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Activation of mammalian retinoid X receptors by the insect growth regulator methoprene

(retinoic acid receptor/retinoic acid/juvenile hormone/isoprenoids/pesticide)

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ABSTRACT **We report that methoprene and its derivatives can stimulate gene transcription in vertebrates by acting through the retinoic acid-responsive transcription factors, the retinoid X receptors (RXRs). Methoprene is an insect growth regulator in domestic and agricultural use as a pesticide. At least one metabolite of methoprene, methoprene acid, directly binds to RXR and is a transcriptional activator in both insect and mammalian cells. Unlike the endogenous RXR ligand, 9-*cis*-retinoic acid, this activity is RXR-specific; the methoprene derivatives do not activate the retinoic acid receptor pathway. Methoprene is a juvenile hormone analog that acts to retain juvenile characteristics during insect growth, preventing metamorphosis into an adult, and it has been shown to have ovicidal properties in some insects. Thus, a pesticide that mimics the action of juvenile hormone in insects can also activate a mammalian retinoid-responsive pathway. This finding provides a basis through which the potential bioactivity of substances exposed to the environment may be reexamined and points the way for discovery of new receptor ligands in both insects and vertebrates.**

With the exception of their role in vision, the manner in which the retinoids exert their biological effects resides in their ability to regulate gene expression. Vitamin A metabolites—i.e., retinoids—play essential roles in many aspects of development, metabolism, and reproduction in vertebrates (1). Some of the end products of vitamin A metabolism have been identified as the molecules responsible for the action of retinoids. Retinol, the major circulating form of retinoid, is converted within cells to all-*trans*-retinoic acid and 9-*cis*-retinoic acid (9cRA) (2–4). The retinoic acids function through two classes of receptors: the retinoic acid receptors (RARs), which bind to both atRA and 9cRA, and the retinoid X receptors (RXRs), which bind only to 9cRA. These receptors modulate ligand-dependent gene expression by interacting as RXR/RAR heterodimers or RXR homodimers on specific target-gene DNA sequences known as hormone response elements. In addition to their role in retinoid signaling, RXRs also serve as heterodimeric partners of nuclear receptors for vitamin D, thyroid hormone, and peroxisome proliferators (reviewed in ref. 5).

Although both RXR and RAR bind and respond to 9cRA, evolutionarily these receptors are quite distinct. RXR and RAR share only 27% amino acid identity in their ligand-binding domains (6). In addition, at least one homolog of RXR has been identified in insects, called ultraspiracle (7). Like RXR, ultraspiracle serves as a heterodimeric partner to other receptors. For example, the ecdysone receptor requires ultraspiracle as its coreceptor to bind and respond to its ligand, 20-hydroxyecdysone (8). Significantly, however, ultraspiracle does not respond to any of the known retinoids, including 9cRA. These results are consistent with the finding that insects do not appear to have a retinoid requirement (except for vision) to maintain viability. In insects, besides ecdysone, there is found another lipophilic hormone, juvenile hormone (JH), that is chemically similar to the retinoids (9, 10). Like the retinoid and steroid hormones, JH is synthesized from the common isoprenoid precursor farnesol pyrophosphate via the mevalonate biosynthetic pathway. Given the shared portions of the biosynthetic pathways of the known insect and vertebrate hormones and the similarity between the insect and mammalian receptors, we investigated the possibility that JH-like ligands may have homologs in mammalian systems. To examine the prospect of alternative RXR ligands, we screened several natural and synthetic isoprenoid compounds for their ability to activate transcription from an RXR response element. This screen identified several RXR-selective agonists that are metabolites of the noncyclic synthetic terpenoid methoprene.

