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Retinoid-Mediated Stimulation of Steroid Sulfatase Activity in Myeloid Leukemic Cell Lines Requires RARα and RXR and Involves the Phosphoinositide 3-Kinase and ERK-MAP Kinase Pathways

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Abstract All-*trans* retinoic acid and 9-*cis*-retinoic acid stimulate the activity of steroid sulfatase in HL60 acute myeloid leukemia cells in a concentration- and time-dependent manner. Neither of these 'natural retinoids' augmented steroid sulfatase activity in a HL60 sub-line that expresses a dominant-negative retinoic acid receptor α (RARα). Experiments with synthetic RAR and RXR agonists and antagonists suggest that RARα/RXR heterodimers play a role in the retinoid-stimulated increase in steroid sulfatase activity. The retinoid-driven increase in steroid sulfatase activity was attenuated by inhibition of phospholipase D (PLD), but not by inhibitors of phospholipase C. Experiments with inhibitors of protein kinase C (PKC) show that PKCα and PKCδ play an important role in modulating the retinoid-stimulation of steroid sulfatase activity in HL60 cells. Furthermore, we show that pharmacological inhibition of the RAF-1 and ERK MAP kinases blocked the retinoid-stimulated increase in steroid sulfatase activity in HL60 cells and, by contrast, inhibition of the p38-MAP kinase or JNK-MAP kinase had no effect. Pharmacological inhibitors of the phosphatidylinositol 3-kinase, Akt, and PDK-1 also abrogated the retinoid-stimulated increase in steroid sulfatase activity in HL60 cells. These results show that crosstalk between the retinoid-stimulated genomic and non-genomic pathways is necessary to increase steroid sulfatase activity in HL60 cells. J. Cell. Biochem. 97: 327–350, 2006. © 2005 Wiley-Liss, Inc.

Key words: retinoid receptors; genomic and non-genomic signaling; steroid sulfatase; myeloid cells

Retinoids are derivatives of vitamin A that cause growth arrest, differentiation, and/or apoptosis in a many types of cell, including leukemic cells and breast, colon, and prostate carcinoma cell lines [reviewed in Ross et al., 2000; Soprano et al., 2004]. Treatment of all of these cell types with retinoids increases the level of expression and activity of steroid metabolizing enzymes. The products of these enzymes play important roles in regulating cell growth and function.

Retinoids increase the expression and activities of aromatase in breast adenocarcinoma, osteoblasts, and placental cell lines [Mu et al.,

Abbreviations used: 4-HPR, 4-hydroxyphenyl retinamide; 9-*cis*-RA, 9-*cis*-retinoic acid; ATRA, all-*trans* retinoic acid; DAG, diacylglycerol; DiC10, didecanoylglycerol; E_1S, estrone sulphate; EC_{50}, concentration required to produce a half maximal response; ED_{50}, binding affinity; IC_{50}, concentration required to produce a half maximal inhibition; FBS, fetal bovine serum; Himo, 1-L-6-hydroxymethyl-*chiro*-inositol-2I-2-O-methyl-3-O-octadecyl-carbonate; MAPK, mitogen-activated protein kinase; *m*-3M3FBS, 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide; MEK, mitogen-activated protein kinase kinase; PA, phosphatidic acid; PAP, phosphatidate phosphohydrolase; PI3K, phosphoinositide 3-kinase; PC-PLC, phosphotidylcholine-specific phospholipase C; PI-PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; PKC, protein kinase C; RAR, retinoic acid receptor; RXR, retinoid-X receptor; SUMF1, sulfatase modifying factor 1.

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2000; Yanase et al., 2001; Zhu et al., 2002], and of oxidative 17β-hydroxysteroid dehydrogenase in myeloid cells, breast carcinoma cell lines, and osteoblasts [Reed et al., 1994; Piao et al., 1997; Mountford et al., 1999; Li et al., 2002; Zhu et al., 2002]. All-*trans* retinoic acid (ATRA) increased the expression of MRNA encoding steroid sulfatase in HL60 cells and this was associated with an increase in enzymatic activity [Hughes et al., 2001]. Retinoid-mediated increases in steroid sulfatase activity have also been observed in MCF-7 breast cancer cells [Ng et al., 2000], several colon cancer cell lines [Hughes et al., 2001], and some prostate cancer cell lines [Hughes and Brown, unpublished observations]. In HL60 cells, the increase in steroid sulfatase activity is a bone fide marker of retinoid-stimulated granulocytic differentiation. The mechanisms that underlie the effects of retinoids on the expression and/or activity of these steroid-metabolizing enzymes have not been fully characterized.

The biological actions of retinoids are mediated by two classes of receptor: the retinoic acid receptors (RARα, β, and γ) and the retinoid X receptors (RXRα, β, and γ). 9-*cis*-retinoic acid (9-*cis*-RA) is a natural agonist for all the three classes of RAR and RXR, whilst ATRA is a natural pan-RAR agonist. RARs form heterodimers with RXR that binds to retinoic acid response elements (RAREs) in the regulatory regions of many genes. Unligated RARα/RXR heterodimers bind to RAREs in association with transcription repressors, such as SMRT- and Nco-R [reviewed in Bastien and Rochette-Egly, 2004]. Ligation of RARα/RXR heterodimers leads to a conformational change that allows binding to DNA with a much higher affinity and the release of co-repressors. This, in turn, permits the recruitment of transcriptional co-activators, histone acetyltransferase, and ATP-dependent chromatin remodeling molecules of the SRC/p160, p300/CBP, and CARM-1 families. Extensive chromatin remodeling then allows access of RNA polymerase II and the general transcriptional machinery to the promoter region of target genes [reviewed in Bastien and Rochette-Egly, 2004].

A significant proportion of 'retinoid-sensitive' genes do not have a recognizable RARE in their promoter region. These genes are unlikely to be directly regulated by retinoids via RAREs [Balmer and Blomhoff, 2002]. Instead, their expression requires an intermediary regulator of transcription that is expressed and/or activated in response to retinoids. For example, retinoids increase signaling from the phosphoinositide 3-kinase (PI3K)/PDK-1/Akt/p70 S6 kinase pathway. The latter pathway is indispensable for efficient ATRA-stimulated differentiation of human myeloid leukemic [Bertagnolo et al., 1999; Lewandowski et al., 2002; Bortul et al., 2003; Ishida et al., 2004; Lal et al., 2005; Lopez-Pedrera et al., 2004], endometrial adenocarcinoma [Carter, 2003], and neuroblastoma cells [Lopez-Carballo et al., 2002; Miloso et al., 2004]. The mitogen-activated protein kinase (MAPK) and CREB signaling cascades are activated during ATRA-induced differentiation of human neuroblastoma and other neuronal cell lines [Chu et al., 2003; Canon et al., 2004]. Activation of the MAPK pathway has been shown to be essential for granulocytic differentiation of myeloid cell precursors [Hong et al., 2001; Miranda et al., 2002; Yen et al., 2004], and for the retinoid-mediated differentiation of embryonic stem cells into adipocytes [Bost et al., 2002].

Here we use a variety of RAR and RXR specific ligands to examine how the activity of steroid sulfatase is regulated by retinoids in HL60 cells. We show that both RARα and RXR are required for the retinoid-stimulated increase in steroid sulfatase activity. Furthermore, we show that the PI3K and MAPK signaling pathways play important roles in modulating the retinoid stimulated increase in steroid sulfatase activity.

EXPERIMENTAL PROCEDURES

Compounds

Retinoid analogs were synthesized by the Retinoid Research Division of Allergan, Inc. (Irvine, CA). The RARα specific compound AM580 [Szondy et al., 2001] and the RARγ agonist 4-hydroxyphenyl retinamide (4-HPR) [Fanjul et al., 1996] were purchased from Sigma-Aldrich (Poole, Dorset, UK). 1α,25-dihydroxyvitamin D_3 was a gift from Dr. Lise Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). Radiolabeled steroids were from Perkin-Elmer Life Science Products-UK. Ltd. (Cambridge, UK). All cell culture medium and supplements were purchased from Invitrogen Ltd. (Paisley, Scotland). Expression vectors for RAR (α, β, and γ) and RXR (α, β, or γ) as well as recombinant baculoviruses containing RARα, β, and γ cDNAs have been described elsewhere

[Heyman et al., 1992]. The ERE-tk-luc and estrogen-receptor-RAR fusion protein expression vectors have also been published elsewhere [Beard et al., 1994].

The following cell permeant inhibitors of intracellular signaling pathways have been used in this study. The RAF kinase inhibitor GW5074, the MEK kinase inhibitors PD098059, U1026 and its inactive analog U1024, the phospholipase D (PLD) inhibitors 1-butanol (and the inactive 2-butanol), 2,3-diphosphoglycerate, the diacylglycerol analog didecanoylglycerol (DiC10), the protein kinase C δ (PKCδ) activator bistratene A, the phosphatidate phosphohydrolase (PAP) inhibitors propranolol and chlorpromazine, the phosphoinositide-specific phospholipase C (PI-PLC) inhibitor U73122 as well as its inactive analog U73344, the PI-PLC activator 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (*m*-3M3FBS), the Src family kinase inhibitor PP2, the Syk tyrosine kinase inhibitor piceatannol, the JAK inhibitor AG490, the PI3K inhibitors wortmannin and LY294002, the mTOR inhibitor rapamycin, as well as the p38 MAPK inhibitor SB230580 were all purchased from the Sigma Chemical Co (Poole Dorset, UK). The PLD inhibitor C2-ceramide and its inactive analog dihydro-C2-ceramide, D-609, the phosphatidylcholine-specific phospholipase C (PC-PLC) inhibitor, the PKCβ inhibitor LY379196, the PI3K inhibitor deguelin, the Akt inhibitors 1-L-6-hydroxymethyl-*chiro*-inositol-2I-2-O-methyl-3-O-octadecyl-carbonate (HIMO) and SH-5, the JNK inhibitor SP600125, and the p38 MAP kinase inhibitor PD169316 were purchased from Calbiochem (Nottingham, England UK). The PKC inhibitors GF 109293X, Gö 6976, HBBDE, and rottlerrin, the NF-κB pathway inhibitors SN-50, parthenolide, and MG-132 were purchased from Affiniti Research products (Exeter, UK). We were guided in our choice of inhibitor combinations and incubation conditions by previous studies performed in HL60 and other myeloid cell lines, in which the pharmacological specificities of the inhibitors had been previously characterized [Bertagnolo et al., 1999, 2004; Cambien et al., 1999; El Marjou et al., 2000; Slosberg et al., 2000; Lopez-Pedrera et al., 2001, 2004; Wang and Studzinski, 2001a,b; Lewandowski et al., 2002; Neri et al., 2002; Martelli et al., 2003, Wang et al., 2003; Glasow et al., 2005; Lal et al., 2005; Yen et al., 2004; Zhao et al., 2004; Fong et al., 2005]. In these experiments, we have, where possible, followed the 'rules' proposed by Davies et al. [2000] for the evaluation of the effects of pharmacological inhibitors on cellular processes. To this end, we determined, in parallel experiments, that the effect of the inhibitors on the retinoid-stimulated increase in steroid sulfatase activity occurred at the same concentrations that blocked the activation of an authentic physiological substrate of the protein kinase and measurements were made using the same cell extract (data not shown). Second, we also ensured that the same effects were observed with at least two structurally unrelated inhibitors of the intracellular target under investigation. Finally, we only used inhibitors whose specificities have been tested towards a wide range of intracellular signaling molecules both in vitro and in vivo.

To ensure that any inhibitory effects produced by the pharmacological inhibitors were not simply produced by a general increase in cell toxicity, lactate dehydrogenase (LDH) release was measured at the start and end of the incubation period with the inhibitor. LDH was assayed using a colorimetric LDH assay kit (Sigma) essentially as described in the manufacturers' protocol. The inhibitors were deemed to have had a toxic effect, and hence any observations made with them discarded, if the percentage LDH release significantly increased during the time course of the experiment or at the end of the incubation period greater than 20% of the total cellular LDH had been released by the agent alone.

Cell Culture

The HL60 acute myeloid leukemia cell line was originally provided by Dr. R.C. Gallo (NIH, Bethesda, MD). The HL60-Pager D cell line was obtained from Prof. Farzin Farzaneh (Division of Cancer Studies, Kings College, London, UK). HL60-Pager D cells express a truncated RARα that exerts a dominant negative effect that blocks retinoid-driven RARα-mediated granulocytic differentiation. The dominant negative effect is achieved by quenching accessory transcription factors.

HL60 cells were grown in RPMI 1640 medium containing glutamine, 10% heat-inactivated foetal bovine serum (FBS) and supplemented with antibiotics in 95% air/5% CO_2 at 37°C. Cells were seeded at 2.5×10^5 cells per ml as 10 ml cultures in 25 cm^2 flasks and treated with

0.1–1,000 nM ATRA or other synthetic retinoid analogs for 72 h, unless otherwise stated. In experiments in which the effects of retinoid receptor antagonists were assessed, the cells were treated for 2 h with the antagonist before addition of the retinoid agonist.

A sub-line of the promyelocyte cell line U937 that contains a dominant negative truncated PI3K p85 subunit (Δp85) under the control of a CMV driven promoter (U937:Δp85-p18 cells) was originally developed by Dr. Frank Cooke and was a kind gift from Dr. Phill Hawkins (Inositide Signalling Laboratory, Babraham Institute, Cambridge, UK). Stock cultures of U937:Δp85-p18 cells were cultured in antibiotic- and FBS-supplemented RPMI 1640 medium containing 0.6 mg/ml G418 and 0.1 mg/ml hygromycin B. To induce expression of Δp85, the stock cell cultures were treated with 20 mM IPTG, 1 nM PMA, and 100 μM $ZnCl_2$ for 16 h prior to experimentation.

Measurement of Steroid Sulfatase Activity in Cell Fractions

Cells were collected by centrifugation at 400*g* for 5 min at 4°C. The cells were re-suspended in 1 ml of hypotonic homogenization buffer (50 mM HEPES, 0.1 mM EDTA, pH 7.4 supplemented with Sigma Protease inhibitor cocktail 1 and Sigma Phosphatase inhibitor cocktail). After 30 min on ice, the cells were disrupted by repeated sonication and centrifuged at 100,000*g* at 4°C for 60 min. The resultant pellet was re-suspended in 0.5 ml of the above buffer.

Steroid sulfatase activity was assayed at 37°C in 0.02 M Tris-HCl and 0.1 mM EDTA (pH 7.5) containing 20 μM E_1S (spiked with ~100,000 dpm of [^{3}H]-E_1S) in a final volume of 100 μl. Reactions were started with 30–80 μg protein and quenched after 60 min with 900 μl of ice-cold 0.1 M sodium bicarbonate containing 5,000 dpm of [^{14}C]-E_1 (to determine the recovery of the extraction process). Nine hundred fifty microliters of the resulting mixture were extracted with 3 ml of toluene, and the organic and aqueous layers were separated. Eight milliliters of Emulsifier Safe scintillant (Canberra Packard, Pangborne, Berks, UK) were added to each fraction, and radioactivity was determined in a Packard 2000CA TriCarb liquid scintillation counter. Blank incubations in the absence of protein were used to correct for carry-over of substrate into the organic layer. The protein concentration of each sample was measured in triplicate using Bio-Rad protein assay kit using bovine γ-globulin as the standard (Bio-Rad, Hemel Hempstead, Herts, UK).

In Vitro Retinoid Receptor Binding Assays and Retinoid Receptor Transactivation Assays

Extracts were prepared from Sf21 insect cells, 48 h after infection with baculoviruses containing constructs encoding human RARα, β, and γ as described by Allegretto et al. [1993] and Nagpal et al. [1995]. Binding affinities of the retinoid analogs were assessed from their ability to compete for [^{3}H]-ATRA binding essentially as described in Heyman et al. [1992]. Results are expressed as the concentration required to inhibit ^{3}H-ATRA binding by 50% (ED_{50}).

