EFFECTS OF METHOPRENE, ITS METABOLITES, AND BREAKDOWN PRODUCTS ON RETINOID-ACTIVATED PATHWAYS IN TRANSFECTED CELL LINES

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Abstract—Methoprene (isopropyl (2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate) is an insect juvenile hormone agonist that blocks metamorphosis in some insects. Recent evidence suggests that a metabolite, methoprene acid, activates vertebrate retinoid X receptors (RXRs), and may interfere with retinoic acid–regulated developmental processes. Methoprene, methoxy-methoprene acid, and two major breakdown products were tested for their ability to interfere with retinoid-regulated pathways when using transfected cells. The CV-1 cells were transiently transfected with genes encoding RXRs and response elements attached to luciferase reporters, and retinoic acid–sensitive F9 cells were stably transfected with retinoic acid receptor (RAR)/RXR response elements attached a β-gal reporter (Sil-REM/β-gal-NEO). Experiments confirmed that methoxy-methoprene acid acted as a ligand for RXRs and was capable of activating transcription through RAR/RXR response elements. However, neither methoprene nor the breakdown products, 7-methoxycitronellal and 7-methoxy-3,7,11-trimethyl-2,4-dodecadienoate, activated transcription in transfected CV-1 or F9 cells. Methoprene and methoxy-methoprene acid may interfere with the conversion of all-trans-retinol and all-trans-retinaldehyde to all-trans-retinoic acid in the F9-derived cell line. Methoprene was as effective as the retinol dehydrogenase inhibitor citral in blocking the retinol-induced transcription of RAR/RXR-regulated reporter genes, whereas methoxy-methoprene acid blocked transcription stimulated by retinaldehyde.

Keywords—Methoprene Retinoic acid Retinoic acid receptor Retinoid X receptor Retinol

INTRODUCTION

Numerous recent reports have described the widespread appearance of malformed frogs and other amphibians in North America (see www.npwrc.usgs.gov/narcam). Most common are hind limb malformations; including partial or total deletions; split limbs or limb segments; duplications resulting in as many as eight complete or partial legs; and a variety of incomplete, twisted, or folded bones [1]. Although similar examples have been found in wild populations in the past [2], comparisons between current and historic rates suggest that malformations are now occurring in higher frequency and that populations with malformed individuals are more broadly distributed [3]. To date, the proximate cause (or causes) of the malformations have not been established, but plausible candidates include trematode parasites, which have been associated with limb malformations in wild frogs and can induce malformations in certain species in laboratory experiments [4]; ultraviolet-B radiation, which has been shown to cause limb deletions in native ranid frogs [5,6]; and xenobiotic chemicals that mimic retinoic acid.

During vertebrate development, retinoids act as ligands for two classes of receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which, in turn, function as transcription factors that regulate gene activity involved in pattern formation in the body axes, brain, and limbs [7]. Among the most potent retinoids are all-trans-retinoic acid (atRA) and 9-cis-retinoic acid (9cRA). The former binds and activates RARs, whereas the latter binds both RARs and RXRs. Application of exogenous retinoids during limb development or regeneration can result in a variety of malformations in vertebrate models [8]. For instance, atRA or 9cRA applied to developing limb buds in beads or paper can cause limb and digit duplications [9], whereas global application of retinoids to regenerating amphibian limbs can induce hypomorphic growth, resulting in truncated or reduced limbs [10]. In addition, Maden [11] has shown that retinoic acid applied to regenerating tails of certain anuran species can induce a homeotic transformation of tail tissue into trunk and limbs. Studies to establish whether developing amphibians have been exposed to natural or anthropogenic agents that cause malformations have been initiated [12], but retinoids or retinoid mimics have not been reported in water or sediment samples from affected areas.

Retinoids share a structural relationship with other terpene-based signaling molecules, such as the plant hormones abscissic acid and gibberellin, vertebrate steroids, invertebrate ecdysteroids, and insect juvenile hormone (JH) [13]. Juvenile hormones regulate the transformation between larval stages in insect development, and manipulation of JH titer by exposure to synthetic analogs produces a lethal disruption of the endocrine system; this has been successfully employed to control insect populations by using a class of pesticides known as insect growth regulators [14]. Methoprene (isopropyl (2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate), an analog of JHIII, is widely applied to wetlands in urban and suburban areas to reduce mosquito populations, as a feed additive for dairy cows to control flies, and in flea collars and sprays [15]. Because insect JHIII has no apparent homolog in vertebrates, methoprene and the other JH analogs are thought to be particularly safe as pesticides for use in domesticated animals and in areas of human habitation. However, methoxy-methoprene acid (methoprene acid), a relatively stable metabolite [16], has been shown to activate transcription of vertebrate RXR-stimulated reporter genes in transfected insect cells [17].

