Effects of Mitogen-Activated Protein Kinase Pathway and Co-Activator CREP-Binding Protein on Peroxisome Proliferator-Activated Receptor-γ-Mediated Transcription Suppression of Angiotensin II Type 1 Receptor Gene

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Peroxisome proliferator-activated receptor (PPAR-γ) and its ligands suppress several genes related to atherogenesis. We previously reported that ligand-activated PPAR-γ suppressed angiotensin II type 1 receptor (AT1R) gene transcription in vascular smooth muscle cells (VSMCs) by the inhibition of Sp1 binding to the -58/-34 GC-box related element in the AT1R gene promoter region via a protein–protein interaction. It has been reported that the mitogen-activated protein (MAP) kinase pathway inhibits PPAR-γ function through its phosphorylation, and co-activator CREB-binding protein (CBP)/p300 interacts with PPAR-γ and modulates its activity. Since both the MAP kinase pathway and CBP have recently been reported to be atherogenic, we examined their effects on PPAR-γ-mediated AT1R gene transcription suppression. We observed that 1) PPAR-γ-mediated AT1R gene transcription suppression was augmented by treatment with the MAP kinase kinase inhibitor PD98059, while treatment with the p38 kinase inhibitor SB203580 showed no effect; 2) the PPAR-γ-mediated AT1R mRNA decrease was also augmented by PD98059 treatment; 3) CBP overexpression partially, but significantly, abrogated PPAR-γ-mediated AT1R gene transcription suppression; and 4) the CBP effect was eliminated when the -58/-34 GC-box related element was disrupted. It is therefore speculated that: 1) PPAR-γ phosphorylation by the MAP kinase pathway may attenuate PPAR-γ-mediated AT1R gene transcription suppression through the inhibition of PPAR-γ activity; and 2) CBP may enhance the activity of the remaining Sp1 on the -58/-34 GC-box related element, resulting in a reduction in PPAR-γ-mediated AT1R gene transcription suppression. The MAP kinase pathway and CBP may thus antagonize against PPAR-γ in AT1R gene transcription, probably leading to the progression of atherosclerosis. (Hypertens Res 2003; 26: 623–628)

Key Words: mitogen-activated protein kinase kinase, prostaglandin, nuclear hormone receptor

Introduction

Angiotensin II (AII) exerts many biological effects such as cell contraction, proliferation, and migration of vascular smooth muscle cells (VSMCs) that contributes to the progression of atherosclerosis and hypertension (1, 2). All these actions are mediated by membrane AII type 1 receptor (AT1R) (3), and activation of AT1R by AII binding triggers a variety of signal transduction pathways including the MAP

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kinase pathway (4). AT1R antagonists have been widely used in the treatment of cardiovascular disease (5).

Insulin-sensitizing thiazolidinediones, including troglitazone and 15-deoxy-D12,14-prostaglandin J2 (PGJ2), are known to activate nuclear peroxisome proliferator-activated receptor (PPAR-γ) as its ligands (6, 7). Although the function of PPAR-γ and its ligands has thus far been studied mainly in adipocytes (8), the anti-atherosclerotic effects of PPAR-γ and its ligands have recently been focused on in the vasculature (9, 10). We recently observed that PPAR-γ ligands, including troglitazone and PGJ2, suppressed AT1R gene expression both at the mRNA and protein levels in VSMCs (11, 12). We also demonstrated that this suppression was mediated by ligand-activated PPAR-γ at the transcription level through the -58/-34 GC-box related element in the AT1R gene promoter region by the inhibition of Sp1 binding to DNA via a protein–protein interaction (12).

In addition to its ligands, the function of PPAR-γ itself is reported to be modulated by several factors, including the mitogen-activated protein (MAP) kinase pathway (13) and co-activators such as CREB-binding protein (CBP) and its homolog p300 (14). The MAP kinase pathway inhibits PPAR-γ activity through its phosphorylation (13). CBP/p300 interacts with PPAR-γ through its LXXLL motif, and modulates PPAR-γ function (14). Interestingly, activation of the MAP kinase pathway and CBP/p300 has recently been reported to be atherogenic (15, 16). We therefore examined the effects of the MAP kinase pathway and CBP on the suppression of PPAR-γ-mediated AT1R gene transcription.

