Liver X Receptor Activator Downregulates Angiotensin II Type 1 Receptor Expression Through Dephosphorylation of Sp1

Ikuyo Imayama, Toshihiro Ichiki, Dan Patton, Keita Inanaga, Ryohei Miyazaki, Hideki Ohtsubo, Qingping Tian, Kotaro Yano, Kenji Sunagawa

Abstract—Atherosclerosis is considered to be a combined disorder of lipid metabolism and chronic inflammation. Recent studies have reported that liver X receptors (LXRs) are involved in lipid metabolism and inflammation and that LXR agonists inhibit atherogenesis. In contrast, angiotensin II is well known to accelerate atherogenesis through activation of the angiotensin II type 1 receptor (AT1R). To better understand the mechanism of LXR on the prevention of atherogenesis, we examined whether activation of LXR affects AT1R expression in vascular smooth muscle cells. T0901317, a synthetic LXR ligand, decreased AT1R mRNA and protein expression with a peak reduction at 6 hours and 12 hours of incubation, respectively. A well-established ligand of LXR, 22-(R)-hydroxycholesterol, also suppressed AT1R expression. The downregulation of AT1R by T0901317 required de novo protein synthesis. AT1R gene promoter activity measured by luciferase assay revealed that the DNA segment between −61 bp and +25 bp was sufficient for downregulation. Luciferase construct with a mutation in Sp1 binding site located in this segment lost its response to T0901317. T0901317 decreased Sp1 serine phosphorylation. Although preincubation of vascular smooth muscle cells with T0901317 for 30 minutes had no effect on angiotensin II–induced extracellular signal–regulated kinase phosphorylation, phosphorylation of extracellular signal–regulated kinase by angiotensin II was markedly suppressed after 6 hours of preincubation. These results indicate that the suppression of AT1R may be one of the important mechanisms by which LXR ligands exert antiatherogenic effects. (***Hypertension*. 2008;51:1631-1636.**)

Key Words: liver X receptor ■ angiotensin II type 1 receptor ■ Sp1

The liver X receptors (LXRs) are member of the nuclear hormone receptor superfamily.[1] Their endogenous ligands are oxidized cholesterol derivatives, such as oxysterols[1] and glucose.[2] LXRs regulate the expression of genes involved in lipid and glucose metabolism. In lipid metabolism, LXRs are known to regulate genes involved in "reverse cholesterol transport," which includes cholesterol efflux, transport, and excretion. ATP binding cassette A1 is involved in cholesterol efflux, and ABCG5 and ABCG8 are involved in cholesterol transport.[3,4] Expression of these ABC proteins is increased by LXR agonists. In mice, LXR agonists were reported to promote biliary and fecal excretion of cholesterol.[5] It was reported recently that glucose directly activated LXRs[2] and regulated the transcription of genes such as GLUT4.[6] In addition, LXR agonists were reported to negatively regulate the expression of inflammatory cytokines[7] and prevent the formation of atherosclerotic lesions in atherosclerosis-prone mice.[8,9]

The effects of angiotensin II (Ang II) are mediated by Ang II receptors, and so far 2 isoforms, type 1 receptor (AT1R) and type 2 receptor, have been identified.[10] AT1R mediates most of the traditional effects of Ang II, such as vasoconstriction and cell proliferation. It is well known that Ang II enhances atherogenesis,[11,12] and an AT1R antagonist attenuated atherogenesis in animal models.[13–15]

Emerging evidence has suggested that Ang II is critically involved in various aspects of inflammation.[16] In line with this notion, Ang II was reported to activate nuclear factor-κB, a transcription factor involved in the regulation of many inflammation-related genes, in vascular smooth muscle cells (VSMCs) through AT1R.[17] Intriguingly, an LXR agonist inhibited nuclear factor-κB activation.[7] Therefore, it may be possible that the LXR and Ang II/AT1R pathways functionally antagonize in terms of the inflammatory response.

A recent study showed that GW3965, a synthetic LXR agonist, increased murine renin gene expression.[18] However, the effect of LXR activation on AT1R expression has not been determined. In the present study, we tested whether LXRs are involved in the regulation of AT1R gene expression.

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Materials and Methods

Materials

DMEM was purchased from GIBCO BRL. FBS was purchased from JRH Biosciences. BSA, T0901317, 22-(R)-hydroxycholesterol (22-R-HC), 22-(S)-hydroxycholesterol (22-S-HC), cycloheximide (CHX), actinomycin D (ActD), trichostatin A (TSA), PD123319, and mouse monoclonal anti-α-tubulin were purchased from Sigma Chemical Co. Rabbit polyclonal antibodies against AT1R[19,20] and p16 were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against Sp1 and phosphoserine were purchased from Upstate, Inc, and Chemicon International, Inc, respectively. Rabbit polyclonal antibodies against extracellular signal–regulated kinase (ERK) and phosphorylated ERK (pERK) were purchased from Cell Signaling Co. Horseradish peroxidase–conjugated secondary antibodies (antirabbit and antimouse IgG) were purchased from Vector Laboratories, Inc. Losartan was kindly provided by Merck Co. [α-^{32}P]dCTP was purchased from Perkin-Elmer Life Sciences. Luciferase assay system was purchased from Promega Biosciences, Inc. Other chemical reagents were purchased from Wako Pure Chemicals unless mentioned specifically.

Cell Culture

VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats by an explant method and maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 95% air-5% CO_2. At passage 2, >95% of cells were positive for α-smooth muscle actin. VSMCs were cultured until grown to confluence. The medium was changed to DMEM with 0.1% BSA, and the cells were cultured for an additional 2 days. Then, the VSMCs were used in the experiment. Cells between passages 4 and 13 were used.

Northern Blot Analysis

Northern blot analysis was performed as described previously.[21] ActD (5 μg/mL) was used to examine the stability of AT1R mRNA. ActD was added after 6 hours of stimulation with T0901317 (10 μmol/L). In a control experiment, only ActD was added. Cells were harvested after 3, 6, 12, and 24 hours of ActD supplementation, and the expression level of AT1R mRNA was examined by Northern blot analysis.

Measurement of AT1R Gene Promoter Activity

Five deletion mutants of the AT1A gene promoter were prepared by digestion with restriction endonucleases and ligated to the luciferase gene. The AT1R promoter-luciferase construct with mutation in the GC-box–related sequence (wild-type: TGCAGAGCAGCGACGC-CCCCTAGGC; mutant: TGCAGAGCAGCGACGTTTCCTAGGC) was a generous gift from Dr Akira Sugawara (Tohoku University, Sendai, Japan).[22]

Confluent VSMCs were split by trypsin/EDTA solution, and cells were prepared in a 6-cm tissue culture dish. At 80% confluence, 5 μg of AT1 promoter-luciferase fusion DNA and 2 μg of β-galactosidase gene (LacZ) were introduced to VSMCs by the DEAE-dextran method according to the manufacturer's instruction (Promega Corporation). AT1R promoter/luciferase DNA construct with a mutation in the GC box (Sp1 binding site) was also introduced to VSMCs with the LacZ expression plasmid. VSMCs were cultured in DMEM with 10% FBS for 18 hours, washed twice with PBS, cultured in DMEM with 0.1% BSA for 24 hours, and then stimulated with T0901317 (10 μmol/L) for 12 hours. The luciferase activity was measured as described previously.[21]

Western Blot Analysis

Western Blot analysis was performed as described previously.[21]

Immunoprecipitation

VSMCs were lysed in a Nonidet P-40 lysis buffer (0.5% Nonidet P-40; 10 mmol/L of Tris-HCl [pH 7.5]; 150 mmol/L of NaCl; 2.5 mmol/L of KCl; 20 mmol/L of β-glycerol phosphate; 50 mmol/L of NaF; 1 mmol/L of Na_3VO_4; 1% aprotinin; 0.5% leupeptin; and 1 mmol/L of dithiothreitol), and the lysates were subjected to immunoprecipitation with an anti-Sp1 antibody as described previously.[23] Western blot analysis was performed with the antiphosphoserine antibody as described previously.[23] The intensity of the bands was quantified with a MacBAS bioimaging analyzer (Fujifilm).

Statistical Analysis

Statistical analysis was performed with either 1-way ANOVA or 2-way ANOVA and Fisher's test, if appropriate. Statistical significance was designated as $P<0.05$. Values are expressed as means±SEMs.

