 μmol/L), a c-Src inhibitor, rottlerin (10 μmol/L), a PKC inhibitor, PD98059 (10 μmol/L), an MEK inhibitor, or Y27632 (10 μmol/L), a ROCK inhibitor. Cells were harvested and subjected to immunoblotting analysis for ACAT-1. Each bottom panel shows a densitometric analysis of ACAT-1 immunoblotting (top panel). Data are expressed as the mean±SEM from 3 independent experiments using monocytes from 3 different donors. *P<0.0001 vs vehicle (U-II 25 nmol/L).

U-II Increases ACAT-1 mRNA in Human Monocyte-Derived Macrophages

To determine whether U-II–induced ACAT-1 upregulation was attributable to increased ACAT-1 mRNA levels, Northern blotting analyses were also performed on day 7 (Figure 4A). Densitometric scanning of Figure 4A revealed that U-II at a concentration of 25 nmol/L significantly increased the levels of the 2.8- and 3.6-kb transcripts by 1.7-fold and 1.75-fold (P<0.01) respectively, but had no significant effect on 4.3- or 7.0-kb transcripts (Figure 4B). These results indicated that ACAT-1 expression is regulated by U-II at mRNA level.

Figure 1. Dose-dependent effect (A) and time-dependent effect (B) of U-II on ACAT-1 expression in human monocytes/macrophages. A, Human monocytes were incubated for 7 days with the indicated concentrations of U-II. B, Human monocytes were incubated for the indicated time periods with or without U-II (25 nmol/L). Cells were harvested and subjected to immunoblotting analysis for ACAT-1. Each bottom panel shows a densitometric analysis of ACAT-1 immunoblotting (top panel). Data are expressed as the mean±SEM from 3 independent experiments using monocytes from 3 different donors. *P<0.0001 vs other concentrations of U-II.

Figure 2. Effect of U-II on ACAT activity in human monocyte-derived macrophages. Human monocytes were incubated for 7 days with or without the indicated concentrations of U-II. Cell homogenates were solubilized and determined for the ACAT activity using the reconstituted assay. Data are expressed as the mean±SEM from triplicate determinations of 3 independent experiments using monocytes from 3 different donors. *P<0.0005 vs U-II 0 nmol/L; †P<0.0001 vs other concentrations of U-II.

Figure 3. Signal transduction pathways of U-II–induced ACAT-1 expression in human monocyte-derived macrophages. Human monocytes were incubated for 7 days with or without U-II (25 nmol/L) in the presence or absence of anti–U-II antibody (20 μL/2 mL plate), urantide (10 nmol/L), a UT receptor antagonist peptide, 4-aminoquinoline (100 nmol/L), a UT receptor antagonist nonpeptide, GDP-β-S (100 μmol/L), a G-protein inactivator, PP2 (1 μmol/L), a c-Src inhibitor, rottlerin (10 μmol/L), a PKC inhibitor, PD98059 (10 μmol/L), an MEK inhibitor, or Y27632 (10 μmol/L), a ROCK inhibitor. Cells were harvested and subjected to immunoblotting analysis for ACAT-1. Each bottom panel shows a densitometric analysis of ACAT-1 immunoblotting (top panel). Data are expressed as the mean±SEM from 3 independent experiments using monocytes from 3 different donors. *P<0.0001 vs vehicle (U-II 25 nmol/L).
U-II Increases Acetyl-LDL–Induced CE Accumulation in Human Monocyte-Derived Macrophages

Effect of U-II on foam cell formation as assessed by acetyl-LDL–induced CE accumulation in monocyte-derived macrophages is shown in Figure 5. Acetyl-LDL induced CE accumulation in a dose-dependent manner. Further, coincubation with U-II (25 nmol/L) resulted in a significant increase in acetyl-LDL–induced CE accumulation.

U-II Does Not Increase Endocytic Uptake of $^{[125]I}$Acetyl-LDL in Human Monocyte-Derived Macrophages

The effects of U-II on SR-A function as assessed by the endocytic uptake of $^{[125]I}$acetyl-LDL by monocyte-derived macrophages are shown in Figure 6. U-II (25 nmol/L) had no significant effect on cell-association or degradation of $^{[125]I}$acetyl-LDL.