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Methoprene undergoes rapid isomerization and degradation when exposed to light and oxygen [18,19], and is readily metabolized by bacteria, insects, and vertebrates [16,18]. Microbial metabolism yields methoprene acid [16], as well as 7-methoxy-citronellal, and its corresponding acid [16,18] as major products. This study sought to establish whether methoprene and its common breakdown products or metabolites could activate transcription mediated by the vertebrate RXR.

MATERIALS AND METHODS

Methoprene and retinoids

All retinoids, citral, and citronellall were obtained from Sigma Chemical (St. Louis, MO, USA). Methoprene was from Chem Service (West Chester, PA, USA). Methoxy-methoprene acid, 7-methoxy-citronellal, and 7-methoxy-citronelllic acid were generously provided by Wellmark International (Bensenville, IL, USA). Retinoids and methoprene products were kept in dark glass bottles and stored at −20°C, and all treatments were conducted under dimmed indirect light.

Cell culture

Cells were grown in cell dishes or multiwell plates (Falcon, Franklin Lakes, NJ, USA) in Dulbecco modified Eagle medium (Sigma) containing pyruvate, glutamine, and charcoal–dextran–stripped fetal bovine serum (Hyclone, Logan, UT, USA) at 37°C in a 5% CO₂ atmosphere. The CV-1 cells (African green monkey kidney, American Type Culture Collection, Rockville, MD, USA) were grown with 10% charcoal–dextran–stripped fetal bovine serum. The F9 cells (mouse teratocarcinoma; American Type Culture Collection) were grown in gelatinized plates with 15% charcoal–dextran–stripped fetal bovine serum. The F9 S1 cells (Stable transfection of F9S:1 cells) were a gift from David Mangelsdorf (Howard Hughes Medical Institute, UT, USA) at 37°C in a 5% CO₂ atmosphere. This stably transfected line was derived [20] from F9 cells by insertion of a gene consisting of the retinoic acid receptor response element from the promoter region of the RARβ 2 gene attached to a structural β-galactosidase gene, contained in a plasmid (pMSG) carrying a gene conferring resistance to the antibiotic gentamicin (G-418). This retinoic acid receptor response element is activated by RAR/RXR heterodimers. The F9S:1 cells were grown in Dulbecco modified Eagle medium containing 15% charcoal–dextran–stripped fetal bovine serum and G-418 at 0.8 mg/ml as a selective agent.

Transient transfections

Expression plasmids for retinoid receptors RAR (CMX-hRAR), RXRγ (CMX-hRXR), reporter constructs consisting of the retinoic acid response element from the cellular retinoic acid binding protein II (CRABP II) gene, which binds RXR/RXR homodimers, attached to structural elements of the luciferase gene (CRBPII-TK-Luc), and a constitutively active expression plasmid for β-galactosidase (CMX-βgal) were gifts from David Mangelsdorf (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, USA). The CV-1 cells were plated in 24- or 48-well plates at 2 × 10⁴ or 1 × 10⁵ cells/well, respectively. After 24 h, medium was changed and the cells were transfected by using FuGene transfection reagent (Roche, Indianapolis, IN, USA). Receptor and reporter plasmids were transfected at 0.25 μg/well or 0.11 μg/well for 24- or 48-well plates, respectively. Plasmids for β-galactosidase were transfected at 0.1 times the luciferase reporter concentration.

Exposures

Experiments were performed in multiwell plates, and each included a set of wells containing medium alone and a set containing carrier solvent (methanol or dimethylsulfoxide; <1% total volume), but no test chemical. Solvent volumes were equalized in all wells within an experiment; neither dimethylsulfoxide nor methanol at these concentrations affected cell growth or reporter activity. Transient transfection experiments also contained sets of wells containing transfection agent with appropriate solvent volumes. Each control or test concentration was replicated in three or four wells within an experiment. Transiently transfected CV-1 cells were incubated for 12 to 24 h, after which the medium was exchanged for medium containing solvents, retinoids, or test chemicals. After an additional 24 to 48 h, the cells were harvested and analyzed for luciferase and β-galactosidase activity. The F9S:1 cells were seeded in 96-well, gelatin-coated plates at 5,000 cells/well in Dulbecco modified Eagle medium containing charcoal–dextran–stripped fetal bovine serum (15%) and G-418 (0.8 mg/ml). After 24 h, the medium was replaced by fresh medium containing retinoids or other test chemicals, the plates were incubated for 24 h, and the cells were harvested and analyzed for β-galactosidase activity and protein content.