Results

LXR Agonist Reduced the Expression of AT1R mRNA and Protein

VMSCs were incubated with T0901317 (10 μmol/L) for various periods, and expression of AT1R mRNA was examined. The expression of AT1R mRNA was reduced with a peak at 6 hours of incubation (Figure 1A). The downregulation was transient, and the expression level resumed to the control level at 12 hours. We tested whether a second challenge with T0901317 affected the AT1R mRNA expression. The medium was replaced with fresh serum-free medium containing 10 μmol/L of T0901317 at 6 hours of stimulation, and VSMCs were further incubated for 6 hours. In this case, AT1R mRNA was still downregulated at 12 hours (Figure S1, available online at http://hyper.ahajournals.org). AT1R mRNA was reduced in a dose-dependent manner at 6 hours of incubation with T0901317 (Figure 1B). T0901317 reduced the AT1R protein level with a peak reduction at 6 hours of incubation (Figure 1C). The expression level of AT1R protein in VSMCs without T0901317 was quite stable during this incubation period (data not shown). T0901317 suppressed AT1R expression in a dose-dependent manner (Figure 1D). Janowski et al[24] had reported that 22-R-HC is a potent agonist of LXR. To confirm whether LXR mediates AT1R downregulation, we examined the effect of 22-R-HC (10 μmol/L, 6 hours) and its S enantiomer, 22-S-HC (10 μmol/L, 6 hours). 22-R-HC decreased the expression of AT1R. However, 22-S-HC, which is not an agonist of LXR, did not change the expression of AT1R (Figure 2A). Even the higher concentrations of 22-S-HC (30 to 50 μmol/L, 6 hours) did not affect AT1R mRNA expression (Figure 2B). These data suggest that AT1R is downregulated by the specific effect of LXR activation.

T0 Inhibits AT1R Expression at the Transcriptional Level

Deletion mutants of AT1 promoter/luciferase fusion DNA were used to determine the specific promoter region responsible for T0901317-induced AT1R suppression. Luciferase activity was suppressed in all of the DNA constructs (Figure 3A). The DNA construct with mutation in Sp1 binding site (AT1R promoter region from −58 to −34 bp) showed reduced basal luciferase activity compared with wild type (−61 bp) luciferase construct (data not shown), as reported previously, and stimulation with T0901317 (10 μmol/L) did not affect the luciferase activity in the Sp1 mutant luciferase construct (Figure 3A). Therefore, we suppose that Sp1 is a positive regulatory element in the AT1R gene promoter and

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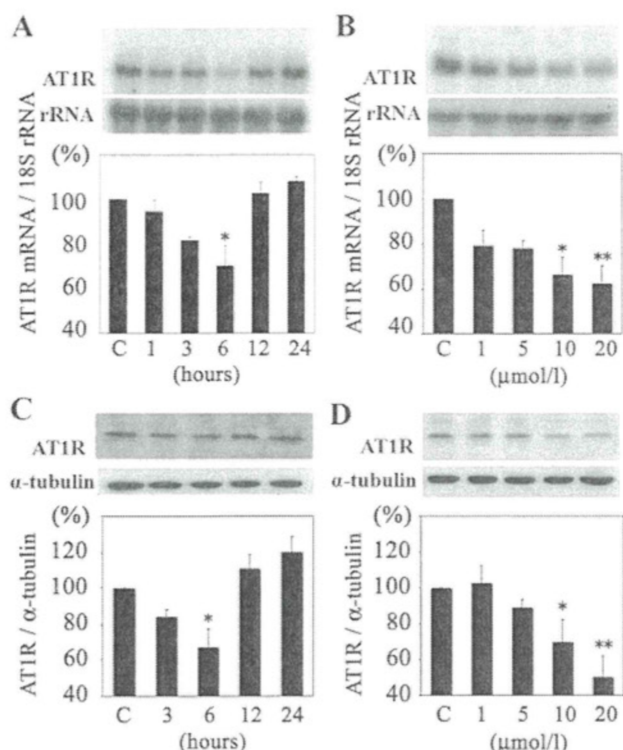

Figure 1. T0901317 suppressed AT1R expression in VSMCs. A, VSMCs were incubated with T0901317 (10 μmol/L) for various periods as indicated in the figure. Total RNA was isolated, and expression of AT1R mRNA and 18S rRNA (rRNA) was determined by Northern blot analysis. Radioactivity of AT1R mRNA was measured with an imaging analyzer and was normalized by radioactivity of rRNA. The ratio of AT1R mRNA to rRNA is shown in the bar graph. B, VSMCs were incubated with T0901317 at concentrations varying from 1 to 20 μmol/L for 6 hours. The expression of AT1R mRNA was determined and analyzed as described above. C, VSMCs were incubated with T0901317 (10 μmol/L) for various periods as indicated. Expression of AT1R protein and α-tubulin was detected by Western blot analysis. The density of the specific band was scanned and quantified with an imaging analyzer. The ratio of AT1R to α-tubulin is shown in the bar graph. D, VSMCs were incubated with T0901317 at various concentrations from 1 to 20 μmol/L for 6 hours. The expression of AT1R protein was determined and analyzed as described above. Values (means±SEMs) are expressed as a percent of control culture (100%; n=6). $^{*}P<0.05$ vs control; $^{**}P<0.01$ vs control. C indicates control.

that T0901317 induced suppression of AT1R gene expression by inhibiting Sp1 function.

T0901317 did not affect the degradation rate of AT1R mRNA (Figure 3B). These data suggest that T0901317 inhibits AT1R gene transcription and does not affect AT1R mRNA stability.

De Novo Protein Synthesis Is Required for T0901317-Induced Downregulation of AT1R Expression

We used CHX (10 μg/mL, 1 hour), a protein synthesis inhibitor, to examine whether T0901317-induced downregulation of AT1R expression depended on de novo protein synthesis. Incubation with CHX alone did not affect AT1R mRNA expression. CHX, however, inhibited the T0901317-induced AT1R mRNA downregulation (Figure 4A). These data suggest that downregulation of AT1R mRNA by LXR requires de novo protein synthesis.

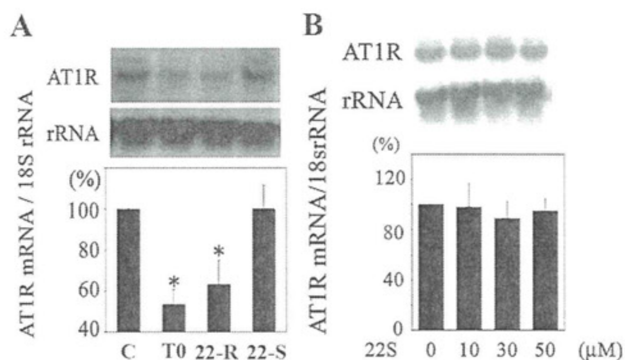

Figure 2. Downregulation of AT1R by 22-R-hydroxycholesterol (HC) but not by 22-S-HC. A, VSMCs were incubated with T0901317 (10 μmol/L), 22-R-HC (10 μmol/L), or 22-S-HC (10 μmol/L) for 6 hours. Expression of AT1R mRNA was determined as described in the legend to Figure 1A. Values (means±SEMs) are expressed as a percentage of control culture (100%; n=5). $^{*}P<0.05$ vs control. B, VSMCs were incubated various concentration of 22-S-HC indicated in the figure for 6 hours (n=3). Expression of AT1R mRNA was determined as described in the legend to Figure 1A.

Histone Deacetylase Activity Is Not Involved in T0901317-Induced Downregulation

It has been reported that the recruitment of histone deacetylase (HDAC) is necessary for gene regulation by a LXR agonist.[25] We used TSA (1 μmol/L), an HDAC inhibitor, to examine the involvement of HDAC in the process. Preincubation with TSA for 24 hours had no effect on T0901317-induced AT1R mRNA suppression (Figure 4B). It is, therefore, suggested that HDAC is not required for T0901317-induced AT1R downregulation.

T0901317 Increased p16 Expression and Suppressed Sp1 Phosphorylation

Previously, Wang et al[26] reported that p16 inhibited Sp1-mediated gene transcription by suppression of cyclin A expression and phosphorylation of Sp1 at the serine residue. We examined the effect of T0901317 (10 μmol/L) on p16 expression and the Sp1 phosphorylation level. T0901317 increased p16 expression and decreased the phosphorylation level of Sp1 at the serine residue. (Figure 4C and 4D).