Discussion

Accumulating evidence reported by Douglas et al\(^1,3,5,23\) indicates that U-II is an important contributor to cardiovascular diseases. U-II is the most potent vasoconstrictor identified to date, with 10-fold and 300-fold greater potency than endothelin-1 and norepinephrine, respectively.\(^26\) U-II causes vasoconstriction in human coronary, pulmonary, mammary, and radial arteries, and saphenous and umbilical veins in vitro.\(^27,28\) Bohm et al\(^28\) reported that peripheral infusion of U-II caused vasoconstriction in the human forearm. Cheung et al\(^6\) showed that plasma concentrations of U-II were elevated in 62 hypertensive patients compared with 62 normotensive controls (13.6±11.00 vs 8.8±0.9 pmol/L) and were positively correlated with systolic and diastolic blood pressures. Their study showed that plasma concentrations of U-II are positively correlated with body weight and fasting plasma glucose.\(^6\) Plasma U-II levels are known to be high in patients with diabetes mellitus.\(^8\) Animal experiments have shown that U-II inhibits glucose-induced insulin release from pancreatic $\beta$-cells,\(^29\) and stimulates hyperlipidemia by channeling glucose to free fatty acid synthesis.\(^30\) These findings suggest that U-II may be associated with metabolic syndrome. Further studies using several selective UT receptor antagonists or urotensin-converting enzyme(s) and knockout or transgenic animals are required to investigate the precise role of U-II in pathological conditions, such as hypertension, atherosclerosis, and metabolic syndrome.

Human U-II–like immunoreactivity was originally reported in human vasculature with diffuse staining in cardiac myocytes and intense staining in the macrophage and VSMC-rich region of human coronary atherosclerotic plaque.\(^1\) Maguire and Davenport\(^2\) reported expression of U-II in endothelial cells of the human aorta and epicardial coronary artery as well as intramyocardial vessels with diameters (60 to 120 $\mu$m) typical of resistance arteries but not in cardiac myocytes or VSMCs. However, UT receptors are present in VSMCs.
throughout the human coronary artery tree, from large epicardial to small resistance arteries, and mediate vasoconstriction. A similar distribution of positive U-II–like immunoreactivity was demonstrated in macrophages for CD68, suggesting that U-II may be produced mainly in macrophages. Bousette et al have recently shown that lymphocytes are by far the largest producers of U-II, whereas monocytes and macrophages are the largest producers of UT receptor, with relatively little expression in foam cells, lymphocytes, or platelets in human carotid arteries and aortae. Recent studies have shown that U-II upregulates the expression of collagen-1 and downregulates the expression of matrix metalloproteinase-1 in human endothelial cells and activates NADPH oxidase and plasminogen activator inhibitor-1 in human VSMCs, leading to atherosclerotic plaque formation. The expression of UT receptor is upregulated by interferon-γ (IFN-γ), a product of the activated T lymphocytes found within the atherosclerotic plaque. These findings indicate that U-II acts in an autocrine or paracrine manner in the setting of atherosclerosis.

ACAT-1, an intracellular enzyme located in the rough endoplasmic reticulum, plays a crucial role in the accumulation of CE as lipid droplets within macrophages in atherosclerotic lesions. A previous immunohistochemical study demonstrated high levels of ACAT-1 expression in macrophage-derived foam cells in human atherosclerotic lesions. ACAT-1 expression is upregulated in human monocytic THP-1 cells by IFN-γ and some factors that induce monocytic differentiation into macrophages, such as 1,25-dihydroxyvitamin D₃, 9-cis-retinoic acid, and phorbol 12-myristate 13-acetate. In primary human monocyte-derived macrophages, ACAT-1 expression is upregulated by dexamethasone, dehydroepiandrosterone, and transforming growth factor-β₁ (TGF-β₁; 2-fold) but is downregulated by adiponectin. Our preliminary studies show that among G-protein agonists, serotonin (2-fold) and angiotensin II (2.2-fold) but not endothelin-1 (1.4-fold) could upregulate significantly ACAT-1 expression in the same cell system. We regard 2-fold increase in ACAT-1 expression by these vasoactive agents as a significant cellular event that accelerates the formation of human macrophage-derived foam cells. In addition, the increase in acetyl-LDL–induced CE accumulation by U-II (1.4-fold) can be regarded as a significant change because it is comparable to the previous reports that showed 1.2-fold increase by dehydroepiandrosterone and 1.6-fold increase by high glucose. The vasoconstrictive or VSMC proliferative actions of U-II have been shown to be mediated by the UT receptor followed by various intracellular signal transduction mechanisms, such as phospholipase C, protein tyrosine kinases, PKC, and ERK, and the RhoA/ROCK–related pathways. However, little information regarding the pathways of ACAT-1 expression in macrophages is available. The present study showed that U-II–induced ACAT-1 upregulation is abolished by selective UT receptor antagonists and the specific inhibitors of G-protein, c-Src tyrosine kinase, PKC, MEK, or ROCK. These observations indicated that UT receptor/G-protein/c-Src/PKC/MEK and ROCK pathways are involved in U-II–induced ACAT-1 upregulation in human monocyte-derived macrophages.