Cell harvesting, extraction, and reporter assays

Cells in culture dishes or multiwell plates were washed twice with ice-cold phosphate-buffered saline, and then treated with lysis buffer (Tropix, Bedford, MA, USA) containing 0.5 mM dithiothreitol. After 15 min at 20°C, the plate was frozen at −80°C, thawed at 20°C, and scraped. Aliquots from each well were analyzed immediately for reporter activity and protein content, or the plate was stored at −80°C until analysis. Luciferase activity was measured in a medium modified from Brasier and Fortin [21], consisting of glycyglycine, 12.5 mM, pH 7.8; KH₂PO₄, 0.5 mM; K₂HPO₄, 6.5 mM; MgSO₄, 7.5 mM; ethyleneglycoltetraacetic acid, 0.5 mM; bovine serum albumin, 0.25 mg/ml; luciferin, 0.5 mM; dithiothreitol, 0.5 mM; and adenosine triphosphate, 1 mM. Luciferase assays were performed in 96-well plates and quantified with a plate-reading luminometer. β-Galactosidase activity was analyzed by using a luminescence assay (Galacto-Light Plus, Tropix) according to the manufacturer’s instructions, and quantified with a plate-reading luminometer. Protein content was determined by the Bradford technique (Bio-Rad, Hercules, CA, USA). Reporter activity was normalized to adjust for unequal cell growth in individual wells and is reported as relative light units. For transiently transfected CV-1 cells, luciferase activity was divided by constitutively expressed β-galactosidase activity in a second aliquot taken from each well; for stably transfected F9S:1 cells, β-galactosidase activity was divided by protein content in a second aliquot taken from each well. The data shown represent at least three experiments with separate passages of F9S:1 or CV-1 cells.

RESULTS

Receptor activation: F9S:1 cells

Stably transfected F9S:1 cells responded to low nanomolar concentrations of both atRA and 9cRA, consistent with the presence of the RAR/RXR-stimulated reporter gene (Fig. 1), in which either ligand can bind to RAR. β-Galactosidase activity was half-maximally stimulated by approximately 1.0 nM atRA and 9.2 nM 9cRA, and was linear through approximately
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Fig. 1. The F9S:1 cells respond to retinoic acid by production of β-galactosidase. Cells were exposed to all-trans-retinoic acid (●) or 9-cis-retinoic acid (▪), incubated for 48 h, then harvested and analyzed for β-galactosidase reporter activity. Data represent mean ± standard deviation of relative light units (RLUs), consisting of β-galactosidase activity divided by protein content. Three wells were used per concentration.

Fig. 2. The F9S:1 cells respond to methoprene acid, but not to methoprene or to its major breakdown products. Cells were incubated with solvent (methanol), retinoic acids, methoprene, methoprene acid, 7-methoxy-citronellal, or 7-methoxy-citronellic acid at the indicated concentrations. After 18 h, the cells were harvested and analyzed for β-galactosidase reporter activity. Data represent mean ± standard deviation of relative light units (RLUs), consisting of β-galactosidase activity divided by protein content. Four wells were used per data point.

Fig. 3. Transiently transfected CV-1 cells respond to 9-cis-retinoic acid and methoprene acid. The CV-1 cells were transiently transfected with genes for retinoid X receptor (RXRα) and RXR homodimer response elements attached to a luciferase reporter, then incubated with solvent (methanol), or 9-cis-retinoic acid, all-trans-retinoic acid, methoprene, methoprene acid, 7-methoxy-citronellal, or 7-methoxy-citronellic acid at the indicated concentrations. After incubation for 24 h, cells were harvested and analyzed for luciferase and β-galactosidase activity. Data from each well are derived as relative light units (RLUs), consisting of luciferase reporter activity divided by constitutive β-galactosidase activity. Four wells were used for each data point in an experiment. Data represent mean ± standard deviation of RLUs for reporter induction above methanol control values for multiple experiments.

Receptor activation: CV-1 cells

In CV-1 cells cotransfected with plasmids for hRXRα and an RXR/RXR-luciferase reporter, both 9cRA and methoprene acid stimulated reporter activity (Fig. 3). The all-trans-retinoic acid, methoprene, 7-methoxy-citronellal, and 7-methoxy-citronellic acid did not stimulate activity beyond that seen in the solvent controls.