MATERIALS AND METHODS

Schneider and CV-1 Cell Cotransfection Assays. Construction of receptor expression and reporter plasmids for expression in *Drosophila* and mammalian cells has been described (4, 6, 11, 12). Schneider cells in 6-well culture plates were cotransfected with 0.5 μg of the luciferase reporter plasmid ADH-CRBPII-LUC, 0.5 μg of the β-galactosidase expression plasmid A5C-βgal, and 1 μg of receptor expression plasmid A5C-mRXRα, A5C-mRXRβ, A5C-mRXRγ, or A5C-hRXRα) by the calcium phosphate precipitation method (4, 11). After 24 h, candidate ligands or solvent control was added to the medium nourishing the transfected cells. The cells were harvested 36 h later, and extracts were prepared and assayed for enzyme activity (4, 11). Luciferase values were normalized for transfection and harvesting efficiency by measuring β-galactosidase activity, and the results reported as average relative light units (RLU) of at least two replicates. Candidate RXR ligands were synthesized at Ligand Pharmaceuticals, except for JH III and farnesol (Sigma), and lauric (dodecanoic), traumatic (2-dodecenedioic), and octanoic acids (Aldrich). All ligands were dissolved in ethanol or methanol and delivered to cells at 1:1000 dilution [0.1% (vol/vol) of solvent in medium]. CV-1 cells were seeded into 48-well culture dishes and cotransfected with 50 ng of TK-CRBPII-LUC reporter plasmid, 50 ng of CMX-βgal expression plasmid, and 25 ng of receptor expression plasmid (CMX-mRXRα, CMX-mRXRβ, or CMX-mRXRγ) by using the calcium phosphate method as described (4, 11). After 8 h, the precipitate was washed off the cells with phosphate-buffered saline (PBS; 138 mM NaCl/2.7 mM KCl/1.2 mM KH_2PO_4/8.1 mM Na_2HPO_4, pH 7.4), and medium containing the appropriate concentration of ligand was added

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Abbreviations: JH, juvenile hormone; 9cRA, 9-*cis*-retinoic acid; RXR, retinoid X receptor; RAR, retinoic acid receptor; RLU, relative light units; hRAR, human RAR.

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to the plates. Luciferase activity was determined 36 h later as described above.

Ligand Binding Assay. Competitive ligand-binding experiments were performed with baculovirus-expressed human RXRα protein by utilizing a hydroxyapatite-binding assay as detailed (13, 14). In this assay, ^{3}H-labeled 9cRA specifically bound to RXRα was displaced by increasing concentrations of unlabeled methoprene or methoprene acid competitor.

GAL4-Receptor Chimera Cotransfection Assays. GAL4-receptor chimera constructs consisted of the GAL4 DNA-binding domain (amino acids 1–147; ref. 15) ligated in-frame to the ligand-binding and C-terminal activation domains of human RARα (hRARα) (amino acids 186–462; ref. 16), hRARβ (amino acids 147–448; ref. 17), hRARγ (amino acids 156–454; ref. 18), or hRXRα (amino acids 203–462; ref. 6). These GAL4-receptor chimeras were then introduced into CMX expression vectors (12). The GAL4-responsive reporter plasmid TK-MH100x4-LUC was constructed by inserting four copies of the yeast upstream activating sequence UAS_G enhancer—i.e., MH100—(19) into the luciferase reporter plasmid TK-LUC. CV-1 cells seeded into 48-well culture plates were cotransfected with 80 ng of the reporter plasmid TK-MH100x4-LUC, 50 ng of CMX-βgal expression plasmid, and 30 ng of receptor expression plasmid (either CMX-GAL4 as a control or CMX-GAL4-hRARα, CMX-GAL4-hRARβ, CMX-GAL4-hRARγ, or CMX-GAL4-hRXRα). Ligand addition and the determination of luciferase activity were performed as described above.

RESULTS AND DISCUSSION

As part of an ongoing search for new hormone-like substances, we screened several natural and synthetic compounds that are exposed to the environment for their ability to activate nuclear hormone receptors. For these studies, we utilized a cotransfection assay similar to that used to identify the RXR ligand, 9cRA (4, 11). Candidate ligands were initially tested in Schneider cells cotransfected with an RXR expression plasmid and a luciferase reporter plasmid containing an RXR-specific response element. This reporter plasmid contains a minimal promoter and the hormone response element from the rat cellular retinol binding protein II gene (CRBPII) which can be activated by RXR but not by RAR (20). Fig. 1*A* shows that transcription from this reporter construct was activated by 9cRA and several analogues of JH, including methoprene [isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate], hydroprene [ethyl (2*E*,4*E*)-3,7,11-trimethyl-2,4-dodecadienoate] (data not shown), and their derivatives, methoprene acid and hydroprene acid. Although methoprene and hydroprene are known for their potent JH-like activity in insects, JH III (Fig. 1*A*) and JH I (data not shown), as well as the JH precursor, farnesol, do not activate RXR in this assay. Chemically similar carbon chain fatty acids also are inactive. Interestingly, all of the RXR activators are derivatives of isoprene (Fig. 1*B*), a structural motif that is found in a variety of biologically important lipophilic molecules (21).