The human ACAT-1 gene encodes 4 different mRNA species (2.8-, 3.6-, 4.2-, and 7.0 kb) and has 2 promoters (P1 and P7) located on chromosomes 1 and 7, respectively. Among the 4 ACAT-1 mRNAs, the levels of the 2.8- and 3.6-kb transcripts were increased selectively by U-II, whereas the 4.3- and 7.0-kb transcripts remained unchanged (Figure 4). The 2.8- and 3.6-kb transcripts are regulated by the P1 promoter, whereas the 4.3-kb transcript is regulated by P1 and P7 promoters. The present study demonstrated increases in expression of 2 shorter transcripts by U-II during differentiation of monocytes into macrophages. The same phenomenon was observed when ACAT-1 was upregulated by TGF-β₁. Our experiments demonstrated that the upregulation of ACAT-1 expression by U-II occurs predominantly during differentiation of human monocytes into macrophages as compared with after differentiation (data not shown).

Other than ACAT-1, the formation of foam cells is modulated by several scavenger receptors that mediate the influx of atherogenic lipoproteins. Previous studies have established that macrophages take up acetyl-LDL mainly through SR-A and undergo transformation into foam cells. Expression of SR-A has been reported to be enhanced by high levels of glucose and macrophage colony stimulating factor but decreased by IFN-γ in human monocyte-derived macrophages. However, the results of the present study showed that U-II did not affect SR-A function in the formation of human primary monocyte-derived macrophages (Figure 6).

**Limitations**

There are several potential limitations in the present study. The pooled human serum was used to maintain the human monocyte–macrophage culture. Human serum U-II concentrations are 2 to 7 pmol/L in the healthy subjects. Our determinants of U-II confirm these data (hU-II EIA kit; Phoenix Pharmaceuticals, Inc.). Therefore, the levels of U-II involved in RPMI medium 1640 containing 10% human serum are 0.2 to 0.7 pmol/L, which are negligible compared with 25 nmol/L of U-II added in this study.

Urantide has been reported recently to be a potent ligand for human UT (hUT) receptor. Patacchini et al showed that urantide binds with nanomolar affinity (pKᵦ 8.3) to the recombinant hUT and antagonizes the contractile effects of U-II in the rat aorta without showing any residual agonist activity. In our study, urantide dose-dependently inhibited the U-II–induced ACAT-1 upregulation in human monocyte-derived macrophages. However, urantide is considered a low-efficacy partial agonist according to the fact that it mimicked the effects of U-II on [Ca²⁺]ᵢ release in Chinese hamster ovary (CHO) cells stably expressing the hUT receptor (CHO₅ cells). The different pharmacological behavior of urantide (pure antagonist in human macrophages as well as rat aortic VSMCs versus partial agonist in CHO₅ cells) could be attributed to the amount of expression of hUT receptor. The efficacy of urantide on [Ca²⁺]ᵢ release might be overestimated in the cell system expressing very high levels of recombinant receptors, whereas in human macrophages, where the density of UT sites is lower, it could.
not be detected. Further studies are needed to confirm the hypothesis.

Perspectives
To the best of our knowledge, this is the first study to demonstrate that U-II plays a novel role in the formation of macrophage-derived foam cells by upregulating ACAT-1 expression via the UT receptor/G-protein/c-Src/PKC/MEK and ROCK pathways, but not by SR-A. These findings provide an understanding of potential molecular mechanisms by which hypertension promotes the development of atherosclerosis. Our results, together with those of other studies, suggest that the U-II/UT receptor system may be a useful new therapeutic target in U-II–mediated vascular responses, such as hypertension and atherosclerosis.

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