T0901317-Induced AT1R Downregulation Reduced Cellular Response to Angiotensin II

It is well known that Ang II induces phosphorylation of ERK in VSMCs through AT1R.[27] First, we confirmed the receptor isoform responsible for Ang II-induced ERK activation. Losartan, an AT1R antagonist, but not PD123319, an Ang II type 2 receptor–specific antagonist, inhibited Ang II-induced ERK phosphorylation, indicating that AT1R is responsible (Figure 5A). Next we determined whether downregulation of AT1R gene expression leads to a reduction of functional response of VSMCs to Ang II stimulation. VSMCs were pretreated with T0901317 (10 μmol/L) for 30 minutes and 3, 6, and 12 hours and then stimulated with Ang II (100 nmol/L) for 5 minutes. The phosphorylation of ERK was examined by Western blot analysis. Ang II-induced ERK phosphorylation

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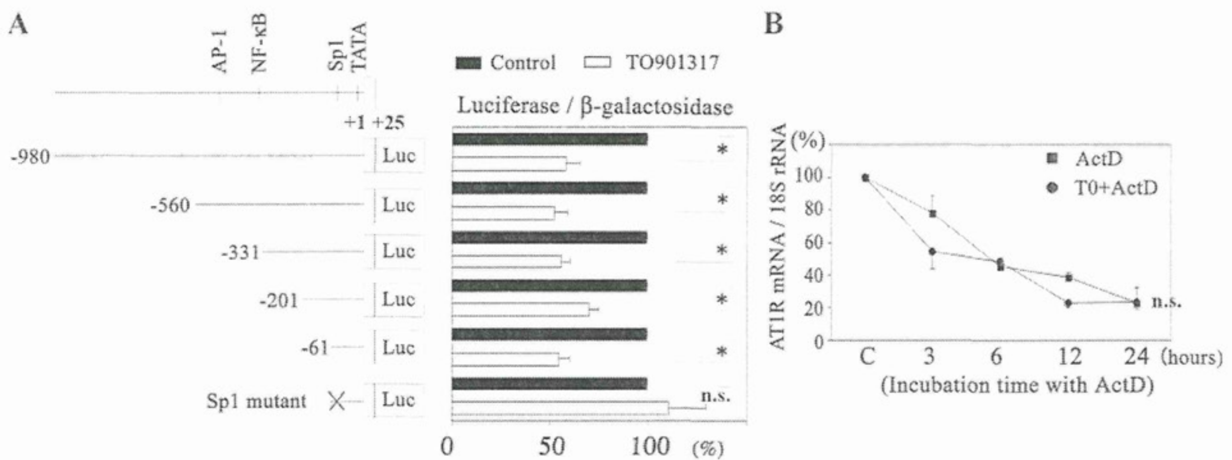

Figure 3. Effect of T0901317 on AT1R gene promoter activity and AT1R mRNA stability. A, The scheme of deletion mutants of AT1R promoter/luciferase fusion DNA construct is indicated on the left side. Relative luciferase activity normalized by β-galactosidase activity is indicated by the bar graph on the right. Relative luciferase activity of unstimulated VSMCs in each construct was set as 100%. Black and open bars indicate the relative luciferase activity of unstimulated and T0901317 (10 μmol/L)-stimulated VSMCs, respectively, which are transfected with the same construct indicated on the left. Values (means±SEMs) are expressed as a percentage of control culture (n=6). *$P<0.05$ vs unstimulated cells. n.s. indicates not significant. B, Total RNA was isolated at the indicated time after ActD (5 μg/mL) supplementation, and expression levels of AT1R mRNA and rRNA were determined with the method described in the legend to Figure 1A. Expression level of AT1R mRNA was normalized with that of rRNA. The normalized AT1R mRNA expression before the addition of ActD in each group was set as 100% (n=4).

was not affected by 30 minutes of preincubation with T0901317, suggesting that T0901317 had no direct effect on Ang II signaling. ERK phosphorylation was remarkably reduced after 6 to 12 hours of preincubation with T0901317 (Figure 5B) when AT1R expression is maximally suppressed (Figure 1A). However, phorbol ester (100 nM) increased ERK phosphorylation after incubation with T0901317 for 6 to 12 hours, suggesting that the ERK activation pathway may not be affected by T0901317 (data not shown). Thus, downregulation of AT1R consequently resulted in the attenuation of the cellular response to Ang II.

Discussion

In the present study, we demonstrated that T0901317, a synthetic LXR agonist, suppressed the expression of AT1R at mRNA and protein levels and that cellular response to Ang II was reduced by AT1R suppression. The results of the luciferase assay suggest that the AT1R promoter region that contains the Sp1 binding site is essential for T0901317-induced AT1R suppression. This is the first study reporting the effect of LXR activation on AT1R expression and its molecular mechanism. We also showed for the first time that LXR agonists upregulated p16 and induced dephosphorylation of Sp1, which may inhibit AT1R gene expression.

It was reported that LXRs regulate gene transcription by 2 mechanisms. One is a DNA-dependent pathway that involves binding of liganded LXR to LXR response element of target genes after the formation of heterodimer with the retinoid X receptor.[1] The other is an LXR response element–independent pathway that involves interference with other transcription factor pathways.[28] Several studies reported various indirect transcriptional regulations by LXR.[29,30] Transcription factors such as AP-1,[31] nuclear factor-κB,[28] c-Jun, and c-Fos[32] are inhibited by LXR. The AT1R gene promoter region does not contain the consensus sequence of LXR response element.[33] It is, therefore, suggested that the effect of LXR on AT1R downregulation may be mediated by the inhibition of other transcription factors.

Based on the deletion and mutation analysis of AT1R gene promoter, it was suggested that the Sp1 binding site located between −58 and −34 bp is crucial for T0901317-induced AT1R suppression. The basal luciferase activity of Sp1 mutant was 50% to 70% of wild-type (−61 bp) luciferase construct (data not shown), which is consistent with the previous study.[22] Therefore, the Sp1 site is a positive regulatory element of the AT1R gene, and T0901317 may suppress AT1R gene expression by inhibiting Sp1 function. Wang et al[26] reported the interaction between Sp1 and cyclinA in Sp1-mediated gene transcription. In this report, they concluded that p16, a cyclin-dependent kinase inhibitor, induced cyclinA/cyclin-dependent kinase downregulation, which resulted in the attenuation of phosphorylation of Sp1, and consequently suppressed Sp1-mediated gene transcription. Our results that showed increased p16 expression and a decreased phosphorylation level of Sp1 at the serine residue by T0901317 are consistent with this previous report. It is reported that T0901317 decreased platelet-derived growth factor–induced expression of cyclinA and D1 and inhibited VSMC proliferation.[34] However, it has not been reported that LXR ligand upregulates p16 as far as we know. Because T0901317-induced AT1R suppression requires de novo protein synthesis, it is suggested that upregulation of p16 expression and resultant inhibition of Sp1 phosphorylation may be responsible for T0901317-induced AT1R downregulation.

A recent report showed that the LXR ligand inhibited cytokine-induced clearance of nuclear receptor corepressor complexes from the C-reactive protein gene promoter.[25] It may be possible that recruitment of nuclear receptor corepressor complexes and HDAC is involved in LXR-induced

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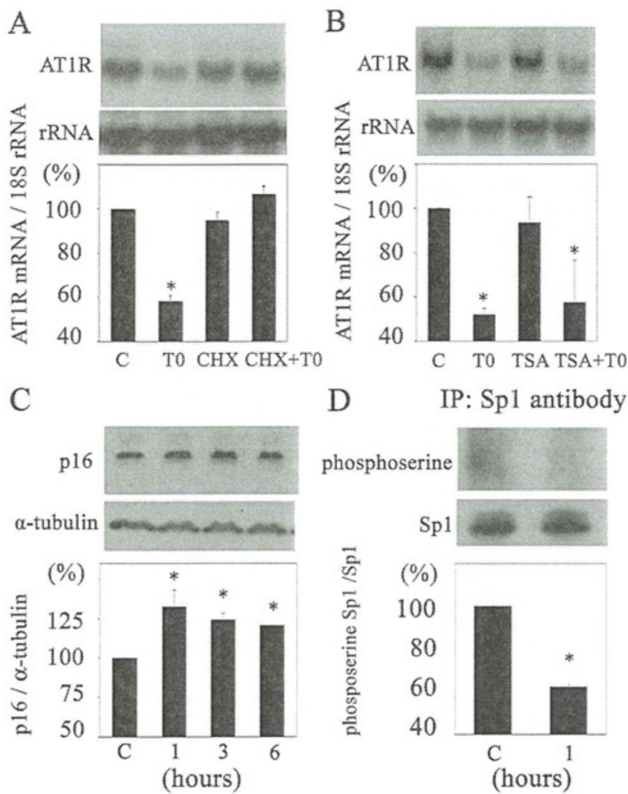

