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Retinoid-induced mu opioid receptor expression by phytohemagglutinin-stimulated U937 cells

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Opioid use may be associated with an increased risk of neurological disease in human immunodeficiency virus (HIV) infection through effects on immune cell function. Studies were performed to examine the effects of specific retinoid receptor activation on mu opioid receptor (MOR) production by phytohemagglutinin (PHA)-stimulated U937 cells, a mononuclear cell line. PHA stimulation increased activation of the MOR promoter as well as levels of MOR mRNA, total receptor protein in cell lysates, and surface and cytoplasmic receptor expression. Retinoid X receptor (RXR) agonist and retinoic acid receptor (RAR) antagonist further increased MOR expression by the PHA-stimulated cells. In contrast, MOR expression was suppressed by RAR agonist and by RXR antagonist. Finally, opioid receptor binding was also increased by RXR agonist and RXR antagonist; no increase in binding occurred in the presence of RAR agonists and RXR antagonist. All together, these studies suggest that MOR expression in U937 cells can be differentially regulated by specific retinoid receptor activation. Such effects may have important clinical relevance for opioid users with HIV infection, including individuals with neurological disease. *Journal of NeuroVirology* (2005) **11,** 157–165.

Keywords: all-*trans* retinoic acid; 9-*cis* retinoid acid; morphine; mu opioid receptor; naloxone; RAR; retinoid receptor; RXR; vitamin A

Introduction

The occurrence of neurological disease in human immunodeficiency virus (HIV) infection has been unequivocally demonstrated to be a cause of significant morbidity and mortality among infected individuals (McCabe, 1990; Simpson and Berger, 1996). The specific role of drugs of abuse, such as the opioid drugs, in increasing the risk of neurological complications is, however, less clear. The effects of opioids are presumably mediated through interactions with opioid receptors, which were originally discovered in the central nervous system. There are now known to be at least three major classes of opioid receptor types, which are designated as mu, kappa, and delta. In the central nervous system, activation of opioid receptors induces prominent effects on functions such as the perception of pain and the regulation of mood and respiration. Opioid receptor activation can also result in a variety of immune effects that occur partially through direct binding to opioid receptors on immune cells, which have been demonstrated to express the all three receptor subtypes. Mu opioid receptor (MOR) transcripts and protein production have been identified in macrophages, T cells, and B cells (McCarthy *et al*, 2001). Immune effects induced by activation of MOR in these cells include, respectively, decreased macrophage-mediated killing, suppression of lymphocyte proliferation, and decreased antibody production (Friedman *et al*, 2003; McCarthy *et al*, 2001).

It was observed that MOR expression by a subclone of SHSY-5Y cells, a human neuroblastoma cell line that produces mu and kappa opioid receptors at an approximately 5:1 ratio, was increased by prolonged exposure to retinoic acid (Jenab and Inturrisi, 2002;

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Zadina *et al*, 1993). The potential effects of retinoids on opioid receptor expression in immune cells have not been previously examined. Retinoids, however, have been demonstrated in numerous studies to have specific effects on immune function, including the suppression of proinflammatory and enhancement of anti-inflammatory cytokine production (Bartlett, 1998; Cantora *et al*, 1994, 1995, 1996; Carman and Hayes, 1991; Chun *et al*, 1992; McLellan *et al*, 1988). These actions are mediated through retinoid binding to retinoic acid receptor (RAR), which binds all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9cis RA), and to retinoid X receptor (RXR), which is bound by 9cis RA (Burri and Kutnick, 1987; Heyman *et al*, 1992; Levin *et al*, 1992; Mangelsdorf *et al*, 1990). The retinoid–retinoid receptor complex then can interact with responsive elements in the regulatory region of target genes and with components of the gene transcriptional complex to directly or indirectly activate or suppress gene expression (Zhang *et al*, 1992). In previous studies, it was that demonstrated that RXR activation suppressed tumor necrosis factor (TNF)-α production by phytohemagglutinin (PHA)-stimulated U937 cells, and that this effect was reversed morphine (Mou *et al*, 2003). Therefore, studies were pursued and are reported here in which U937 cells exposed to selective retinoid receptor agonists and antagonists were examined to determine whether RAR and RXR activation can also differentially regulate MOR expression.

