Prostaglandin A₂ Acts as a Transactivator for NOR1 (NR4A3) within the Nuclear Receptor Superfamily

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Within the nuclear receptor superfamily, Nur77, Nurr1, and NOR1 constitute the nuclear receptor subfamily 4 group A. Modulation of NOR1 function would be therapeutic potential for diseases related to dysfunction of NOR1, including extraskelatal myxoid chondrosarcoma and autoimmune diseases. By screening arachidonate metabolites for their capacity of transcriptional activation, we have identified prostaglandin (PG) A₂ as a transactivator for NOR1. PGA₂ acted as a potent activator of NOR1-dependent transcription through the GAL4-based reporter system. The putative ligand-binding domain (LBD) of the receptor directly bound PGA₂, and LBD-deleted receptor showed little transcriptional activation by PGA₂. Primary cultured spleen cells derived from transgenic mice overexpressing NOR1, showed higher sensitivity to PGA₂ compared to those from wild-type mice. These observations suggest that PGA₂ can serve as a transactivator of NOR1, and thus suggest a possibility of pharmacological modulation of the NOR1 pathways by PGA₂-related compounds.

Key words prostaglandin A; orphan nuclear receptor; NOR1; transactivator; ligand-binding domain; retinoic X receptor α

Nuclear receptors comprise a superfamily of ligand-activated transcription factors that regulate gene expression involved in reproduction, development, and adult physiology. Currently, ligands have been identified for only half of the known nuclear receptors; the remaining receptors, known as orphan nuclear receptors, form a promising area for research and development. The orphan receptors Nur77,²⁾ Nurr1,³⁾ and NOR1,⁴⁾ constitute the nuclear receptor subfamily 4 group A (NR4A) within the superfamily.⁵⁾ They have been classified as immediate-early genes whose expression is tightly regulated by extracellular signals. In addition, Nur77 and Nurr1, but not NOR1, can heterodimerize with retinoic X receptor α (RXR α) and mediate efficient trans-activation through a DR-5 element in response to the RXR-specific ligand, 9-*cis* retinoic acid.^{6–9)} Although NR4A shares features of the typical steroid receptor organization comprising an N-terminal trans-activation domain, a central DNA-binding domain and a C-terminal putative ligand-binding domain (LBD), there are no specific ligands that may regulate NR4A-dependent transcriptional activity. Moreover, no function has yet been ascribed to the LBDs of the NR4A family.

It is well known that most nuclear receptors have been implicated in human diseases. Dysfunction of NR4A receptors has likewise been shown to associate with human disorders. NOR1 is implicated in oncogenesis as part of the EWS fusion protein, resulting from chromosomal translocation found in human extraskelatal myxoid chondrosarcoma tumors.¹⁰⁾ In addition, NOR1, as well as Nur77, play a key role in apoptosis of T lymphocytes, eosinophils, and various cell types,^{11–15)} and thus may be implicated in disorders related to genetically- or environmentally-induced defects of activation-induced apoptosis, including autoimmune diseases and allergic diseases. As such, transactivators for NOR1 repre-

sent potential agents for therapeutic intervention to combat human disorders associated with dysfunctional NR4A signaling.

We have examined a large number of bioactive lipid-soluble mediator pathway metabolites, including those produced by the arachidonate and glycerolipid pathways, which are possibly induced in various cells following pathological stimuli such as antigen-induced apoptotic signaling, and found that only prostaglandin (PG) A₂ has the ability to activate NOR1-dependent transcription, and can bind directly to NOR1 LBD. Furthermore, primary cultured spleen cells derived from transgenic mice overexpressing NOR1, showed higher sensitivity to PGA₂ compared to those from wild-type mice. These findings suggest a specific molecular linkage between PGA₂ and NOR1, and thus suggest possible therapeutic targets for the discovery of important new pharmaceutical agents.