Figure 4. Effects of CHX and TSA on T0901317-induced downregulation of AT1R and effects of T0901317 on p16 expression and phosphorylation level of Sp1. A, VSMCs were incubated with or without CHX (10 μg/mL) for 1 hour and then incubated with T0901317 (10 μmol/L) for 6 hours. Expression of AT1R mRNA was determined as described in the legend to Figure 1A. Values (means±SEMs) are expressed as a percentage of control culture (100%; n=5). *$P<0.05$ vs control. B, VSMCs were incubated with TSA (1 μmol/L) for 24 hours and then incubated with T0901317 (10 μmol/L) for 6 hours. Expression of AT1R mRNA was determined as described in the legend to Figure 1A. Values (means±SEMs) are expressed as a percent of control culture (100%; n=5). *$P<0.05$ vs control or TSA. C, VSMCs were incubated with T0901317 (10 μmol/L) for various periods, as indicated. The expressions of p16 protein and α-tubulin were determined and analyzed as described in the legend to Figure 1C. Values (means±SEMs) are expressed as a percentage of control culture (100%; n=6). *$P<0.05$ vs control. D, VSMCs were incubated with T0901317 for 1 hour. The cell lysates were subjected to immunoprecipitation with an anti-Sp1 antibody, followed by Western blot analysis with an antiphosphoserine antibody (top). The membrane was stripped and reprobed with an anti-Sp1 antibody (bottom). *$P<0.05$ vs control. C indicates control.

AT1R downregulation. However, in our study, we found that TSA did not affect the T0901317-induced AT1R suppression, which may exclude the possible involvement of HDAC in LXR agonist-induced AT1R downregulation.

The expression of AT1R returned to the control level after 12 hours of stimulation with T0901317. However, replacement of the medium with a fresh serum-free medium containing 10 μmol/L of T0901317 at 6 hours of stimulation resulted in the suppression of AT1R at 12 hours. These data suggested that recovery of the AT1R expression at 12 hours was because of degradation or metabolism of the T0901317 rather than the desensitization of AT1R gene expression to T0901317. We also showed that T0901317 reduced Ang II-induced ERK phosphorylation. Intriguingly, ERK phosphorylation was still suppressed after 12 hours of stimulation when the expression of the AT1R level returned to the control level. This may suggest that AT1R protein in the surface of VSMCs had not been fully recovered, although the mRNA or protein level of AT1R was recovered.

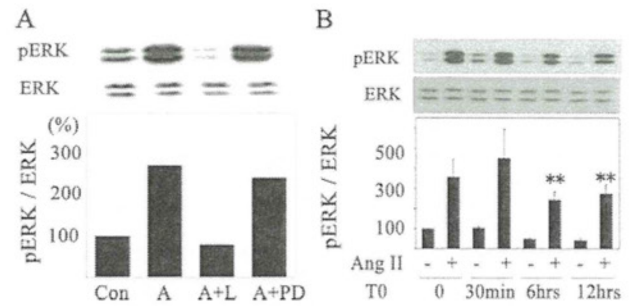

Figure 5. Reduction of Ang II-induced ERK phosphorylation by T0901317. A, VSMCs were pretreated with either losartan (10 μmol/L) or PD123319 (10 μmol/L) for 30 minutes and then stimulated with Ang II (100 nmol/L) for 5 minutes (n=3). B, VSMCs were pretreated with T0901317 (T0: 10 μmol/L) for 30 minutes and 3, 6, and 12 hours and then stimulated with Ang II (100 nmol/L) for 5 minutes. pERK and ERK protein were detected by Western blot analysis. The density of the specific band was scanned and quantified with an imaging analyzer. The ratio of pERK to ERK is shown in the bar graph. Values (means±SEMs) are expressed as a percentage of control culture (100%; n=5). ##$P<0.01$ vs control; **$P<0.01$ vs Ang II without T0901317.

LXR activators are reported to prevent the development and progression of atherosclerosis in animal models.[8,9] The important molecular mechanisms involve the reduction of inflammatory responses, such as cytokine production and improvement of glucose and lipid metabolism. Joseph et al[7] reported that LXR activators inhibit inflammation by downregulating the expression of inducible NO synthase, cyclooxygenase-2, and interleukin-6. In contrast, the enhanced Ang II signaling pathway causes atherosclerosis,[11] which results from activation of inflammatory responses, such as cytokine production, matrix deposition, and induction of adhesion molecules. Therefore, it may be possible that the antiatherosclerotic effects of the LXR activator involve AT1R downregulation.

Perspectives

Our results showing that LXRs agonists downregulate AT1R expression and attenuate the cellular response to Ang II indicate another anti-inflammatory property of LXR activators through inhibition of Ang II signaling. Ang II plays an important role in various pathological conditions and is effective through Ang II receptors. The cellular response to Ang II depends on the expression level of AT1R; thus, downregulation of AT1R can be one way to avoid the vicious effect of Ang II. Because LXRs are expressed ubiquitously, activation of LXRs may be a novel and an effective therapy to attenuate pathological effects of Ang II.

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Disclosures

None.

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Effects of Valsartan or Amlodipine on Endothelial Function and Oxidative Stress after One Year Follow-up in Patients with Essential Hypertension

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Endothelial function is impaired in hypertensive patients. Decreased nitric oxide production and increased oxidative stress are involved in this abnormality. The aim of the present study was to evaluate whether endothelial function and oxidative stress differ following long-term antihypertensive treatment with an angiotensin type 1 receptor blocker, valsartan, or a calcium channel blocker, amlodipine, in patients with essential hypertension. Hypertensive patients were treated with valsartan (80–160 mg/day) or amlodipine (5–10 mg/day) for one year (n = 9 for each). The baseline blood pressure was similar between groups, and the magnitude of the decreases in blood pressure did not differ during treatment at three months, six months, or one year. Endothelial function and oxidative stress markers were examined before and after treatment. Endothelial function, assessed by flow-mediated vasodilation, was significantly improved in hypertensive patients treated with valsartan (5.8 ± 1.2 to 10.7 ± 1.4 %, $p < 0.01$*) but not in those treated with amlodipine. The percent increase in vasodilation induced by sublingual nitroglycerin did not differ between the two groups. As markers of oxidative stress, urinary excretion of 8-isoprostane and 8-hydroxy-2′-deoxyguanosine was significantly reduced in patients treated with valsartan, but not in those treated with amlodipine. These findings suggest that the treatment of hypertensive patients with valsartan for at least one year improves endothelial function in association with reduced oxidative stress. The improved endothelial function and reduced oxidative stress might be involved in the benefits of anti-hypertensive treatment beyond simply lowering blood pressure, although the effects of treatment with valsartan or amlodipine over a much longer period are unknown.*

Keywords blood pressure, endothelium, angiotensin receptors, calcium channel blockers

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Introduction

Valsartan, an angiotensin type 1 (AT1) receptor blocker, and amlodipine, a long-acting dihydropyridine calcium channel blocker (CCB), are widely used for the treatment of hypertension. Large clinical trials have confirmed their usefulness for preventing cardiovascular events mainly by lowering blood pressure (1,2). These relatively new classes of antihypertensive drugs are expected to have some beneficial effects beyond their blood pressure-lowering actions due to their nitric oxide (NO)-producing, anti-oxidant, and anti-inflammatory effects, which were established in experimental animal studies (3,4).

Endothelial dysfunction occurs at the early stage of hypertension and leads to hypertensive vascular diseases (5,6). The precise mechanisms involved are complicated, but involve reduced NO production and increased generation of reactive oxygen species (ROS; 7,8). The effects of antihypertensive drugs on endothelial function have been studied clinically with a relatively small population (9–12). Some studies demonstrated that inhibitors of the renin-angiotensin system, such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (ARBs), improve impaired endothelial function in the forearm in hypertensive patients (9,10,12). To the best of our knowledge, however, there are no studies comparing the effects of valsartan and amlodipine on endothelial function together with their anti-oxidant effects in hypertensive patients. Therefore, the aim of the present study was to examine whether long-term treatment with valsartan or amlodipine improves endothelial function and reduces ROS generation in hypertensive Japanese patients.

