Selective Stimulation by Ceramide of the Expression of the \(\alpha\) Isoform of Retinoic Acid and Retinoid X Receptors in Osteoblastic Cells

**A ROLE OF SPHINGOSINE 1-PHOSPHATE-MEDIATED AP-1 IN THE LIGAND-DEPENDENT TRANSCRIPTIONAL ACTIVITY OF THESE RECEPTORS**

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Recent studies have demonstrated that sphingosine 1-phosphate (SPP) plays a functional role as a signaling molecule in gene expression in several kinds of cells. The present study demonstrates selective expression by ceramide of retinoic acid receptor-\(\alpha\) (RAR-\(\alpha\)) and retinoic X receptor-\(\alpha\) (RXR-\(\alpha\)) in osteoblastic MC3T3-E1 cells and a functional role of SPP-mediated AP-1 in the signaling mechanism of ligand-dependent transcriptional activity of heterodimers of these receptors in the cells. C\(_2\)- and C\(_6\)-ceramides selectively stimulated the expression of RAR-\(\alpha\) and RXR-\(\alpha\) genes, but not that of \(\beta\) and \(\gamma\) isomeric genes of RAR and RXR, in the cells. The C\(_2\)-ceramide-induced stimulation was clearly inhibited by \(\text{N}-\text{threo}\)-dihydrosphingosine, an inhibitor of sphingosine kinase. SPP also selectively stimulated the expression of both receptors and increased the specific binding of the nuclear proteins to direct repeat 5 (DR-5), a consensus sequence of RAR-RXR. In addition, SPP markedly stimulated transient chloramphenicol acetyltransferase (CAT) activity of retinoic acid-dependent transcriptional activity in the cells transfected with a DR-5-CAT reporter gene. The SPP stimulation was activation protein-1 (AP-1)-dependent, because the SPP stimulatory action toward these nuclear gene expressions and the transient CAT activity were inhibited by antisense c-fos and c-jun oligonucleotides. We observed that SPP actually stimulated AP-1 transcriptional activity in the cells. This study suggests an important role of SPP-mediated AP-1 in the selective expression of RAR-\(\alpha\) and RXR-\(\alpha\) in osteoblastic cells via the sphingosine pathway.

Breakdown of sphingomyelin (SM)\(^1\) through SMase results in the formation of ceramide, which is subsequently metabolized to sphingosine and sphingosine 1-phosphate (SPP) (1–6). TNF-\(\alpha\), IL-1, platelet-derived growth factor, and vitamin D\(_3\) are potent regulators of bone remodeling (7–12), and many studies (12–17) have suggested that these cytokines use sphingosine metabolites such as ceramide or SPP as a second messenger in their signal transduction via activation of SMase. These studies suggest that sphingosine metabolites play an important role as intracellular signaling molecules in osteoblasts and osteoclasts. In fact, these metabolites are able to mimic the biological actions of these cytokines in osteoblasts (12, 18–20). On the other hand, in mature osteoclasts, we also showed that ceramide strongly inhibits bone resorption by these cells (21).

Retinoic acid (RA) is an important regulator hormone for the proliferation and differentiation of a variety of human and mouse cells (22, 23). RA displays multiple biological actions through the transcriptional function of two distinct classes of receptors RAR (RAR-\(\alpha\), -\(\beta\), and -\(\gamma\)) and RXR (RXR-\(\alpha\), -\(\beta\), and -\(\gamma\)) that bind as heterodimers to their respective target DNA sequences (24–29). Because it has been well documented that RXR, as an auxiliary protein, forms heterodimers with receptor of vitamin D\(_3\) and thyroid hormone (28, 29), RXRs play an important role in the signal transduction of RA, of vitamin D\(_3\), and of thyroid hormone.

In view of the above, it is of interest to investigate whether sphingosine metabolites function as a signaling molecule in the expression of RARs and RXRs in osteoblasts and, if so, to explore the signaling mechanism involved in their expression. We demonstrate herein that ceramide selectively stimulated the expression of the \(\alpha\) isoform of RAR and RXR and that the transcriptional activity of these receptors in osteoblastic MC3T3-E1 cells occurred via an SPP-mediated AP-1 pathway.