Briefly, CV-1 cells were transiently transfected with a plasmid containing ERE-tk-Luc promoter-reporter construct using Lipofectamine. ERE-tk-Luc transcript comprises a luciferase reporter under control of an estrogen receptor response element. The cells were also transiently transfected with expression vector encoding a fusion protein containing the ligand-binding domain of the RAR, either α, β, or γ, fused to an estrogen receptor DNA binding domain. Twenty four hours after transfection, the cells were treated cells with retinoid analogs under test for 16 h in culture medium containing 2.5% charcoal-treated FBS and lysed for determination of luciferase and β-galactosidase activities. Luciferase activity was measured using the Dual-Luciferase Reporter 100 Assay System (Promega), and β-galactosidase activity was determined by colorimetric assay. The reporter activity was normalized against β-galactosidase activity. The transactivation data are presented as the percentage ± SEM of the maximal response produced by 1 μM AGN191183.

Data Manipulation and Statistical Analysis

Multiple experiments were performed with quadruplicate replicates. Results are expressed as means ± SEM. The statistical significance between groups of data was analyzed by the Student's *t*-test or where appropriate by the Mann–Whitney rank sum or Kruskal–Wallis one-way analysis of variance tests using either the Minitab™ or the SigmaStat™ statistical software packages. Irrespective of the test used, a *P*-value <0.05 was considered to be a significant difference. Dose-response curves were

fitted to a standard sigmoidal dose-response model curve using either the Enzfit™ non-linear curve fitting software package or the 'pharmacology' module of the Sigmaplot™ (version 8.0) graphical software package.

RESULTS

Characterization of the Receptor Specificity of Retinoid Analogs Used in This Study

AGN191183 (TTNPB) is a pan RAR agonist that binds to all three RAR isoforms with similar low nanomolar affinities (Table I) and can drive transactivation via RARα, β and γ (Fig. 1a). Other workers have shown that AGN191183 drives transcription via the formation of RAR/RXR heterodimer formation and is incapable of driving RXR/RXR-mediated transactivation [Rusten et al., 1996]. AGN195183 and AM580 are RARα-selective agonists, as they only bind to RARα (Table I) and drive transcription exclusively via RARα (Fig. 1a). AGN190168 ('tazarotene') is a pro-drug that is rapidly metabolized in cells into tazarotenic acid (AGN190299), both of these retinoid analogs have moderate binding affinities for RARβ and RARγ, but only binding weakly to RARα (Table I) and, are best described as a RARβγ-selective agonist. Since, HL60 cells do not express RARγ at detectable levels [De The et al., 1989; Hashimoto et al., 1989, 1990; Nervi et al., 1989; Kizaki et al., 1993; Nagy et al., 1995], we have used AGN190168 and AGN190299 as RARβ selective agonists. AGN205327 preferentially binds to and activates RARγ (Table I and Fig. 1a), and was used as a RARγ agonist. AGN19404 does not bind to any of the RAR isoforms (Table I) but does bind to each of the RXR isoforms with equal affinity (Table I). Vuligonda et al. [2001] have shown that on its own AGN194204 drives transcription via RXR homodimers but fails to drive RAR/RXR- or RAR/RAR-mediated transactivation. Hence this compound is used as a pure RXR agonist.

AGN194310 blocked TTNPB-driven transactivation from RARα, β, and γ (Fig. 1b); hence, AGN194310 is a RARαβγ antagonist. AGN 196996 blocked RARα-mediated transactivation, had no effect on transcription mediated by RARβ and RARγ (Fig. 1b), and was used as a RARα-specific antagonist. Transactivation via RARβ and RARγ, but not via RARα, was blocked by AGN194431 (Fig. 1b); therefore this compound can be considered to be a RARβγ antagonist. AGN205728 is a RARγ-specific antagonist, since transactivation mediated by RARγ, but not RARα or RARβ, was inhibited by this compound (Fig. 1b).

All-*trans*-Retinoic Acid and 9-*cis*-Retinoic Acid Increase Steroid Sulfatase Activity in HL60 Cells but not in the Retinoid Resistant HL60-Pager D Cells

Figure 2a shows that HL60 cells require exposure to 100 nM ATRA for at least 16–24 h before any significant increase in membrane-associated steroid sulfatase activity is observed. After this lag period, steroid sulfatase activity rose steadily until a maximum value was reached ~48 h after the addition of ATRA. A similar pattern of increased steroid sulfatase activity was observed when HL60 cells were

TABLE I. Binding Affinities (ED_{50} in nM) of Selected Retinoids Against Baculovirus Expressed RAR or RXR Isoforms

Retinoids	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
RAR agonists						
AGN191183 (pan-RAR agonist)	15.7	7.2	6.7	9,113	4,093	2,556
AGN195183 (RARα agonist)	20.1	>5,000	>5,000	>10,000	>10,000	>10,000
AGN190168 (RARβγ agonist)	>1,000	14.2	135	>10,000	>10,000	>10,000
AGN190299 (RARβγ agonist)	616	41	57	>10,000	>10,000	>10,000
AGN205327 (RARγ agonist)	3,766	734	32	>10,000	>10,000	>10,000
RXR agonist						
AGN194204 (pan-RXR agonist)	>10,000	>10,000	>10,000	2	2	2
RAR antagonists						
AGN194310 (pan-RAR antagonist)	4.3	5	2	>10,000	>10,000	>10,000
AGN196996 (RARα antagonist)	3.9	4,036	>10,000	>10,000	>10,000	>10,000
AGN194431 (RARβγ antagonist)	300	6	20	>10,000	>10,000	>10,000
AGN205728 (RARγ antagonist)	2,400	4,248	3	>10,000	>10,000	>10,000
RXR antagonist						
AGN195393 (pan-RXR antagonist)	793	950	1,498	1	1	2

Nuclear extracts were prepared from baculovirus infected Sf21 insect cells engineered to express either human RARα, -β, or -γ or RXRα, -β, or -γ as described by Allegretto et al. [1993]; Nagpal et al. [1995]. The equilibrium binding affinities of each retinoid analog (ED_{50} in nM) were estimated by the [^{3}H]-ATRA displacement method as described in Heyman et al. [1992].

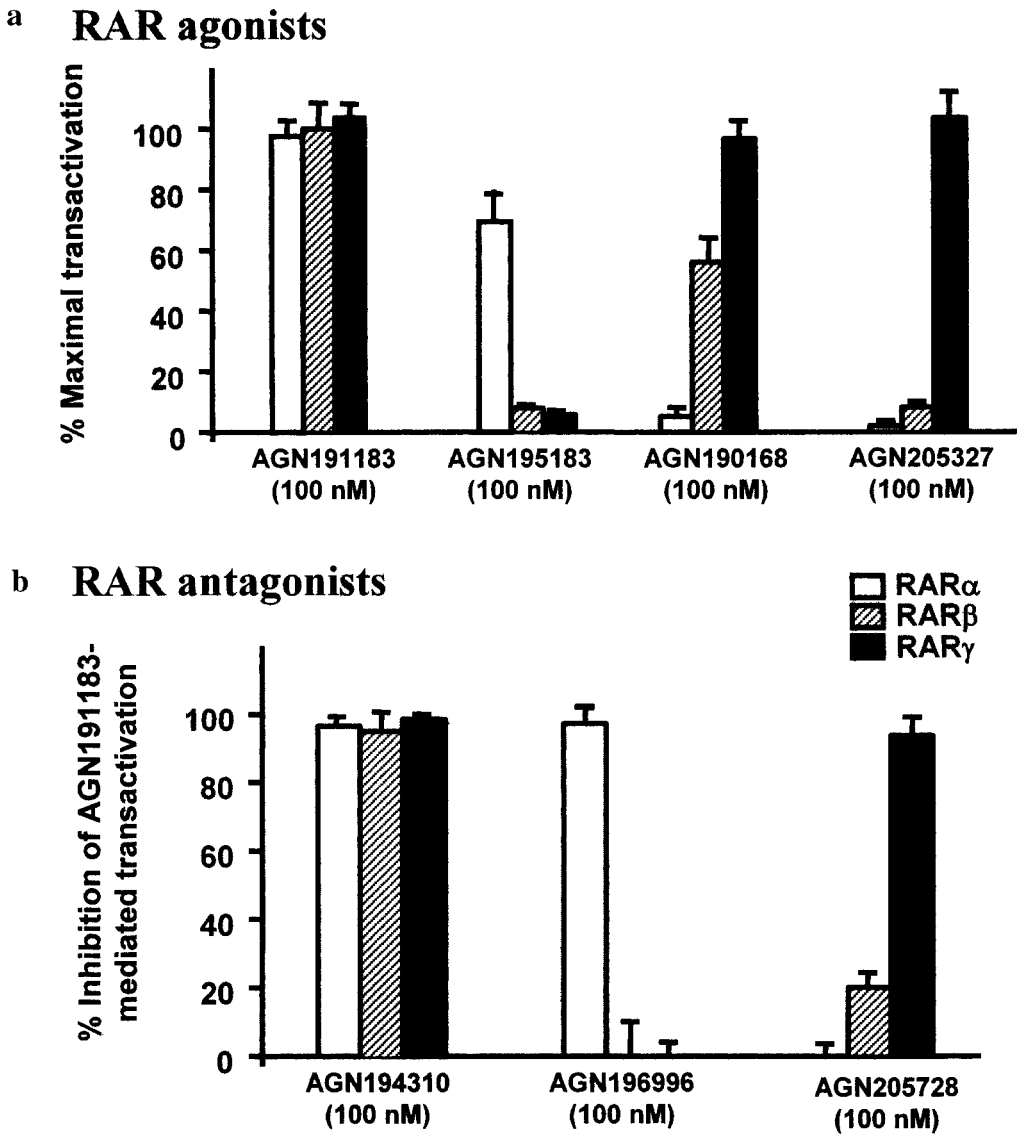


Fig. 1. Characterization of the receptor specificity of retinoid analogs used in this study. The capacities of retinoid analogs to bind and antagonize RARs were assessed essentially as described by Nagpal et al. [1995]. Briefly, CV-1 cells were transiently transfected with a plasmid containing ERE-tk-Luc promoter-reporter construct and an expression vector encoding a fusion protein containing the ligand binding domain of RARα, -β, or -γ, fused to an estrogen receptor DNA binding domain. **a**: It shows cells treated with 100 nM of the RAR agonist under test for 18 h. Data are expressed as a percentage of the maximal response produced with 100 nM ATRA. **b**: It shows cells that were treated with RAR antagonist (100 nM) and then stimulated with 100 nM AGN191183. Data are expressed as the percentage inhibition of the maximal transactivation produced by AGN191183.

treated with 100 nM 9-*cis*-RA (data not shown). In the second experiment shown in Figure 2b, HL60 cells were treated with various concentrations of ATRA (○) or 9-*cis*-RA (□) for 72 h prior to measuring steroid sulfatase activity. Steroid sulfatase activity was increased by both agents in a dose responsive fashion; at the maximal concentration of each agent used, steroid sulfatase activity had been increased ~fourfold. Both dose-response curves were monophasic and fitted a standard sigmoidal curve. The concentration of ATRA required to induce a half-maximal increase in steroid sulfatase activity in HL60 cells was 37.1 ± 9.3 nM ($n = 4$), and the Hill slope of the dose-response curve was 0.93 ± 0.14 ($n = 4$). 9-*cis*-RA induced a half-maximal increase in steroid sulfatase activity in HL60 cells at a concentration of 101.4 ± 13.5 nM ($n = 4$), and the Hill slope was 1.17 ± 0.18 ($n = 4$).

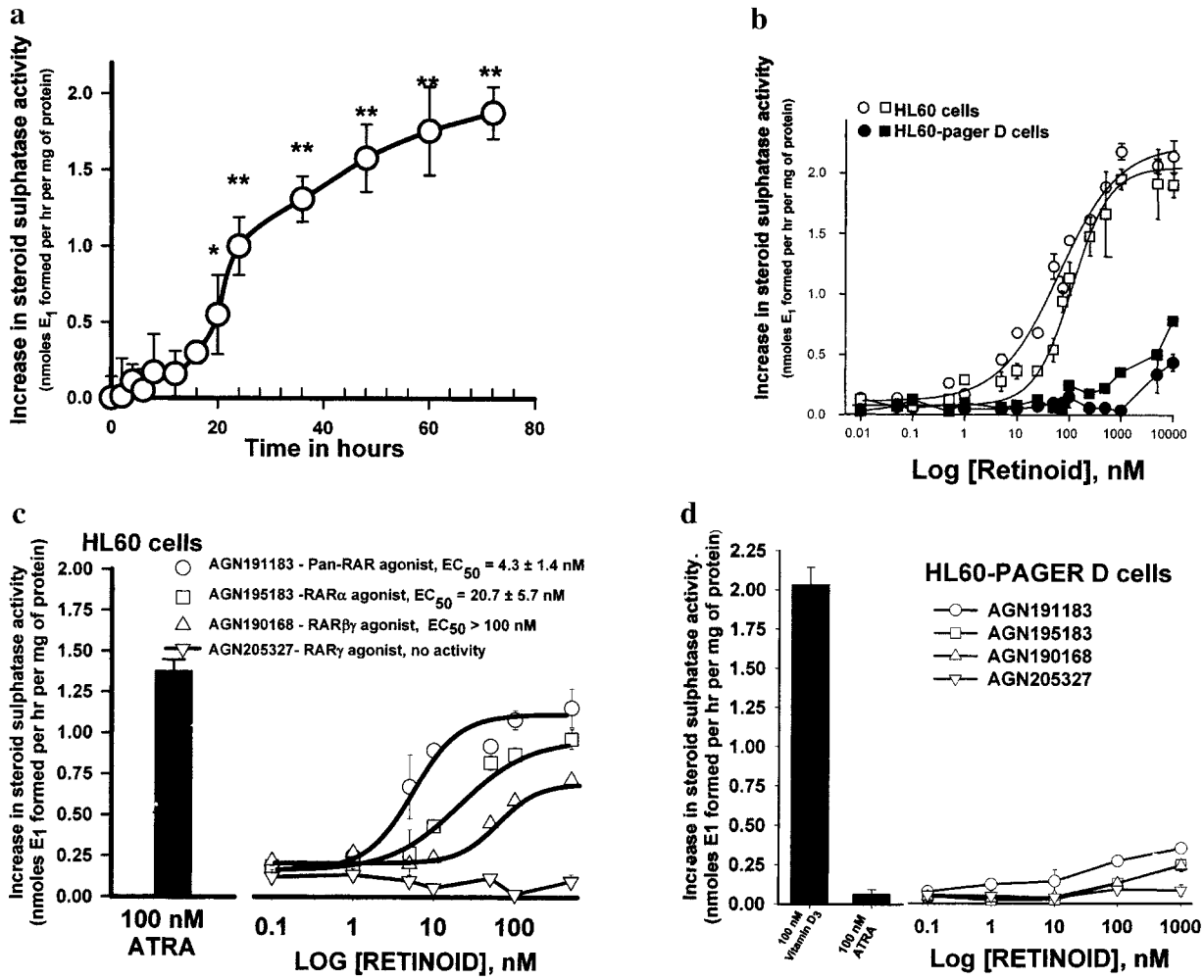


Fig. 2. All-*trans*-retinoic acid and 9-*cis*-retinoic acid increase steroid sulfatase activity in HL60 cells but not in the retinoid resistant HL60-Pager D cells. **a**: It shows the time course of the increase in steroid sulfatase activity in HL60 cells stimulated with 100 nM ATRA (n = 9). **b**: It shows the dose-response curves for the increase in steroid sulfatase activity produced by 72 h exposure of HL60 (open figures, n = 4) or HL60-Pager D cells (closed figures, n = 4) to increasing concentrations of ATRA (○, ●) or 9-*cis*-RA (□, ■). **c**: It shows dose-response curves for the increase in steroid sulfatase activity produced by 72 h exposure of HL60 to the pan-RAR agonist AGN191183 (○, $EC_{50} = 4.3 \pm 1.4$ nM, n = 4), the RARα-selective agonist AGN195183 (□, $EC_{50} = 20.7 \pm 5.7$ nM, n = 4), the RARβγ-selective agonists AGN190168 (△, $EC_{50} \gg 100$ nM, n = 4) AGN190299 (◇, $EC_{50} \gg 100$ nM. n = 3), or the RARγ-selective agonist AGN205327 (▽, n = 4). **d**: It shows an identical experiment to the one shown in (c), but this experiment was performed in HL60-Pager D cells. All data points are means ± SEM of quadruplicate determinations, and each experiment was performed a minimum of three or four times.