These results indicate that RXR can respond to both the ester and acid forms of the JH analogues shown in Fig. 1*B*. To determine which of these compounds may bind RXR as ligands, competitive ligand-binding assays were performed. For these experiments we used a hydroxyapatite assay (14) to measure the ability of the RXR agonists to compete with 9cRA for binding to baculovirus-produced RXRα protein (13). As shown in Fig. 2, methoprene acid competes with [^{3}H]9cRA for binding to RXRα in a concentration-dependent manner, demonstrating that the acid derivatives bind directly to RXR. In contrast, methoprene is unable to compete with 9cRA for RXR binding at any concentration. The ability of methoprene to activate but not bind RXR suggests that the ester is metabolically converted to the active acid form in cells. This hypothesis is supported by previous studies showing that methoprene is metabolized within cells to several products and that one of the major forms is the methoxy acid derivative, methoprene acid (22). Esterases that may facilitate this conversion are found in many cell types; organs and cells that display high levels of esterase activity include the pancreas, liver, and macrophages.

To further test the ability of methoprene acid to function as an RXR ligand, we investigated its transactivation properties on all three RXR subtypes. Methoprene acid transactivates RXR in both Schneider cells (Fig. 3*A*) and in CV-1 cells (Fig. 3*B*). The dose response for methoprene acid on each of the three RXR subtypes demonstrates a difference in maximal response but approximately the same EC_{50} for each of the receptors (2 μM in Schneider cells and 20 μM in CV-1 cells). Similar to the effects seen with 9cRA (11), RXRα and -γ

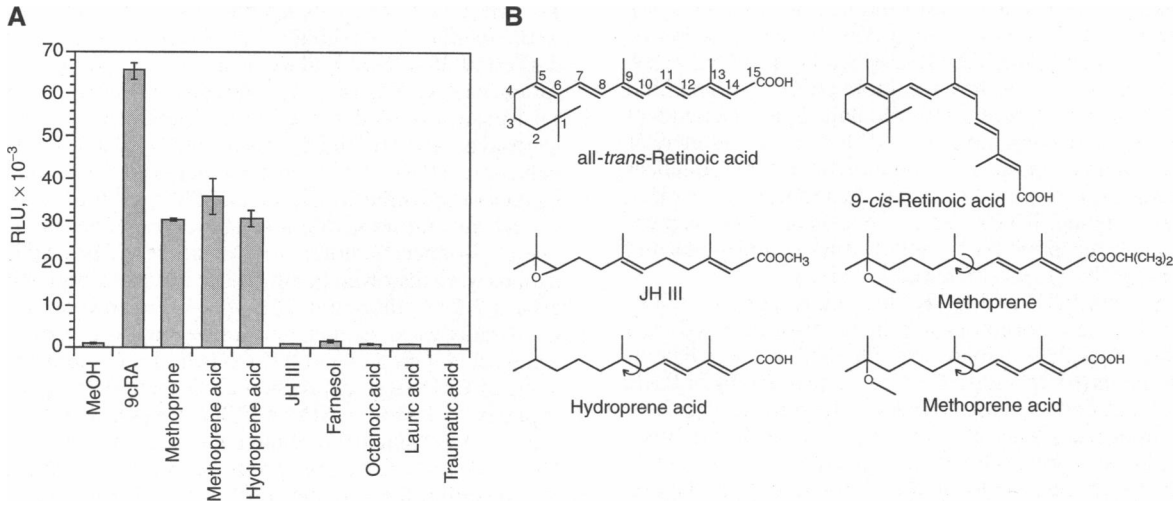

FIG. 1. Isoprenoid activators of RXR. (*A*) Ligand-screening assay. Schneider cells were cotransfected with the expression plasmid A5C-hRXRα (6) and the luciferase reporter plasmid ADH-CRBPII-LUC (6) and then incubated with either methanol as a solvent control or with one of the indicated compounds. Cell lysates were then assayed for luciferase activity, which is expressed as RLU and represents the mean of triplicate assays (± SEM) normalized to β-galactosidase activity as an internal control. Relative inductions by RXR activators above methanol control were 175-fold for 10^{-5} M 9cRA, 80-fold for 10^{-4} M methoprene, 95-fold for 10^{-5} M methoprene acid, and 81-fold for 10^{-4} M hydroprene acid. The concentration of other compounds was 10^{-4} M. (*B*) Structures of retinoid and juvenoid analogues. The arrow shown on the structures of methoprene and its derivatives indicates that rotation around this bond allows a conformation similar to the 9-*cis* bond in retinoic acid.