**MATERIALS AND METHODS**

Reagents—N-Acetylsphingosine (C\(_2\)-ceramide), N-hexanoylsphingosine (C\(_6\)-ceramide), SPP, \(\text{N}-\text{threo}\)-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPP), and \(\text{N}-\text{threo}\)-dihydrosphingosine (DHS) were purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Sphingomyelinase, fatty acid-free bovine serum albumin (BSA), and pyrrolidine-dithio-carbamate (PDTC) were obtained from Sigma (St. Louis, MO). Curcumin was purchased from Nacalai tesque (Kyoto, Japan). \(\alpha\)-MEM was obtained from Flow Laboratory (McLean, VA), and fetal calf serum was obtained from Hyclone (Logan, UT). 32\(^{\text{P}}\)dCTP, 32\(^{\text{P}}\)ATP, and the megaprime DNA labeling system element; CAT, chloramphenicol acetyltransferase; RA, retinoic acid receptor; RXR, retinoic X receptor; AP-1, activation protein-1; TGF-\(\beta\), transforming growth factor-\(\beta\); vitamin D\(_3\), 1a,25-dehydroxvitamin D\(_3\); DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.
were from Amersham Pharmacia Biotech Japan (Tokyo, Japan). The oligonucleotide 5'-end-labeling scheme was purchased from PerkinElmer (Boston, MA).

Osteoblastic Cell Line and Its Culture—Cells of the clonal osteoblastic MC3T3-E1 cell line were cultured to the subconfluence state in α-MEM supplemented with 10% fetal calf serum at 37 °C in 5% CO2, and then washed, and incubated overnight in serum-free α-MEM. They were then washed and treated with test samples at various concentrations. Sphingolipids were added to cells as a complex with 0.4% fatty acid-free BS in serum-free α-MEM.

cDNA Hybridization Probe—Each plasmid containing the mouse RAR or RXR-β or γ DNA sequences was provided by Dr. P. Chambon. A plasmid containing β-actin DNA was obtained from Oncor (Gaithersburg, MD).

Northern Blot Analysis—Total cellular RNA was extracted by the guanidine isothiocyanate procedure (30). As described previously (8, 9), the RNA was subjected to 1% agarose electrophoresis and blotted onto a nylon membrane (MSI Magnagraph, Westboro, MA). The membranes were baked, prehybridized, and then hybridized to each cDNA probe labeled with [α-32P]dCTP by use of a megaprime DNA labeling system. After hybridization, the membranes were washed, dried, and exposed to x-ray film (Eastman Kodak Co., Rochester, NY) at −70 °C. β-Actin was used as an internal standard for quantification of total mRNA in each lane of the gel.

Western Blot Analysis with RAR-α and RAR-β Antibodies—Rabbit polyclonal antibodies specific for RAR-α and RAR-β were provided by Dr. P. Chambon. MC3T3-E1 cells (1 × 105 cells) were lysed with lysis buffer (10 mM Tris, pH 7.9, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 20 mM EDTA, 0.25 mM PMSF, 10 μg/ml aprotinin). Protein concentration was measured by the method of Bradford (31). The samples (50 μg) were electrophoresed on 10% polyacrylamide slab gels, with Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS). The proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) by means of a semidiody transfer system (ATTO Co., Tokyo, Japan), employing transfer buffer composed of 0.025 Tris, 0.192 μl, and 20% methanol. Next, the membrane was washed with 0.1% Tween 20 in Tris buffer saline (TBS; 100 mM Tris, pH 7.9, 150 mM NaCl) and blocked for 12 h with 2% skim milk in TBS containing 0.05% Tween 20. The membrane was subsequently washed with TBS containing 0.1% Tween 20 and next incubated for 18 h with antibody against RAR-α or RAR-β diluted 1:1000 in TBS containing 0.1% Tween 20 and 5% BSA. After further washing with TBS containing 0.1% Tween 20, the membrane was incubated for 3 h with secondary horseradish peroxidase-conjugated anti-rabbit antibody (Bio-Rad Laboratories, Inc., Beverly, MA; Diluted: 1:1000 in TBS containing 0.1% Tween 20). After having been washed with 0.1% Tween 20 in TBS, the membrane was incubated with LumiGLO chemiluminescent reagent (New England BioLabs, Inc.) and peroxide. Finally, the membrane was exposed to x-ray film.

