Peroxisome Proliferators Activate Extracellular Signal-regulated Kinases in Immortalized Mouse Liver Cells*  

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Peroxisome proliferators (PPs) are a class of nongenotoxic carcinogens in the rodent liver. The induction of immediate-early gene expression in immortalized mouse liver cells by the PPs Wy-14,643, monoethylhexyl phthalate, ciprofibrate ethyl ester, and clofibrate suggested that they may be activating growth-regulatory signal transduction pathways. We report that incubation of quiescent ML457 cells with Wy-14,643 resulted in the appearance of two tyrosine-phosphorylated bands of approximately 44 and 42 kDa with maximal phosphorylation at 20 min. These two proteins were identified as extracellular signal-regulated kinases (ERKs) ERK1 and ERK2 (also known as mitogen-activated protein kinases, or MAPKs). Stimulation of quiescent ML457 cells with monoethylhexyl phthalate, ciprofibrate ethyl ester, and clofibrate also resulted in tyrosine phosphorylation of ERK1 and ERK2; however, the steroid PP dehydroepiandrosterone sulfate, which does not induce immediate-early gene expression, did not induce phosphorylation of ERK1 and ERK2. Kinase activity of ERK1 and ERK2 was stimulated by the PPs, consistent with their phosphorylation. The PPs also induced phosphorylation of the upstream regulator MAPK/ERK kinase (MEK). Preincubation of quiescent cells with MEK inhibitor PD98059 blocked activation of ERK1 and ERK2 by the PPs, implicating MEK activation as a requirement for PP-induced ERK activation. In addition, pretreatment with PD98059 greatly reduced the PP-induced expression of immediate-early genes c-fos, egr-1, and to a lesser extent junB. Induction of ERK phosphorylation and junB expression by Wy-14,643 was also seen in rat hepatocytes. These results attribute many of the effects of PPs on immediate-early gene expression to the activation of the MEK/ERK signal transduction pathway and add the PPs to the growing number of tumor promoters that modulate signaling proteins.

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**Materials and Methods**  

No specific materials and methods were described in the text. It is unclear what experimental details were provided in the original article. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PP, peroxisome proliferator; MEHP, monoethylhexyl phthalate; DHEA, dehydroepiandrosterone; DHEA-S, DHEA-sulfate; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PPAR, peroxisome proliferator-activated receptor; RIPA, radioimmune precipitation buffer; FACO, fatty acyl-CoA oxidase; CYP4A1, cytochrome P450-A1; BHTOOh, butylated hydroxytoluene hydroperoxide.

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EXPERIMENTAL PROCEDURES  

Materials—Wy-14,643 was purchased from ChemSyn Science Laboratories (Lenexa, KS). Ciprofibrate ethyl ester was provided by Sanofi Research Division (Malvern, PA). MEHP was provided by Richard B.
Moore of Zeneca Central Toxicology Laboratory (Cheshire, UK). Cofactor, dehydroepiandrosterone sulfate (DHEA-S), and myelin basic protein were from Sigma. PD98059 was from Calbiochem. Antibodies against ERK1 and ERK2 and monoclonal anti-phosphotyrosine antibody PY20 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology, Inc. (Lake Placid, NY). Phospho-specific MAPK and MEK1/2 antibodies were from New England Biolabs Inc. (Beverly, MA). Plasmid clones of c-fos and egr-1 were from American Type Culture Collection (Rockville, MD). The cDNA clone of jun B was provided by Daniel Nathans, The John Hopkins University, Baltimore, MD. Fatty acyl-CoA oxidase (FACO) cDNA probe was from Diane Umbenhauer, Merck Research Laboratories (West Point, PA). Cytochrome P450-A1 (CYP4A1) cDNA was from G. Gordon Gibson, University of Surrey (Guildford, UK).

Cell Culture and Stimulation—ML-457 is an immortalized cell line derived from a CD-1 mouse liver tumor that is not-tumorigenic in nude mice. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin. Before stimulation, confluent cultures were serum-starved by incubation in 0.1% serum-containing medium for 3 days. The quiescent cells were then subjected to the various compounds diluted in medium containing 0.1% serum and 5 mM HEPES, pH 7.1. In experiments using the inhibitor PD98059, cells were incubated with the inhibitor for 1 h before stimulation. Hepatocytes were isolated from Harlan Sprague Dawley rats by standard two-stage perfusion and incubated overnight in serum-free William’s Medium E before treatment. Stock solutions of PPs and PD98059 were dissolved in Me2SO. Upon dilution of stocks in culture media, Me2SO concentrations were 0.2% or lower.