Figure 2b shows that both ATRA (●) and 9-*cis*-RA (■) failed to increase steroid sulfatase activity in HL60-Pager D cells. HL60-Pager D cells are resistant to ATRA-stimulated differentiation, but do differentiate when treated with dimethylsulphoxide, 1α,25-dihydroxyvitamin D_3, sodium butyrate, or phorbol ester [Farzaneh and Brown, unpublished observations]. Figure 2b shows that 1α,25-dihydroxyvitamin D_3-stimulated an increase in steroid sulfatase activity in HL60-Pager D cells. The inability of the natural retinoids to increase steroid sulfatase activity in HL60-Pager D cells suggests an absolute requirement RAR-derived signals.

The all-*trans*-Retinoic Acid-Driven Increase in Steroid Sulfatase Activity Is Mediated by a RARα-Dependent Signaling Pathway

To identify the RAR receptor isoform(s) that is (are) responsible for the retinoid-mediated regulation of steroid sulfatase activity, we examined the effects of various receptor selective retinoid analogs. The pan RAR agonist AGN191183 (TTNPB, ○) produced a dose-responsive increase in steroid sulfatase activity

in HL60 cells (Fig. 2c), and had no effect in HL60-Pager D cells (Fig. 2d). A maximal stimulatory effect was achieved with 100 nM AGN191183 when steroid sulfatase activity had been increased ~three- to fourfold. The AGN191183 dose-response curve was monophasic and fitted a standard sigmoidal curve. The concentration of AGN191183 required to induce a half-maximal increase in steroid sulfatase activity in HL60 cells was 4.3 ± 1.4 nM, and the Hill slope of the dose-response curve was not significantly different from unity being 1.23 ± 0.29 ($n = 4$). These observations confirm the importance of RARs in the retinoid-mediated increase in steroid sulfatase activity.

The RARα selective agonist AGN195183 (□) augmented steroid sulfatase activity in HL60 cells in a dose-responsive fashion (Fig. 2c), and had no effect in HL60-Pager D cells (Fig. 2d). The AGN195183 dose-response curve was monophasic and fitted a standard sigmoidal curve. A half-maximal increase in steroid sulfatase activity in HL60 cells was achieved with 20.7 ± 5.7 nM AGN195183 ($n = 4$), and the Hill slope of the dose-response curve was not significantly different from unity being 0.94 ± 0.17 ($n = 4$). The RARα specific compound AM580 [Szondy et al., 2001] has a potent differentiating effect on promyelocytic leukemia cells [Gianni et al., 1996]. Stimulation of HL60 with increasing doses of AM580 for 72 h produced a dose-responsive increase in steroid sulfatase activity. The concentration of AM580 required to half-maximally increase steroid sulfatase activity in HL60 cells was 11.4 ± 1.9 nM, and the slope of the dose-response curve was not significantly different from unity being 1.16 ± 0.14 (data not shown).

RARγ is expressed at very low levels in HL60 cells, so we anticipated that RARγ-specific agonists would fail to increase steroid sulfatase activity in HL60 cells. This was the case as concentrations as high as 500 nM AGN205327 (△, Fig. 2c), and 4-HPR (data not shown) did not increase steroid sulfatase activity in HL60 cells. The RARβγ agonist AGN190168 (tazarotene, ◇) and its metabolite AGN190299 (tazarotenic acid, ▽) increased steroid sulfatase activity in HL60 cells in a dose-responsive fashion, and had no effect in HL60-Pager D cells (Fig. 2d). However, the AGN190168 and AGN190299 dose-response curves in HL60 cells were incomplete at 500 nM. At this concentration used, the increase in steroid sulfatase activity was only 60–70% of that produced by 100 nM ATRA. The EC_{50}'s could only be estimated to be $\gg$100 nM. Hence, AGN190168 and AGN190299 only affect steroid sulfatase activity in HL60 cells at concentrations that are substantially higher than their reported binding affinities for either RARγ or RARβ.

The Retinoid-Stimulated Increase in Steroid Sulfatase Activity Is Blocked by Pan-RAR and RARα Antagonists, but not by RARβγ or RARγ Antagonists

Figure 3 shows the results of an experiment in which HL60 cells were pre-treated with increasing concentrations of either a pan RAR-antagonist (AGN194310, ○), a RARα-specific antagonist (AGN196996, □), a RARβγ antagonist (AGN194331, △), or a RARγ-specific antagonist (AGN205728, ▽) for 2 h prior to addition of 100 nM ATRA or the pan-RAR agonist AGN191183 or the RARα-specific agonist AGN195183 or the RARβγ-specific agonist AGN190168. The pan-antagonist AGN194310 blocked the increase in steroid sulfatase activity produced by each RAR agonists with half maximal inhibitory concentrations of ~10 nM (IC_{50} vs. 100 nM ATRA $= 4.7 \pm 0.9$ nM (Fig. 3a), vs. 100 nM AGN191183 $= 8.6 \pm 1.7$ nM (Fig. 3b), vs. 100 nM AGN195183 $= 3.9 \pm 0.7$ nM (Fig. 3c), and vs. AGN190168 $= 2.9 \pm 0.9$ nM (Fig. 3d)). Similarly, the RARα-specific AGN196996 antagonist inhibited the RAR agonist-mediated increase in steroid sulfatase activity with a half maximal inhibitory concentration of ~10 nM (IC_{50} vs. 100 nM ATRA $= 4.9 \pm 0.7$ nM (Fig. 3a), vs. 100 nM AGN191183 $= 10.3 \pm 0.7$ nM (Fig. 3b), vs. 100 nM AGN195183 $= 12.3 \pm 0.9$ nM (Fig. 3c), and vs. AGN190168 $= 5.6 \pm 1.4$ nM (Fig. 3d)). In contrast, the RARβγ-specific antagonist AGN 194331 and the RARγ-specific antagonist AGN205728 both failed to have any significant effect on the increase in steroid sulfatase activity produced by any of the RAR agonists (Fig. 3a–d).

RXR Plays an Essential Role in the RARα-Mediated Increase in Steroid Sulfatase Activity in HL60 Cells

HL60 cells express RXRα and RXRβ, but not RXRγ [De The et al., 1989; Nagy et al., 1995; Wang and Yen, 2004]. Activation of RXR alone does not appear to be sufficient stimulus for the retinoid-induced increase in steroid sulfa-

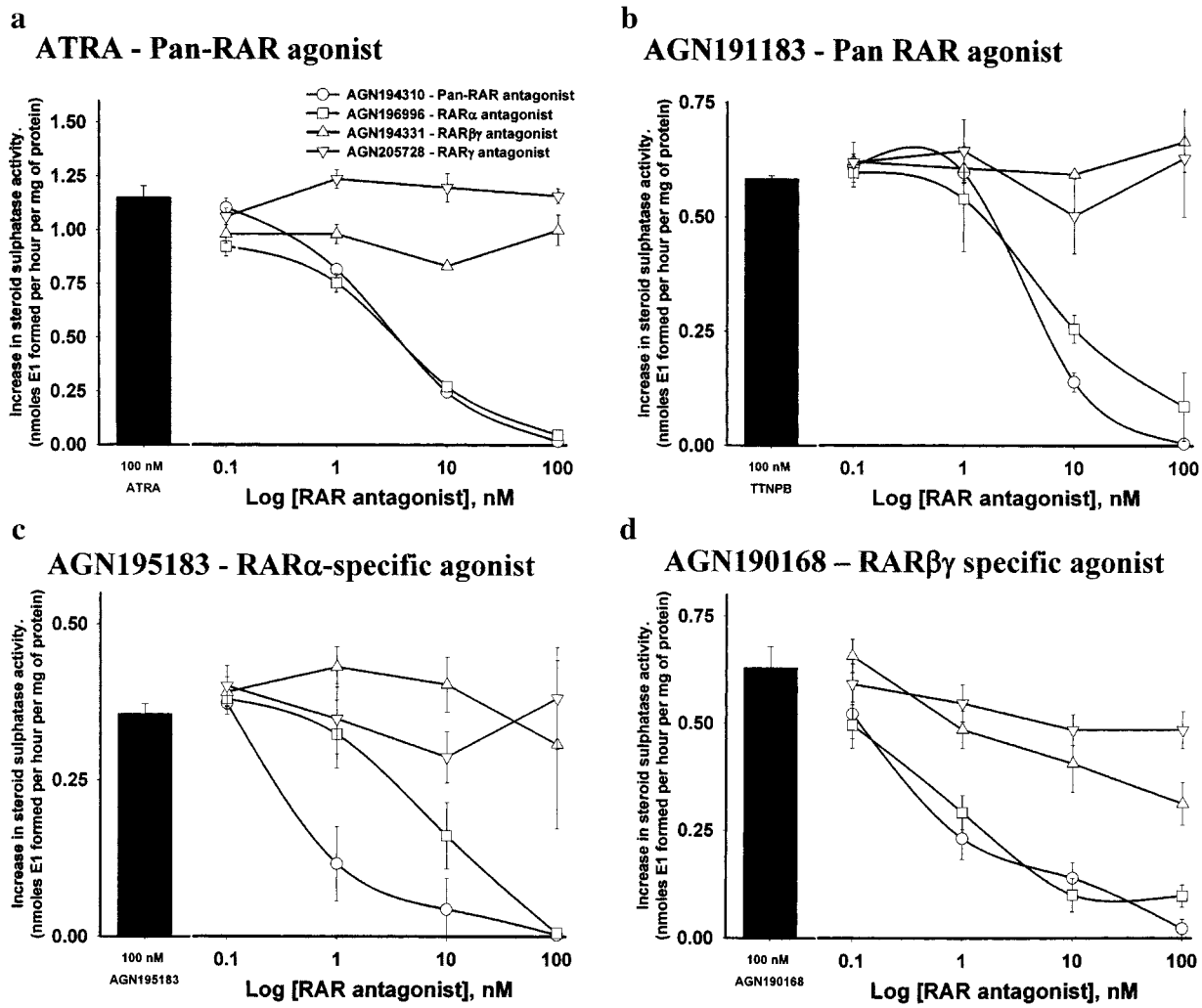


Fig. 3. The retinoid-stimulated increase in steroid sulfatase activity is blocked by pan-RAR and RARα antagonists but not by RARβγ or RARγ antagonists. **a**: It shows the effects of pre-treatment of HL60 cells for 2 h with increasing concentrations of the pan-RAR antagonist AGN194310 (○), the RARα-selective antagonist AGN196996 (□), the RARβγ antagonist AGN194331 (△), or the RARγ antagonist AGN205728 (▽) on the increase in steroid sulfatase activity produced by stimulation with 100 nM all-*trans* retinoic acid. **b–d**: They show similar experiments using the pan-RAR agonist AGN191183, the RARα-selective agonist AGN195183, and the RARβγ-selective agonist AGN190168, respectively. All data points are means ± SEM of quadruplicate determinations, and each experiment was performed a minimum of four times.

tase activity. Treatment of HL60 cells with the synthetic pan-RXR agonists, AGN194204 and AGN195203, and the 'natural' RXR ligands phytanic acid or methoprene acid [Radominska-Pandya and Chen, 2002] at concentrations as high as 500 nM did not have any significant effect on steroid sulfatase activity (Fig. 4a). RXR agonists can stimulate the formation of RXR homodimers and these RXR homodimers can be transcriptionally active in some cell types [Ijpenberg et al., 2004]. However, RXR agonists failed to increase steroid sulfatase activity in the RARα-insensitive HL60-Pager D cell line (data not shown). Hence, in the absence of a RAR signaling component, synthetic and 'natural' RXR agonists are unable to stimulate an increase in steroid sulfatase activity.

Pre-treatment of HL60 cells with the pan-RXR antagonist AGN195393 [Balasubramanian et al., 2004] inhibited the increase in steroid sulfatase activity induced by 100 nM ATRA ($IC_{50} = 23.9 \pm 6.7$ nM, Fig. 4b), the pan RAR agonist AGN191183 ($IC_{50} = 110.8 \pm 9.4$ nM, Fig. 4c), the RARα-selective agonist AGN195183 ($IC_{50} = 41.9 \pm 17.3$ nM, Fig. 4d), or the RARβγ-selective agonist AGN190168 ($IC_{50} = 79.8 \pm 13.7$ nM, Fig. 4e) in a dose-

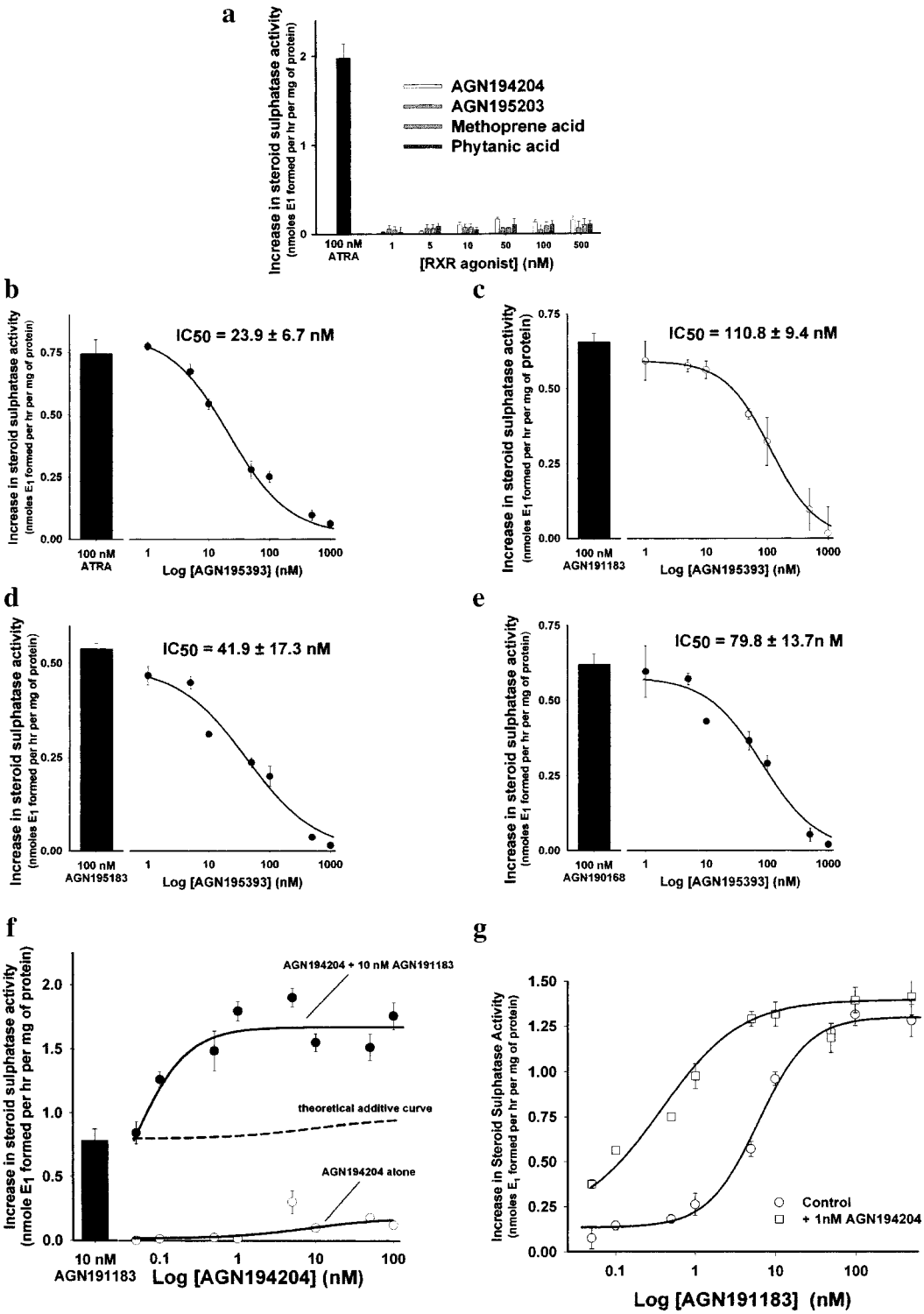


Fig. 4. RXR is required for the RARα-mediated increase in steroid sulfatase activity in HL60 cells. **a**: It shows the effects of increasing concentrations (1–500 nM) of the synthetic RXR agonists AGN194204, AGN195204, and the 'natural RXR ligands methoprene acid and phytanic acid on steroid sulfatase activity in HL60 cells. Cells were stimulated with the 'rexinoids' for 72 h prior to assay. As a control, one batch of HL60 cells was treated with 100 nM all-*trans* retinoic acid. **b**: It shows steroid sulfatase activity in HL60 cells treated for 2 h with increasing concentrations of the pan-RXR antagonist AGN195393 (●) prior to stimulation with 100 nM ATRA. Similar experiments using the pan-RAR agonist AGN191183, the RARα-selective agonist AGN195183, and the RARβγ-selective agonist AGN190168 are shown in (**c**–**e**), respectively. All data points are means ± SEM of quadruplicate determinations, and each experiment was performed a minimum of four times. **f**: It shows the effect on steroid sulfatase activity of co-treatment of HL60 cells with a fixed concentration of the pan-RAR agonist AGN191183 (10 nM) and increasing concentrations (0.05–100 nM) of the pan-RXR agonist AGN194204. **g**: It shows HL60 cells that were co-treated with a fixed dose of AGN194204 (1 nM) and increasing concentrations (0.05–500 nM) of AGN191183. All data points are means ± SEM of quadruplicate determinations, and each experiment was performed a minimum of four times.

responsive fashion. Therefore, blockade of RXR signaling abrogates the RARα-mediated increase in steroid sulfatase activity.