Preparation of Nuclear Protein Extracts—Subconfluent cell monolayers in 15-cm diameter dishes were treated or not with test samples as indicated by legends, and then their cell nuclei were isolated as described earlier (10, 32). Next, the nuclei were treated with buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT), and further treated by stirring for 60 min at 4 °C in buffer B (for DR-5: 10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 0.6 mM KCl, 0.5 mM DTT; for AP-1: 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 0.4 mM NaCl, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF). Nuclear extracts were obtained by centrifugation for 60 min at 25,000 × g and demineralized by passage through a Sephadex G-25 column equilibrated with buffer C (5 mM HEPES, pH 7.9, 0.02 mM KCl, 0.04 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). Protein concentration was measured by the method of Bradford (31).

Gel Mobility Shift Assay—As described previously (10, 32), binding reactions were performed for 20 min on ice with 5 μg of sample protein in 20 μl of binding buffer (for DR-5 binding: 2 mM HEPES, pH 7.9, 100 mM KCl, 12% (v/v) glycerol, 1 mM DDT, 0.5 mM PMSF, for AP-1 binding to TRE: 2 mM HEPES, pH 7.9, 8 mM NaCl, 0.2 mM EDTA, 12% (v/v) glycerol, 5 mM DDT, 0.5 mM PMSF, and 1 μg poly(dI-dC) containing 20,000 cpm of each 32P-labeled oligonucleotide in a final volume of 20 μl. Poly(dI-dC) and nuclear extracts were first incubated at 4 °C for 10 min before adding the labeled oligonucleotide. Double-stranded oligonucleotides for 5′-AGCTTCAGTGTCACCAAGGAGTGTCAGAGCAT-3′ of the DR-5 binding site with high affinity for RAR-RXR heterodimers and 30-mer double-stranded oligonucleotides containing -TGACTCA- (Oncogene Science, Inc.) of the AP-1 binding site, i.e. TRE, were end-labeled with (γ-32P)ATP by the oligonucleotide 5′-end-labeling method.

In some experiments, after a further incubation for 5 min on ice, antibodies specific for RAR and RXR were added, and the reaction mixture was maintained on ice for 15 min before being loaded onto polyacrylamide gels. DNA-protein complexes were electrophoresed on native 5% polyacrylamide gels in 0.5× TBE buffer (22 mM Tris, 22 mM boric acid, and 0.5 mM EDTA, pH 8.0). Gels were vacuumed, dried, and exposed to Kodak x-ray film at −70 °C.

Plasmid Construction for Transient Expression Assay—The plasmid pDR-5-β-globulin-CAT was constructed by inserting a synthetic oligonucleotide containing the DR-5 sequence, which encodes two copies of the CAT gene (pDR-5-β-globulin-CAT and pβ-globulin-CAT plasmids were kindly provided by Dr. S. Kato). The plasmid pTRE-TK-CAT was constructed by inserting a synthetic oligonucleotide containing the -TGACTCA- motif with HindIII-XbaI sites into the corresponding sites of pTK-CAT, which contains the herpes simplex virus thymidine kinase promoter enhancer region located upstream of the CAT gene (pTRE-TK-CAT and pTRE-TK-CAT plasmids were kindly provided by Dr. S. Kato). pG3L-Enhancer vector was obtained from Promega (Madison, WI).