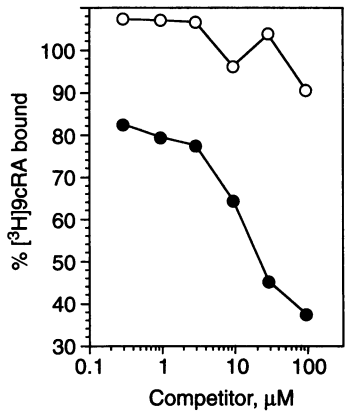

FIG. 2. Methoprene acid binds RXR. Competition for 9cRA binding to RXRα by methoprene acid was accomplished by incubating human RXRα protein with 40 nM [^{3}H]9cRA in the presence of increasing concentrations of unlabeled methoprene (○) or methoprene acid (●). Specific binding is expressed as percent [^{3}H]9cRA bound to RXRα, where 100% is the amount of specific 9cRA binding in the absence of competitor. All data points represent the mean of triplicate assays. In other experiments we have also shown that hydroprene acid but not hydroprene is an RXR ligand. Experiments with RXRβ and -γ have revealed similar binding results.

respond strongly, whereas RXRβ responds only weakly to methoprene acid.

To demonstrate that the action of methoprene acid is specific for RXR, cotransfection experiments were performed with chimeric receptors in which the GAL4 DNA-binding domain was fused to the ligand-binding domains of RXR or one of the three RAR subtypes (Fig. 4). These chimeric proteins can bind to a GAL4 upstream activation sequence (UAS_G) in the promoter of a luciferase reporter construct but can only activate transcription in the presence of the hybrid receptor's ligand. The distinct advantage of using the GAL4-receptor system instead of the wild-type receptors and response elements is that the GAL4 hybrids provide a sensitive and effective means for assaying receptor–ligand interactions, even in the presence of the cell's endogenous wild-type receptors. As expected, 9cRA can activate transcription with GAL4-RXR and all three of the GAL4-RAR subtypes (Fig. 4). The GAL4 fusion proteins retain the ligand specificity of their wild-type receptor counterparts. This is demonstrated by the ability of the RAR-selective ligand TTNPB (6) to specifically

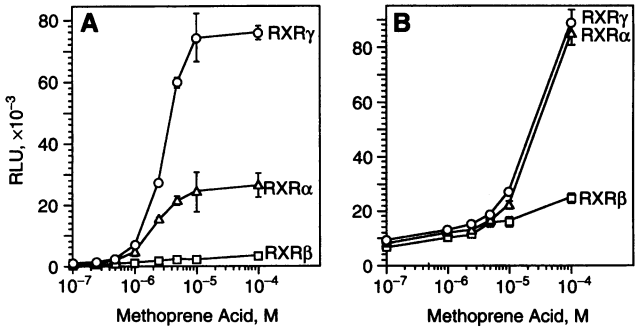

FIG. 3. Methoprene acid is a ligand activator for all three RXRs. Shown are the dose responses of the three RXR subtypes to methoprene acid in insect Schneider cells (*A*) or mammalian CV-1 cells (*B*) cotransfected with expression plasmids for mouse RXRα, -β, or -γ and the reporter plasmid ADH-CRBPII-LUC (for Schneider cells) or TK-CRBPII-LUC (for CV-1 cells). In control experiments (not shown), no ligand-dependent transactivation was observed at any concentration when RXR expression plasmids were excluded from the transfection assay. Transactivation of RXRs is expressed in RLU as described in the legend to Fig. 1.

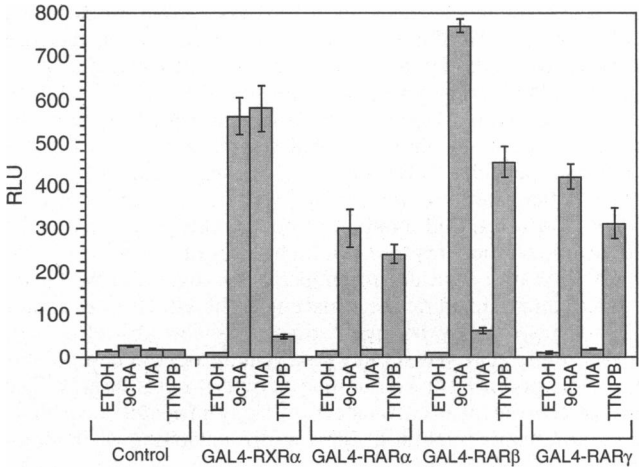

FIG. 4. Methoprene acid is an RXR-specific ligand. CV-1 cells were cotransfected with the expression plasmid CMX-GAL4-hRXRα, CMX-GAL4-hRARα, CMX-GAL4-hRARβ, or CMX-GAL4-hRARγ and the reporter plasmid TK-MH100x4-LUC. Cells were then incubated with ethanol, 10 μM 9cRA, methoprene acid (MA), or the RAR-selective ligand ethyl *P*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoic acid (TTNPB) (6). Receptor transactivation of RXRs is expressed in RLU as described in the legend to Fig. 1.