Cell morphology

The F9 cells differentiate into primitive and parietal endodermlike cells in response to retinoic acids [22]. In the absence of retinoids, F9 cells formed dense clusters of relatively small cells (Fig. 4). Cells exposed to atRA or 9cRA developed large, irregular shapes, with long processes between cells. In addition, retinoic acid–treated cells were dispersed and distinctly separated, whereas control cells grew in dense clusters. Methoprene had no apparent effect on cell morphology or pattern, resulting in small, compact colonies that resembled untreated cells. Methoprene acid treatment produced some colonies that appeared to have differentiated into primitive endodermlike cells, reminiscent of retinoic acid–treated cells, but the effect was less pronounced than that seen with the retinoids, with many cells remaining in the compacted, undifferentiated form.

5 nM and 20 nM of the two retinoic acids, respectively. Enzyme expression was also linear through 48 h of incubation with either 1 μM atRA or 0.1 μM 9cRA (data not shown).

Methoprene acid also stimulated transcription of β-galactosidase in F9S:1 cells (Fig. 2). Maximal activity was achieved at 75 μM methoprene acid, and activity was generally 25 to 40% of that maximally stimulated by the two retinoic acids. Methoprene and the breakdown products, 7-methoxy-citronellal and 7-methoxy-citronellic acid, did not stimulate activity beyond that seen in the solvent controls.

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Retinol and retinal

Citral is a monoterpene aldehyde that blocks the formation of retinoic acid from retinol and retinaldehyde (retinal) [23], acting as a competitive inhibitor for several enzymes in both pathways [24]. Because the methoprene photolytic product, 7-methoxy-citronellal, is structurally similar to citral, the effects of methoprene, methoprene acid, and 7-methoxy-citronellal were analyzed in F9S:1 cells treated with retinol and retinal.

Retinol and retinal both stimulated β-galactosidase transcription in F9S:1 cells, demonstrating that they contain the metabolic mechanisms to convert both compounds to retinoic acid. Half-maximal activation was approximately 0.1 μM for retinol and 0.3 μM for retinal (data not shown). Citral at 100 mM reduced β-galactosidase production stimulated by retinol by approximately 60%, and that stimulated by retinal by 50% (Fig. 5). This is consistent with the scale of citral inhibition seen in other tissues [23]. 7-Methoxy-citronellal, its corresponding acid, and citronellal had no effect on β-galactosidase production stimulated by either retinoid (data not shown). However, methoprene appeared to be as potent an inhibitor of retinol-induced activity as citral, reducing β-galactosidase activity by approximately 60% at 50 mM and 80% at 100 mM (Fig. 5A). Conversely, methoprene acid did not reduce retinol-stimulated activity. The inhibitory activities of methoprene and methoprene acid were reversed in F9S:1 cells treated with retinal (Fig. 5B). Methoprene had no effect on retinal-stimulated β-galactosidase activity, but methoprene acid inhibited transcription to approximately the same degree as did citral. Methoprene, methoprene acid, and 7-methoxy-citronellal had no effect on activation stimulated by atRA (data not shown).

DISCUSSION

In vitro techniques can be very useful for isolating biochemical interactions and identifying toxic mechanisms of action that might be obscure in whole tissues or organisms. Transiently transfected cells have been extensively used to detect interactions between various nuclear hormone receptor, cofactors, and ligands, as well as to detect biologically active chemicals [25]. Stably transfected cell lines offer a highly reproducible method of detecting low levels of biologically active ligands that act as hormone agonists or antagonists. Murine embryonal carcinoma F9 cells stably transfected with RARβ-lacZ reporters have been used to detect retinoic acid–producing tissues of developing embryos [20,26], as well as for testing retinoic acid production stimulated by various chemicals [27], and for testing complex mixtures for components that interact with retinoid-associated pathways [28]. The F9 cells are naturally sensitive to retinoic acid, responding by activating genes, morphological changes, and differentiation into several
different cell types [22]. In addition, F9 cells express RNA messages for isoforms of the predominant RAR and RXR subtypes [29], making them sensitive to a wide variety of retinoic acids [27].