Western Blot Analysis—After stimulation, cells were washed in cold phosphate-buffered saline and lysed in SDS lysis buffer (1% SDS, 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl, pH 7.5). Lysates (50 μg) were separated by 4–20% SDS-polyacrylamide gel electrophoresis and subsequently transferred to Immobilon-P membranes. For anti-phosphotyrosine blotting, membranes were blocked in 1% bovine serum albumin in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.1% Tween 20) for 1 h and then incubated with a 1:2000 dilution of monoclonal antibodies PY20 and G410 in block for 1 h. Identification of ERK1 and ERK2 was accomplished using a 1:2000 dilution of polyclonal anti-ERK1 antibody plus polyclonal anti-ERK2 antibody in 5% nonfat milk in TBST. Phospho-specific ERK (MAPK) and MEK1/2 antibodies were used as specified by the manufacturer. Immunoreactive bands were detected using enhanced chemiluminescence reagents (Amersham Life Science, Inc.).

MAPK Activity—Treated cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, 5 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotinin, 10 μg/ml leupeptin, 20 mM β-glycerophosphate in phosphate-buffered saline). Lysates containing 100 μg of protein were immunoprecipitated by incubation with polyclonal anti-ERK1 and anti-ERK2 antibodies (5 μl of each antibody) for 1 h followed by a second hour with protein A-agarose. Immunocomplexes were washed three times with RIPA buffer and three times with kinase buffer (50 mM Tris-HCl, pH 8, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA). Immunocomplexes were incubated for 20 min at 30 °C in 30 μl of kinase buffer containing 20 μM ATP, 4 μCi of [γ-32P]ATP, and 0.5 mg/ml myelin basic protein. Reactions were terminated by the addition of Laemmli buffer, and 10 μl of each sample was subjected to 10–18% SDS-polyacrylamide gel electrophoresis and then visualized by autoradiography.

Northern Blot Analysis—After stimulation, cells were washed with cold phosphate-buffered saline, and total RNA was isolated by guanidinium isothiocyanate lysis as described by Chomczynski and Sacchi. Total RNA was quantitated by absorbance at 260 nm, and 10 μg of each sample was electrophoresed on a 1.2% agarose, 6% formaldehyde gel. Equal loading of RNA was confirmed by ethidium bromide staining. RNA was transferred to nylon-supported nitrocellulose by capillary blotting, and blots were hybridized overnight with 32P nick-translated DNA (5 × 106 cpm/ml) in 50% formamide, 1 × NaCl, 1% SDS, 100 μg/ml denatured salmon sperm DNA, and 10% dextran sulfate. Blots were washed in 0.1 × SSC (150 mM NaCl, 15 mM sodium citrate), 0.5% SDS at 37 °C and then exposed to Kodak Biomax film.

RESULTS

To determine if the PPs activated a tyrosine kinase cascade, quiescent ML457 cells were treated with 1 mM Wy-14,643 for various lengths of time. This concentration causes optimal induction of immediate-early gene expression while avoiding toxicity (17, 18). Western blotting of whole cell lysates with anti-phosphotyrosine antibodies showed a transient increase in tyrosine phosphorylation of two proteins in response to Wy-14,643 (Fig. 1). The two proteins were approximately 44 and 42 kDa in size and were maximally phosphorylated at 20 min. A low level of phosphorylation in control cells (re-fed with no addition) was seen at 0 and 10 min but disappeared by 20 min, possibly due to the removal of extracellular factors by the media change. Interestingly, these proteins were the same as the downstream regulators of the Ras pathway, ERK1 and ERK2. Immunoprecipitation and Western blotting with anti-ERK antibodies confirmed the identity of p42 and p44 as ERK1 and ERK2 (Fig. 2, see below).