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Methods

Patients and Design

Twenty-five patients with hypertension participated in this study. The definitions used for hypertension were those of the Japanese Society of Hypertension: systolic blood pressure of at least 140 mmHg and/or diastolic blood pressure of at least 90 mmHg. Valsartan (80 mg) or amlodipine (5 mg) was administered. The study was an open trial study. After confirming the blood pressure level and the absence of side effects, some patients received higher doses of each drug (160 mg [valsartan] or 10 mg [amlodipine]) to achieve an optimal blood pressure level. The patients were observed at 30-day intervals during the study. None of the patients were diabetic or current smokers. Baseline total cholesterol, triglyceride, and high-density lipoprotein cholesterol levels are shown in Table 1. The study was approved by the Ethics Committee of Kyushu University, and written informed consent was obtained from each patient.

Measurement of Flow-Mediated Vasodilation (FMD)

Imaging studies of the right brachial artery were performed using a Toshiba Power Vision 8000 ultrasound machine equipped with a 11-MHz linear array transducer. The brachial artery diameter was measured in response to increased flow (flow-mediated vasodilation, FMD) and in response to nitroglycerin spray (400 μg; 13,14). Increased flow was achieved by inflating a pneumatic tourniquet placed around the forearm to a pressure of 50 mmHg above the systolic pressure for 5 minutes followed by rapid cuff deflation. Blood flow velocity was measured using the Doppler echo method. All ultrasound

Table 1
Patient characteristics

	Valsartan	Amlodipine
Number of patients	9	9
Age (y)	57 ± 3	66 ± 3
Male/female	2/7	4/5
Height (cm)	157 ± 3	156 ± 3
Weight (kg)	63 ± 4	60 ± 4
BMI (kg/m2)	25.7 ± 1.5	24.5 ± 1.1
FBS (mg/dL)	91.7 ± 2.1	93.8 ± 3.1
HbA1c (%)	4.98 ± 0.04	4.99 ± 0.12
Total cholesterol (mg/dL)	212.6 ± 16.3	203.9 ± 10.4
Triglyceride (mg/dL)	144.6 ± 31.5	97.3 ± 6.9
HDL-C (mg/dL)	59.7 ± 5.0	59.8 ± 5.1

Values are mean ± SE. Abbreviations: BMI = body mass index, FBS = fasting blood sugar.

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measures were performed by one experienced well-trained ultrasonographer who was blind to the treatment. The intra-observer variability was 0.79 ± 0.23%.

Measurement of Oxidative Stress Markers

Serum concentrations of cholesterol, triglycerides, HDL-cholesterol, glucose, etc., were measured before and during the treatment as needed. The urinary concentrations of 8-iso-prostane and 8-hydroxy-2′-deoxyguanosine (8-OHdG) were assayed before and after 12 months of treatment using an enzyme-linked immunosorbent assay (SRL, Tokyo, Japan). Plasma concentrations of renin, angiotensin II, aldosterone, and brain natriuretic peptide were measured using an enzyme-linked immunosorbent assay (SRL).

Statistical Analysis

Baseline values were examined using an unpaired Student's t-test, and the basal values before and after treatment were compared using a paired t-test. Changes in blood pressure and FMD were analyzed using a two-way analysis of variance. Data are expressed as mean ± SE. A p value of less than 0.05 was considered significant.

Results

Study Population Characteristics

The clinical characteristics of the hypertensive patients (n = 18, 7 men and 11 women; mean age: 61 ± 2 years; ages between 39–76 years) are summarized in Table 1. Body mass index, fasting blood sugar, and lipid profiles did not differ between the valsartan- and amlodipine-treated groups. Plasma hormone profiles are shown in Table 2. Plasma brain natriuretic peptide levels were significantly lower in the valsartan-treated group than in the amlodipine-treated group at 6 and 12 months.

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Table 2
Plasma hormone and inflammatory markers before and during the treatment

	Valsartan				Amlodipine			
	Baseline	6-m	12-m	*p* value	Baseline	6-m	12-m	*p* value
Renin (pg/mL)	4.4 ± 0.6	9.1 ± 1.7	9.9 ± 1.6	0.024	6.8 ± 0.8	7.7 ± 2.4	7.3 ± 1.6	0.921
Ang II (pg/mL)	9.0 ± 2.6	11.2 ± 2.4	10.0 ± 2.2	0.808	6.0 ± 0.9	7.8 ± 1.1	6.6 ± 1.3	0.548
Aldosterone (pg/mL)	124.9 ± 15.6	88.9 ± 13.9	97.2 ± 11.6	0.176	105.0 ± 12.5	114.9 ± 15.3	108.2 ± 16.7	0.896
Noradrenaline (pg/mL)	418.4 ± 41.2	487.0 ± 34.9	500.9 ± 72.6	0.499	549.8 ± 53.2	654.6 ± 80.0	555.0 ± 74.6	0.510
Adrenaline (pg/mL)	31.7 ± 6.7	34.9 ± 10.3	34.2 ± 10.1	0.966	50.7 ± 7.9	39.3 ± 8.3	43.2 ± 8.2	0.609
BNP (pg/mL)	23.4 ± 6.0	15.6 ± 4.0*	13.0 ± 2.9*	0.250	48.7 ± 15.4	43.8 ± 12.0	51.3 ± 15.6	0.934
hs-CRP (μg/mL)	641 ± 368	611 ± 248	541 ± 268	0.971	620 ± 134	1355 ± 600	1178 ± 517	0.513

p value shows intra-group comparison.*$p < 0.05$ vs. amlodipine at the same month.
Abbreviations: Ang II = angiotensin II, BNP = brain natriuretic peptide, hs-CRP = high sensitivity C-reactive protein.

Blood Pressure and Heart Rate Changes During the Treatment

Time courses of blood pressure and heart rate are shown in Figure 1. The blood pressure reduction following treatment did not differ between groups. Heart rate did not change during either treatment (see Figure 2).

Effect of Valsartan or Amlodipine on FMD

Baseline brachial arterial diameter before treatment did not differ between the valsartan- and amlodipine-treated groups (3.24 ± 0.32 vs. 3.35 ± 0.23 mm). The baseline diameter after the treatment also did not differ between the two groups (4.03 ± 0.15 vs. 4.43 ± 0.30 mm). The baseline diameter in both groups tended to increase, although the increase was not statistically significant. The percent change in FMD did not differ between the groups before the treatment. Treatment with valsartan for 12 months, however, increased the percent change in

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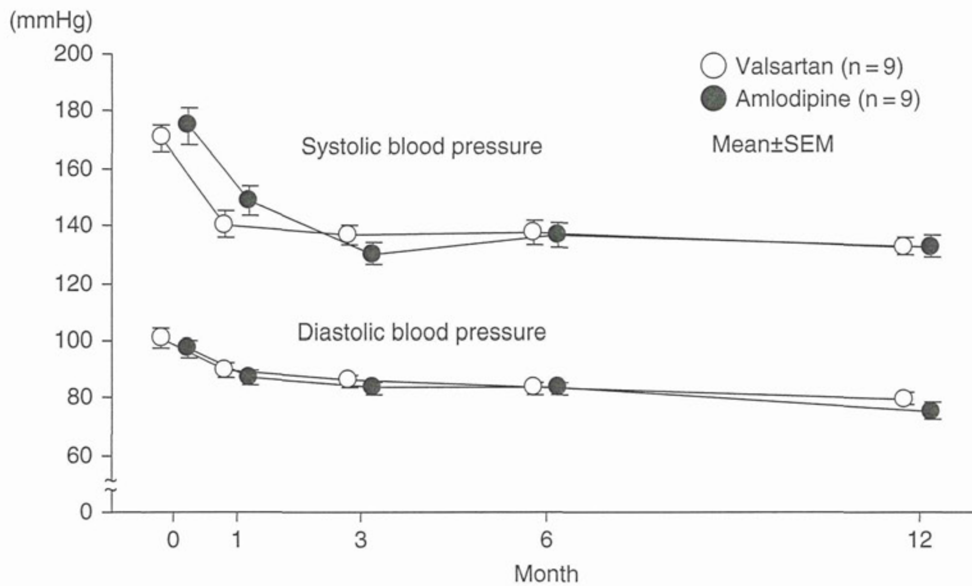

Figure 1. Time course of systolic and diastolic blood pressure in hypertensive patients treated with valsartan (open circle) or amlodipine (closed circle) (n = 9 for each).