Transient Expression Assay—This assay was performed according to the method of Felgner et al. (32–34). As described previously (10), the cells (1 × 105) were inoculated in 5-cm diameter dishes and incubated for 12 h in 10% fetal calf serum containing α-MEM. Then, the cells were washed three times with α-MEM (Life Technologies, MD), transfected with a total of 7 μg of DNA by use of 10 μg of LipofectAMINE (Life Technologies, Inc.), and incubated for 6 h in serum-free Opti-MEM. The cells were transfected with 2 μg of reporter plasmid or control plasmid (pDR-5-β-globulin-CAT or pβ-globulin-CAT plasmids for measuring RAR-RXR transcriptional activity; pTRE-TK-CAT or pTRE-TK-CAT plasmids for measuring AP-1 transcriptional activity). The assay was performed in the presence of 1 μg of pG3L-3-Enhancer vector, a luciferase expression plasmid, used as an internal control to normalize for variations in transfection efficiency. Bluescribe M13-Stratagene, La Jolla, CA) was used as a carrier to adjust the total amount of DNA. The cells were washed three times after the transfection. The cellular extracts were prepared by use of reporter lysis buffer (Promega) and subjected to CAT assay after normalizing luciferase activity. CAT activity was determined by autoradiography of thin-layer chromatography plates following completion of the CAT reaction by using an equivalent concentration of v-threo-dichloroacetate-1,4-[14C]chloramphenicol (Amersham Pharmacia Biotech Japan) as described previously (35, 36).

Preparation of c-fos, c-jun Antisense or Sense Oligonucleotides—c-fos antisense (5′-TGC GTT GAA GCC CGA GAA-3′) and c-jun antisense (5′-CGT TTC CAT TGT TGC AGT-3′) phosphorothionate oligonucleotides were synthesized and purified as described previously (8, 10). These nucleotide sequences were complementary to the first 18 bases following the AUG sequence of mouse c-fos and c-jun mRNAs. Also, the corresponding sense oligonucleotides were prepared and used as a control.

RESULTS

SMase Selectively Stimulates Expression of RAR-α and RAR-β Genes in MC3T3-E1 Cells—We were interested in determining whether SMase regulates gene expression of RAR and RXR subclasses (α, β, and γ) in osteoblastic cells. Thus, first, we examined by Northern blot assay the expression of these genes in cells treated with SMase. Fig. 1A illustrates the kinetics of the expression of these genes. SMase strongly stimulated the expression of RAR-α and RAR-β genes as early as 1 h after the initiation of the treatment but did not affect the levels of the other subclasses. And, as shown in Fig. 1B, SMase strongly expressed expression of RAR-α and RXR-β genes in the cells was dose-dependent. These results thus showed that SMase selectively stimulated the expression of RAR-α and RAR-β genes in MC3T3-E1 cells.

Stimulatory Effect of Cell-permeable Ceramide on Expression of RAR-α and RAR-β Genes in MC3T3-E1 Cells—Ceramide, generated by the hydrolysis of sphingomyelin with SMase, acts as a second messenger in several cells in response to various stimuli (1–8). Thus, using cell-permeable C25 and Ceramide, we investigated whether these ceramides also stimulated the
expression of RAR-α and RXR-α genes in the cells. As shown in Fig. 2 (A and B), C_2- and C_6-ceramides also stimulated the expression of the α gene of both nuclear receptors in a dose-dependent manner. These ceramides did not have any effect on the gene expression of the β and γ subclasses (data not shown). Taken together with the results described in Fig. 1, these results suggest that SMase stimulates the expression of RAR-α and RXR-α genes in the cells through ceramide action.

**SPP Mediates Ceramide-stimulated Expression of RAR-α and RXR-α Genes in MC3T3-E1 Cells**—With respect to the SMase pathway, several studies (1, 2, 5) have shown that ceramide displays several of its biological activities via a SPP-dependent pathway. Therefore, we next addressed whether ceramide-stimulated expression of RAR-α and RXR-α genes in the cell was SPP-dependent. We used DHS, which is a potent inhibitor of sphingosine kinase, to examine the effect of kinase inhibition on ceramide-stimulated expression of these genes in the cells. The C_2-ceramide stimulation was eliminated by DHS in a dose-dependent manner (Fig. 3, A and B). Because these results suggested the involvement of SPP in ceramide-stimulated expression of RAR-α and RXR-α genes in the cells, in addition, we examined whether SPP itself induces actually the expression of these genes in the cells. Fig. 3C shows that SPP also was a potent stimulator of the expression of these genes in the cells. On the other hand, SPP did not have any effect on the gene expression of other RAR and RXR subclasses (data not shown).