To see if phosphorylation of ERK1 and ERK2 could be found with other PPs, quiescent ML457 cells were treated with 1 mM concentrations of MEHP, ciprofibrate ethyl ester, clofibrate, and DHEA-S. The 20-min time point for each PP is shown in three separate experiments in Fig. 2. ERK tyrosine phosphorylation was examined by Western blotting with anti-phosphotyrosine (Fig. 2A) and anti-phospho ERK (Fig. 2B) antibodies or by immunoprecipitation of ERK proteins followed by anti-phosphotyrosine Western blotting (Fig. 2C). Consistent with its strong induction of immediate-early gene expression (17), MEHP caused a marked increase in the phosphorylation of ERK1 and ERK2 as compared with no addition (Fig. 2, A–C). Two other inducers of immediate-early gene expression, ciprofibrate ethyl ester and clofibrate, also caused a phosphorylation of ERK1 and ERK2. However, the extent of ERK phosphorylation induced by ciprofibrate ethyl ester and clofibrate varied during experiments and is probably due to the oily nature of the compounds, resulting in variable solubilities at the time of treatment. DHEA-S is the more soluble and active metabolite of DHEA, a naturally secreted adrenal steroid (20). Unlike the other PPs, DHEA-S does not resemble a fatty acid, does not cause a transient increase in liver cell proliferation in vivo (21), and does not induce immediate-early gene expression (17). Consistent with these findings, DHEA-S did not induce phosphorylation of ERK1 and ERK2 (Fig. 2, A–C).

To determine if the tyrosine phosphorylation of the ERKs resulted in induction of their kinase activity, ERK1 and ERK2

![Fig. 1. Wy-14,643 induces tyrosine phosphorylation of p44 and p42. Quiescent ML457 cells were treated with no addition (NA) or 1 mM Wy-14,643 for the times indicated before SDS lysis. Whole cell lysates were subjected to 4–20% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride. Tyrosine-phosphorylated proteins were detected by immunoblotting using anti-phosphotyrosine antibodies and visualized by enhanced chemiluminescence.](image-url)
were immunoprecipitated from quiescent ML457 cells stimulated with increasing doses of the PPs and used in an immunocomplex kinase assay. ERK activation by the PPs was consistent with the results seen for tyrosine phosphorylation. The 1 mM concentrations of Wy-14,643, MEHP, ciprofibrate ethyl ester, and clofibrate all increased ERK activation over control levels (no addition), whereas DHEA-S did not increase ERK activity. In addition, increasing ERK activity could be seen with increasing doses of each of the PPs with the exception of DHEA-S (Fig. 3). ERK activity continued to increase with PP doses of up to 2 mM; however, cytotoxicity was apparent above 1 mM (data not shown).

In response to growth factors and other agonists, receptor tyrosine kinases initiate a signaling cascade leading to Ras activation followed by a sequential activation of Raf and MEK (22). MEK is a dual-specific kinase that phosphorylates ERK1 and ERK2, leading to their activation (23). MEK is also activated by phosphorylation. To determine if the PPs activated MEK, quiescent ML457 cells were stimulated with 1 mM concentrations of the PPs or with 20% serum. Western blotting using a phospho-specific MEK antibody showed that MEK was phosphorylated in response to PP stimulation (Fig. 4).

Phosphorylation by activated MEK is the main pathway of ERK activation although other mechanisms of ERK activation have been demonstrated (24, 25). To determine if ERK activation by the PPs is mainly through their activation of MEK, a specific inhibitor of MEK, PD98059, was employed (26). PD98059 prevents the activation of MEK by upstream regulators (27). Incubation of quiescent ML457 cells with PD98059 before stimulation with the PPs greatly reduced both the tyrosine phosphorylation (data not shown) and kinase activity of the ERKs (Fig. 5). A dose of 10 µM PD98059 reduced the levels of ERK activation by the PPs to approximately that of no addition, confirming a role for MEK in PP-induced ERK activation.

Activation of the ERKs leads to the phosphorylation of a variety of substrates including transcription factors (28, 29) that ultimately regulate gene expression. Induction of immediate-early gene expression by the PPs has been shown previously (17, 18). To determine if activation of ERK1 and ERK2 by the PPs is responsible for their induction of immediate-early gene expression, quiescent cells were preincubated with PD98059 followed by PP stimulation and RNA isolation. The evaluation of three immediate-early genes, c-fos, egr-1, and junB, is shown in Fig. 6. As seen previously (17), the expression of c-fos, egr-1, and junB mRNA was induced by 1 mM concentrations of Wy-14,643, MEHP, ciprofibrate ethyl ester, and clofibrate. In the presence of PD98059, a marked reduction in PP-induced gene expression was observed. Expression of c-jun (data not shown) expression was generally less inhibited.
treated with 100 \( \mu M \) in other liver and non-liver cell lines (17, 18). Hepatocytes were of immediate-early gene expression by the PPs has been shown

induction by PPs at 1 mm concentrations for 20 min. ERK kinase activity was evaluated using myelin basic protein (MBP) as described under “Experimental Procedures.” NA, no addition; MEHP, monoethylhexyl phthalate; MBP, myelin basic protein.