MATERIALS AND METHODS

Constructions for Mammalian Hybrid Systems Mammalian one-hybrid and two-hybrid systems (CheckMate™ Mammalian Two-Hybrid System, Promega, Madison, WI, U.S.A.) were used to high-throughput screen low molecular natural compounds against NOR1, whose transactivation activity was detected by firefly luciferase activity. The pACT-RXR α vector was made by insertion of a 1386 base full length RXR α cDNA into a multiple cloning region downstream of the herpes simplex virus VP16 activation domain of pACT vector. The pBIND-full-length or -LBD of NOR1 (Met¹-Phe⁶²⁶ or Arg³⁴⁸-Phe⁶²⁶) was also made by insertion of each cDNA into a multiple cloning region downstream of the GAL4 DNA-binding domain of pBIND vector.

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Cell Culture, Transient Transfection, and Luciferase Assays NIH3T3 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were kept in Dulbecco's modified Minimal Essential Medium supplemented with 10% fetal calf serum in 5% CO₂. Cells grown to 60–70% confluence in 96-well dishes were transiently transfected with pACT-RXR α or pACT control vector, pBIND-LBD, or pBIND-full length cDNA for NOR1, or pBIND control vector, and pG5 *luc* vector using FuGene6 transfection reagent (Roche Diagnostic Corporation, IN, U.S.A.). After a few hours, low molecular natural compounds were introduced (Most reagents were obtained from Cayman Chemical, Ann Arbor, MI, U.S.A.). Cells were harvested for 48 h and then assayed for luciferase activity using Dual Luciferase Reporter Assay System (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. To standardize the transfection efficiency, the relative luciferase unit was calculated by the ratio of firefly luciferase activity to *Renilla* luciferase activity transcribed from the pBIND vector. Each experiment represented at least three sets of independent quadruplicates.

Purification of Glutathione-S-transferase (GST)-LBD Fusion Proteins in *E. coli* The GST fusion protein expression plasmid, GST-NOR1 LBD was constructed in pGEX-6P (Amersham Biosciences, Piscataway, NJ, U.S.A.), by insertion of the NOR1 (Pro³⁹⁵-Phe⁶²⁶) cDNA. *E. coli*, transformed by the expression plasmid, was cultured overnight at 25 °C after introduction of 10 mM isopropyl-thio- β -D-galactopyranoside. The 10000 $\times$ g supernatant fraction collected after solubilization by triton X-100 and sonication of the collected cells, was applied to a GST-sepharose affinity column, and the bound GST-LBD fusion protein was eluted with 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM glutathione. More than 90% pure GST-fusion protein was used in the following binding experiments.

Biacore S51 Binding Assay The binding experiments of chemical compounds were performed at 25 °C, using the Biacore S51 surface plasmon resonance (SPR) sensor (Biacore, Uppsala, Sweden). Purified GST-LBD of NOR1 was immobilized on the series S sensor chip CM5 with the amine coupling chemistry. PG-related compounds were diluted in the PBS buffer (pH 6.85) containing 5% dimethylsulfoxide to make 500, 250, 125, 62.5, and 31.25 μ M concentrations of analyte. A solution of each compound was applied to the flow cells of the sensor chips at the same time for 60 s with a flow rate of 90 μ l/min. The binding of analyte compound to the immobilized GST-LBD was automatically measured for 60 s, and dissociation was also monitored for 60 s. The binding signals were recorded as sensorgrams, and each sensorgram was corrected for that obtained from the reference spot.

Production of NOR1 Transgenic Mouse Line The NOR1 transgene was constructed by insertion of the full length human NOR1 cDNA into the pCAGGS vector (obtained from RIKEN DNA bank, Tsukuba, Ibaraki, Japan) which has a CMV enhancer and chicken β -actin promoter. The transgenic mouse lines were produced by injecting the purified DNA fragment into male pronuclei of fertilized eggs obtained from C57BL/6N females (Charles River Japan, Yokohama, Kanagawa, Japan). The integration of transgene into the mice genome was determined by Southern blot analysis, probed by human NOR1 cDNA by comparison with 1, 3, 10, 30, 100 copies of purified human NOR1 gene. A

transgenic founder with 10 copies integrated were obtained from 23 new-born micro-injected mice. Some F1 mice obtained by mating the 10 copies-integrated male transgenic founder with female C57BL/6N mice were used for the following experiments.