**SPP Stimulates the Expression of RAR-α and RXR-α at the Protein Level in MC3T3-E1 Cells**—To confirm this SPP-stimulated expression of RAR-α and RXR-α genes in the cells, we analyzed by Western blot assay whether SPP also stimulated their expression at the protein level. As shown in Fig. 4 (A and B), our Western blot assay showed that SPP clearly increased the expression of these receptor proteins. Although the data are not shown, control antibody (normal rabbit serum) did not recognize any bands in the blot assay.

**SPP Stimulates the Binding of Nuclear Protein to DR-5, the Core Response Element for RARs-RXRs, in MC3T3-E1 Cells**—Several studies (24–29) have demonstrated that members of the nonsteroid nuclear receptor family can recognize the consensus half-site sequence of AGGTCA in the form of a direct repeat. RAR-RXR heterodimers bind efficiently to the DR-5 sequence that contains two copies of AGGTCA as a direct repeat separated by five nucleotides and promote transcriptional activation in a ligand-dependent manner. SPP stimulation of RAR-α and RXR-α expressions in the cells suggested to us that SPP is able to stimulate binding of nuclear protein to DR-5. To explore this possibility, using the gel mobility shift assay, we examined the binding activity of nuclear proteins toward DR-5 in SPP-treated cells. As expected, SPP stimulated the binding activity of the nuclear proteins in a dose-dependent fashion. This stimulatory binding was completely prevented by an unlabeled DR-5 used as a competitor (Fig. 5A). To confirm that SPP stimulation of the binding activity was due to RAR-α and RXR-α, we investigated the specificity by the gel mobility shift assay with specific antibodies to each receptor. As shown in Fig. 5B, the DNA-protein complexes were shifted to a position indicating slower migration when the nuclear extracts were treated with specific antibody against each receptor. However, such a shift in the DNA-protein complex was not detected when control rabbit serum was used. Thus, these observations strongly suggest that SPP stimulates the binding of RAR-α and RXR-α contained in the nuclear protein preparation to DR-5.

**SPP Stimulates the Transcriptional Activity of RAR-RXR Heterodimers in MC3T3-E1 Cells**—Because SPP stimulation of RAR-α and RXR-α binding to DR-5 suggested to us that the sphingosine metabolite also may be able to stimulate the transcriptional activity of RAR-RXR heterodimers in a ligand (RA)-dependent manner, we explored this point by using the transient CAT assay on cells transfected with plasmids containing the DR-5-CAT reporter gene (pDR-5-β-globulin-CAT). As expected, in the presence of RA at the doses tested, SPP significantly stimulated the CAT activity of RAR-RXR heterodimers in the cells (Fig. 6, A and B). However, the significant stimulation of CAT activity was not observed in the cells transfected...
ments to validate this suspicion. As shown in Fig. 7 (A), SPP-stimulated expression of RAR-c-/RXRA and RXR-α was mediated via AP-1. Therefore, using antisense c-/RXRA and/or NF-κB, although not by PDTC, a NF-κB inhibitor having high specificity (data not shown). In view of these observations, we suspected that the SPP-stimulated expression of these receptors and their transcriptional activity might have been mediated via AP-1. Therefore, using antisense c-fos and c-jun oligonucleotides, we carried out the following experiments to validate this suspicion. As shown in Fig. 7 (A and B), SPP-stimulated expression of RAR-c and RXR-α genes in the cells was clearly reduced by treatment with antisense c-fos and c-jun oligonucleotides but not by that with the sense ones. Although we do not show the data, we also observed the inhibition of C2-ceramide-stimulated expression of these genes by antisense oligonucleotide of these oncogenes. In addition, these antisense oligonucleotides also inhibited SPP stimulation of expression of RAR-c and RXR-α genes in MC3T3-E1 cells. A, cells were treated with specific antibody against RAR-c or RXR-α. The gel mobility shift assay was performed with 32P-labeled oligonucleotide containing the DR-5 sequence in the presence of the nuclear proteins. The lower arrow indicates the position of DNA-protein complexes. B, the nuclear extracts prepared as described in panel A were treated or not with specific antibody against RAR-c or RXR-α. The gel mobility shift assay was performed with 32P-labeled oligonucleotide containing the DR-5 sequence in the presence of the nuclear proteins. The lower arrow indicates the position of DNA-protein complexes, and the upper arrow, the shifted complex formed in the presence of antibody to RAR-c or RXR-α.