Results

MOR gene promoter activation in U937 cells is increased by RXR agonists and RAR antagonists

To examine the effects of specific retinoid receptor activation on the activity of the MOR gene promoter, the pXP2 construct containing the full-length MOR promoter and a firefly luciferase reporter was transfected into the U937 cells, and then the cells were exposed to the retinoid receptor agonist and antagonist agents as described. These studies showed that PHA stimulation alone, as demonstrated by an increase in firefly luciferase:renilla ratios, induced activation of the MOR promoter (Figure 1). Ratios were even higher for cells exposed to 9cis RA (RAR and RXR agonist), LG101305 (RXR agonist), and LG100815 (RAR antagonist), suggesting an even greater level of activation of the promoter by these agents. In contrast, cells exposed to ATRA (RAR agonist) and LG101208 (RXR antagonist) demonstrated levels of promoter activation that were similar to what was measured for nonstimulated cells.

RXR activation increases mu opioid receptor gene expression in U937 cells

Transcriptional activation of the MOR gene promoter was increased in the presence of RXR agonists and RAR antagonists and inhibited by RXR antagonist and RAR agonist. Therefore, levels of MOR mRNA produced by the cells were examined using real-time polymerase chain reaction (PCR), with the results expressed relative to levels produced by cells exposed to PHA alone. These studies showed that relative MOR mRNA levels produced by nonstimulated cells and by stimulated cells incubated with LG101208 were lower than that observed for PHA-stimulated cells. In contrast, relative levels for stimulated cells also incubated either with 9cis RA, LG100815, or LG101305 were higher than for cells that were incubated with PHA alone. Relative MOR mRNA levels for cells incubated with PHA plus ATRA were similar to those for cells incubated with PHA alone and (Figure 2).

RAR activation decreases total MOR protein production by PHA-stimulated U937 cells

U937 cell lysates were examined for immunoreactive MOR protein on Western blots with detection of β-actin performed as an internal control (Figure 3). These studies showed a low level of MOR protein detection in lysates from nonstimulated control cells and from PHA-activated cells exposed to ATRA.

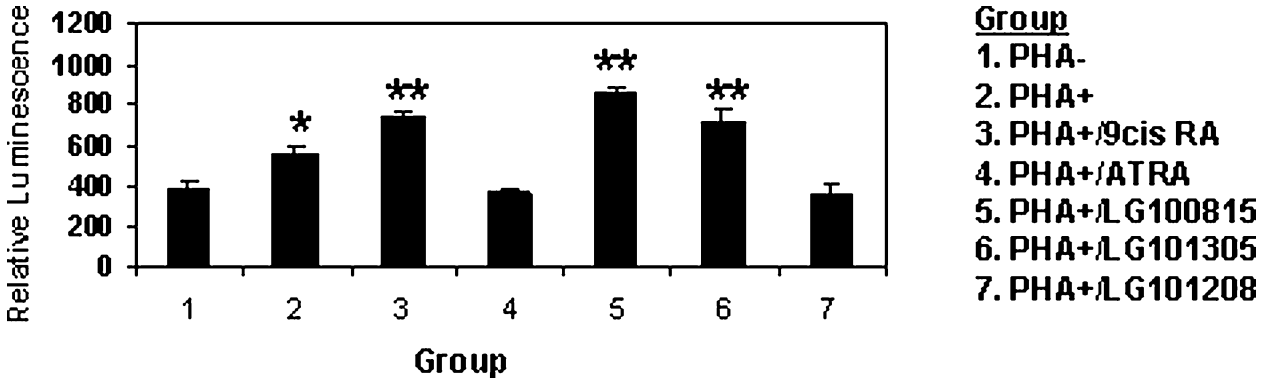

Figure 1 MOR promoter activation by RXR. The cells were transfected with hMOR-Pxp2–firefly luciferase, phRL-SV40–renilla luciferase control vector, and pIRES2-eGFP. Human MOR promoter activation is expressed as a ratio of firefly to renilla luminescence. $^{*}P < .05$; $^{**}P < .01$; $N = 3$ per group.