RT-PCR and Viability Assays of Mice Spleen Cells Spleens were isolated from both NOR1 transgenic and wild-type mice and placed in RPMI 1640 medium supplemented with 10% FCS. Suspensions of spleen cells were incubated with 10 or 30 μ M PGA₂ in 96-well plates for 48 h. The expression levels of NOR1, cyclin D1, p21^{Cip1}, and p27^{Kip1} were determined by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was also measured as a control. Total RNA was isolated from harvested cells using Isogen solution (Nippon Gene, Tokyo, Japan). Complementary DNA was generated with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) and amplified using various primer sets as follows: NOR1 (5'-CTGCAATGGACTT-GTCCTGC-3' and 5'-GAGCTTGTCGATGACAGAAG-3'); cyclin D1 (5'-TGAGAACAAGCAGACCATCC-3' and 5'-AGATGTCCACATCTCGCACG-3'); p21^{Cip1} (5'-CGTGGACAGTGAGCAGTTGC-3' and 5'-AGACCAATCTGCGCT-TGGAG-3'); p27^{Kip1} (5'-ATGGAAGAAGCGAGTCAGCG-3' and 5'-CTGATTGTCTGACGAGTCAG-3'); G3PDH (5'-TGGCCAAGGTCATCCATGAC-3' and 5'-TCAGATGCC-TGCTTCACCAC-3'). PCR was performed for 25–40 cycles using Taq polymerase (TaKaRa Bio Inc., Tokyo, Japan), and analyzed on 2% agarose gels after ethidium bromide staining. Spleen cell viabilities after 48 h culture with PGA₂ were measured by trypan-blue dye-exclusion tests.

RESULTS

Screening of Transactivators for NOR1 Nuclear Receptor by Mammalian Hybrid Systems To identify possible transactivators for one of the NR4A nuclear receptor family, NOR1, we screened bioactive metabolites derived from lipid-soluble mediator pathways, such as those of the arachidonate and glycerolipid metabolic pathways. These metabolites are in the mammalian lipid metabolic pathway databases in the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.ad.jp/kegg/>) and are possibly induced in activated or apoptotic leukocytes. To delineate these compounds, we established mammalian one and two hybrid systems using GAL4-LBD or -full-length NOR1 receptor-connected chimera plasmids, since the mechanisms for transcriptional activation through the NGFI-B responsive element (NBRE: AAAGGTCA), the putative NR4A-target sequence identified by genetic screening in yeast,¹⁶⁾ might be different from through the endogenous target sequences.¹⁷⁾

By screening dozens of arachidonate and glycerolipid metabolites for their capacity of transcriptional activation using this assay system, we found that at concentrations of 10 μ M, PGA₂ stimulated NOR1-dependent transcriptional activity (Fig. 1). The transactivation activity of PGA₂ for full-length NOR1 was induced both in the presence and absence of RXR α , indicating that RXR α was not essential for the PGA₂ transactivation activity to the full-length of NOR1. RXR α -independency in NOR1 transactivation is correlated well with the previous reports showing that NOR1 do not heterodimerize with RXR α .⁷⁾ Regardless of the presence or

absence of RXR α , PGA₂ did not behave as a transactivator for the N-terminal AF-1 domain-deleted construct of NOR1 (NOR1 LBD) in the assay.