Methods

Plasmids

The following, previously described reporter plasmids containing rat AT1R gene promoter fragments and luciferase cDNA (12) were used for transient transfection studies: -1969/-104-luc (1969-base pairs (bp) 5′UTR and 104-bp 5′untranslated region (UTR) of rat AT1R gene); -58/-1-luc (58-bp 5′FL); and mutated -58/-1-luc whose GC-box related sequence within the -58/-34 region was disrupted (changed from TGCAGACGACGACGCCCCCTAGGC to TGCAGACGACGACCGATTGTCAGCAA-3′; reverse primer 5′AGA TCCACAAGTTCGACATCATGT-3′ (22)). RT-PCR of AT1R and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (forward primer: 5′GCATTACATTGCCAGTGTG-3′ and reverse primer 5′TCCACAAGTTCGACATCATGT-3′ (22)). RT-PCR of AT1R and GAPDH mRNAs was performed simultaneously as follows: 30 min at 50°C and 2 min at 94°C for RT, followed by 30 cycles of 45 s at 94°C, 45 s at 53°C, and 1 min 30 s at 72°C. A linear correlation between PCR cycles and densitometry intensity of PCR products was confirmed (data not shown). The intensity of PCR products was calculated using a Luminous Imager (AI-C, Japan).

Transgenic Transfection Study/Luciferase Assay

When cultured rat VSMCs reached 70% confluence, the media were changed to stripped media (18) and incubated for 5–6 h. The cells were then subjected to transfection using lipofectin according to the manufacturer’s instructions (Gibco BRL, Gaithersburg, USA). Briefly, 1.2 μg reporter plasmids and 0.8 μg β-galactosidase control plasmid were mixed with 5 μl lipofectin per 3.5 cm plate. In some experiments, several concentrations of CBP expression plasmid were also co-transfected. Twelve h after transfection, the media were changed to stripped media and the cells were incubated for an additional 12 h. The cells were then incubated either with or without 50 μmol/l troglitazone (provided by Sankyo Co., Ltd., Tokyo, Japan) or 2.5 μmol/l PGJ2 (Cayman Chemical, Ann Arbor, USA) for 12 h, and their luciferase activities were measured. In one experiment, the cells were incubated with 30 μmol/l PD98059 (Calbiochem, La Jolla, USA), a highly specific inhibitor of MAP kinase kinase (19), or 10 μmol/l SB203580 (Calbiochem), a highly specific inhibitor of p38 kinase (20), for 12 h with 2.5 μmol/l PGJ2 (the inhibitors were added to the media 30 min before adding PGJ2). After harvesting, the cell extracts were analyzed for both luciferase and β-galactosidase activities (18) to determine the expression of both genes. The transfection efficiency was normalized by the β-galactosidase expression.

RNA Preparation/Semi-Quantitative Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

When rat VSMCs became 70% confluent, media were changed to stripped media (18) and incubated for 5–6 h. The cells then were incubated either with or without 2.5 μmol/l PGJ2 (Cayman Chemical) for 12 h. In one experiment, 30 μmol/l PD98059 (Calbiochem) (19) was also used (PD98059 was added to the media 30 min before adding PGJ2). Their total RNAs were then extracted using an RNasy mini kit (Qiagen, Hilden, Germany), and were subsequently treated with RNase-free DNase (Takara Shuzo, Kyoto, Japan). The extracted RNAs (1 μg) were subjected to RT-PCR using specific primers either for rat AT1R (forward primer: 5′TGAGACCAACTCAACCCAGA-3′; reverse primer 5′GCATTACATTGCCAGTGTG-3′ (21)) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (forward primer: 5′TCCCTCAAGTTCGACATCATGT-3′; reverse primer 5′TCCACAAGTTCGACATCATGT-3′ (22)). RT-PCR of AT1R and GAPDH mRNAs was performed simultaneously as follows: 30 min at 50°C and 2 min at 94°C for RT, followed by 30 cycles of 45 s at 94°C, 45 s at 53°C, and 1 min 30 s at 72°C. A linear correlation between PCR cycles and densitometry intensity of PCR products was confirmed (data not shown). The intensity of PCR products was calculated using a Luminous Imager (AI-C, Japan).