Methoprene has been applied as a mosquito control agent in metropolitan and suburban areas, to control flies in dairy operations, and as an active ingredient in domestic flea sprays [15]. As a JH mimic, methoprene could be expected to affect insects and other invertebrates that depend upon changes in JH concentration to progress through metamorphosis, but not affect vertebrates and other organisms that lack JH sensitivity. Such selectivity, combined with comparatively low vertebrate toxicity [30], makes methoprene amenable for use in residential and agricultural settings. An additional advantage is that methoprene does not persist in the environment. Applied as a liquid to freshwater microcosms [19], or in water under laboratory conditions [5], methoprene has a half-life of less than 24 h. Photolysis appears to be the most prominent degradative mechanism in aqueous systems [18], followed by microbial metabolism [15,16]. When exposed to sunlight for 7 d in sterile water, methoprene decomposed into four major and more than 40 minor products [15]. The parent molecule was not detected, and the predominant photodecomposition products consisted of 7-methoxy-citronellal (9%), and its acid (7-methoxy-citronelic acid, 7%), along with an epoxide (4%), and a methyl ketone (4%), none of which are active JH mimics. Microbial degradation is dependent on the constitution of the microbial population, as demonstrated by studies in which water samples from different natural sources yielded vastly different compositions of breakdown products [15,16]. Notably, the retinoid mimic methoprene acid constituted 6 to 24% of the breakdown products in microcosm tests [16].

The rapid conversion of methoprene into inactive compounds under environmental conditions led to the development of sustained delivery vehicles, such as granules and briquets, which slowly release the active compound as they degrade in water [19]. When granules are applied at the recommended rates, the expected environmental methoprene concentration is 10 μg/L; however, Hershey et al. [31] reported that in a series of 30 surface dips in three ponds treated at a nominal application rate of 2 μg/L, 50% had values below detection limits (0.4 μg/L), whereas values in the remaining samples ranged from <2.5 μg/L to as high as 510 μg/L. In a similar study, Knuth [32] measured methoprene in 10 replicates in three water bodies; six had values below the detection limit of 0.2 μg/L, and the remaining four had values from 0.39 to 8.8 μg/L. These studies indicate that actual methoprene concentrations in the field, as well as the composition of breakdown products, will depend on the physical, chemical, and biological characteristics of the area.

Retinoic acid is derived predominantly from retinal that is either obtained from nutritive β-carotenes, or from retinol (vitamin A) that is formed from stored retinyl esters [33]. Retinol is converted to retinal by a variety of alcohol dehydrogenases or by short-chain dehydrogenases and reductases. Retinal is then oxidized to retinoic acid through by a variety of aldehyde dehydrogenases [33]. In this study, both retinol and retinal stimulated β-galactosidase reporter activity in F9S:1 cells, indicating that these cells contain the enzymatic pathways necessary to convert these precursors into retinoic acid. Although retinol and retinal have been shown to bind to RAR in competition experiments [34], this occurred only at nonphysiological temperatures, and other studies have shown little or no activation of retinoid receptors with either compound [35], indicating that metabolism to retinoic acid is essential.

Oxidation of retinol to retinal and of retinal to retinoic acid both can be blocked in vitro and in vivo by citral [23], a monoterpene aldehyde that acts as a substrate with a low Michaelis constant and low maximal velocity for retinol and retinal dehydrogenases [24,36]. Testing the effects of methoprene and its products on retinol- and retinal-stimulated reporter activity revealed a complex situation. As expected, citral blocked the stimulation of reporter activity by both retinol or retinal. However, methoprene inhibited retinol-stimulated reporter activity to a degree similar to that produced by equimolar concentrations of citral, whereas 7-methoxy-citronellal had no effect in tests with either retinoid. Methoprene had no effect on reporter activity stimulated by retinal, suggesting that it inhibited the conversion of retinol to retinal. Conversely, methoprene acid did not inhibit retinol-stimulated activity, but it effectively blocked retinal-stimulated activity. Activity levels in the presence of methoprene acid also may reflect direct stimulation of the reporter through RXR, and thus, any inhibition of retinol- and retinal-stimulated activity may be partially masked. Neither methoprene nor methoprene acid inhibited activity stimulated by atRA or 9cRA, so inhibition at the receptor level probably is not the cause of inhibition of retinol- and retinal-stimulated activity.

Further tests of individual enzyme activities, as well as cellular studies, are needed to determine whether methoprene and methoprene acid operate directly on retinol- and retinal-oxidizing enzymes or on transport systems, whether the inhibitory activity is caused by methoprene metabolites, and whether methoprene can block the production of retinoic acid from precursors in vivo. Blocking the conversion of retinol to retinoic acid can have profound developmental effects. When chick wing buds were first exposed to citral, then grafted to unexposed wing buds, the resulting limb developed abnormally, with shortened skeletal elements and cartilage bending [37]. Although similar experiments have not been performed with methoprene or methoprene acid, studies testing the effects of methoprene and its breakdown products and metabolites on developing Rana pipiens and Xenopus [5,38] indicated that methoprene and methoprene acid could cause malformations, but only at concentrations that proved to be toxic.

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REFERENCES
of trematode infection on amphibian limb development and survival. Science 284:802–804.