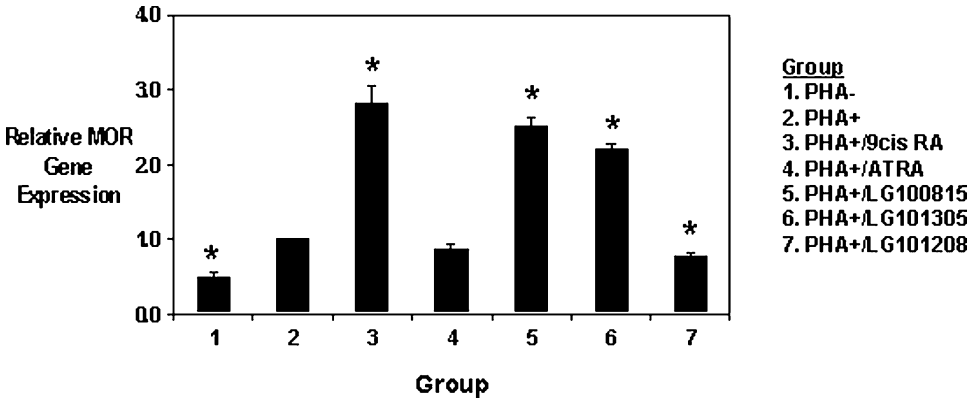

Figure 2 Real-time PCR analysis of relative MOR gene expression by retinoid-exposed cells. The cells were incubated either in medium alone, PHA, or PHA plus either 9cis RA, ATRA, LG101305, LG100815, or LG101208. $^{*}P < .05$; $N = 3$ for each group.

Relative to these cell groups, detection of MOR protein was greater in lysates from cells exposed to PHA alone and from PHA-stimulated cells exposed to either 9cis RA or to LG101305.

RXR activation increases surface and cytoplasmic MOR protein expression

Total U937 MOR protein production, as detected on Western blots, was decreased in the presence of ATRA. In these studies, levels of MOR protein production by the U937 cells were assessed quantitatively by indirect immunofluorescence staining with anti-MOR antibodies followed by flow cytometric analysis using two approaches. In the first, surface expression of MOR was examined (Figure 4A). These studies showed that surface MOR expression by nonstimulated cells was less than that produced by PHA-stimulated cultures. In contrast, stimulated cells also exposed to either 9cis RA, LG101305, or LG100815 resulted in significantly increased surface MOR expression.

In the second approach, intracytoplasmic staining for MOR was examined after incubating the cells with brefeldin, a protein transport inhibitor that induces cytoplasmic accumulation of newly produced protein, followed by fixation and permeabilization of the cells (Figure 4B). These studies also showed that, compared to PHA-stimulated control cultures, decreased MOR levels in cytoplasm for nonstimulated cells and increased levels for cells exposed to PHA plus either 9cis RA, LG100815, or LG101305.

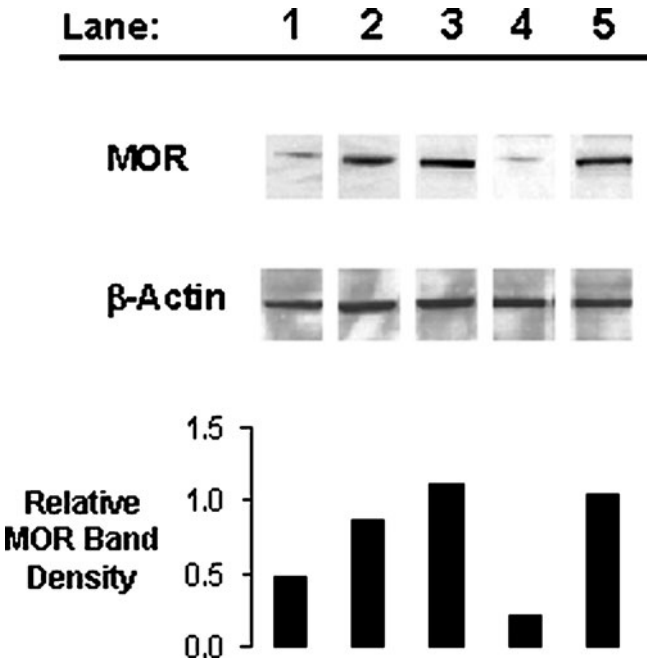

Figure 3 Western blot for MOR. Lysates from the U937 cell groups were examined on Western blots for MOR and for β-actin. The cells were incubated either with medium alone (lane 1), PHA (lane 2), PHA plus either 9cis RA (lane 3), ATRA (lane 4), or LG101305 (lane 5). The bar graph at the bottom shows the density of the MOR band relative to that of the actin control.

Surface MOR binding is increased by RXR activation

MOR promoter activity, mRNA levels, and protein expression were differentially altered by exposure of the cells to the specific retinoid agents. Therefore, studies were performed to examine the effects of such exposure on surface MOR binding. For these experiments, intact cells were incubated with either 0.5 nM fluorescein-labeled naloxone alone or labeled naloxone plus 100 nM morphine sulfate. These studies showed higher levels of surface binding for PHA-stimulated cells than what was observed for nonstimulated cells and stimulated cells incubated with ATRA or LG101208 (Figure 5). Binding, however, was similar for PHA stimulated cells and stimulated cells also incubated either with 9cis RA, LG100815, and LG101305. The noted increased binding, as well as binding noted for nonstimulated control cells, was inhibited by exposure of the cells to morphine.