Involvement of AP-1 in SPP Stimulation of RAR-c and RXR-α Gene Expressions and of RA-dependent Transcriptional Activity of RAR-RXR Heterodimers in MC3T3-E1 Cells—Several studies (1–8, 12–17) have shown that sphingomyelin metabolites induce the appearance of AP-1 and NF-κB transcriptional factors in several kinds of cells, including fibroblasts and osteoblasts. Our previous studies (32) demonstrated that TGF-β1 stimulated the transcriptional activity of RAR-RXR heterodimers in MC3T3-E1 cells via AP-1. In this regard, our preliminary experiments showed that SPP-stimulated expression of RAR-c and RXR-α genes in MC3T3-E1 cells was dramatically inhibited by curcumin, a potent inhibitor of AP-1 and/or NF-κB, although not by PDTC, a NF-κB inhibitor having high specificity (data not shown). In view of these observations, we suspected that the SPP-stimulated expression of these receptors and their transcriptional activity might have been mediated via AP-1. Therefore, using antisense c-fos and c-jun oligonucleotides, we carried out the following experiments to validate this suspicion. As shown in Fig. 7 (A and B), SPP-stimulated expression of RAR-c and RXR-α genes in the cells was clearly reduced by treatment with antisense c-fos and c-jun oligonucleotides but not by that with the sense ones. Although we do not show the data, we also observed the inhibition of C2-ceramide-stimulated expression of these genes by antisense oligonucleotide of these oncogenes. In addition, these antisense oligonucleotides also inhibited SPP stimulation of RA-dependent transcriptional activity of RAR-RXR heterodimer in CAT assay as described above (Fig. 7, C and D). Although the data are not shown, the CAT activity in the cells transfected with the β-globulin-CAT reporter plasmid actually was not significantly affected when the transfected cells were treated with antisense oligonucleotides of c-fos and c-jun. In addition, RA-dependent transcriptional activity of RAR-RXR heterodimer without SPP also was not affected by treatment of the antisense oligonucleotides. These results strongly suggest that SPP stimulation of expression of RAR-c and RXR-α genes and of RA-dependent transcriptional activity of RAR-RXR heterodimers in MC3T3-E1 cells occurs in an AP-1-dependent manner.
Sphingosine 1-Phosphate Stimulates RAR and RXR Expression

**DISCUSSION**

Several studies (37–39) demonstrated bone abnormalities in mice with targeted disruption of RAR-α and RAR-β genes, strongly suggesting that the nuclear receptors encoded by these two genes play an important role in morphogenesis and development of bone in vivo. Therefore, it was of interest to us to investigate the possible mechanism(s) of calcitropic hormone and cytokine action for the expression of these receptor genes in osteoblastic cells. Because it has been shown that sphingosine metabolites such as ceramide and SPP act as a second messenger of cytokines in several kinds of cells, including osteoblastic cells (1–6, 12–20), we focused here on the signaling mechanism of such metabolites in the expression of RAR and RXR genes in MC3T3-E1 cells. Interestingly, our present study demonstrated that ceramide selectively stimulated the expression of the α isoform of both RAR and RXR in osteoblastic cells and that the transcriptional activity of these receptors occurred via an SPP-mediated AP-1 pathway.

SMase selectively stimulated the gene expression of α isoform of RAR and RXR in MC3T3-E1 cells. The SMase stimulation was observed to be significant after at least 1 h of pretreatment and was dose-dependent. However, such stimulatory action was not observed for other subclasses (β and γ) of RAR and RXR. These observations suggested to us that ceramide, a sphingosine metabolite of SMase, is able to stimulate the expression of RAR-α and RAR-β genes in the cells. Therefore, we addressed this point by using cell membrane-permeable C2 and C6 ceramides. As expected, each ceramide stimulated the gene expression of the α isoform of RAR and RXR in the same manner as SMase. Because it is well known that TNF-α uses ceramide as a second messenger in its signaling system, we examined the effect of this cytokine and observed that the cytokine clearly stimulated the gene expression of the α isoform of RAR and RXR, but not RAR-β, γ, and RXR-β, γ genes, in the osteoblastic cells. This stimulatory effect of TNF-α clearly increased when the cells were treated simultaneously with PPPP, which causes accumulation of intracellular ceramide (data not shown). Taken together, these results suggest that the cytokine activated SMase, which then formed ceramide, which in turn selectively stimulated the gene expression of the α isoform of RAR and RXR in MC3T3-E1 cells.