activate only the GAL4-RARs. In contrast, methoprene acid is specific for activation of only the GAL4-RXR. The discovery of ligands selective for RXRs and RARs should provide useful tools for dissecting these two receptor pathways.

One paradox of the retinoid receptors is the ability of 9cRA to function as a ligand for both RARs and RXRs. Evolutionarily, the two receptor systems are distinct, and inspection of their ligand-binding domains reveals that they are quite dissimilar (only 27% identity between RARα and RXRα; ref. 6). This observation, taken together with the ability of RXR to be specifically activated by noncyclic terpenoids, such as methoprene acid, has led us to speculate the existence of other naturally occurring RXR-specific ligands. The larger concentrations of methoprene acid needed to elicit a response may reflect differences in cell permeability to this synthetic compound or it may indicate that the compound undergoes further metabolism. In other preliminary experiments, we have demonstrated that methoprene may be converted in cells to metabolites that are more potent than methoprene acid and indeed may be higher affinity ligands. Such results are reminiscent of the original observation that all-*trans*-retinoic acid could activate RXR in cells because of its metabolic conversion to the higher affinity ligand 9cRA. Although methoprene acid is not a naturally occurring compound, the structure of methoprene acid might provide clues toward finding its natural counterpart in vertebrates. Inspection of the chemical structures shown in Fig. 1*B* permits a comparison between the RXR-active and -inactive compounds. For example, one notable difference between JH and methoprene is the lack of a double bond at the position equivalent to the 9-10 carbons of the retinoic acids. The equivalent *trans* configuration in JH is shifted in methoprene, allowing for free rotation around this bond. The *cis* or *trans* form of retinoic acid is the critical feature that determines its ability to bind to RXR and is likely the reason methoprene but not JH can activate RXRs. Likewise, the cyclohexene ring found in retinoic acid but absent in methoprene acid may be an important determinant for RAR binding.

Methoprene was the first insect growth regulator (IGR) to be approved by the Environmental Protection Agency for experimental use against mosquitos (23). Methoprene is now used in a variety of domestic and agricultural products. Potential uses include its use as an additive in carpet flea

products, tobacco, cattle feed and water, fruit waxes, and stored grain (23–25). IGRs were approved on the basis of studies conducted by the U.S. Department of Agriculture and private laboratories (for licensing purposes) in the 1970s, which showed minimal adverse effects and established methoprene as a safe means of insect control when compared with conventional pesticides (24). From the studies presented here, it is clear that methoprene may be considered both a JH analogue and a retinoid analogue. Indeed, this may explain the reported teratogenic effects of high doses of methoprene that have been observed during mouse embryogenesis, which included limb deformities reminiscent of the effects of retinoids (26). Thus, it may be interesting to review the effects of methoprene acid and similar noncyclic terpenoid compounds for their potential hormone-like activity in vertebrates. The use of the receptor cotransfection assay provides a sensitive and noninvasive method for rapidly screening such compounds.

One consequence of the ability of RXR to be activated by methoprene is the implication that a parallel pathway exists in insects for the mechanism of action of JH or other related terpenoids. To date, there is no definitive evidence for a nuclear JH receptor, although several studies have begun to address the issue (reviewed in ref. 27). The nuclear orphan receptor ultraspiracle would appear to be an ideal JH-receptor candidate, since it is the insect homologue of RXR and shares many of the functional properties of RXR (7, 8). However, under the conditions in which RXR is responsive, ultraspiracle does not respond to any of the retinoids or juvenoids tested, including methoprene acid (ref. 11 and data not shown). Although the true JH receptor may have yet to be discovered, our studies suggest that metabolism may also play an important part in generating the ligand of the JH receptor. The further study of the biological connection between these terpenoids should provide important clues toward the elucidation of the molecular mode of action of juvenoids, retinoids, and other yet-to-be-discovered regulatory lipophilic compounds.

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