Discussion

The cellular effects of retinoids have been demonstrated to be of potential benefit in the treatment

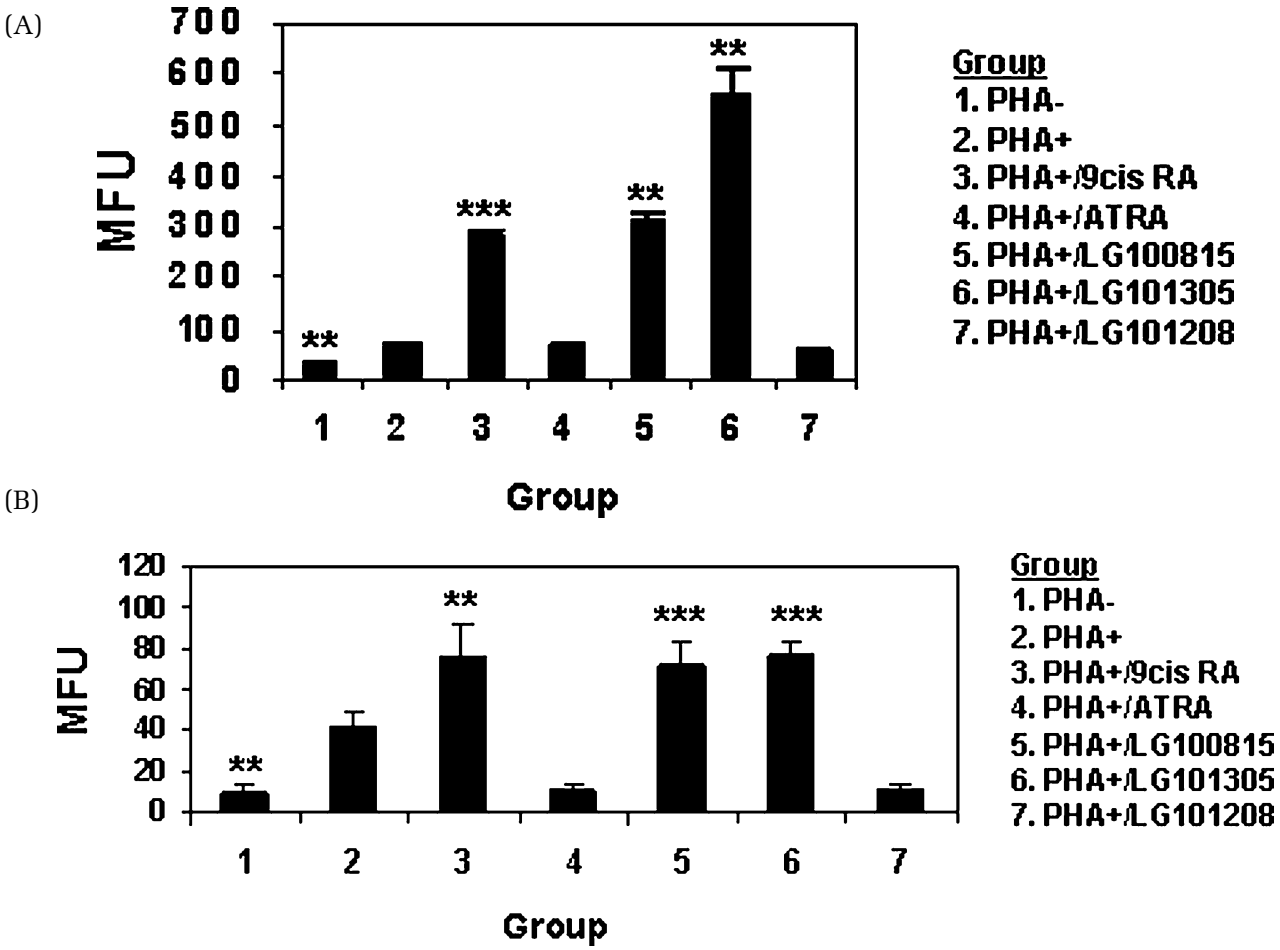

Figure 4 Detection of MOR expression using indirect immunofluorescence and flow cytometry. **A**, Surface MOR expression by U937 cells: Intact cells were stained using rabbit anti-MOR primary and Alexa Fluor-conjugated donkey anti-rabbit secondary antibodies, then analyzed using flow cytometry. **B**, Intracytoplasmic expression of MOR: Cells were permeabilized with detergent (brefeldin A), then stained and analyzed as described in (**A**). $^{**}P < .01$; $^{***}P < .001$; $N = 3$ per group. MFU = mean fluorescence units.