One of the nonendogenous cyclopentenone PG derivatives, PGA₁, also proved to have similar levels of transactivator activity to PGA₂ in the presence of the GAL4-full-length NOR1 receptor chimera, regardless of the presence or absence of RXR α . The dose-dependence for PGA₁ and PGA₂ are shown in Fig. 2. At 10 μ M, both PGA₁ and PGA₂ did not enhance luciferase transcriptional activities in the GAL4-NOR1 LBD. On the other hand, both PGA derivatives enhanced the transcriptional activities in the GAL4-full-length NOR1 receptor chimera at >5 μ M. However, 10 μ M dose of both compounds is critical concentration, because concentrations >30 μ M were cytotoxic, expressed as decreases of luciferase transcriptional activities (data not shown). These transactivation activities by PGA derivatives were also ob-

served in human HeLa cells (data not shown) as well as mouse NIH3T3 cells.

In order to investigate the necessity of the C-terminal LBD of NOR1 for the transcriptional activation by PGA₂, we constructed chimeric plasmids with GAL4 binding protein and the full-length NOR1 receptor gene from which the LBD region was deleted. On transfection of the construct into cells, 10 μ M dose of PGA₂ showed little transcriptional activation to the LBD-deleted NOR1 (Fig. 3). This result indicates that the C-terminal LBD is essential for the PGA₂ transactivation activity to NOR1.

Direct Binding of PGA Derivatives to the LBD of NOR1 Nuclear Receptor We tested direct binding of these compounds to the LBD protein of NOR1 receptor in a cell-free system. We expressed GST-LBD protein of NOR1 in *E. coli* and measured the direct binding of three PGA derivatives, PGA₁, PGA₂, and a negative control compound, 13,14-dihydro-15-keto PGA₂, to the NOR1-LBD using the Biacore S51 SPR sensor. This sensor has a higher sensitivity and the improved fluidics that enables monitoring of the small signal

Fig. 1. Mammalian One Hybrid and Two Hybrid Systems for Screening of Transactivators of NOR1 Nuclear Receptor and the Effects of PGA₂ on the Transcriptional Activation

Fold activations of luciferase activity by PGA₂ (10 μ M) in mammalian hybrid systems using pBIND GAL4 DNA-binding domain-LBD and full-length (FL) of NOR1 chimera plasmids are shown. In the presence or absence of pACT-RXR α , control blank (vehicle only) and 10 μ M PGA₂ addition are represented by open and closed bars, respectively. The mean luciferase fold activation values vs. pBIND without PGA₂ $\pm$ S.E. (bars) are derived from a minimum of one experiment in quadruplicate. Similar results were obtained in more than two independent experiments.

Fig. 3. Effects of LBD Deletion in NOR1 Nuclear Receptor on Transcriptional Activation by PGA₂

The transcriptional activation by PGA₂ (10 μ M) in the construct of full-length (FL) NOR1 and its LBD deletion is shown in the presence of pACT-RXR α . The deletion constructs used are indicated in the left column. Arbitrary units of transactivation against pBIND negative control levels are indicated in the abscissa. Open bar: control blank (vehicle only), closed bar: 10 μ M PGA₂ addition. The mean luciferase fold activation values $\pm$ S.E. (bars) are derived from a minimum of one experiment in quadruplicate. Similar results were obtained in two independent experiments.

Fig. 2. Dose Response Relationships for Effects of PGA₁ and PGA₂ on Transcriptional Activation by NOR1 Nuclear Receptor

Transcriptional activation by PGA₁ and PGA₂ in mammalian hybrid systems using pBIND GAL4 DNA-binding domain-NOR1 LBD and -full length (FL) NOR1 chimera plasmids is shown in the presence of pACT-RXR α . Closed rhombus: pBIND control, open circle: pBIND-NOR1 LBD, closed circle: pBIND-NOR1 FL. The mean luciferase fold activation values $\pm$ S.E. (bars) are derived from a minimum of one experiment in quadruplicate. Similar results were obtained in more than two independent experiments.