Generation of Anti-PPAR-γ Antibodies/Western Immunoblot Analysis

Anti-PPAR-γ antibody recognizing both PPAR-γ1 and PPAR-γ2 was raised against synthetic peptides containing mouse PPAR-γ1 (60–79) (23), and anti-PPAR-γ2 antibody recognizing PPAR-γ2 alone was raised against synthetic peptides containing mouse PPAR-γ2 (14–30) (23) as previ-
ously described (24) (Sato et al., unpublished data). The specificity of each antibody was confirmed by Western immunoblot analyses using in vitro translated PPAR-γ1 and -γ2 proteins (Sato et al., unpublished data). Western immunoblot analyses with these antibodies were performed as previously described (12, 24) using 20 µg of nuclear extracts prepared from VSMCs cultured either in regular media (with 10% fetal bovine serum), regular media plus 30 µmol/l PD98059 (Calbiochem) (19), or serum-free media (with 0% fetal bovine serum) for 24 h. Western immunoblot analyses of whole cell extracts of VSMCs using anti-CBP antibody (Santa Cruz Biotechnology, Santa Cruz, USA) were also performed.

Statistical Analyses

Statistical analyses of the transient transfection studies were performed by one-factor analysis of variance (ANOVA) using StatView 4.0 software (ABACUS Concepts, Berkeley, USA).

Results

Effects of MAP Kinase Inhibitors on AT1R Gene Transcription Suppression

Since the MAP kinase pathway is known to inhibit PPAR-γ activity through its phosphorylation (13), we first examined the effects of the MAP kinase inhibitors on PPAR-γ-mediated AT1R gene transcription suppression. We treated - 1969/ + 104-luc transfected rat VSMCs with 30 µmol/l PD98059, a specific inhibitor of MAP kinase kinase (19), and 2.5 µmol/l PGJ2 for 12 h. Interestingly, the effect of PGJ2 on the suppression of - 1969/ + 104-luc transcription was augmented by PD98059 treatment (Fig. 1, lines 2 and 4). In
that inhibition of the MAP kinase pathway augmented the involvement in the serum. Taken together, these data suggest kinase pathway, which may be activated by growth factors γular media (with 10% fetal bovine serum) were used. Al-
pected molecular size (邺 scouting with PGJ2 (line 3) comparing with PGJ2 alone (line 2) was confirmed by semi-
titative RT-PCR. In order to examine the basal phosphoryla-
tion state of PPAR-γ in VSMCs, Western immunoblot analyses using anti-PPAR-γ and anti-PPAR-γ2 antibodies were performed. As shown in lane 1 of Fig. 3, anti-PPAR-γ antibody detected a band (designated as band P; arrowhead) larger than that for the PPAR-γ1 protein, which was the expected molecular size (ær52 kDa) (23) (designated PPAR-
γ1; arrow) when nuclear extracts of VSMCs cultured in reg-
ular media (with 10% fetal bovine serum) were used. Al-
though the amounts of the lower band (PPAR-γ1) and the upper band (P) were almost equal when VSMCs were cul-
tured in regular media (Fig. 3, lane 1), the amounts of the upper band (P) dramatically decreased (~40% of the lower band in lane 2, and ~30% of the lower band in lane 3) when VSMCs were treated with MAP kinase pathway inhibitor PD98059 (Fig. 3, lane 2) or cultured in serum-free media (with 0% fetal bovine serum) (Fig. 3, lane 3). Anti-PPAR-γ2 antibody recognizing only the PPAR-γ2 protein could not detect both of these bands (Fig. 3, lane 4), indicating that the larger band (P) did not represent the PPAR-γ2 protein. These data strongly suggest that the larger band (P) re-
resents phosphorylated PPAR-γ1 at the basal state by the MAP kinase pathway, which may be activated by growth factors involved in the serum. Taken together, these data suggest that inhibition of the MAP kinase pathway augmented the suppression of PPAR-γ-mediated AT1R gene transcription, most likely by activation of PPAR-γ through inhibition of its phosphorylation.