of neoplastic, inflammatory, and infectious disorders such as breast cancer and head and neck squamous cell cancer (Cantora *et al*, 1995, 1996; Jiang *et al*, 1998; Klaassen *et al*, 2001; Soprano and Soprano, 2002), rheumatoid arthritis (Brinckerhoff *et al*, 1980), multiple sclerosis (Royal *et al*, 2002), and measles infection (Hussey and Klein, 1990). Among HIV-1–infected injection drug users, including individuals with a history of opioid abuse, vitamin A deficiency has been associated with lower CD4 counts, more

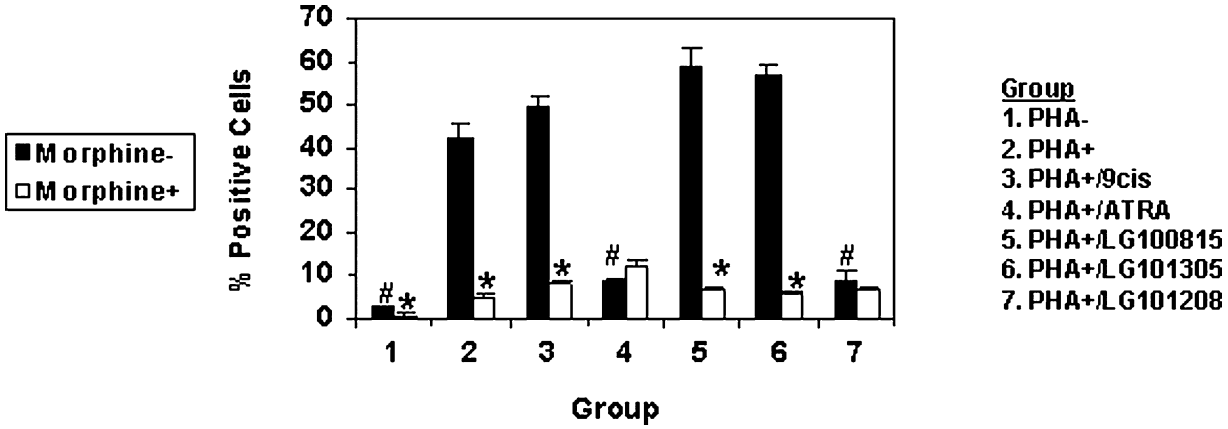

Figure 5 MOR surface binding by U937 cells. Intact nonstimulated control cells and cells incubated with PHA with or without retinoid agonist or antagonist were stained with fluorescein-conjugated naloxone and analyzed by flow cytometry. $^{*}P < .05$; $N = 3$ for each group.

rapid progression of HIV disease, and higher mortality (Semba *et al*, 1993, 1995). However, clinical trials involving replacement therapy with vitamin A have demonstrated no effect on the clinical course of HIV disease (Semba *et al*, 1998). Studies performed *in vitro* suggest that retinoids either can increase or suppress replication of HIV in immune cell lines (Hanley *et al*, 2004; Kitano *et al*, 1990; Poli *et al*, 1992; Turpin *et al*, 1992; Yamaguchi *et al*, 1994). Opioids can induce lymphocytes and astrocytes to express the CCR5 chemokine receptor (Mahajan *et al*, 2002; Miyagi *et al*, 2000; Suzuki *et al*, 2002), which is also a HIV coreceptor, and have been shown to promote immune infection by HIV (Chuang *et al*, 1993; Guo *et al*, 2002; Mahajan *et al*, 2002; Peterson *et al*, 1990, 1999).