To examine the role of RXR further, we tested the effect of co-treatment of HL60 cells with the pan-RAR agonist AGN191183 and the pan-RXR agonist AGN194204. As already shown, AGN194204 itself did not have any significant effect on steroid sulfatase activity in HL60 cells (Fig. 4f), but co-administration of 10 nM of AGN191183 and AGN194204 (0.05–500 nM) revealed a significant synergy between these agents in increasing steroid sulfatase activity. Similar effects were observed when ATRA, AGN195183, and AGN190168 were used as the RAR agonist, and RXR was agonized with AGN195203 (data not shown). Figure 4g shows experiments in which a fixed dose of the pan-RXR agonist AGN194204 was co-administered with increasing doses of the pan-RAR agonist AGN191183. The EC_{50} of the AGN191183 dose-response curve was reduced ~30-fold ($P < 0.003$, $n = 9$) in the presence of 1 μM AGN194204. Similar synergistic effects were observed when RARs were agonized with ATRA, AGN195183, or AGN190168, and RXR was agonized with either AGN194204 or AGN195203 (data not shown). This synergy was blocked by pre-incubating cells with RXR pan-antagonist (data not shown).

Inhibition of PLD Blocks the ATRA-Stimulated Increase in Steroid Sulfatase Activity in HL60 Cells

ATRA induces a slow-to-start but long-lasting increase in the expression of phospholipase D1 (PLD1) and phospholipase D2 (PLD2) in myeloid leukemic cells undergoing granulocytic differentiation [Nakashima et al., 1998; El Marjou et al., 2000]. Pre-treatment of HL60 cells with the primary alcohol PLD1/PLD2 inhibitor 1-butanol (0.3%, v/v) significantly decreased the ATRA-stimulated increase in steroid sulfatase activity (Fig. 5a, $n = 5$, $P < 0.002$). In contrast, pre-treatment with the inactive secondary alcohol 2-butanol (0.3%, v/v) did not have any significant effect on ATRA-stimulated increase in steroid sulfatase activity (Fig. 5a, $n = 5$, $P > 0.7$). Other non-specific effects of 1-butanol were ruled out by showing that the ATRA-stimulated increase in steroid sulfatase activity was also blocked by pre-treating HL60 cells with the competitive PLD inhibitor 2,3-diphosphoglycerate (5 mM, Fig. 5a, $n = 4$, $P < 0.002$) [Kanaho et al., 1993]. Cell permeant short-chain ceramides can specifically inhibit PLD in myeloid cells [Mansfield et al., 2004], and treatment of HL60 cells with 50 μM C_2-ceramide for 2 h prior to the addition of ATRA reduced the ATRA-mediated increase in steroid sulfatase activity by approximately 70% (Fig. 5a, $n = 5$, $P < 0.002$). Dihydro-C_2-ceramide (50 μM), which does not inhibit PLD, failed to have any significant effect (Fig. 5a, $n = 5$, $P > 0.6$).

The initial product of PLD activity is phosphatidic acid (PA). Co-incubation of HL60 cells with 100 μM PA had no significant effect on basal steroid sulfatase activity (Fig. 5b) and produced a small 20–25% augmentation of ATRA-stimulated steroids sulfatase activity (Fig. 5b, $n = 4$, $P > 0.05$). Inhibition of PAP-mediated degradation of PA by pre-treatment HL60 cells with either 100 μM propranolol ($n = 3$, $P < 0.02$) or 100 μM chlorpromazine ($n = 3$, $P < 0.01$) [Rabano et al., 2004] inhibited the ATRA-mediated increase in steroid sulfatase activity by ~70% [Fig. 5b].

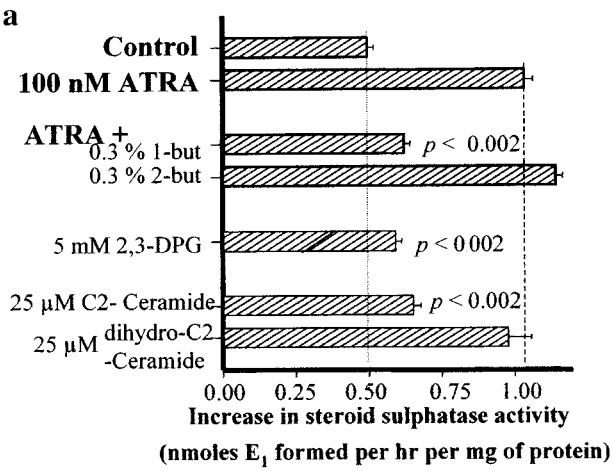
Treatment of HL60 cells with up to 5 μM of the PI-PLC inhibitor U73122 and its inactive analog U73433 had no effect on either basal or ATRA-stimulated steroid sulfatase activity (Fig. 5c). The PI-PLC-Ca^{2+} signaling can be activated in myeloid cells [Bae et al., 2003] and other cell types [Krjukova et al., 2004] by 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (*m*-3M3FBS). However, treatment of HL60 cells with 25 μM *m*-3M3FBS had no significant effect on either basal steroid sulfatase activity or the increase in steroid sulfatase activity that is induced by ATRA (Fig. 5c). Similarly, the phosphotidylcholine-specific phospholipase C (PC-PLC) inhibitor D-609 (100 μM) failed to have any significant effect on the increase in steroid sulfatase activity in HL60 cells induced by 100 nM ATRA (Fig. 5c).

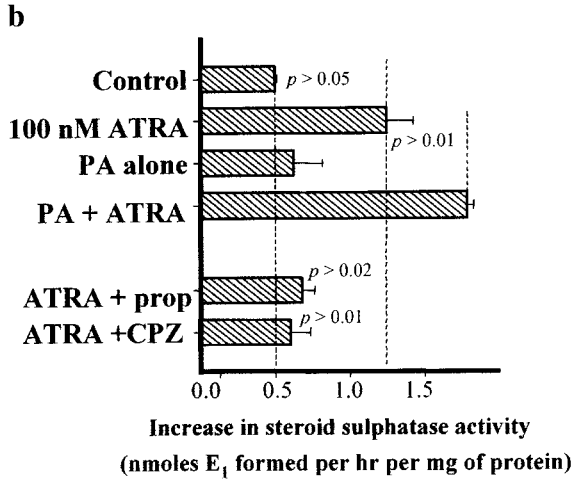
Taken together, these observations reveal that the concerted actions of PLD and PAP and the subsequent generation of diacylglycerol (DAG) are required for the ATRA-mediated increase in steroid sulfatase activity in HL60 cells.

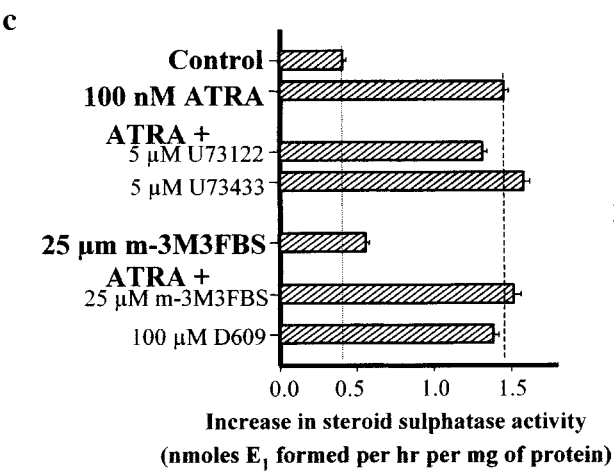
PKCα and PKCδ Blocks have Important Roles in the Retinoid-Stimulated Increase in Steroid Sulfatase Activity in HL60 Cells

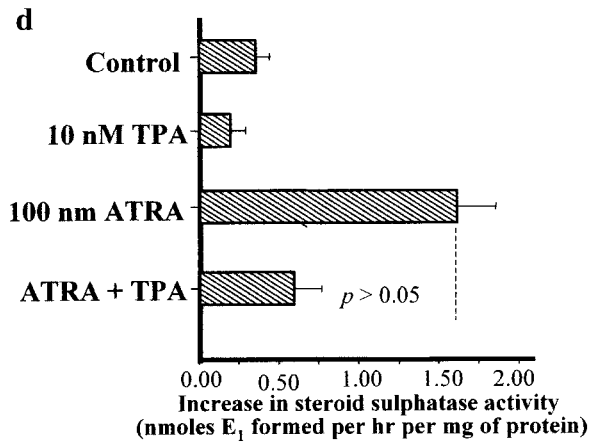
Major cellular DAG targets include the calcium-dependent, diacylglycerol-dependent conventional PKC (cPKC) isoforms α, βI, βII,

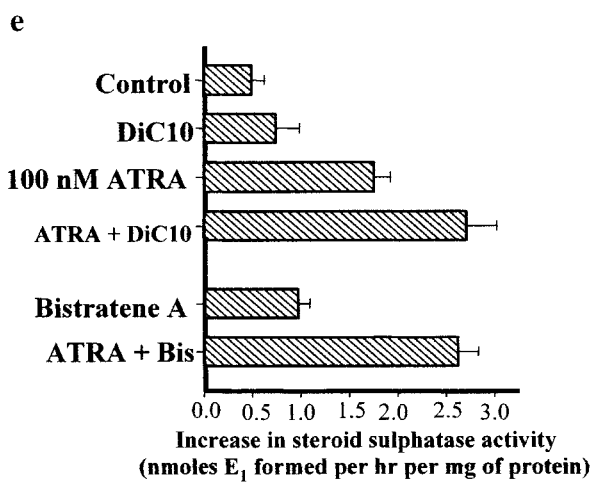
and γ and the calcium-independent, and diacylglycerol-dependent novel PKC (nPKC) isoforms δ, ϵ, η, θ [Yang and Kazanietz, 2003]. HL60 cells express most of the known PKC isoforms [Miguel et al., 2000] and several cPKC or nPKC isoforms are postulated to be drivers of ATRA-mediated differentiation of HL60 cells [Nakashima et al., 1996].

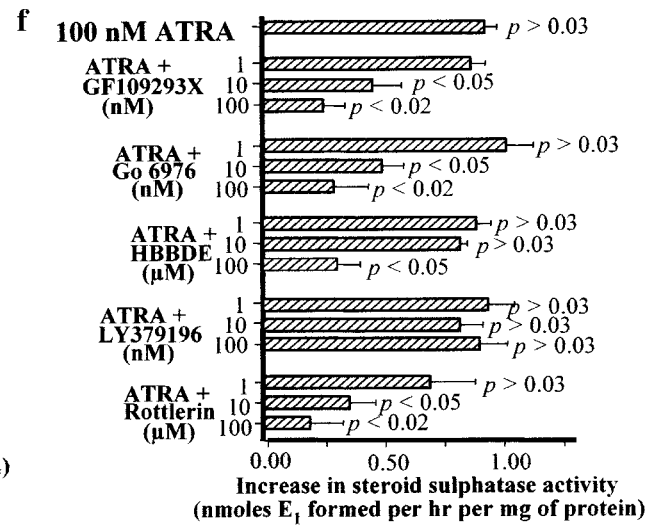












Prolonged treatment of cells with PKC activators is a standard technique for downregulating the expression of classical and novel PKC isoforms [Leontieva and Black, 2004]. Treatment of HL60 cells with 10 nM phorbol ester for 24 h prior to exposure to ATRA completely blocked the ATRA-stimulated increase in steroid sulfatase activity (Fig. 5d, $n = 3$, $P > 0.002$). Co-treatment of HL60 cells with the diacylglycerol analog, didecanoylglycerol (DiC10, 200 μM), and ATRA (100 nM) produced a small but significant augmentation of the ATRA-mediated stimulation of steroid sulfatase activity (Fig. 5e, $n = 3$, $P > 0.05$). These observations point to a role for cPKC and nPKC isoforms in regulating the ATRA-driven rise in steroid sulfatase activity.

Selective PKC inhibitors were used to probe the role of individual PKC isoforms in the ATRA-driven rise in steroid sulfatase in HL60 cells. Figure 5f shows that the ATRA-stimulated increase in steroid sulfatase activity was abrogated by the pan-specific PKC inhibitor GF 109293X (1–100 nM), the cPKCα and cPKCβ specific inhibitor Gö 6976 (1–100 nM), and the cPKCα-specific inhibitor HBBDE. Conversely, the cPKCβI and βII inhibitor LY379196 failed to have any significant effect on the ATRA-stimulated increase in steroid sulfatase activity. The ATRA-stimulated increase in steroid sulfatase activity was also inhibited by rottlerin at concentrations (>10 μM) that are reported to specifically inhibit nPKCδ. The polyether bistratene A preferentially activates nPKCδ in HL60 cells [Griffiths et al., 1996], and co-treatment of HL60 cells with ATRA and bistratene A (50 nM) produced a significant increase in the ATRA-stimulated increase in steroid sulfatase activity (Fig. 5e, $n = 3$, $P > 0.01$).

Inhibition of Tyrosine Kinase Activity Blocks Retinoid-Mediated Increase in Steroid Sulfatase Activity in HL60 Cells

The retinoid-mediated increase in steroid sulfatase activity was abrogated by the relatively non-specific non-receptor tyrosine kinase inhibitors genistein and Herbimycin A (data not shown). Retinoids activate members of the Src tyrosine kinase family, and the non-receptor tyrosine kinase Syk plays an important role in ATRA-mediated granulocytic differentiation of HL60 cells. Piceatannol is a selective inhibitor of the Syk non-receptor tyrosine kinase [Bertagnolo et al., 2004], and this agent inhibited the retinoid-mediated increase in steroid sulfatase activity in HL60 cells in a dose-related fashion ($IC_{50} = 0.63 \pm 0.3$ μM, $n = 4$) (Fig. 6). The Src family kinase inhibitor PP2 also inhibited the retinoid-mediated increase in steroid sulfatase activity in HL60 cells in a dose-related fashion (IC_{50} ~50 μM, $n = 4$) (Fig. 6). The Jak tyrosine kinse inhibitor, AG490, did not have any significant effect on retinoid-mediated stimulation of steroid sulfatase activity, even at concentrations as high as 100 μM.

