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

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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 members of the nuclear hormone receptor superfamily.1 Their endogenous ligands are oxidized cholesterol derivatives, such as oxysterols1 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 to regulate the expression of genes such as GLUT4.6 In addition, LXR agonists were reported to negatively regulate the expression of inflammatory cytokines2 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.
Materials and Methods

Materials

DMEM was purchased from Gibco BRL, FBS was purchased from JRH Biosciences. BSA, T0901317, 22-(R)-hydroxysterol (22-R-HC), 22-(S)-hydroxysterol (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,20 and p16 were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against α-SMA 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-32P]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% CO2. 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 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: TGCAGAGCGAGCCTCACCA; mutant: TGCAGAGCGAGCCTCACCT) 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 Na3VO4; 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.24 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

VSMCs 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 al24 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
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 expression by LXR requires de novo protein synthesis.

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 al26 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
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, down-regulation 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 regulatory element of the AT1R gene, and T0901317 may suppress AT1R gene expression by inhibiting Sp1 function. Wang et al. 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. In this report, it was reported that LXR agonists upregulated p16 and induced dephosphorylation of Sp1, which may inhibit AT1R gene expression.

It was reported that LXR ligands 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. The other is an LXR response element–independent pathway that involves interference with other transcription factor pathways. Several studies reported various indirect transcriptional regulations by LXR. Nuclear factors such as AP-1, nuclear factor-κB, c-Jun, and c-Fos are inhibited by LXR. The AT1R gene promoter region does not contain the consensus sequence of LXR response element. 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. Therefore, the Sp1 site is a positive regulatory element of the AT1R gene, and T0901317 may suppress AT1R gene expression by inhibiting Sp1 function.
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.

LXR activators are reported to prevent the development and progression of atherosclerosis in animal models. The important molecular mechanisms involve the reduction of inflammatory responses, such as cytokine production and improvement of glucose and lipid metabolism. Joseph et al reported that LXR activators inhibit inflammation by down-regulating the expression of inducible NO synthase, cyclooxygenase-2, and interleukin-6. In contrast, the enhanced Ang II signaling pathway causes atherosclerosis, 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 cycle of inflammation. Therefore, activation of LXRs may be a novel and an effective therapy to attenuate pathological effects of Ang II.

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None.

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