Fig. 4. Direct Binding of PGA Derivatives to LBD of NOR1 Nuclear Receptor in Cell-Free Biacore Systems

The sensorgrams show the association and dissociation of PGA_1 , PGA_2 , and the negative control PGA derivative, 13,14-dihydro-15-keto- PGA_2 , to GST control and GST fusion protein of NOR1 LBD (indicated at the top of the column). The compounds tested are indicated on the left. Compounds are injected over the LBD surfaces for 60 s, and then dissociation of the bound molecules is monitored for 60 s. The black, purple, blue, and red lines indicate the sensorgrams obtained with 500 μM , 250 μM , 125 μM , and 62.5 μM concentrations of each compound, respectively. Similar results were obtained in two independent experiments.

changes derived from the binding of compounds to proteins. The response signals obtained with GST were always smaller than those obtained with GST-NOR1 LBD, showing that there were specific binding signals of these compounds to NOR1-LBD (Fig. 4). Both PGA_1 and PGA_2 showed specific binding to GST-NOR1 LBD in a dose-dependent manner. Little or no binding of the compounds was observed when the same concentrations were applied to GST immobilized in identical molar quantities, on the sensor chips. A PGA derivative, 13,14-dihydro-15-keto PGA_2 , which have no transactivation activity for the NOR1 dependent transcription (data not shown), did not bind specifically to the GST-NOR1 LBD.

Effects of PGA_2 in NOR1 Transgenic Mice Spleen Cells NOR1 has been shown to be involved in apoptotic cell death in T lymphocytes.^{11–14} PGA_2 was also shown to induce apoptotic cell death and affect the expression levels of cyclin D1 and p21^{Cip1} .^{18–20} To clarify whether the effects of PGA_2 are influenced by the expression levels of NOR1, we investigated the effects of PGA_2 on spleen cells derived from the NOR1 transgenic mice. We generated the NOR1 transgenic mice, into which 10 copies of the actin promoter-constructed NOR1 gene had been incorporated. These transgenic mice were small, with a body weight approximately half that of the wild-type mice, and their thymus and spleens showed dramatic atrophy (data not shown). The live birth ratio of such transgenic mice was less than 50%.

When we compared the effect of PGA_2 in the spleen cells of the NOR1 transgenic mice to that in the wild-type strains, NOR1 expression was markedly elevated in the NOR1 transgenic mouse spleen cells (Fig. 5A). When spleen cells were

Fig. 5. The Effects of PGA_2 on Isolated Spleen Cells of NOR1 Transgenic (TG) Mice

(A) RT-PCR of NOR1, some cell cycle-related genes (CyclinD1, p21^{Cip1} , and p27^{Kip1}), and G3PDH in the spleen cells of NOR1 TG and wild-type (WT) mice after treatment with PGA_2 . (B) Viability of the spleen cells of NOR1 TG and WT mice after treatment with PGA_2 . White bar: WT mice, gray bar: TG mice. The mean levels $\pm$ S.E. (bars) are derived from a minimum of one experiment in quadruplicate, and the statistical results of Student's *t*-test are shown (** $p < 0.01$). Similar results were obtained in two independent experiments.

cultured in the presence of 10 or 30 μM PGA_2 , expression of cyclin D1 was decreased in the NOR1 transgenic mouse spleen cells than in wild-type mice. On the contrary, expression of the cyclin D1 inhibitory gene, cyclin dependent kinase inhibitor p21^{Cip1} , was elevated by PGA_2 -dose-dependently. In wild-type mouse spleen cells, we observed little effect of PGA_2 on p21^{Cip1} .

Next, we examined the PGA_2 -induced cell death in the NOR1 transgenic mice. When wild-type mouse spleen cells were cultured in the presence of 30 μM PGA_2 , approximately 10% of the cells underwent cell death (Fig. 5B). Surprisingly, in the spleen cells derived from the NOR1 transgenic mice, PGA_2 showed strong effects on the cell viability. When the cells were cultured in the presence of 10 or 30 μM PGA_2 , approximately 30% or 50% of the cells underwent cell death, respectively. This effect of PGA_2 observed in the NOR1 transgenic mice was significantly higher than that in the wild-type mice.