Fig. 5. The role of phospholipase D, phospholipase C, and protein kinase C isoforms in the all-*trans* retinoic acid-mediated increase in steroid sulfatase activity in HL60 cells. **a**: It shows the effects inhibitors of phospholipase D on the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. Cells were pre-treated with 0.3% (v/v) 1-butanol (1-but), 0.3% (v/v) 2-butanol (2-but), 5 mM 2,3-diphosphoglycerate (2,3-DPG), 25 μM C_2-ceramide, or 25 μM dihydro-C_2-ceramide for 2 h prior to addition of 100 nM ATRA. **b**: It shows the effects of phosphatidic acid (PA. 100 μM) alone or in combination with 100 nM ATRA on steroid sulfatase activity in HL60 cells. b: It also shows the effect of inhibition of PAP on the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. Cells were treated with 100 μM propranolol (prop) or 100 μM chlorpromazine (CPZ) for 2 h prior to stimulation with 100 nM ATRA. **c**: It shows the effects inhibitors of phosphoinositide-phospholipase C (U73122, 5 μM) and its inactive analog (U73433, 5 μM) as well as phosphatidylcholine-specific phospholipase C (D609, 100 μM) on the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. Cells were pre-treated with agents for 2 h prior to addition of the 100 nM ATRA. Steroid sulfatase activity was assessed 72 h after ATRA addition. **c**: It also shows the effect of the phospholipase C activator 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzene-sulfonamide (*m*-3M3FBS) when added alone or in combination with 100 nM ATRA on steroid sulfatase activity in HL60 cells. **d**: It shows the effects of treatment of HL60 cells with 10 nM phorbol dibutyrate (TPA) for 24 h on the ATRA-stimulated increase in steroid sulfatase activity. Cells were washed twice at the end of the TPA incubation period and then stimulated with 100 nM ATRA. **e**: It shows the effects the cPKC/nPKC activator didecanoylglycerol and the PKCδ activator bistratene A added alone or in combination with 100 nM ATRA on steroid sulfatase activity in HL60 cells. **f**: It shows the effects inhibitors of protein kinase C on the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. Cells were pre-treated with the pan-PKC inhibitor GF 109293X (1–100 nM), the cPKCα/β specific inhibitor Gö 6976 (1–100 nM), the cPKCα–specific inhibitor HBBDE (1–10 μM), the cPKCβ inhibitor LY379196 (1–100 nM), or the cPKCδ-, α-selective inhibitor rottlerrin (1–100 μM) for 2 h prior to addition of 100 nM ATRA. All data points are means ± SEM of quadruplicate determinations, and each experiment was performed a minimum of four times.

PI3K Activity Is Essential for Retinoid-Mediated Increase in Steroid Sulfatase Activity in HL60 Cells

PI3K activity can be inhibited in intact cells by wortmannin [Arcaro and Wyman, 1993], LY294002 [Vlahos et al., 1994], and deguelin [Chun et al., 2003]. All three PI3K inhibitors inhibited the ATRA-mediated increase in steroid sulfatase activity in a dose-responsive fashion: wortmannin ($IC_{50} = 0.26 \pm 0.11$ μM, $n = 3$), LY294002 ($IC_{50} = 4.69 \pm 1.5$ μM, $n = 5$), and deguelin ($IC_{50} = 0.99 \pm 1.06$ μM, $n = 3$) (Fig. 7a). LY294002 also inhibited the increase in steroid sulfatase activity in HL60 cells stimulated by the pan-RAR agonist AGN191183 ($IC_{50} = 5.5 \pm 1.6$ μM, $n = 4$), the RARα-agonist AGN195183 ($IC_{50} = 2.9 \pm 1.2$ μM, $n = 4$), and the RARβγ-agonist AGN190168 (IC_{50} 1.7 ± 0.99 μM, $n = 4$) (Fig. 7b). Thus, PI3K activation plays an important role in the retinoid-stimulated, RARα-driven stimulation of steroid sulfatase activity in HL60 cells.

A role for PI3K in the ATRA-stimulated increase in steroid sulfatase activity was confirmed using U937:Δp85-p18 cells, which carry an inducible dominant negative truncated p85 subunit that competes with endogenous p85. ATRA, the pan-RAR agonist, AGN191183, and the RARα agonist, AGN195183, produced a modest increase in steroid sulfatase activity in uninduced U937:Δp85-p18 cells (Fig. 7c, $n = 3$). The retinoid-induced rise in steroid sulfatase activity was significantly lowered by inducing the expression of Δp85 by 18 h incubation with Zn^{2+} and IPTG (Fig. 7c, $n = 3$, $P < 0.02$ for each agonist). Pre-treatment of the parental U937 cells with the induction mixture did not affect the retinoid-mediated increase in steroid sulfatase activity (data not shown).

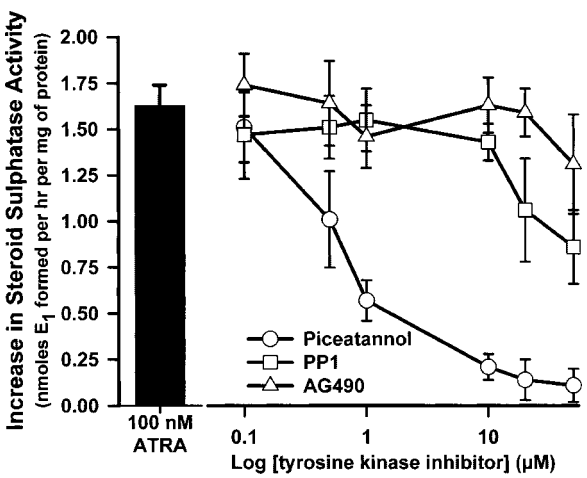


Fig. 6. Inhibition of the Src and Syk non-receptor tyrosine kinases blocks retinoid-mediated increase in steroid sulfatase activity in HL60 cells. Figure shows the effect of tyrosine kinase inhibition on the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. Cells were treated with either the Syk inhibitor piceatannol (○, 0.1–50 μM), the Src-family kinase inhibitor PP2 (▽, 0.1–100 μM), or the JAK inhibitor AG490 (△, 0.1–100 μM) for 2 h prior to stimulation with 100 nM ATRA. All data points are means ± SEM of quadruplicate determinations, and each experiment was performed a minimum of four times.

The phosphoinositide-dependent kinase-1 (PDK-1) is an immediate downstream target of PI3K activation in many cell types. Pre-treatment of HL60 cells with one of the prototypical PDK-1 inhibitors UCN-01 [10 μM, Sato et al., 2002], celecoxib [50 μM, Kulp et al., 2004], or n-α-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) [50 μM, Ballif et al., 2001] at concentrations close to their reported IC_{50}'s inhibited the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells by >50% (Fig. 7d, $n = 3$). Akt/PKB is a primary target of PI3K-PDK-1. Inhibition of Akt, by the phospholipid analogs 1-L-6-hydroxymethyl-*chiro*-inositol-2I-2-O-methyl-3-O-octadecyl-carbonate (HIMO, 5 μM) [Martelli et al., 2003] and SH-5 (5 μM) [Kozikowski et al., 2003], inhibited the ATRA-stimulated increase in steroid sulfatase activity by ~50–60% (Fig. 7d). These pharmacological experiments show that the PI3K/PDK-1/Akt signaling cascade plays an important role in retinoid-mediated increase in steroid sulfatase activity.

ATRA activates p70 S6 kinase in HL60 cells and this involves PI3K, Akt, and mTOR [Lal et al., 2004]. The ATRA-stimulated PI3K-dependent activation of p70 S6 kinase in HL60 cells can be blocked by rapamycin [Lal et al., 2005]. Pre-treatment of HL60 cells with rapamycin (500 nM) inhibited the ATRA-stimulated increase in steroid sulfatase activity by ~30% (Fig. 7d). It has recently been shown that stimulation of Akt by ATRA in HL60 cells leads to activation of NF-κB [Bortul et al., 2003; Witcher et al., 2003]. Figure 7d shows that the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells was blocked by the NF-κB inhibitors SN50, MG132, and partnenolide. These pharmacological experiments show that several PI3K/PDK-1/Akt targets have a role in retinoid-mediated increase in steroid sulfatase activity.

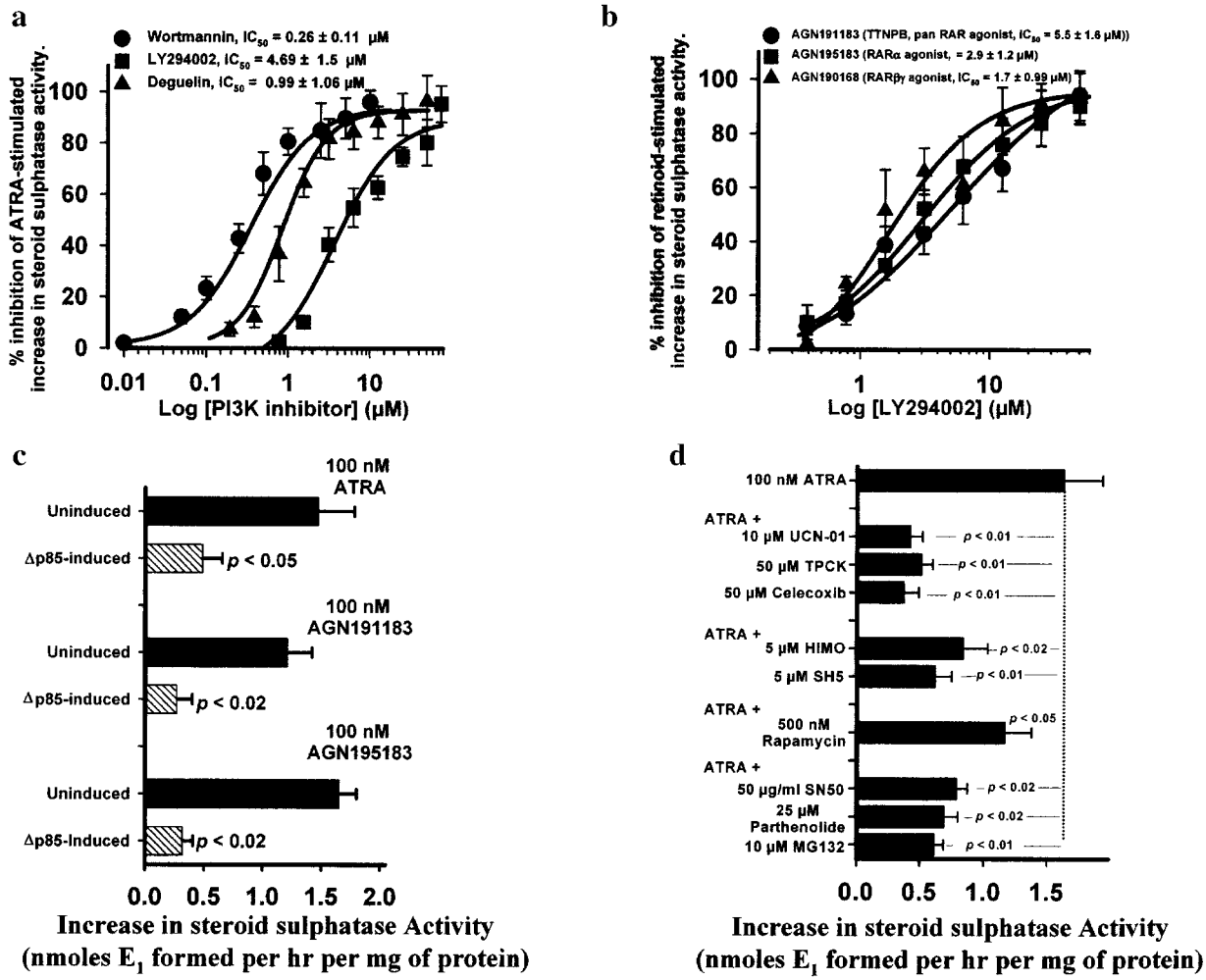


Fig. 7. The phosphoinositide 3-kinase/PDK-1/Akt signalling pathway is required for retinoid-mediated increase in steroid sulfatase activity in HL60 cells. **a**: It shows the effects of inhibition of phosphatidylinositol 3-kinase activity on the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. Cells were treated with either wortmannin (●, 0.05–50 nM), LY294002 (■, 0.1–100 μM), or deguelin (▲, 0.05–20 μM) for 2 h prior to stimulation with 100 nM ATRA. **b**: It shows the effects of inhibition of phosphatidylinositol 3-kinase with LY294002 (0.1–100 μM) on the increase in steroid sulfatase activity in HL60 cells stimulated with 100 nM AGN191183 (●), 100 nM AGN195183 (■), or 100 nM AGN190168 (▲). **c**: It shows the effects of ATRA on steroid sulfatase activity in the U937:Δp85-p18 cells that contain an inducible dominant negative p85 subunit of phosphatidylinositol 3-kinase (Δp85). Stock cultures of U937:Δp85-p18 cells were cultured in antibiotic- and FBS-supplemented RPMI 1640 medium containing 0.6 mg/ml G418 and 0.1 mg/ml hygromycin B. Expression of Δp85 was induced by 20 mM IPTG, 1 nM PMA, and 100 μM $ZnCl_2$ and 16 h later the cells were treated with 100 nM ATRA. **d**: It shows the effect of inhibition of PDK-1, Akt, the p70 S6 kinase and NF-κB pathways on the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. Cells were treated with either the PDK-1 inhibitors UCN-01 (10 μM), TPCK (50 μM), Celecoxib (50 μM), the Akt inhibitors HIMO (5 μM) and SH5 (5 μM), the p70 S6-kinase inhibitor rapamycin (500 nM) or the NF-κB inhibitors SN50 (50 μg/ml), parthenolide (25 μM), or MG132 (10 μM) for 2 h prior to stimulation with 100 nM ATRA. All data points are means ± SEM of quadruplicate determinations, and each experiment was performed a minimum of three or four times.

The RAF-MEK-ERK MAPK Signaling Pathway Is Essential for Retinoid-Mediated Increase in Steroid Sulfatase Activity in HL60 Cells

Other workers have shown at concentration ranging from 1 to 100 nM, ATRA, acting via RAR/RXR heterodimers, stimulates a slow but long-lasting RAF-1 driven increase in the activity of the MEK-ERK map kinase signaling pathway in HL60 and other myeloid leukemic cell lines [Marchinkowska et al., 1997; Yen et al., 1999, 2004; Hong et al., 2001; Miranda et al., 2002; Glasow et al., 2005]. To test whether the RAF-MEK-ERK kinase pathway plays any role

in the retinoid driven upregulation of steroid sulfatase activity, HL60 cells were pre-incubated with the specific RAF inhibitor GW-5074 or the MEK inhibitors U0126 and PD098059 for 2 h prior to treatment with 100 nM ATRA. Figure 8 shows that inhibition of RAF with the specific inhibitor GW5074 blocked the ATRA-stimulated increase in steroid sulfatase activity (●, $IC_{50} = 0.008 \pm 0.0015$ μM). Similarly, MEK specific inhibitors PD098059 (■, $IC_{50} = 14.4 \pm 1.7$ μM) or U0126 (▲, $IC_{50} = 5.1 \pm 2.6$ μM), but not its inactive analog U1024 (△, $IC_{50} \gg 100$ μM), blocked the ATRA-stimulated increase in steroid sulfatase activity. These observations are highly suggestive that an ATRA-driven increase in the RAF-MEK-ERK kinase pathway plays an important role in the retinoid-stimulated increase in steroid sulfatase activity.

Inhibition of p38 MAP kinase has been shown to induce granulocytic differentiation of HL60 cells [Ishii et al., 2001; Zhang et al., 2003]. However, inhibition of p38 MAP kinase with PD203580 did not have any effect on basal steroid sulfatase activity in HL60 cells (data not shown). Figure 8 shows that the p38 inhibitors SB203580 (▽) or PD169316 (◇) had no effect on ATRA-stimulated steroid sulphatase activity in HL60 cells. Therefore, the p38-MAP kinase pathway does not appear to be involved in the ATRA-mediated upregulation of steroid sulfatase activity in HL60 cells. The c-jun N-terminal kinase (JNK) MAP kinase signaling pathways does not appear to be essential for ATRA-induced differentiation of HL60 cells [Yen et al., 1999; Battle et al., 2001]. As perhaps might have been expected, treatment of HL60 cells with the JNK inhibitor SP600125 (△, 1–100 μM) had no effect on the ATRA-stimulated increase in steroid sulfatase activity (Fig. 8).

