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

Takuya Watanabe, Toshiaki Suguro, Tomoko Kanome, Yu-ichiro Sakamoto, Syuusuke Kodate, Tamio Hagiwara, Shigeaki Hongo, Tsutomu Hirano, Mitsuru Adachi, Akira Miyazaki

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 macrophage-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 (rotterlin), 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 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, thus contributing to the relatively rapid development of atherosclerosis in hypertension. (Hypertension. 2005;46:738-744.)

Key Words: cholesterol ■ metabolism ■ human ■ macrophages ■ atherosclerosis ■ vasoconstriction

Human urotensin II (U-II) is the most potent vasoconstrictor identified to date, with a potency 1 to 2 orders of magnitude greater than that of endothelin-1. U-II acts through the orphan G-protein–coupled receptor-14, recently cloned and renamed UT receptor. Human U-II is a cyclic peptide of 11 amino acids, with a molecular weight of ~1388, and is expressed in the cardiovascular system as well as the central nervous system, kidney, spleen, small intestine, thymus, prostate, pituitary, and adrenal gland. Interestingly, U-II as well as UT receptor are found within the regions of monocyte/macrophage infiltration in atherosclerotic plaques of coronary and carotid arteries and abdominal aortic aneurysms. U-II circulates in human plasma, and its plasma concentration is increased in hypertension, congestive heart failure, diabetes mellitus, renal failure, and portal hypertension caused by liver cirrhosis. U-II also induces vascular smooth muscle cell (VSMC) proliferation and cardiac fibrosis and hypertrophy via the extracellular signal-regulated kinase (ERK) pathway. Cheung et al reported that U-II is positively correlated with systolic blood pressure and has an etiologic role in hypertension and its complications. In addition, higher levels of urinary excretion of U-II have been demonstrated in patients with essential hypertension. These findings suggest that the U-II/UT receptor system may involve candidate genes for hypertension. The presence of massive clusters of macrophage-derived foam cells in situ in the subendothelial spaces is one of the characteristic features seen in the early stages of atherosclerotic lesions. Foam cells produce various bioactive molecules, such as cytokines, growth factors, and proteases, which play crucial roles in the progression of atherosclerosis. Macrophages take up acetylated LDL (acetyl-LDL) through the scavenger receptor class A (SR-A) and transform into foam cells, which are characterized by intracellular accumulation of cholesterol ester (CE). Acyl-coenzyme A (CoA): cholesterol acyltransferase (ACAT), an intracellular enzyme located in the rough endoplasmic reticulum, catalyzes CE formation from cholesterol and fatty acyl-CoA. Of the 2
human ACAT isozymes ACAT-1 and ACAT-2. ACAT-1 is the dominant isozyme in monocyte-macrophages.17 ACAT-1 is expressed at high levels by macrophage-derived foam cells in atherosclerotic lesions in vivo18 and upregulated during differentiation from monocytes into macrophages in vitro.19 Therefore, ACAT-1 plays a crucial role in the formation of macrophage-derived foam cells.14 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, rottlerin, and PD98059 were obtained from Sigma. 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.16 Purified monocytes were suspended in RPMI medium 1640 and seeded onto 6-cm dishes (4 × 10^5 cells per dish) for immunoblotting analysis, cholesterol esterification assay, and endocytic uptake of ^[^3H]acetyl-LDL, and 10-cm dishes (1 × 10^6 cells per dish) for ACAT assays and Northern blotting analysis. After 1 hour of incubation (37°C; 5% CO₂) for adherence, the medium was replaced with RPMI medium 1640 supplemented with 10% pooled human serum, streptomycin (0.1 mg/mL), and penicillin G (100 units/mL).

Adhered monocytes were incubated at 37°C in 5% CO₂ for 7 days to induce differentiation into macrophages in the presence or absence of the indicated concentrations of U-II. Anti–U-II antibody (20 μL/2 mL plate),21 urantide (10 nmol/mL),22 the selective UT receptor antagonist peptide, or 4-aminooquinoline (100 nmol/mL),23 the selective UT receptor antagonist nonpeptide, were added 1 hour before the addition of U-II. To evaluate the postreceptor intracellular signal transduction pathways of U-II–induced ACAT-1 expression, GDP-β-S (100 μmol/L),2 a specific G-protein inactivator, PP2 (1 μmol/L),2,13 a specific c-Src protein tyrosine kinase inhibitor, rottlerin (10 μmol/L),24 a specific protein kinase C (PKC) inhibitor, PD98059 (10 μmol/L),2 a specific mitogen-activated protein kinase kinase (MEK) inhibitor, or Y27632 (10 μmol/L),13 a specific Rho kinase (ROCK) inhibitor, were added together with U-II to ensure that the blocking activities of the antagonists or inhibitors were not attributable to their cytoxic effects, dose-response studies were performed with only the antagonists or inhibitors. From these studies, we selected a concentration that can be attained physiologically if the compound is not cytotoxic and a concentration that did not disturb monocytic differentiation into macrophages.

Western Blotting Analysis

Cells were extracted with 100 μL of 10% sodium dodecyl sulfate (SDS) as described previously.19 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).19 The densities of the bands were measured using Light-Capture and Densitograph software (AE-6962FC; CS Analyzer ver2.0; ATTO Corp.).