DISCUSSION

Among a large number of natural lipid mediators in the mammalian lipid metabolic pathway database KEGG, we found that only PGA_2 enhanced transcriptional activity of NOR1, one of orphan receptors within the nuclear receptor superfamily. Although most of the naturally occurring PGs have been shown to bind cell surface receptors such as G

protein-coupled receptors, PGA_2 has been shown to accumulate in the nucleus and to affect the transcription of a number of genes.²¹⁾ These observations are consistent with our findings that PGA_2 activated NOR1-dependent transcription and bound to NOR1 localized in the nucleus.

Classically, ligand binding to LBD of nuclear receptors is thought to induce conformational changes in the receptors, resulting in association of the C-terminal AF-2 domain with coactivators. On these occasions, nuclear receptor-ligand complexes contact the basal transcriptional machinery to increase transcriptional activity. Given this concept of ligand-dependent transactivation, our observations that PGA_2 transactivated NOR1-dependent transcription, that PGA_2 bound to the LBD of NOR1, and that the NOR1 mutant devoid of LBD was no more transactivated by PGA_2 suggest that PGA_2 has the potential to be a "ligand" of NOR1. However, the effects of PGA_2 on NOR1-dependent transcription is relatively low compared to those of the other ligands on their cognate receptors, such as estradiol on ER, and retinoic acids on RAR (data not shown). The recent reports demonstrated that LBD of Nurr1, the other member of NR4A within the superfamily, lacked a binding site for low molecular ligands.^{22,23)} Although there are no information of NOR1 LBD structure, NOR1 might lack a typical binding site conserved among the steroid receptors. In addition, NOR1 has been shown to be active in transcription when expressed in cells without any adding ligands.^{8,13)} Thus, we concluded that PGA_2 acts as a transactivator of NOR1, rather than a specific ligand regulating the NOR1-dependent transcription. We also demonstrated that the N-terminal AF-1 domain-deleted construct of NOR1 (NOR1 LBD in Fig. 1) lost the transcriptional activation by PGA_2 , suggesting that the effects of PGA_2 may require both LBD and AF-1 domains of NOR1. Wansa *et al.* demonstrated that NR4A N-terminal AF-1 domain supports the efficient recruitment of the coactivator complex, which is necessary for transcriptional activity.^{24,25)} Furthermore, the AF-1 domain was shown to interact directly with the C-terminal LBD domain.²⁶⁾ Our results and these reports suggest an alternative explanation that PGA_2 transactivates NOR1 through AF-1 dependent coactivator recruitment and a crosstalk between the AF-1 and LBD domain.

NOR1 has been shown to be involved in leukocyte apoptosis, neural differentiation, and cell growth.^{4,9,11–15)} Similarly, PGA derivatives are reported to be involved in cell cycle regulation, apoptosis and growth inhibition, not *via* cytosolic proliferative signaling pathways.²⁷⁾ While the target molecules or receptors for PGA_2 have not yet been identified, previous reports have shown that PGA_2 induced p21^{Cip1} expression, suppressed cyclin D1 expression, and induced G1 arrest in proliferating cells.^{18–20)} Our observations that the effects of PGA_2 ; induction of p21^{Cip1} , suppression of cyclin D1, and inducibility of cell death, were enhanced in spleen cells derived from NOR1-overexpressing transgenic mice, suggest that the effects of PGA_2 are influenced, at least in part, by the expression levels of NOR1.

Nuclear receptors are among the most promising pharmaceutical target genes. Specific compounds that regulate NOR1-dependent transcriptional activity have the potential to be used as treatments for cancers and immunological disorders. Although the NR4A family are thought to be constitutive activators, our findings suggest that NR4A nuclear re-

ceptor pathways are subject to pharmacological modulation by low molecular weight compounds. Promising PGA_2 -related compounds active at NOR1 nuclear receptor will inform future therapeutic strategies for NR4A-related human disorders.

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