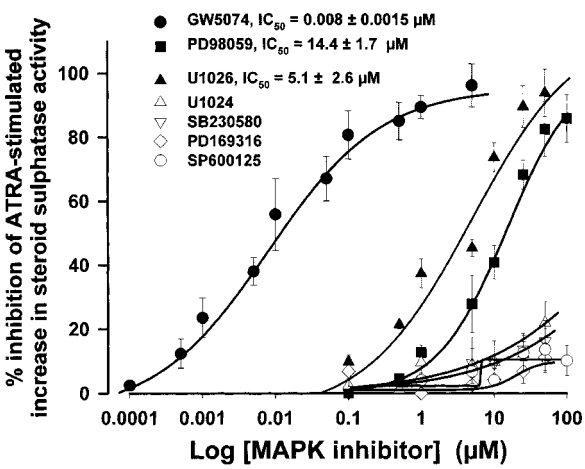


Fig. 8. Activation of a RAF-MEK-ERK mitogen-activated protein kinase signaling cascade, but not the p38-MAP kinase and JNK-MAP kinase, signaling pathway is essential for the ATRA-mediated increase in steroid sulfatase activity in HL60 cells. Figures show the effect of inhibition of RAF-1, ERK-MAP kinase, the p38-MAP kinase, and JNK-MAP kinase pathways on the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. Cells were treated with either the RAF-1 inhibitor GW5074 (●, 1–1,000 nM), the ERK-MAP kinase inhibitors PD098059 (■, 0.1–100 μM), U1026 (▲, 0.1–100 μM) or its inactive analog U2014 (△, 0.1–100 μM), the p38-MAP kinase inhibitors SB230580 (▽, 0.1–100 μM,) or PD169316 (◇, 0.1–100 μM), and the JNK-MAP kinase inhibitor SP600125 (△, 1–100 μM) for 2 h prior to stimulation with 100 nM ATRA. All data points are expressed as the percentage inhibition of the increase in steroid sulfatase activity stimulated by 100 nM ATRA and are expressed as means ± SEM of quadruplicate determinations, and each experiment was performed a minimum of four times.

DISCUSSION

Steroid sulfatase (EC 3.1.6.2) is a member of a large family of evolutionary conserved hydrolases that catalyze the cleavage of sulphate esters from a wide range of substrates, including glycosaminoglycans, sulphated lipids, and steroid sulphates [Hanson et al., 2004]. Steroid sulfatase catalyzes the first step of the local conversion of circulating inactive steroid sulphates into bioactive steroids and provides a major source of estrogens and androgens in many peripheral tissues [reviewed in Reed et al., 2004]. Excessive steroid sulfatase activity plays an important role in the pathogenesis of hormone-dependent breast cancer [reviewed in Nussbaumer and Billich, 2004; Reed et al., 2004]. In the skin, increased steroid sulfatase activity is associated with androgenic alopecia and acne [reviewed in Chen et al., 2002], and inactivating mutations in the gene for steroid sulfatase are responsible for recessive x-linked icthyosis, a disease characterized by abnormal desquamation and permeability barrier dysfunction [Elias et al., 2004]. Low steroid sulfatase activity is associated with a skewing of the development of T lymphocytes into a T_H2-type cytokine profile, and may be a pre-disposition factor for allergic diseases [Daynes et al., 1990]. Furthermore, steroid sulfatase is a major contributor to the formation of neuroactive steroids that modulate cognitive function [reviewed in Hanson et al., 2004; Nussbaumer and Billich, 2004]. Little is known about the biochemical mechanisms that control the expression of steroid sulfatase [Reed et al., 2004].

We have previously shown that treatment of HL60 cells with ATRA and 9-*cis* produces a marked increase in both the expression of the mRNA encoding steroid sulfatase and its enzymatic activity [Hughes et al., 2001]. Therefore, an increase the level of enzyme expression is an important component in the retinoid-stimulated increase in steroid sulfatase activity in HL60 cells. However, steroid sulfatase, like other members of the sulfatase family, undergoes a post-translational modification that increases catalytic efficiency of the enzyme. The enzyme sulfatase-modifying factor 1 (SUMF1) catalyzes the conversion of a conserved cysteine residue in the active site of steroid sulfatase into a α-formylglycine residue [Cosma et al., 2003; Dierks et al., 2003]. Overexpression of SUMF-1 causes an increase in the activity of steroid sulfatase and other members of the sulfatase family [Cosma et al., 2004], therefore, the extent of post-translational modification appears to be a limiting factor that determines the activity of the sulfatase family members. This implies that changes in the expression/activity of SUMF1 may indirectly increase cellular steroid sulfatase activity without necessitating an increased expression of the sulfatase. In fact, IL-6 and TNFα increase the activity of steroid sulfatase in MCF-7 breast cancer cells but does not augment the expression of steroid sulfatase mRNA [Newman et al., 2000]. Therefore, the cytokine-mediated upregulation of steroid sulfatase activity appears to be post-translationally regulated and does not appear to be mediated by an increase in gene transcription of mRNA stabilisation. It is not known whether IL-6 or TNFα modulates the expression or activity of SUMF1, but it provides an attractive explanation of the effects of these cytokines on steroid sulfatase activity in breast tissue. In our hands, the retinoid-stimulated increase in the enzymatic activity of steroid sulfatase was characterized by an increase in the enzyme's V_{max} with the K_m remaining constant [Hughes et al., 2001]. Therefore, at present, we cannot rule out the possibility that part of the retinoid-stimulated increase in steroid sulfatase activity in myeloid cells is indirect and could be mediated by a retinoid-mediated increase in either the expression or activity of SUMF1. We are currently investigating whether retinoids can regulate the expression/activity of SUMF1in myeloid cells.

In this study, we have examined the receptor mechanism(s) that underlie the retinoid-stimulated increase in the activity of steroid sulfatase in HL60 cells. Retinoids are thought to act primarily through nuclear receptor heterodimers to trans-activate gene expression. Indeed, studies using RAR and RXR agonists and antagonists have shown that both RAR and RXR are required for retinoid-mediated stimulation of steroid sulfatase activity. Both the 'natural' RAR agonists ATRA and 9-*cis*-RA stimulated an increase steroid sulfatase activity in HL60 cells. However, these retinoids are relatively nonspecific in that they bind to each of the three RAR isoforms with similar affinities. Therefore, we have used a range of pharmacological reagents to determine the role each RAR isoforms plays in the retinoid-stimulated increase in steroid sulfatase activity.

Transcripts encoding RARγ have been detected in HL60 cells by RT-PCR and Northern blotting [Wang and Yen, 2004], but whether these are translated into functional protein is debatable since the majority of published studies report that RARγ protein cannot be detected in HL60 cells by Western blotting [De The et al., 1989; Hashimoto et al., 1989, 1990; Nervi et al., 1989; Kizaki et al., 1993; Nagy et al., 1995]. However, one recent report has described low levels of RARγ protein expression in HL60 cells [Ozpolat et al., 2004]. However, a role for RARγ–RXR heterodimers in driving the retinoid-mediated increase in steroid sulfatase activity can be discounted by observations that AGN205327, the RARγ-specific agonist, failed to increase steroid sulfatase and AGN205728, a RARγ-specific antagonist, failed to block the ATRA-mediated increase in steroid sulfatase activity.

Undifferentiated HL60 cells do not express RARβ, but the latter is rapidly expressed upon the addition of ATRA [De The et al., 1989; Gaub et al., 1989; Hashimoto et al., 1989, 1990; Nervi et al., 1989; Kizaki et al., 1993; Nagy et al., 1995; Ozpolat et al., 2004; Wang and Yen, 2004]. Since HL60 cells do not appear to express high levels of RARγ, we can use AGN190168 and AGN190299 as RARβ agonists to investigate possible roles for RARβ-RXR heterodimers in driving the retinoid-stimulated increase in steroid sulfatase activity. Both AGN190168 and AGN190299 increase in steroid sulfatase activity in HL60 cells, but this only occurs at concentrations much higher than their reported

binding affinities for either RARβ or RARγ. Therefore, at high concentrations, it is possible that the effects of AGN190168 and AGN190229 on steroid sulfatase activity are a consequence of 'non-specific' binding to RARα and are mediated by RARα/RXR-mediated transactivation. This is borne out by the observations that the increase in steroid sulfatase activity produced by AGN190168 was blocked by the pan-RAR and RARα antagonists but was unaffected by the RARβ and RARγ specific antagonists.

RARα is abundantly expressed in HL60 cells [De The et al., 1989; Gaub et al., 1989; Hashimoto et al., 1989, 1990; Nervi et al., 1989; Kizaki et al., 1993; Nagy et al., 1995; Ozpolat et al., 2004; Wang and Yen, 2004], and is the most important RAR isoform involved in the RAR/RXR heterodimer-driven granulocytic differentiation of myeloid cell lines. For example, HL60 granulocytic differentiation is induced by RARα-specific, but not RARβ- or RARγ-specific, agonists [Dawson et al., 1994; Apfel et al., 1995; Brooks et al., 1996; Gianni et al., 1996; Kizaki et al., 1996; Tocci et al., 1996; Ozpolat et al., 2004] and blocked by a RARα specific antagonist [Apfel et al., 1992; Metha et al., 1997]. Similarly, retinoids do not induce differentiation in HL60 cells that had spontaneously acquired a dominant negative truncated RARα in culture, [Robertson et al., 1992], and this resistance is reversed by overexpression of RARα. Retinoid responsiveness was also lost in a myeloid cell line that was engineered to express a dominant negative RARα [Tsai and Collins, 1993]. A key role for RARα in the retinoid-mediated stimulation of steroid sulfatase activity is indicated by the observation that both ATRA and 9-*cis*-RA and synthetic RARα-selective analogs were unable to stimulate an increase in steroid sulfatase activity in the RARα-insensitive HL60-Pager D cell line. This observation was confirmed by the pharmacological experiments, which demonstrated that a RARα isoform selective agonist could increase steroid sulfatase activity in HL60 cells, and this was blocked by the pan-RAR and RARα-selective antagonists but was unaffected by RARβγ- and RARγ-selective antagonists.

Agonism of RXR receptors alone is not a sufficient stimulus to drive an increase in steroid sulfatase activity. This is evidenced by the observation that in RARα-resistant HL60-pager D cells both natural (ATRA, 9-*cis*, methoprene acid, or phytanic acid) and synthetic (AGN194204 or AGN195203) RXR ligands failed to increase steroid sulfatase activity. Both RARα and RXR are required for retinoids to efficiently upregulate steroid sulfatase activity in HL60 cells, since RXR-specific antagonists blocked the RAR agonist-stimulated increase in steroid sulfatase activity. A combination of RARα and RXR agonists produced a synergistic increase in RAR-stimulated increase in steroid sulfatase activity. It is well-established RXR agonists synergize with RAR agonists in driving RXR-RAR transactivational signaling via RARE's [Roy et al., 1995; Germain et al., 2002] and growth arrest and differentiation of myeloid leukemic cell lines [Apfel et al., 1995; Botling et al., 1997; Hida et al., 2001; Pendino et al., 2003]. These data emphasize the importance of signaling through both retinoid receptor subunits are required to produce a physiological response to retinoids.

However, recent evidence suggests that many retinoid-driven cellular processes also require activation of a variety of 'non-genomic' intracellular signaling cascades. We show that this is the case in the retinoid-stimulated increase in steroid sulfatase activity in HL60 cells. To this end, we show that several signaling pathways, particularly the PI3K and MAPK signaling cascades, play an important role in the ability of retinoids to upregulate steroid sulfatase activity in HL60 cells.

Retinoids drive a slow but persistent increase in the expression of PLD1 and PLD2 in HL60 and NB4 myeloid leukemic cell lines, presumably by a direct genomic mechanism [El Marjou et al., 2000]. This increase in PLD expression is mirrored by a progressive increase in basal PLD activity as the myeloid cells undergo differentiation. Experiments with pharmacological inhibitor show that the increase in PLD expression/activity is essential for granulocytic differentiation [Nakashima et al., 1998; El Marjou et al., 2000]. In a similar fashion, PLD inhibitors blocked the ATRA-stimulated increase in steroid sulfatase activity indicating a role for PLD. The lipid product of PLD activity is phosphatidic acid (PA), and the bulk of cellular PA is converted to DAG by PAP. Inhibition of the PAP-mediated generation of DAG in HL60 cells blocked the ATRA-stimulated increase in steroid sulfatase activity suggesting that DAG plays an essential role in this process. Recently, Batista et al. [2004] have shown that inhibition of diacylglycerol kinase, the enzyme that

recycles DAG into PA, accelerates the time course of HL60 granulocytic differentiation, again alluding to the importance of DAG in the differentiation of HL60 cells. The failure of PI-PLC and PC-PLC specific inhibitors to have any effect on retinoid-stimulated increase in steroid sulfatase activity suggests that neither of these enzymes are major sources of DAG in retinoid-stimulated HL60 cells. In fact, ATRA has complex effects on PI-PLC activity in HL60 cells. During the first 24–36 h following exposure to ATRA PI-PLC activity appears to be inhibited and this is followed by an increase in nuclear PI-PLC activity 48–72 h after exposure [Geny et al., 1991: Porfiri et al., 1991; Iirie et al., 1995; Ohoka et al., 1995; Lopez-Pedrera et al., 2001].

DAG regulates the activities of cPKC and nPKC isoforms. Experiments with selective PKC isoform inhibitors showed that PKCα and PKCδ play roles in the retinoid-stimulation of steroid sulfatase activity in HL60 cells. PKCα and PKCδ can promote retinoid receptor-mediated gene transactivation. For example, Cho and Talmage [2001] have shown that overexpression of PKCα in the PKCα-deficient MDA-MB-231 breast cancer cell line restores retinoid receptor-mediated transactivation [Cho and Talmage, 2001], perhaps by facilitating RAR/RXR heterodimer formation [Delmotte et al., 1999]. Moreover, Kambhampati et al. [2003] have shown that PKCδ can form a complex with RARα that binds to RAREs to drive transcription. A role for PKCδ in retinoid-dependent initiation of mRNA translation is suggested by the observation that ATRA-treatment of HL60 leads to phosphorylation of mTOR and increases the activity of p70 S6 kinase [Lal et al., 2005]. The exact roles of PKCα and PKCδ in retinoid-mediated stimulation of steroid sulfatase are studies for the future.

Experiments using three structurally unrelated PI3K inhibitors revealed a role for PI3K in the RARα/RXR-mediated increase in steroid sulfatase activity in HL60 cells. Similarly, the ATRA-stimulated increase in steroid sulfatase activity was attenuated in an U937-cell line in which class IA PI3K-drived signals are blocked by overexpression of a dominant negative p85 subunit. Other workers have shown that PI3K inhibitors block retinoid-stimulated granulocytic differentiation [Bertagnolo et al., 1999, 2004; Lopez-Pedrera et al., 2004] and that down-regulation of PI3K expression with a p85 antisense oligonucleotide blocks the ATRA-mediated differentiation of HL60 cells [Bertagnolo et al., 1999]. Inhibitors of PI3K-p110β and PI3K-p100δ failed to have any significant effect on the ATRA-stimulated increase in steroid sulfatase activity (data not shown). It is, therefore, likely that ATRA activates the PI3K p85-p110α heterodimer. The non-receptor tyrosine kinase Syk may play a key role in the activation of p85-p110 PI3K heterodimers for the following reasons. Inhibition of Syk activity blocks ATRA-mediated granulocytic differentiation of HL60 cells [Qin and Yamamura, 1997], and as shown here the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. Syk activity is rapidly stimulated by ATRA in HL60 cells [Bertagnolo et al., 2004], and the guanine nucleotide exchange factor Vav is then phosphorylated by Syk. The freshly phosphorylated Vav forms an active signaling complex with the p85-p110 PI3K heterodimer. Commensurate with all this is the observation that the Syk inhibitor piceatannol disrupts the Vav/p85 interaction and blocks the ATRA-induced PI3K activity and HL60 granulocytic maturation [Bertagnolo et al., 2004].

The ATRA-stimulated increase in steroid sulfatase activity was blocked by inhibitors of the PI3K targets PDK-1 and Akt. Many of the known cellular PI3K/PDK-1/Akt targets are involved in the regulation of gene transcription or protein translation [Vara et al., 2004]. Lal et al. [2005] have recently shown that ATRA-stimulates mTOR-p70 S6 kinase activity in a PI3K/Akt-dependent manner in HL60 cells. The mTOR-p70 S6 kinase pathway does not influence retinoid-stimulated RARE-mediated transcriptional activation in HL60 cells, but it does play an important role in the initiation of mRNA translation [Lal et al., 2005]. The mTOR-p70 S6 kinase inhibitor rapamaycin had a small but significant inhibitory effect on ATRA-mediated increase in steroid sulfatase activity. ATRA stimulates the NF-κB transcription factor in HL60 cells in a PI3K/Akt-dependent fashion [Bortul et al., 2003; Witcher et al., 2003]; inhibition of the NF-κB blocked the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells.