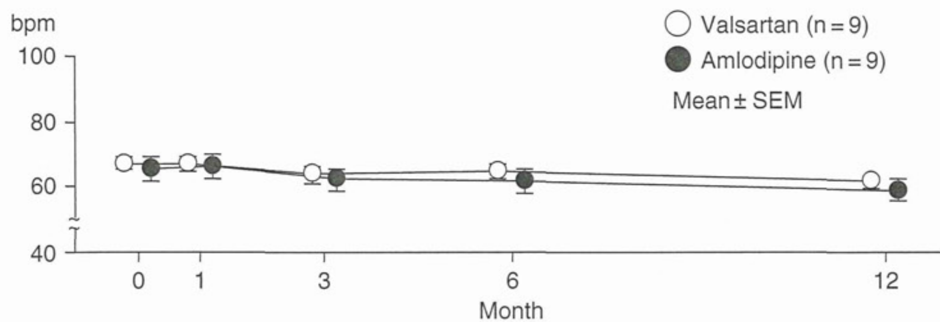

Figure 2. Time course of heart rate in hypertensive patients treated with valsartan (open circle) or amlodipine (closed circle) (n = 9 for each).

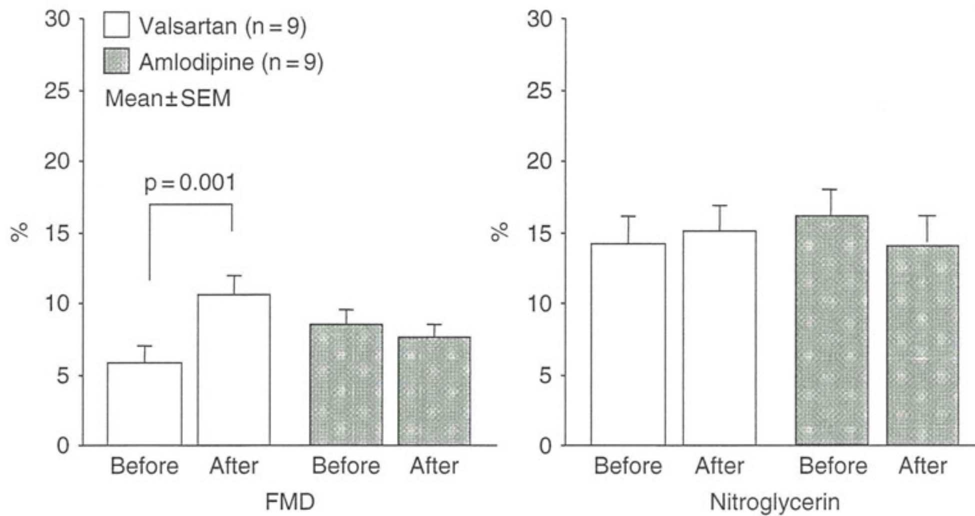

Figure 3. Percent change in the brachial diameter in response to FMD (left panel) or nitroglycerin (right panel) before and after the treatment with valsartan (open bar) or amlodipine (shadow bar).

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FMD, but treatment with amlodipine did not (see Figure 3). The percent change in Doppler flow velocity evoked by reactive hyperemia did not differ between groups or before and after each treatment (before and after valsartan: 236 ± 18 vs. 213 ± 17%; before and after amlodipine: 183 ± 9 vs. 197 ± 12%). The percent change in the brachial artery diameter evoked by nitroglycerin did not differ between before and after either treatment (see Figure 3).

Effect of Valsartan or Amlodipine on Oxidative Stress Markers

Urinary levels of 8-isoprostane and OHdG were significantly decreased in patients treated with valsartan, but not in patients treated with amlodipine (see Figure 4). The baseline values of these markers did not differ between the valsartan- and amlodipine-treated groups.

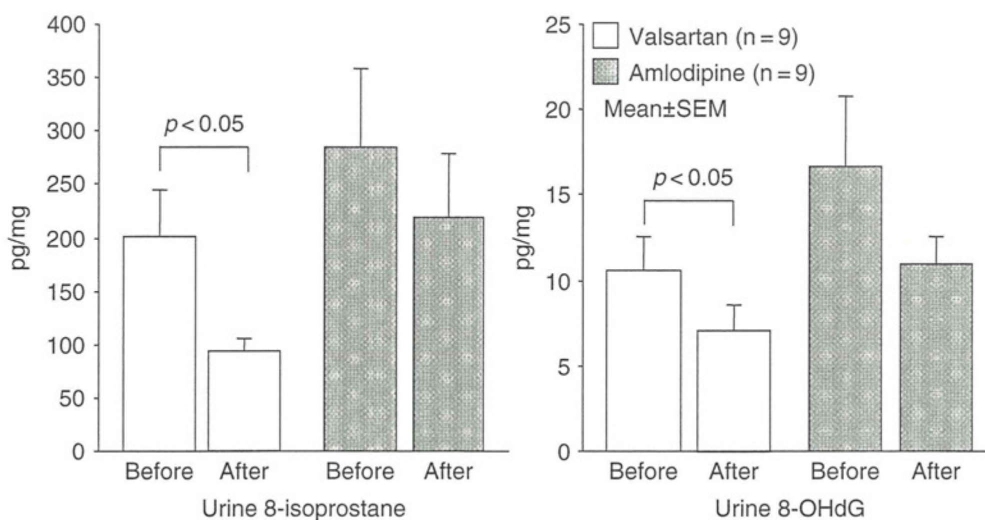

Figure 4. Urinary concentration of 8-isoprostane or 8-OHdG before and after treatment with valsartan (open bar) or amlodipine (shadow bar).

Discussion

The major findings of the present study were that treatment of patients with hypertension for 12 months with valsartan, but not amlodipine, increased the percent change in FMD in association with reduced urinary levels of 8-isoprostane and OHdG. These results suggest that valsartan, an AT1 receptor blocker, improves endothelial function (probably due to reduced oxidative stress) in hypertensive patients.

We evaluated the endothelial function of the brachial artery (conduit artery, not resistance artery) by FMD in the present study. This method is rapidly achieving widespread use because it is non-invasive (13,14). The extent of the change in dilatation, however, is small, and endothelial function evaluated using this method is based on the assumption that increases in flow are due to reactive hyperemia because the stimuli are the same. Guidelines or statements regarding the implications and limitations for assessing endothelial function were recently proposed (13,14). In addition, we measured urinary excretion of 8-isoprostane and 8-OHdG, which are relatively sensitive markers of ROS generation (15–17). An evaluation of oxidative stress levels in humans is somewhat difficult due to variation in the measurements. Therefore, the validity of these measurements and whether they truly reflect oxidative stress, particularly in outpatients, is unknown. Urinary excretion levels of 8-isoprostane and 8-OHdG were reduced after treatment with valsartan, but not after treatment with amlodipine, at least at the one-year follow-up. These results suggest that oxidative stress was reduced by the treatment with valsartan in our hypertensive patients.

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Endothelial function is determined by the balance between NO and superoxide production (6). Bioavailable NO is scavenged by superoxide (6). An important source of superoxide production is NAD(P)H oxidase, which is activated by AT1 receptor stimulation (18). Thus, it is possible that ARB improves endothelial dysfunction by this mechanism. There are few reports, however, describing this action of ARB in humans (12,19–21). In particular, to the best of our knowledge, there are no reports describing valsartan-induced improvement of endothelial dysfunction in hypertensive patients. Our observations suggest that valsartan increased FMD in hypertensive patients and reduced oxidative stress markers.

Endothelial dysfunction in hypertension precedes the development of atherosclerotic processes in the vasculature, which lead to the hypertensive vascular diseases and organ damage (5,6). Although strict long-term treatment with antihypertensive drugs is the most important therapy, evidence from large clinical trials suggests that both ARB and CCB are widely used for the treatment of hypertension without consideration of their effects beyond lowering blood pressure (1,2). In particular, both classes of drugs improve endothelial dysfunction (12,22). ARBs, such as losartan, irbesartan, and candesartan, ameliorate endothelial dysfunction in hypertensive patients (12,19–21). CCBs, such as long-acting nifedipine, amlodipine, and efonidipine, also improve endothelial dysfunction (10,22–25), although there is some controversy (26). We do not have a clear explanation for our finding that amlodipine did not improve endothelial dysfunction, because it has antioxidant effects in vitro (4).