Assay for ACAT Activity

The enzyme activity was determined by the reconstituted assay.18,20,25 Cells were treated with 1 mmol/L Tris and 1 mmol/L EDTA at pH 7.4 for hypotonic shock followed by homogenization with buffer A (50 mmol/L Tris-HCl and 1 mmol/L EDTA at pH 7.8 with protease inhibitors). Cell homogenates were mixed with 4 mol/L KCl and 20% 3-[3-cholamidopropyl]dimethylammonio]propanesulfonate in buffer A to obtain the final concentration of 1 mol/L and 2%, respectively. These samples (80 μg in 20 μL) were reconstituted with 140 μL of sodium taurocholate-cholesterol-phosphatidylcholine (PC)–mixed micelles (0.2 as cholesterol/PC molar ratio). The enzyme reaction was initiated by adding 20 μL of the substrate mixture containing 250 μmol/L of ^[^3H]oleoyl-CoA (20 dpm/pmol), followed by incubation for 15 minutes at 37°C. Lipids were extracted and the radioactive cholesterol[^3H]oleate was determined by thin-layer chromatography.

Northern Blotting Analysis

A 1469-bp fragment of human ACAT-1 cDNA was amplified by polymerase chain reaction with sense (5'-GACAAGCGCTC-TCTTTAGATGAC-3') and antisense (5'-CTGTGGAGCT-TCACTGGAGTCCTGAG-3') primers using a human cDNA library (Stratagene) as the template. The cloned cDNA fragment was verified as a part of human ACAT-1 sequence and labeled with α-[^32P]dCTP to use as probe. Total RNA was extracted from cultured cells and subjected to electrophoresis on 1.25% agarose gels containing 2.2 mol/L formaldehyde. The RNA was blotted onto nylon membranes and hybridized with the heat-denatured cDNA probe in Express Hyb solution (Clontech) at 68°C for 1 hour. After washing, the membranes were exposed to X-ray film at −80°C with intensifying screens. The densities of the bands on the autoradiographs were measured by the same manner as described above.

Assay for Cholesterol Esterification

Human LDL (d=1.019 to 1.063 g/mL) and acetyl-LDL were prepared as described previously.16,20 Monocytes were incubated for 7 days with or without U-II (25 nmol/L), followed by incubation for 24 hours with various concentrations of acetyl-LDL in the presence of 0.1 mmol/L ^[^3H]oleate conjugated with BSA.16,20 Cellular lipids were extracted and the radioactivity of cholesterol[^3H]oleate was determined by thin-layer chromatography.16,20

Cellular Assay for Endocytic Uptake of ^[^3H]Acetyl-LDL

Monocytes were incubated for 7 days with or without U-II (25 nmol/L). The cells in each dish were incubated for 15 hours with 5 or 10 μg/mL ^[^3H]acetyl-LDL.2,16,20 An aliquot (0.75 mL) of the culture medium was mixed with 0.25 mL of 40% trichloroacetic acid. To this solution was added 0.2 mL of 0.7 mol/L AgNO₃, followed by centrifugation at 2500 rpm for 10 minutes. Trichloroacetic acid–soluble radioactivity in the supernatant and cell-associated radioactivity were determined as described previously.16

Statistical Analysis

Values expressed as the mean±SEM were statistically analyzed using 1-way ANOVA followed by Bonferroni’s post hoc tests for multiple comparisons or the Student’s t test for unpaired data. Differences were considered statistically significant at P<0.05.

Results

U-II Upregulates 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.

**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 analyses 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).

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), 4-aminoquinoline (100 nmol/L), GDP-β-S (100 μmol/L), PD98059 (10 μmol/L), PP2 (1 μmol/L), rottlerin (10 μmol/L), PD98059 (10 μmol/L), or Y27632 (10 μmol/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.
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 [125I]Acetyl-LDL in Human Monocyte-Derived Macrophages

The effects of U-II on SR-A function as assessed by the endocytic uptake of [125I]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 [125I]acetyl-LDL.

Discussion

Accumulating evidence reported by Douglas et al1,3,5,23 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 al28 reported that peripheral infusion of U-II caused vasoconstriction in the human forearm. Cheung et al6 showed that plasma concentrations of U-II were elevated in 62 hypertensive patients compared with 62 normotensive controls (13.6±1.4 versus 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 β-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 Davenport2 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 μm) 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 monocyte-derived 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-β (TGF-β); and is downregulated by adiponectin. Our preliminary studies show that among G-protein agonists, serotonin (5-HT₂-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.

Urante has been reported recently to be a potent ligand for human UT (hUT) receptor. Patacchini et al. showed that urante binds with nanomolar affinity (pKᵦ 8.3) to the recombinant hUT and antagonizes (pKᵦ 8.3) the contractile effects of U-II in the rat aorta without showing any residual agonist activity. In our study, urante dose-dependently inhibited the U-II–induced ACAT-1 upregulation in human monocyte-derived macrophages. However, urante 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 (CHO₃UT cells). The different pharmacological behavior of urante (pure antagonist in human macrophages as well as rat aortic VSMCs versus partial agonist in CHO₃UT cells) could be attributed to the amount of expression of hUT receptor. The efficacy of urante 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.

**Acknowledgments**

This work was supported in part by grant-in-aid for scientific research of Science and the High Technology Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**References**


Human Urotensin II Accelerates Foam Cell Formation in Human Monocyte-Derived Macrophages
Takuya Watanabe, Toshiaki Suguro, Tomoko Kanome, Yu-ichiro Sakamoto, Syuusuke Kodate, Tamio Hagiwara, Shigeki Hongo, Tsutomu Hirano, Mitsuru Adachi and Akira Miyazaki

Hypertension. 2005;46:738-744; originally published online September 19, 2005;
doi: 10.1161/01.HYP.0000184226.99196.b5

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/46/4/738

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/