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A. Takeshita, A. Watanabe, Y. Takada, and S. Hanazawa, unpublished data.
Sphingosine 1-Phosphate Stimulates RAR and RXR Expression

Several studies (3–5) have demonstrated that ceramide displays some of its biological activities through the SPP pathway. Therefore, using DHS, a potent inhibitor of sphingosine kinase, we explored whether the selective stimulation of ceramide for the expression of RAR-α and RXR-α genes in MC3T3-E1 cells was mediated by SPP. DHS clearly inhibited the ceramide-stimulated expression of both genes in the cell, suggesting that the ceramide stimulation may be SPP-dependent. Interestingly, we observed that SPP itself could selectively stimulate the expression of both genes in the cells. In addition, we observed that DHS also was capable of inhibiting TNF-α-stimulated expression of these nuclear receptor genes in the cells. Other investigators (18, 19) have shown that SPP functions as a signal-transducing molecule in TNF-α-treated MC3T3-E1 cells. Therefore, we believe that SPP plays an important role as a signaling molecule in ceramide-stimulated expression of RAR-α and RXR-α genes in these cells.

RAR-RXR heterodimers bind efficiently to the DR-5 sequence, which regulates, at the transcriptional level, the profound effects of RA seen in a wide variety of systems (21–29). Therefore, it was very important to examine whether SPP actually increased the binding activity of nuclear proteins toward DR-5 and stimulated their transcriptional activity in MC3T3-E1 cells in a ligand-dependent manner. Our gel mobility shift assay showed that SPP increased the binding of nuclear proteins to DR-5 in a dose-dependent manner. In this regard, using specific antibodies against RAR-α and RXR-α, we confirmed the identity of RAR-α and RXR-α as the binding proteins in question. In addition, RA-dependent transcriptional activity of RAR-RXR heterodimers was investigated by use of the DR-5-β-globulin-CAT assay. As expected, SPP clearly stimulated the ligand-dependent transcriptional activity in the cells. Therefore, these results demonstrate that SPP plays an important role as an intracellular signaling molecule in stimulating the transcriptional activity of these nuclear proteins in MC3T3-E1 cells. In this regard, we decided to investigate whether SPP actually stimulates gene expressions of proteins that might contribute to osteoblastic cell functions. Because it has been shown that a hormone response element (DR) sequence is located in the promoter region of the gene of murine osteopontin, a marker protein of osteoblastic cell differentiation (25, 40), we examined the effect of SPP on RA-induced expression of the osteopontin gene in MC3T3-E1 cells. Interestingly, SPP synergistically stimulated this gene expression. This observation adds credence to our conclusion that SPP acts as a signal transducer in RA-dependent differentiation of osteoblastic cells via stimulation of RAR-RXR heterodimers.

Many studies (1–5, 15–17) have shown that sphingosine metabolites induce transcriptional factor AP-1 and NF-κB in several kinds of cells. Therefore, we sought to determine which transcriptional factor plays an important role in SPP stimulation of RAR-α and RXR-α genes in MC3T3-E1 cells. Although the SPP stimulation was not prevented by PDTC, a potent inhibitor of NF-κB, it was inhibited by curcumin, an inhibitor of AP-1 and NF-κB. These observations suggested to us the involvement of AP-1 in the SPP stimulation. The validity of this possibility was strengthened by the following observations: 1) SPP stimulated the expression of c-fos and c-jun genes in MC3T3-E1 cells, 2) SPP markedly stimulated AP-1 transcriptional activity in the cells transfected with TRE-TK-CAT vectors, 3) c-fos and c-jun antisense oligonucleotides inhibited SPP-stimulated expression of RAR-α and RXR-α genes in the cells.

In conclusion, this study demonstrates the participation of the SPP-mediated AP-1 pathway in the selective expression by ceramide of the α isofrom of RAR and RXR genes in mouse osteoblastic MC3T3-E1 cells.

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