A slow-to-start but long-lived activation of the ERK signaling cascade seems to play an important role in retinoid-stimulated granulocytic differentiation of HL60 cells [Wang and Studzinski, 2001a; Miranda et al., 2002].

ERK-MAP kinase signaling is also important to the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells. The mechanism by which the ERK-MAP kinase pathway is activated by ATRA in HL60 cells is still unclear, but it requires RARα-RXR-dependent activation of both RAF-1 [Hong et al., 2001] and PI3K [Baier et al., 1999; Lopez-Pedrera et al., 2004]. Activated ERK kinases can translocate to the nucleus and activate several transcription factors [Yang et al., 2003; Edmunds and Mahadevan, 2004]. Alternatively, Glasow et al. [2005] have shown that ATRA-stimulated ERK-MAPK signaling pathway potentiates ATRA-induced RARα-RXR driven transactivation in U937 promyelocytic cells. The p38-MAPK or JNK-MAPK pathways do not play a role in the ATRA-stimulated increase in steroid sulfatase activity in HL60 cells.

Here, we show that the retinoid-driven increase in steroid sulfatase activity in HL60 cells is mediated by RAR–RXR heterodimers. At the present, we do not know whether this relates to the presence of an RARE in the promoter region of the steroid sulfatase gene. Retinoids have been reported to activate several intracellular signaling cascades in myeloid leukemic cells. However, direct activation of the non-genomic signaling pathways by retinoids may not be essential, since retinoids may sensitize cells to the effects of agents that activate non-genomic signaling pathways, by increasing the expression of signaling intermediates. A better understanding of the interplay between the non-genomic signaling cascades and retinoid-stimulated genomic signaling will provide a clearer insight into the mechanism(s) by which retinoids mediate their effects on cell proliferation, differentiation, and apoptosis in myeloid cells.

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REFERENCES

Allegretto E, McClurg M, Lazarchik S, Clemm D, Kerner S, Elgort M, Boehm M, White S, Pike J, Heyman R. 1993. Transactivation properties of retinoic acid and retinoid X receptors in mammalian cells and yeast. J Biol Chem 268:26625–26633.

Apfel C, Bauer F, Crettaz M., Forni L, Kamber M, Kaufmann F, Lemotte P, Pirson W, Klaus M. 1992. A retinoic acid receptor α antagonist selectively counteracts retinoic acid effects. Proc Natl Acad Sci USA 89: 7129–7133.

Apfel C, Kamber M, Klaus M, Mohr P, Keidel S, LeMotte P. 1995. Enhancement of HL-60 differentiation by a new class of retinoids with selective activity on retinoid X receptor. J Biol Chem 270:30765–30772.

Arcaro A, Wyman M. 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor. Role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. J Biochem 296:297–301.

Bae Y, Lee T, Park J, Hur J, Kim Y, Heo K, Kwak J, Suh P, Ryu S. 2003. Identification of a compound that directly stimulated phospholipase C activity. Mol Pharmacol 63: 1043–1050.

Baier R, Bondeva T, Klinger R, Bondev A, Wetzger R. 1999. Retinoic acid induces selective expression of phosphoinositide kinase γ in myelomonocytic U937 cells. 10:447–456.

Balasubramanian S, Chandraratna R, Eckert R. 2004. Suppression of human pancreatic cancer cell proliferation by AGN194202, an RXR-selective retinoid. Carcinogenesis 25:1377–1385.

Ballif B, Shimamura A, Pae E, Blenis J. 2001. Disruption of 3-phosphoinositide-dependent kinase 1 (PDK1) signalling by the anti-tumorigenic and anti-proliferative agent N-α-tosyl-L-phenylalanine chloromethyl ketone. J Biol Chem 276:12466–12475.

Balmer J, Blomhoff R. 2002. Gene expression regulation by retinoic acid. J Lipid Res 43:1773–1808.

Bastien J, Rochette-Egly C. 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 328:1–16.

Batista E, Warbington M, Badwey J, Van Dyke T. 2004. Differentiation of HL-60 cells to granulocytes involves regulation of select diacyglycerol kinases (DGKs). J Cell Biochem 94:774–793.

Battle T, Roberson M, Zhang T, Varayanis S, Yen A. 2001. Retinoic acid-induced blr2 expression requires RARα, RXR and MAPK activation and uses ERK2 but not JNK/SAPK to accelerate cell differentiation. Eur J Cell Biol 80: 59–67.

Beard R, Gil D, Marler D, Henry E, Colon D, Gillett S, Arefieg T, Breen T, Krauss H, Davies P, Chandraratna R. 1994. Structural basis for the differential RXR and RAR activity of stilbene retinoid analogs. Bioorg Med Chem Letts 4:1447–1452.

Bertagnolo V, Neri L, Marchisio M, Mischiati C, Capitani S. 1999. Phosphoinositide 3-kinase activity is essential for all-*trans*-retinoic acid-induced granulocytic differentiation of HL-60 cells. Canc Res 59:542–546.

Bertagnolo V, Brugnoli F, Marchisio M, Celeghini C, Carini C, Capitani S. 2004. Association of PI 3-K with tyrosine phosphorylated VAV is essential for its activity in neutrophil-like maturation of myeloid cells. Cell Signal 16:423–433.

Bortul K, Tazzari P, Cappellini A, Tabellini G, Billi A, Bareggi R, Manzoli L, Cocco L, Martelli A. 2003. Constitutively active Akt1 protects HL60 leukaemia cells from trail-induced apoptosis through a mechanism

involving NF-κB activation and $cFLIP_L$ up-regulation. Leukaemia 17:379–389.

Bost F, Caron L, Marchetti I, Dani C, Marchand-Brustel Y, Binetruy B. 2002. Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. Biochem J 361: 621–627.

Botling J, Castro D, Oberg F, Nilsson K, Perlmann T. 1997. Retinoic acid receptor/retinoid X receptor heterodimers can be activated through both subunits providing a basis for synergistic transactivation and cellular differentiation. J Biol Chem 272:9443–9449.

Brooks S, Kazmer S, Levin A, Yen A. 1996. Myeloid differentiation and retinoblastoma phosphorylation changes in HL-60 cells induced by retinoic acid receptor- and retinoid X-receptor-selective retinoic acid analogs. Blood 87:227–237.

Cambien B, Millet M-A, Schmid-Antomarchi H, Brosette N, Rossi B, Schmid-Alliana A. 1999. Src-regulated extracellular-regulated kinase and syk-regulated c-jun N-terminal kinase pathways act in conjunction to induce IL-1 synthesis in response to microtubule disruption in HL60 cells. J Immunol 163:5079–5085.

Canon E, Cosagaya J, Scsucova S, Aranda A. 2004. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol Biol Cell 15:5583–5592.

Carter C. 2003. Retinoic acid signalling through PI 3-kinase induces differentiation of human endometrial adenocarcinoma cells. Exp Mol Pathol 75:34–44.

Chen W, Thiboutot D, Zoublis C. 2002. Cutaneous androgen metabolism: Basic research and clinical perspectives. J Invest Dermatol 119:992–1007.

Cho Y, Talmage D. 2001. Protein kinase Cα expression confers retinoic acid sensitivity on MDA-MB-231 human breast cancer cells. Exp Cell Res 10:97–109.

Chu P, Cheung W, Kwong Y. 2003. Differential effects of 9-cis, 13-cis and all-trans retinoic acid in the neuronal differentiation of human neuroblastoma cells. Neuroreport 14:1935–1939.

Chun K, Kosmeder J, Sun S, Pezzuto J, Lotan R, Hong W, Lee H. 2003. Effects of deguelin on the phosphatidylinositol 3-kinase /Akt pathway and apoptosis in premalignant human bronchial epithelial cells. J Natl Canc Inst 95:291–302.

Cosma M, Pepe S, Annunziata I, Newbold R, Grompe M, Parenti G, Ballabio A. 2003. The multiple sulphatase deficiency gene encodes an essential and limiting factor for the activity of sulfatases. Cell 113:445–456.

Cosma M, Pepe S, Parenti G, Settembre C, Annunziata I, Wade-Martins R, Di Domenico C, Di Natale P, Mankad A, Cox B, Uziel G, Mancini G, Zammarchi E, Donati M, Kleijer W, Filocama M, Carozzo R, Carella M, Ballabio A. 2004. Molecular and functional analysis of SUMF1 mutations in multiple sulphatase deficiency. Hum Mut 23:576–581.

Davies S, Reddy H, Caivano M, Cohen P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105.

Dawson M, Elstner E, Kazaki M, Chen D, Pakkala S, Kerner B, Koeffler H. 1994. Myeloid differentiation mediated through retinoic acid receptor/retinoic X receptor (RXR) not RXR/RXR pathway. Blood 84:446–452.

Daynes R, Araneo B, Dowell T, Huang K, Dudley D. 1990. Regulation of murine lymphokine production in vivo. III. The lymphoid tissue microenvironment exerts regulatory influences over T-helper function. J Exp Med 171:979–996.

De The H, Marchio A, Tiolais P, Dejean A. 1989. Differential expression and ligand regulation of the retinoic acid receptor α and β genes. EMBO J 8:429–433.

Delmotte M-H, Tahayato A, Formstecher P, Lefebvre P. 1999. Serine 157, a retinoic receptor α residue phosphorylated by protein kinase C in vitro, is involved in RXR·RARα heterodimerization and transcriptional activity. J Biol Chem 274:38225–38231.

Dierks T, Schmidt B, Borissenko L, Peng J, Preusser A, Mariappan M, Von Figura K. 2003. Multiple sulphatase deficiency is caused by mutations in the gene encoding the human Cα-formylglycine generating enzyme. Cell 113:435–444.

Edmunds J, Mahadevan L. 2004. MAP kinases as structural adaptors and enzymatic activators in transcription complexes. J Cell Sci 117:3715–3723.

El Marjou M, Montalescot V, Buzyn A, Geny B. 2000. Modification in phospholipase D activity and isoform expression occur upon maturation and differentiation *in vivo* and *in vitro* in human myeloid cells. Leukaemia 14: 2118–2127.

Elias P, Crumrine D, Rassner U, Hachem J, Menon G, Man W, Choy M, Leypoldt L, Feingold K, Williams M. 2004. Basis for abnormal desquamation and permeability barrier dysfunction in RXLI. J Invest Dermatol 122: 314–319.

Fanjul A, Delia D, Pierotti M, Rideout D, Yu J, Pfahl M, Qiu J. 1996. 4-hydroxyphenyl retinamide is a highly selective activator of retinoid receptors. J Biol Chem 271:22441–22446.

Fong W-F, Tse A, Poon K-H, Wang C. 2005. Magnolol and honokiol enhamce HL-60 human leukaemia cell differentiation induced by 1,25-dihydroxyvitamin D_3 and retinoic acid. Internal. J Biochem Cell Biol 37:427–441.

Gaub M, Lutz Y, Ruberte E, Petkovich M, Brand N, Chambon P. 1989. Antibodies specific to the retinoic acid receptors α and β. Proc Natl Acad Sci USA 86:3089–3093.

Geny B, Cost H, Barreau P, Basset M, Le Peuch C, Abita J, Cockcroft S. 1991. The differentiating agent, retinoic acid, causes an early inhibition of inositol lipid-specific phospholipase C activity in HL-60 cells. Cell Signal 3: 11–23.

Germain P, Iyer J, Zechel C, Gronemeyer H. 2002. Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. Nature 415:187–192.

Gianni M, Li Calzi M, Terao M, Guiso G, Caccia S, Barbui T, Rambaldi A, Garattini E. 1996. AM580, a stable benzoic derivative of retinoic acid, has a powerful and selective cyto-differentiating effect on promyelocytic cells. Blood 87:1520–1531.

Glasow A, Prodromou N, Xu K, Von Lindern M, Zelent A. 2005. Retinoids and myelomonocytic growth factors cooperatively activate RARα and induce human myeloid leukaemia cell differentiation via MAP kinase pathways. Blood 105:341–349.

Griffiths G, Garrone B, Deacon E, Owen P, Pongracz J, Mead G, Bradwell A, Watters D, Lord J. 1996. The polyether Bistratene A activated protein kinase C-δ and induces growth arrest in HL60 cells. Biochem Biophys Res Commun 22:802–808.

Hanson S, Best M, Wong C-H. 2004. Sulfatases: Structure, mechanism, biological activity, inhibition and synthetic utility. Angew Chem Int Ed 43:5736–5763.

Hashimoto Y, Petkovich M, Gaub M, Kagechika H, Shudo K, Chambon P. 1989. The retinoic acid receptors α and β are expressed in the human promyelocytic leukaemia cell line HL-60. Mol Endocrinol 3:1046–1052.

Hashimoto Y, Kagechika H. Shudo K. 1990. Expression of retinoic acid receptor genes and the ligand-binding selectivity of retinoic acid receptors (RAR's). Biochem Biophys Res Commun 166:1300–1307.

Heyman R, Mangelsdorf D, Dyck J, Stein R, Eichele G, Evans R, Thaller C. 1992. 9-*cis*-retinoic acid is a high affinity ligand for the retinoid-X receptor. Cell 68:397–406.

Hida T, Tai K, Tokuhara N, Ishibashi A, Kikuchi K, Hibi S, Yoshimura H, Nagai M, Yamauchi T, Kobayashi S. 2001. Existence of retinoic acid receptor-independent retinoid X-receptor-dependent pathway in myeloid cell function. Jpn J Pharmacol 85:60–69.

Hong H, Varvayanis S, Yen A. 2001. Retinoic acid causes MEK-dependent RAF phosphorylation through RARα plus RXR activation in HL-60 cells. Differentiation 68: 55–66.

Hughes P, Twist L, Durham J, Choudhry M, Drayson M, Chandraratna R, Michell R, Kirk C, Brown G. 2001. Up-regulation of steroid sulphatase activity in HL60 promyelocytic cells by retinoids and 1α,25-dihydroxyvitamin D_3. Biochem J 365:361–371.

Iirie T, Homma Y, Ohaka Y, Robishaw J, Katada T, Bourne H. 1995. Potentiation of Gi-mediated phospholipase C activation by retinoic acid in HL-60 cells. Possible role of Gγ2. J Biol Chem 270:5901–5908.

Ijpenberg A, Tan A, Gelman L, Kersten S, Seydoux J, Xu J, Metzger D, Canaple L, Chambon P, Wahl W, Desvergne B. 2004. In vivo activation of PPAR target genes by RXR homodimers. EMBO J 23:2083–2091.

Ishida S, Shigemoto-Mogami Y, Shinozaki Y, Kagechika H, Shudo K, Ozawa S, Sawada J-I, Ohno Y, Inoue K. 2004. Differential modulation of PI3-kinase/Akt pathway during all-*trans* retinoic acid- and AM580-induced HL-60 cell differentiation revealed by DNA microarray analysis. Biochem Pharmacol 68:2177–2186.

Ishii Y, Sakai S, Honma Y. 2001. Pyridinyl imidazole inhibitor SB203580 activates p44/p42 mitogen-activated protein kinase and induces differentation of human myeloid leukaemia cells. Leuk Res 25:813–820.

Kambhampati S, Li Y, Verma A, Sassano A, Majchrzak B, Deb D, Parmar S, Giafis N, Kalvakolanu D, Rahman A, Uddin S, Minucci S, Tallman M, Fish E, Platanias L. 2003. Activation of protein kinase Cδ by all-*trans*-retinoic acid. J Biol Chem 278:32541–32551.

Kanaho Y, Nakai Y, Katoh M, Nozawa Y. 1993. The phospholipase inhibitor 2,3-diphosphoglycerate interferes with phospholipase D activation in rabbit neutrophils. J Biol Chem 268:1249–12497.

Kizaki M, Ikeda Y, Tanosaki R, Nakajima H, Morikawa M, Sakashita A, Koeffler H. 1993. Effects of novel retinoic acid compound 9-cis-retinoic acid, on proliferation, differentiation and expression of retinoic acid–α and retinoid X receptor-α RNA by HL60 cells. Blood 82:3592–3599.