In addition to ARB and CCB, some studies show effects of various antihypertensive drugs on endothelial function, although there are differences among drugs in their effects on endothelial function (23–26). Acute blood pressure reduction itself does not improve endothelial function (25), suggesting that long-term treatment with antihypertensive drugs is needed to improve endothelial dysfunction. It also suggests that some classes of drugs have beneficial effects on endothelial function beyond lowering blood pressure, although

this is difficult to demonstrate in humans. In general, inhibitors of the renin-angiotensin system, such as angiotensin-converting enzyme inhibitors or ARBs, are superior to other treatments (9,24,27). β-blockers or diuretics do not effectively improve endothelial function (24,26). Indeed, angiotensin II increases oxidative stress in human forearm vessels (28). Caution is necessary, however, in interpreting the results of those studies, as differences in the methods used, the duration of the treatment, and the study population must be considered.

There are some limitations of the present study. The number of study subjects was small due to difficulties enrolling patients according to the guidelines established by the Ethics Committee of our University. In addition, some patients dropped out from the study due to the necessity of a 12-month follow-up and the repetitive nature of the protocol. Another limitation was that the clinical characteristics varied among the patients enrolled in the present study. This was an open trial study, and each drug was administered in the order in which the patients were enrolled.

In conclusion, the present study demonstrates that valsartan improves endothelial function in hypertensive patients in association with a reduction in oxidative stress markers after a one-year follow-up. Amlodipine, however, did not have this effect in the patients we studied. More long-term follow-up and large multicenter clinical trials are necessary to confirm the improvement of endothelial dysfunction and the prevention of future cardiovascular events.

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INTERNAL MEDICINE

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CARTO™ Images after Heart Transplantation

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Key words: heart transplantation, electroanatomical mapping, arrhythmia

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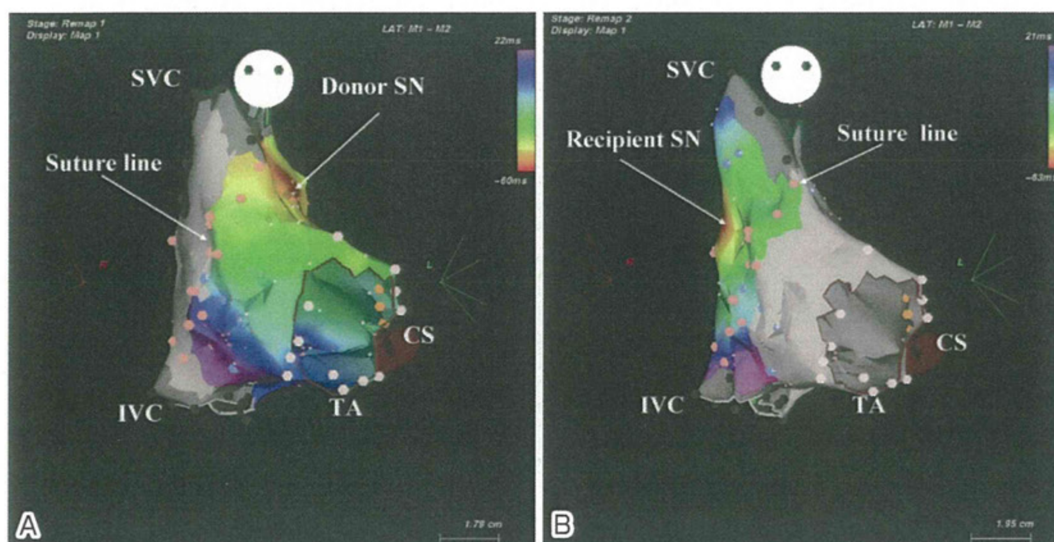

Figure 1. RAO view of CARTO™-activation maps showing the donor right atrium (A) and the recipient right atrium (B). The donor and recipient atriums were completely disconnected and dissociated. SN: sinus node, SVC: superior vena cava, IVC: inferior vena cava, CS: coronary sinus, TA: tricuspid valve annulus.

A 51-year-old man presented to the electrophysiological laboratory with a 3-week history of nonsustained narrow QRS tachycardia 6 weeks after an orthotopic heart transplantation for his dilated cardiomyopathy. Acute and chronic rejections had been already excluded by coronary angiography, echocardiography, and myocardial biopsy on a regular basis. Both the donor and the recipient right atrial (RA) activations were mapped during sinus rhythm using conventional and electroanatomical mapping system (CARTO™, Biosense Webster Ltd., Tokyo, Japan). Intracardiac electrograms and CARTO™-activation maps (Picture 1) revealed that the donor and recipient atrias were completely dissociated. Electrophysiological studies demonstrated evidence of neither the accessory pathways nor the dual atrio-ventricular nodal pathways. Furthermore, programed stimulations of the RAs or the ventricle could not induce any tachyarrhythmias even under isoproterenol administration. Thus, the patient continued medical treatment with beta-adrenergic receptor antagonist. He has remained well without any symptoms 2 years after the heart transplantation. This is the first reported case of CARTO™ images after a heart transplantation in Japan.

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SIRT1, a Longevity Gene, Downregulates Angiotensin II Type 1 Receptor Expression in Vascular Smooth Muscle Cells

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Objective—Resveratrol (3,5,4′-trihydroxystilbene), a polyphenol found in red wine, is known to activate sirtuin1 (SIRT1), a longevity gene. Previous studies have demonstrated that resveratrol extends the life span of diverse species through activation of SIRT1. It was also reported that inhibition of angiotensin II function by angiotensin II type I receptor (AT1R) antagonist prolonged rat life span. We, therefore, hypothesized that resveratrol may inhibit the renin-angiontein system and examined whether resveratrol affects AT1R expression in vascular smooth muscle cells (VSMCs).

Methods and Results—Northern and Western blot analysis revealed that resveratrol significantly decreased the expression of AT1R at mRNA and protein levels in a dose- and time-dependent manner. Overexpression of SIRT1 reduced AT1R expression whereas nicotinamide, an inhibitor of SIRT1, increased AT1R expression and reversed the resveratrol-induced AT1R downregulation. AT1R gene promoter activity was decreased by resveratrol, but resveratrol did not affect the AT1R mRNA stability. Deletion analysis showed that the most proximal region of AT1R gene promoter containing Sp1 site is responsible for downregulation. Administration of resveratrol suppressed AT1R expression in the mouse aorta and blunted angiotensin II–induced hypertension.

Conclusion—Resveratrol suppressed AT1R expression through SIRT1 activation both in vivo and in vitro. The inhibition of the renin-angiotensin system may contribute, at least in part, to the resveratrol-induced longevity and antiatherogenic effect of resveratrol. (***Arterioscler Thromb Vasc Biol*. 2008;28:1263-1269**)

Key Words: resveratrol ■ SIRT1 ■ angiotensin II receptor ■ vascular smooth muscle cell

Resveratrol (3,5,4′-trihydroxystilbene) is one of the polyphenols contained in red wine,[1] pomegranates, and *Polygonum Caspidatum* used in traditional Chinese and Japanese medicine.[2] Resveratrol has various beneficial effects on cardiovascular diseases,[3] cerebral ischemic injuries,[4] and cancer.[5] Previous studies have demonstrated that resveratrol extends the life span of diverse species through activation of silent information regulator 2 (SIR2)[6,7] even on a high-calorie diet.[8] Mammals have 7 SIR2 homologs (sirtuins, SIRT1 to 7), and sirtuins belong to class III histone/protein deacetylases (HDACs). Class I and II HDACs consume a water molecule for direct hydrolysis of the acetyl group, and they are inhibitable by trichostatin A (TSA).[9] On the other hand, sirtuins require nicotinamide adenine dinucleotide (NAD^+) as a cosubstrate for the deacetylation reaction: Sirtuins catalyze a reaction in which the cleavage of NAD^+ and protein deacetylation are coupled to the formation of *O*-acetyl-ADP-ribose, nicotinamide and deacetylated lysine residue.[10] Sirtuins are inhibited by nicotinamide but not by TSA.[11,12] Sirtuins are highly preserved among numerous species and are associated with longevity, cell cycle regulation, apoptosis, DNA damage repair, and muscle differentiation.[13,14] It is generally believed that longevity is mainly promoted by SIRT1,[15] and resveratrol affects various aspects of cell function through SIRT1 in mammalian cells.