Our studies demonstrate that retinoids induce receptor-specific effects on MOR gene expression and protein production. MOR gene expression, intracellular and surface protein production, and MOR binding were induced by PHA stimulation, and gene and protein expression were further increased by exposure to 9cis RA, LG101305, or LG100815. In contrast, exposure to ATRA or to RXR antagonist inhibited these responses. The likely reason that MOR expression is preferentially induced 9cis RA is that the retinoid has a higher affinity for RXR than for RAR (Heyman *et al*, 1992). Morphine, unlike other MOR agonists, does not induce MOR internalization, an occurrence that has been associated, in part, with the development of opioid tolerance (Carlezon *et al*, 1997; Keith *et al*, 1996). Naloxone, like morphine, binds to mu, delta, and kappa opioid receptors, an interaction that also does not result in receptor internalization (Zaki *et al*, 2000). Therefore, although the binding that we observed in our studies was not specific for MOR, it did involve specific interaction with opioid receptor, and retinoid receptor activation induced changes in binding that were consistent with levels of MOR expression detected using MOR-specific antibodies. Notably, the percentage of fluorescein-naloxone–stained cells that were incubated with PHA alone was similar to that noted for cultures also incubated either with LG101305 or LG101208. In contrast, cells incubated with these retinoids produced significantly higher levels of surface and cytoplasmic MOR. This may either reflect the fact that naloxone alone can increase MOR expression by cells (Zadina *et al*, 1993), that MOR binding affinity may have been increased for these cells, or that, indeed, PHA stimulation may have also increased production of delta and kappa opioid receptors. These are questions that also require further study.

The significant role of opioids and opioid receptors in infection is illustrated by studies in which mice implanted with morphine pellets developed bacterial colonization of the peritoneum, liver, and spleen, with death due to sepsis occurring subsequently in a high percentage of the mice (Hilburger *et al*, 1997). It was hypothesized that the infection resulted from colonization of the gastrointestinal tract of the animals with bacteria, followed by seeding of the organisms. This process may have been aided by morphine, which, in addition to decreasing gut motility, has been demonstrated to have a suppressive effect on macrophage phagocytic function (Friedman *et al*, 2003; McCarthy *et al*, 2001; Szabo *et al*, 1993). Morphine can also enhance the production of interleukin (IL)-12 and TNF-α by murine macrophages that are activated with lipopolysaccharide (LPS) and interferon (IFN)-γ in vitro (Peng *et al*, 2000). Therefore, *in vivo* immune responses to components of infection can be potentially directly altered by morphine in association with deleterious consequences to the host. The critical role of MOR in such responses is demonstrated in studies in which wild-type and MOR knockout mice were implanted with morphine pellets (Wang *et al*, 2002). Splenic lymphocytes and peritoneal macrophages were isolated from these animals and examined for effects of morphine on cytokine and nitric oxide production following stimulation with, respectively, concanavalin A and LPS. Morphine suppressed IFN-γ and IL-2 production by splenic lymphocytes stimulated with Con A. In contrast, LPS-activated peritoneal macrophages secreted increased levels of TNF-α, IL-1β, and nitric oxide with morphine exposure. These effects were not observed in the knockout mice, demonstrating that the morphine-induced responses were mediated by binding to MOR. These experimental findings are also reminiscent of our previous studies, utilizing an experimental paradigm that was identical to the one described in this report, which showed that RXR agonist and RAR antagonist suppression of TNF-α production by PHA-stimulated U937 is reversed by exposure to morphine (Mou *et al*, 2003). Here we show that these retinoids increase MOR expression by these cells, presumably sensitizing the cells to the effects of morphine.

The biologically active form of RAR exists as a heterodimer with RXR, and the complex can bind characteristic motifs in gene promoters to either increase or suppress specific gene expression (Mangelsdorf *et al*, 1994). RAR activation induced suppressive effects in our system. It is therefore likely that that the observed RXR-induced enhancement of MOR mRNA and protein expression were due either to effects of RXR homodimers or to heterodimers comprised of RXR and its nuclear receptor partners, which include the peroxisomal proliferator activated receptor (PPAR), thyroid hormone receptor, and vitamin D receptor. The regulatory region of the human MOR has not been reported to contain retinoid receptor response elements, but it does contain those for the glucocorticoid receptor (Wendel and Hoehe, 1998). The glucocorticoid receptor, like other members of this nuclear receptor family, contains DNA and