Kizaki M, Dawson M, Heyman R, Elster E, Morosetti R, Pakkala S, Chen D, Ueno H, Chao W, Morikawa M, Ikeda Y, Heber D, Pfahl D, Koeffler H. 1996. Effects of novel retinoid X receptor-selective ligands can induce myeloid leukaemia differentiation and proliferation in vitro. Blood 87:1977–1984.

Kozikowski A, Sun H, Brignard J, Dennis P. 2003. Novel PI analogues selectively block activation of the pro-survival serine/threonine kinase Akt. J Am Chem Soc 125:1144–1145.

Krjukova J, Holmqvist T, Danis A, Akerman K, Kukkonen J. 2004. Phospholipase C activator m-3M3FBS affects Ca2+ homeostasis independently of phospholipase C activation. Br J Pharmacol 143:3–7.

Kulp S, Yang Y, Hung C, Chen K, Lai J, Tseng P, Fowble J, Ward P, Chen C. 2004. 3-phosphoinositide-dependent protein kinase-1/Akt signals represents a major cyclooxygenase-2-independent targets for celecoxib in prostate cancer cells. 64:1444–1451.

Lal L, Li Y, Smith J, Sassano A, Uddin S, Parmar S, Tallman M, Minucci S, Hay N, Platanias L. 2005. Activation of the p70 S6 kinase by all-*trans*-retinoic acid in acute promyelocytic leukaemia cells. Blood 105:1669–1677.

Leontieva O, Black J. 2004. Identification of two distinct pathways of protein kinase Cα down-regulation in intestinal epithelial cells. 279:5788–5801.

Lewandowski D, Linassier C, Iochman S, Degenne M, Domench J, Colombat P, Binet C, Herault O. 2002. Phosphatidylinositol 3-kinases are involved in all-*trans* retinoic acid-induced upregulation of CD38 antigen on human haematopoietic cells. Br J Haematol 118:535–544.

Li X, Li H, Xiao Z, Piao Y. 2002. Divergent effects of retinoic acids on the expression of ERα and 17β-hydroxysteroid dehydrogenase type 1 in endometrial carcinoma cells (RL 95-2). J Clin Endocrinol Metab 87: 640–649.

Lopez-Carballo G, Moreno L, Masia S, Perez P, Barettino D. 2002. Activation of the phosphatidylinositol 3-kinase/Akt signalling pathway by retinoic acid is required for neural differentiation of SH-SYU5Y human neuroblastoma cells. 277:25297–25304.

Lopez-Pedrera C, Dobado-Berrios P, Ros R, Torres A, Garcia-Navarro S, Jardi M, Felez J, Velasco F. 2001. Signal transduction pathways underlying the expression of tissue factor and thrombomodulin in promyelocytic cells induced to differentiate by retinoic acid and dibutyrl camp. Thromb Haemost 85:1031–1036.

Lopez-Pedrera C, Barbarroja N, Buendia P, Torres A, Dorada G, Velasco F. 2004. Promyelocytic leukemia retinoid signalling targets regulate apoptosis, tissue factor and thrombomodulin expression. Haematologica 89:286–295.

Mansfield P, Carey S, Hinkovska-Galcheva V, Shayman J, Boxer L. 2004. Ceramide inhibition of phospholipase D and its relationship to RhoA and ARF1 translocation in GTPγS-stimulated polymorphonuclear leukocytes. Blood 103:2363–2368.

Marchinkowska E, Wiedlocha A, Radzikowski C. 1997. 1,25-dihydroxyvitamin D_3 induced activation and supbsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells. Biochem Biophys Res Commun 241:419–426.

Martelli A, Tazzari P, Tabelini G, Bortul R, Billi A, Manzoli L, Ruggeri A, Conte R, Cocco L. 2003. A new selective AKT pharmacological inhibitor reduces resistance to chemotherapeutic drugs, TRAIL, all-*trans*-retinoic acid, and ionising radiation of human leukaemia cells. Leuk 17:1794–1805.

Metha K, McQueen T, Manshouri T, Andreef M, Collins S, Aibtar M. 1997. Involvement of retinoic acid receptor-alpha-mediated signalling pathway in the induction of CD38 cell surface antigen. Blood 89:3607–3614.

Miguel B, Calcerrade M, Mata F, Aller P, Clemente R, Catalan R, Martinez A. 2000. Differential redistribution of protein kinase C isoforms by cyclic AMP in HL60 cells. Biochem Biophys Res Commun 274:596–602.

Miloso M, Villa D, Crimi M, Galbiati S, Donzelli E, Nicolini G, Tredici G. 2004. Retinoic acid-induced neuritogenesis of human neuroblastoma SH-SY5Y cells is ERK independent and PKC dependent. J Neurosci Res 75:241–252.

Miranda M, McGuire T, Johnson D. 2002. Importance of MEK-1/-2 signalling in monocytic and granulocytic differentiation of myeloid cell lines. Leukaemia 16:683–692.

Mountford J, Bunce C, Hughes S, Drayson M, Webb D, Hewison M. 1999. Estrone potentiates myeloid cell differentiation a role for 17β-hydroxysteroid dehydrogenase in modulating hemopoiesis. Exp Haematol 27: 451–460.

Mu Y, Yanase T, Nishi Y, Hirase N, Goto K, Takayanagi R, Nawata H. 2000. A nuclear receptor system constituted by RAR and RXR induces aromatase activity in MCF-7 human breast cancer cells. Mol Cell Endocrinol 166: 137–145.

Nagpal S, Athanikar J, Chandraratna R. 1995. Separation of transactivation and AP1 antagonism functions of retinoic acid receptor α. J Biol Chem 270:923–927.

Nagy L, Thomazy V, Shipley G, Fesus L, Lamph W, Heyman R, Chandraratna R, SNM> S P. 1995. Activation of retinoid X receptors induces apoptosis in myeloid cell lines. Mol Cell Biol 15:3540–3551.

Nakashima S, Ohguchi K, Frohman M, Nozawa Y. 1998. Increased mRNA expression of phospholipase D (PLD) isoenzymes during granulocytic differentiation of HL60 cells. Biochim Biophys Acta 1389:173–177.

Nakashima S, Iwasaki Y, Mizutani T, Ohuguchi K, Nagata K, Kitajima Y, Nozawa Y. 1996. Differential expression of protein kinase C isozymes and small FTP-binding proteins during HC-60 differentiation by retinoic acid and cyclic AMP: relation with phospholipase D (PLD) activation. Immunobiology 196:588–598.

Neri L, Bortul R, Borgatti P, Tabellini G, Baldini G, Capitani S, Martelli A. 2002. Proliferating or differentiating stimuli act on different lipid-dependent signalling pathways in nuclei of human leukaemia cells. Mol Biol Cell 13:947–964.

Nervi C, Grippo J, Sherman M, George M, Jetten A. 1989. Identification and characterisation of nuclear retinoic acid-binding activity in human myeloblastic leukaemia HL-60 cells. Proc Natl Acad Sci 86:5854–5858.

Newman S, Purohit A, Ghilchik M, Potter B, Reed M. 2000. Regulation of steroid sulphatase expression and activity in breast cancer. J Ster Biochem Mol Biol 31: 259–264.

Ng J-H, Nesaretnam K, Reiman K, Lai L. 2000. Effect of retinoic acid and palm oil carotenoids on oestrone sulphatase and oestradiol-17β hydroxysteroid dehydrogenase activities in MCF-7 and MDA-MB-231 breast cancer cell lines. Int J Cancer 88:135–138.

Nussbaumer P, Billich A. 2004. Steroid sulphatase inhibitors. Med Res Rev 24:529–576.

Ohoka Y, Kontani K, Iirie T, Nishana H, Katada T. 1995. Involvement of pertussis toxin-sensitive mechanism in retinoic acid-induced differentiation of human leukaemic HL-60 cells. J Biochem 117:190–196.

Ozpolat B, Tari A, Mehta K, Lopez-Berestein G. 2004. Nuclear retinoid receptors are involved in N-(4-hydroxyphenyl)retinamide (Fenretinide)-induced gene expression and growth inhibition in HL-60 acute myeloid leukaemia cells.. Leuk Lymphoma 45:979–985.

Pendino F, Dudognon C, Delhommeau F, Sahraoui T, Flexor M, Bennaceur-Griscelli A, Lanotte M, Segal-Bendirdjian E. 2003. Retinoic acid receptor α and retinoid-X receptor agonists synergistically target telomerase expression and induce tumor cell death. Oncogene 22:9142–9150.

Piao Y, Peltoketo H, Jouppila A, Vihko R. 1997. Retinoic acids increase 17β-hydroxysteroid dehydrogenase type 1 expression in JEG-3 and T47D cells, but the stimulation is potentiated by epidermal growth factor, 12-O-tetradecanoylphorbol-13-acetate, and cyclic adenosine monophosphate only in JEG-3 cells. Endocrinology 138:898–904.

Porfiri E, Hoffbrand A, Wickremasinghe R. 1991. Retinoic-acid granulocytic differentiation of HL60 human promyelocytic leukaemia cells is preceded by downregulation of autonomous generation of inositol lipid-derived second messengers. Blood 78:1069–1077.

Qin S, Yamamura H. 1997. Up-regulation of Syk activity during HL60 cell differentiation into granulocyte but not into monocyte/macrophage-lineage. Biochem Biophys Res Commun 236:697–701.

Rabano M, Pena A, Brizuela L, Macarulla J, Gomez-Munoz A, Trueba M. 2004. Angiotensin II-stimulated cortisol secretion is mediated by phospholipase D. Mol Cell Endocrinol 222:9–20.

Radominska-Pandya A, Chen G. 2002. Photoaffinity labelling of human retinoid X receptor-β (RXRβ) with 9-*cis*-retinoic acid: identification of phytanic acid, docosahexanoic acind and lithocholic acid as ligands of RXRβ. J Biochem 41:4883–4890.

Reed M, Rea D, Duncan L, Parker M. 1994. Regulation of estradiol 17β-hydroxysteroid dehydrogenase expression and activity by retinoic acid in T47D breast cancer cells. Endocrinol 135:4–9.

Reed M, Purohit A, Woo L, Newman S, Potter B. 2004. Steroid sulphatase: Molecular biology, regulation and inhibition. Endocr Rev 26:171–202.

Robertson K, Emami B, Collins S. 1992. Retinoic acid-resistant HL-60R cells harbor a point mutation in the retinoic acid receptor ligand-binding domain confers dominant negative activity. Blood 80:1885–1889.

Ross S, McCafery P, Drager U, De Luca L. 2000. Retinoids in embryonal development. Physiol Rev 80:1021–1054.

Roy B, Taneja R, Chambon P. 1995. Synergistic activation of retinoic acid (RA)-responsive genes and induction of embryonal carcinoma cell differentiation by RA receptor α (RARα)-, RARβ-, or RARγ-selective ligand in combination with a retinoid X receptor-specific ligand. Mol Cell Biol 15:6461–6487.

Rusten L, Dybedal I, Blomhoff H, Blomhoff R, Smeland E, Jacobsen S. 1996. The RAR-RXR as well as the RXR-RXR pathway is involved in signalling growth inhibition of human $CD34^{+}$ erythroid progenitor cells. Blood 87:1728–1736.

Sato S, Fujita N, Tsuruo T. 2002. Interference with PDK1-Akt survival signalling pathway by UCN-01 (7-hydroxystaurosporine). Oncogene 21:1727–1738.

Slosberg E, Yao Y, Xing F, Ikui A, Jirousek M, Weinstein I. 2000. The protein kinase Cb-specific inhibitor LY379296 blocks TPA-induced monocytic differentiation of HL60 cells. Mol Carcinog 27:166–176.

Soprano D, Qin P, Soprano K. 2004. Retinoic acid receptors and cancer. Ann Rev Nutr 24:201–221.

Szondy Z, Reichert U, Bernardon J, Michel S, Toth R, Karaszi E, Fesus L. 2001. Inhibition of activation-induced apoptosis of thymocytes by all-*trans*- and 9-*cis*-retinoic acid is mediated by retinoid receptor-alpha. Biochem J 331:767–774.

Tocci A, Parolini I, Gabbianelli M, Testa U, Luchetti L, Samoggia P, Massella B, Russo G, Valtieri M, Peschle C. 1996. Dual action of retinoic acid on human embryonic/fetal hematopoiesis: Blockade of primitive progenitor proliferation and shift from multipotent/erythroid/monocytic to granulocytic differentiation program. Blood 88: 2878–2888.

Tsai S, Collins S. 1993. A dominant negative retinoic acid receptor blocks differentiation at the promyelocyte stage. Proc Natl Acad Sci USA 90:7153–7157.

Vara J, Casado E, De Castro J, Cejas P, Belda-Iniesta C, Gonzalez-Baron M. 2004. PI3K/Akt signalling pathway and cancer. Canc Treatment Revs 30:193–204.

Vlahos C, Matter W, Hui K, Brown R. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 269:5241–5248.

Vuligonda V, Thacher S, Chandraratna R. 2001. Enantioselective synthesis of potent retinoid X receptor ligands: Differential biological activities of individual antipodes. J Med Chem 44:2298–2303.

Wang X, Studzinski G. 2001a. Activation of the extracellular signal-regulated kinases (ERKS) defines the first phase of 1,25-dihydroxyvitamin D_3-in differentiation of HL60 cells. J Cell Biochem 80:471–482.

Wang X, Studzinski G. 2001b. Phosphorylation of Raf-1 by kinase suppessor of Ras is inhibited by "MEK-specific" inhibitors PD 098059 and U1026 in differentiating HL60 cells. Exp Cell Res 268:294–300.

Wang J, Yen A. 2004. A novel retinoic acid-responsive element regulated retinoic acid-induced BLR1 expression. Mol Cell Biol 24:2423–2443.

Wang Q, Wang X, Studzinski G. 2003. Jun N-terminal kinase pathway enhances signalling of monocytic differentiation of human leukaemia cells induced by 1,25-dihydroxyvitamin D_3. J Cell Biochem 89:1087–1101.

Witcher M, Ross D, Rosseau C, DeLuca L, Miller W. 2003. Synergy between all-*trans* retinoic acid and tumor necrosis factor pathways in acute leukaemia cells. Blood 102:237–245.

Yanase T, Mu Y-M, Nishi Y, Goto K, Nomura M, Okabe T, Takayanagi R, Nawata H. 2001. Regulation of aromatase by nuclear receptors. J Steroid Biochem Molec Biol 79: 1870192.

Yang C, Kazanietz M. 2003. Divergence and complexities in DAG signalling: Looking beyond PKC. Trends Pharm Sci 24:602–608.

Yang S-H, Sharrocks A, Whitmarch A. 2003. Transcriptional regulation by the MAP kinase signalling cascades. Gene 320:3–21.

Yen A, Roberson M, Varvayanis S. 1999. Retinoic acid selectively activates the ERK2 but not JNK or p38 MAP kinases when inducing myeloid differentiation. In Vitro Cell Dev Biol Anim 35:527–532.

Yen A, Fenning R, Chandraratna R, Walker P, Varvayanis S. 2004. A RARβ/γ selective prodrug (Tazarotene) plus RXR ligand induces ERK activation, RB hypophosphorylation, G0 arrest and cell differentiation. Mol Pharm 66: 1727–1737.

Zhang J, Zhuang W, Poon K, Yang M, Fong W. 2003. Induction of HL-60 cell differentiation by the p38 mitogen-activate protein kinase inhibitor SB203580 is mediated through the extracellular signal-regulated kinase signalling pathway. Anticanc Drugs 14:31–38.

Zhao K-W, Li X, Zhao Q, Huang Y, Li D, Peng Z-G, Shen W-Z, Zhao J, Zhou Q, Chen Z, Sims P, Wiedmer T, Chen G-Q. 2004. Protein kinase Cδ mediates retinoic acid and phorbol nyristate aceteate-induced phospholipid scramblase I gene expression: Its role in leukaemic cell differentiation. Blood 104:3731–3738.

Zhu S, Li Y, Wang Y, Xiao Z, Vihko P, Piao Y. 2002. Retinoic acids promote the action of aromatase and 17β hydroxysteroid dehydrogenase type 1 on the biosynthesis of 17β-estradiol in placental cells. J Endocrinol 172: 31–43.