Angiotensin II (Ang II) plays important roles in the pathogenesis of atherosclerosis and hypertension.[16] Mammalian cells express 2 types of Ang II receptors, Ang II type 1 receptor (AT1R)[17] and Ang II type 2 receptor (AT2R).[18] AT1R and AT2R belong to the 7-transmembrane, G protein–coupled receptor family. These receptors exert opposite effects in terms of cell growth and blood pressure regulation.[19] However, most of the traditional cardiovascular effects of Ang II such as vasoconstriction and water and sodium retention are mediated by AT1R.[20,21] It was also reported that inhibition of Ang II function by AT1R antagonist prolonged the life span of hypertensive rats.[22]

We, therefore, hypothesized that resveratrol may inhibit the renin-angiontein system and examined whether resvera-

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trol affects AT1R expression in vascular smooth muscle cells (VSMCs).

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM) was purchased from GIBCO BRL. Fetal bovine serum (FBS) was from JRH Biosciences Inc. Resveratrol, bovine serum albumin (BSA), actinomycin D, phorbol-12-myristate 13-acetate (PMA), TSA, and nicotinamide were purchased from Sigma Chemical Co. Ang II was purchased from PEPTIDE Inc. Rabbit polyclonal antibodies against AT1R and α-tubulin were from Santa Cruz Biotechnology. An anti-SIRT1, anti–extracellular signal regulated protein kinase (ERK), and anti–phospho ERK (pERK) antibodies were purchased from Cell Signaling. [α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from Perkin-Elmer Life Sciences. Other chemical reagents were purchased from Wako Pure Chemicals unless mentioned specifically.

Cell Culture

VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats and maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 95% air-5% CO_2. VSMCs were grown to confluence, cultured in DMEM with 0.1% BSA for additional 2 days, and used in the experiment. Cells between passages 5 and 12 were used.

Northern Blot Analysis

Total RNA was prepared by an acid guanidinium thiocyanate-phenol-chloroform extraction method, and Northern blot analysis of AT1R and 18s ribosomal (r) RNA was performed as described previously.[23] The radioactivity of the AT1R mRNA bands was counted with an imaging analyzer and was normalized with the radioactivity of rRNA. The expression level of AT1R was indicated as a ratio of AT1R mRNA to rRNA. To analyze mRNA stability of AT1R, Actinomycin D (5 μg/mL) was added after 6 hours of stimulation with resveratrol (100μmol/L). VSMCs were harvested at 3, 6, 12, 24 hours after addition of actinomycin D. The expression level of AT1R mRNA was determined by Northern blot analysis.

To determine whether resveratrol-induced AT1R mRNA down-regulation requires de novo protein synthesis, VSMCs were pretreated with or without cycloheximide (CHX, 10μmol/L) for 30 minutes and incubated in the presence or absence of resveratrol (RV, 100 μmol/L) for 12 hours. Then the AT1R mRNA level was determined by Northern blot analysis.

Western Blot Analysis

Western blot analysis of AT1R was performed as described previously.[24] The specific band was scanned with an imaging analyzer, and α-tubulin was used as a loading control. The expression level of AT1R was indicated as a ratio of AT1R to α-tubulin. Expression of SIRT 1 was examined by the same method.

Measurement of AT1R Gene Promoter Activity

Five deletion mutants of AT1a gene promoter were prepared by digestion with restriction endonucleases and ligated to luciferase gene as described previously.[25] AT1R promoter/luciferase DNA constructs and LacZ gene were intrduced to VSMCs with the DEAE-dextran method. Then VSMCs were stimulated with resveratrol (100μmol/L) for 12 hours. Promoter activity of AT1R gene was measured by luciferase assay and normalized by β-galactosidase activity as described previously.[26] The AT1R promoter-luciferase construct with mutation in the GC-box-related sequence (wild-type: TGCAGAGCAGCGACGCCCCCTAGGC mutant: TGCAGAGCAGCGA CGTTTCCTAGGC) was a generous gift from Dr Sugawara (Tohoku University, Japan).

Measurement of Cell Viability

Confluent VSMCs were serum deprived for 48 hours and then treated with resveratrol. After 24 hours incubation, attached cells were harvested with trypsin-EDTA. Cells in the medium were collected by centrifugation. These cells were stained with 0.4% trypan blue. The number of total and dead cells was counted with an hemocytometer.[27]

Adenovirus Vector Expressing SIRT1

A recombinant adenovirus vector expressing a wild-type SIRT1 (AdSIRT1) was described previously.[28] VSMCs were grown to 80% confluence, washed twice with phosphate-buffered saline (PBS), and incubated with AdSIRT1 or empty viral vector (AdEmpty) under gentle agitation for 2 hours at room temperature. Then the cells were washed 3 times with PBS, cultured in DMEM with 0.1% BSA for 2 days, and used for the experiments. Multiplicity of infection (moi) indicates the number of virus per cell added to culture dish.

Preparation of Nuclear Extracts and Gel Mobility Shift Assay

Cells were scraped off, washed in ice-cold PBS followed by ice-cold hypotonic buffer (buffer A: 10mmol/L HEPES, pH7.9, 1.5mmol/L $MgCl_2$, 10mmol/L KCl, 0.5mmol/L PMSF, 0.5mmol/L DTT), and then lysed for 10 minutes on ice in the buffer A containing 0.1% Nonident P-40. The lysates were centrifuged for 10 minutes at 10 000*g*. The pelleted nuclei were suspended in lysis buffer (20 mmol/L HEPES, pH 7.9, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 25% glycerol, 0.5 mmol/L PMSF, 0.5 mmol/L DTT), incubated for 15 minutes at 4°C, and centrifuged for 10 minutes at 10 000*g*. The supernatant was used as nuclear extracts.

A synthetic DNA probe (AT1R gene promoter: −40 bp to −6 bp; GGAACCTGCAGAGCA GCGACGCCCCCTAGGCTATA: containing Sp1 site) was labeled with ^{32}P by using [γ-^{32}P] ATP and T4 polynucleotide kinase, and purified by Sephadex G-50 column. And A synthetic DNA probe with mutation in Sp1 site (GGAACCTGCAGAGCAGCGACGTTTCCTAGGCTATA) was also prepared and used as a competitor. Twenty μg of nuclear extracts was incubated with 1×10^5 cpm of labeled DNA probe and 2 μg of poly (dI-dC) in a buffer containing 10 mmol/L Tris-HCl, pH7.5, 1mmol/L EDTA, 4% glycerol, 100 mmol/L NaCl, 2.5mmol/L DTT, 100 mg/L bovine serum albumin for 15 minutes at room temperature. Then the samples were electrophoresed on 5% acrylamide/0.25×TBE gel (1×TBE 90mmol/L of tris borate, 2mmol/L of EDTA). After electrophoresis, gels were dried and exposed to x-ray film at −70°C.

Animal Experiment

All procedures were approved by the institutional animal use and care committee, and were conducted in conformity with institutional guidelines. Nine-week-old C57/B6 mice were purchased from Kyudo Co Ltd (Japan). Resveratrol was suspended in water at 0.1 mg/mL and administered ad libitum. The estimated dose of orally ingested resveratrol was 10 mg/kg/d. In Ang II group, 490 ng/min/kg of Ang II was administered subcutaneously via osmotic minipump (Alzet). Blood pressure and heart rate were measured using tail-cuff method (UR-5000, UEDA). After 1 week, mice were euthanized under pentobarbital anesthesia. Aortas were quickly removed, minced into small pieces, and homogenized on ice in a buffer (0.25 mol/L sucrose, 5 mmol/L Tris-HCl at pH7.5, 1 mmol/L $MgCl_2$). The homogenates were centrifuged at 2000 rpm for 15 minutes at 4°C, and the supernatants were centrifuged at 100 000*g* for 30 minutes at 4°C. The pellets were used as a membrane fraction and subjected to Western blot analysis of AT1R and α-tubulin.

Statistical Analysis

Statistical analysis was performed with performed 1- or 2-way ANOVA and Fisher test, if appropriate. Data are shown as mean±SEM. $P<0.05$ was considered to be statistically significant.

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