ligand-binding regions that are homologous to those belonging to the retinoid receptors. No direct interaction, however, has been demonstrated to occur between glucocorticoid and retinoid receptors (Mangelsdorf *et al*, 1994). The question is then raised regarding the mechanisms by which RXR and RAR activation might lead to, respectively, enhancement or inhibition of MOR expression. A possible mechanism that may be involved in effects from RAR is cross-inhibition between the receptor and activator protein (AP)-1, as demonstrated for the osteocalcin gene promoter, which occurs because the respective response elements lie in close proximity, prohibiting simultaneous binding to both sites (Schule *et al*, 1990). Alternatively, RAR may compete with other partner receptors for available RXR, sequester available ligand, or bind components of the transcriptional machinery without directly interacting with DNA (Goldman *et al*, 1997; Mangelsdorf *et al*, 1994). RAR activation has been also demonstrated to suppress kappa opioid receptor expression in P19 neuroblastoma cells (Chen *et al*, 1999). This effect results from ATRA increasing production of the corepressor, ikaros, which is a protein that is encoded by the first intron of the receptor promoter (Hu *et al*, 2001). It is certainly possible that a similar mechanism may underline the suppressive effects of ATRA that we observed, and mechanisms with opposing influences can be evoked to explain effects from RXR. Our report is the first to demonstrate that MOR production is increased in association with activation of specific retinoid receptor subtypes. As such, further studies are required to identify those specific mechanisms that are involved in the regulation of MOR expression in this cell line, in terminally differentiated cells, and *in vivo*.

Materials and methods

Cell cultures and reagents

U937 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in medium containing RPMI 1640 (BioWhittaker, Frederick, MD) supplemented with 10% fetal bovine serum (BioWhittaker), 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 2 mM L-glutamine (ICN Biomedical, Costa Mesa, CA). Cell activation was performed with 12.5 μg/ml PHA (ICN Biomedical). The retinoids that were utilized were ATRA, a RAR agonist (Sigma-Aldrich, St. Louis MO), 9cis RA a RAR and RXR agonist (Sigma-Aldrich), and the synthetic retinoids LG101305, a RXR agonist, LG100815, a RAR antagonist, and LG101208, a RXR antagonist (all from Ligand Pharmaceuticals, San Diego, CA).

Retinoid receptor activation

For these studies, 2×10^5 cells were incubated in a total volume of 200 μl in triplicate cultures in 96-well plates. Nonselective agonist activation of retinoid receptor was performed by incubating the cells with 9cis RA, and selective activation was performed utilizing either a specific receptor agonist alone or by first incubating with an antagonist for either receptor followed by exposure to 9cis RA. Briefly, the cells were first incubated with medium alone or with either 10 nM LG100815 or 10 nM LG101208. Subsequently, to cultures incubated in medium alone was added either 100 nM 9cis RA, 100 nM ATRA, or 10 nM LG101305. Simultaneously, 9cis RA was added to cultures containing the retinoid receptor antagonist agents. After 1 h PHA was added to retinoid-exposed cultures, and all cultures were then incubated for an additional 4 h.

Transient transfection and luciferase reporter assay

Transient transfections were performed in triplicate for each cell treatment in 24-well plates in O_{PTI}–MEM I medium (Invitrogen, Carlsbad, CA) using DMRIE-C reagent (Invitrogen). Each well was transfected with 130 nM of pXP2-hMOR-luc plasmid expressing human mu opioid promoter (kind gift by Dr. Lucinda Carr, University of Indiana, Indianapolis, IN), 130nM of phRL-SV40 (Promega, Madison, WI), which contains the renilla luciferase gene, as an internal control, and 130 nM of pIRES2-eGFP (BD Biosciences), which expresses green fluorescent protein, as a transfection efficiency reference; control wells were transfected with the promoterless pXP2 parent vector (Promega). Additions to each well were adjusted to maintain a constant DNA concentration. A total of 2×10^5 cells were seeded into each well and gently mixed with the transfection reagent and DNA. After incubation for 4 h at 37°C in 5% CO_2, fresh culture medium with 15% fetal bovine serum was added in each well. Following 40 h of incubation, the cells were treated with retinoids and PHA as described above. The cells were then harvested and lysed, and the extracts were assayed for firefly luciferase and renilla luciferase activities using the Dual-Glo Luciferase Assay system (Promega), according to the manufacturer's instructions, in an Orion Microplate Luminometer (Berthold Detection Systems, Pforzheim, GER). The luminescence measurements were used to calculate a luciferase:renilla activity ratio.

MOR polymerase chain reaction assays

MOR mRNA was examined in real-time PCR assays after documentation of an amplification efficiency for the reaction of <0.1. Total RNA was extracted from the cells in culture using RNeasy Mini Columns with Qiashredder column inserts (Qiagen, Valencia, CA) and treated with RNase-free DNAse according to the manufacturer's instructions. Reverse transcriptase (RT)-PCR and subsequent PCR amplification were performed in an iCycler (Bio-Rad, Hercules, CA) using the Qiagen OneStep RT-PCR Kit (Qiagen) according to the manufacturer's directions. Reverse transcription was performed for 30 min at 50°C.