**Effect of CBP Overexpression on AT1R Gene Transcription Suppression**

Since CBP is known to interact with PPAR-γ and modulate its function (14), we next examined the effect of CBP over-
expression on PPAR-γ-mediated AT1R gene transcription suppression. As shown in Fig. 4, PGI2-mediated suppression of the transcription of -1969/+104-luc (line 2) was partially, but significantly, abrogated by CBP overexpression in proportion to the transfected amounts (lines 3 and 4). Since the suppression of PPAR-γ-mediated AT1R gene transcription was mediated through the -58/-34 GC-box related el-
ement (12), we next studied the effect of CBP overexpres-
sion on the -58/-1-luc transcription suppression. Although CBP overexpression showed little effect on the basal tran-
scription level of -58/-1-luc (Fig. 5, lines 1 and 4), tran-
scription suppression of -58/-1-luc either by PGJ2 (Fig. 5, lines 2 and 5) or by troglitazone (Fig. 5, lines 3 and 6) was significantly abrogated by CBP overexpression. We next transected mutated -58/-1-luc whose GC-box related se-
quency within the -58/-34 region was disrupted (12). The transcription activity of mutated -58/-1-luc was not af-
ected by both PPAR-γ ligands (Fig. 5, lines 7–9) as previ-
ously observed (12), further confirming that the -58/-34 GC-box related element was responsible for the PPAR-γ-
mediated suppression of AT1R gene transcription. Interest-
ingly, CBP overexpression did not affect the mutated -58/-1-luc activity either in the absence (Fig. 5, line 10) or presence (Fig. 5, lines 11 and 12) of PPAR-γ ligands, sug-
uggesting that the -58/-34 GC-box related element was also necessary for the CBP effect. An increase of CBP protein by CBP overexpression was confirmed by Western immunoblot analyses using anti-CBP antibody (data not shown). These data suggest that the abrogation of PPAR-γ-mediated AT1R gene transcription suppression by CBP may be mediated through the -58/-34 GC-box related element, most likely by activation of the remaining Sp1.

**Discussion**

In VSMCs, the MAP kinase pathway plays major roles in AII-induced c-fos induction, cell migration, and DNA syn-
thesis (15). In addition to AII signaling, the MAP kinase pathway is also involved in insulin (25), platelet derived growth factor (PDGF) (26), and tumor necrosis factor
(TNF)-α (27) signaling in VSMCs, leading to the progression of atherosclerosis. The PPAR-γ ligand troglitazone has recently been reported to inhibit AII-induced DNA synthesis and migration of VSMCs through the inhibition of MAP kinase translocation and activation (28, 29). In the present study, we observed that inhibition of the MAP kinase pathway, but not inhibition of the p38 kinase pathway, augmented the suppression of PPAR-γ-mediated AT1R gene transcription. Phosphorylation of PPAR-γ by the MAP kinase pathway is known to inhibit PPAR-γ activity (13). Therefore, it is reasonable to assume that the observed augmentation of PPAR-γ-mediated AT1R gene transcription suppression by inhibition of the MAP kinase pathway may have been due to the activation of PPAR-γ by the inhibition of its phosphorylation. Since many growth factors, including AII (15), insulin (25), PDGF (26), and TNF-α (27), are known to activate the MAP kinase pathway in VSMCs, PPAR-γ may be prone to be phosphorylated by the MAP kinase pathway under regular culture conditions (10% fetal bovine serum) (12) and in vivo. Half amounts of PPAR-γ1 in 3T3-L1 cells are reported to be phosphorylated by MAP kinase pathway in the basal state (13), which is consistent with our observation in VSMCs. It is therefore speculated that vascular PPAR-γ function may be attenuated by MAP kinase pathway-mediated phosphorylation in patients with atherosclerosis, hypertension, obesity, and hyperinsulinemia whose growth factors described above may be elevated. PPAR-γ phosphorylation by the MAP kinase pathway may thus attenuate PPAR-γ-mediated AT1R gene transcription suppression through the regulation of AT1R.

Co-activator CBP/p300 is known to interact with PPAR-γ through its LXXLL motif, and modulate PPAR-γ function (14). In addition to interacting with transcription factors, CBP/p300 also induces transcription activation through its intrinsic histone acetyltransferase (HAT) activity (30). Recently, HAT activity of CBP has been reported to be enhanced in atherosclerotic lesions in VSMCs (16). Interestingly, the HAT activity is activated by MAP kinase-induced CBP phosphorylation (31). Moreover, CBP/p300 is well known to activate the transcription of nuclear factor-κB, which may lead to atherogenesis (32). Therefore, activation of CBP in VSMCs may be more atherogenic rather than anti-atherogenic. In the present study, we observed that CBP overexpression could abrogate PPAR-γ-mediated AT1R gene transcription suppression through the -58/ -34 GC-box related element. We previously reported that Sp1 could bind to and activate the -58/ -34 GC-box related element (12). Since CBP/p300 is reported to interact with Sp1 (33, 34) and enhance its DNA binding (33), it is reasonable to assume that CBP may abrogate PPAR-γ-mediated AT1R gene transcription suppression through the enhancement of the Sp1 activity remained on the -58/ -34 GC-box related element. The MAP kinase pathway and CBP may thus antagonize against PPAR-γ in AT1R gene transcription, probably leading to the progression of atherosclerosis.

References
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