Amplification was initiated by enzyme activation for 15 min at 95°C followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s with SYBR green detection at 37°C. The following primers were used: MOR forward, 5′-GGTACTGGGAAAACCTGCTGAA GATCTGTG-3′, MOR reverse, 5′-GGTCTCTAGTGTT CTGACGAATTCGAGTGG-3′ (Chuang *et al*, 1995); 18S primers primer pairs were purchased from Ambion (Austin, TX). Relative MOR and 18s gene expression in treated versus control samples was calculated using the ΔΔCT method (Winer *et al*, 1999).

Flow cytometry

PHA-stimulated U937 cells were analyzed for surface and cytoplasmic expression of MOR protein by indirect immunofluorescence staining of the cells with specific antibody. For surface MOR detection, the cells were washed twice in phosphate-buffered saline (PBS) then incubated for 30 min with a 1:500 dilution of rabbit anti-human MOR antibody, generated against a recombinant protein corresponding to amino acids 1 to 80 of the amino terminus of human MOR-1 (Santa Cruz Biotechnology, Santa Cruz, CA), then washed twice in PBS, pH 7.4, and incubated for 30 min with a 1:1000 dilution of Alexa Fluor 488–conjugated mouse anti-rabbit polyclonal antibodies (Molecular Probes, Eugene, OR). After a final wash, the stained samples were analyzed using a FACSCalibur equipped with CellQuest Software (BD Biosciences, San Jose, CA) and calibrated using Calibrite Beads (BD Biosciences). Cell populations were identified on forward scatter versus side scatter dot plots with gates set based on signal intensity obtained with nonstained nonstimulated, nonstained stimulated, and stained nonstimulated cells. Alexa Fluor 488–positive cell populations were analyzed using histogram analysis. For analysis of intracytoplasmic MOR, the cells were incubated with GolgiStop (BD Biosciences), then permeabilized and fixed with Cytoperm/Cytofix solution (BD Biosciences) as per the products' instructions. The cells were then stained and analyzed as described for the surface-stained cells.

MOR surface binding was assessed using naloxone fluorescein (Molecular Probes). For these studies, the cells were washed then incubated for 30 min at room temperature with 0.5 M naloxone fluorescein in PBS. After a second wash, the cells were analyzed using flow cytometry as described above.

Western blot analysis

U937 cells were washed in PBS then lysed using lysis buffer (50 mM Tris-HCl pH 8.0, 1% Triton X-100, 0.1% Sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). Samples containing 40 μg protein were then analyzed by SDS-polyacrylamide gel electrophoreses (PAGE) on 12% gels with prestained SDS-PAGE protein standards (Bio-Rad) running in parallel, then transferred onto a nitrocellulose membrane. After incubation of the membranes in a blocking buffer (5% dry milk, 10-mM Tris-HCl, pH 7.5, 150-mM NaCl, 0.05% Tween-20) for 1 h at room temperature to block nonspecific binding, the membrane was incubated with a 1:300 dilution of polyclonal rabbit anti-MOR immunoglobulin G (IgG) (Santa Cruz Biotechnology). The membranes were then washed in PBS, incubated with a 1:2000 dilution of horseradish peroxidase–conjugated donkey anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and, after a second wash, incubated with ECL Western Blotting Detection Reagent (Amersham Biosciences; Piscataway, NJ) as directed by the manufacturer, then visualized by development on KODAK BioMax Light Film (Eastman Kodak, Rochester, N.Y.) in a Kodak automatic film processor. The membrane was then stripped in stripping buffer (100 mM 2-mercaptoethanol, 2% [*w*/*v*] SDS, 62.5 mM Tris-HCl, pH-6.7) as per the recommended protocol (Amersham, Piscataway, NJ), incubated with polyclonal rabbit anti-β-actin IgG (Sigma) and horseradish peroxidase–conjugated donkey anti-rabbit IgG (Vector Laboratories), then visualized with ECL Western Blotting Detection Reagent as above.

Statistical analyses

Statistical analyses were performed using StatView statistical software (Cary, NC). Relative MOR promoter and gene expression values and percentages of positively stained cells identified on flow cytometry for the sample groups were compared using the Mann-Whitney *U* test. Mean fluorescence and relative luminescence measurements were compared using the unpaired *t* test.

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