To ensure that our results were not specific to this in vitro system, immediate-early gene expression and ERK phosphorylation were examined in primary rat hepatocytes. Induction of immediate-early gene expression by the PPs has been shown in other liver and non-liver cell lines (17, 18). Hepatocytes were treated with 100 \( \mu M \) or 1 mm Wy-14,643 for 20 min for protein isolation or for 1–24 h for RNA isolation. Western blotting using a phospho-specific ERK antibody showed an increase in ERK1 and ERK2 phosphorylation upon treatment with 1 mm Wy-14,643 (Fig. 7A), whereas induction of immediate-early gene expression was seen after a 1-h treatment with 1 mm Wy-14,643 (junB is shown in Fig. 7B), consistent with the results seen in ML457 cells. Although a side-by-side comparison is not shown, the induction of both ERK phosphorylation and immediate-early gene expression was lower in hepatocytes than in ML457 cells. In addition to junB expression, the expression of two genes involved in peroxisome proliferation, FACO and CYP4A1, are shown (Fig. 7B). Both FACO and CYP4A1 are induced by treatment with 100 \( \mu M \) Wy-14,643 but not by the 1 mm dose of Wy-14,643. Conversely, junB expression was induced at 1 mm but not 100 \( \mu M \) Wy-14,643. This is consistent with the fact that peroxisome proliferation is induced at lower doses of PPs (2, 3) while immediate-early gene expression in vitro (17, 18) and cell proliferation in vivo (2, 3) require higher doses.

**DISCUSSION**

We have shown that the PPs Wy-14,643, MEHP, ciprofibrate ethyl ester, and clofibrate transiently activate the MAPK family members ERK1 and ERK2 in the immortalized mouse liver cell line ML457. The steroid PP DHEA-S did not activate the ERKs, which is consistent with its inability to induce immediate-early gene expression (17). These compounds had minimal effects on the activation of the MAPK family members JNK and p38 (data not shown). All of the PPs tested (except DHEA-S) produced a dose-dependent increase in ERK phosphorylation between 100 mm and 2 mm concentrations. The 1 mm dose induced strong levels of ERK activation for each of the PPs without significant toxicity. Although a 1 mm dose is much higher than needed for induction of peroxisomal enzymes, it is consistent with the doses necessary for optimal immediate-early gene induction and regulation of cell cycle progression (17). Several studies have suggested that regulation of cell growth by PPs may be independent of peroxisome proliferation, can require higher doses, and may be more relevant to hepatic carcinogenesis (2–4, 30, 31). In vivo, the plasma levels of both ciprofibrate and clofibrate are in the range necessary for ERK activation in vitro. Continuously feeding rats approximately 12.5 mg of ciprofibrate/kg of body weight resulted in 100% tumor incidence (1) while rats gavaged with 10 mg of ciprofibrate/kg daily for 1 week gave average drug plasma levels of 0.5–0.6 mm (33). Since ciprofibrate accumulates in the liver (34), the liver concentration of ciprofibrate is likely to be higher than plasma levels. Studies with clofibrate have shown a 90% tumor incidence when rats were continually fed approximately 250 mg/kg (1). Plasma drug levels after daily doses of ciprofibrate for 1 week were at a mean of 0.37–0.47 mm in rats fed 250–300 mg/kg and peaked at 1.4 mm in rats gavaged with 125 mg/kg (35).

MEK, the upstream regulator of the ERKs, is also activated by the PPs as shown by the induction of MEK phosphorylation following PP treatment. Inhibition of MEK activity by the MEK-specific inhibitor PD98059 greatly reduced the induction of ERK activity by the PPs. This reduction of ERK activity by PD98059 was utilized to assess the role of ERK activation in the induction of immediate-early gene expression by the PPs. Although the results varied depending on the specific gene and PP evaluated, a small to significant reduction of PP-induced
gene expression was generally observed in the presence of PD98059. Since PD98059 did not block all PP-induced gene expression, other signaling pathways may be modulated by some of the PPs. However, the induction of immediate-early gene expression by the PPs can be largely attributed to the activation of ERK1 and ERK2.

Induction of ERK phosphorylation was also seen in primary rat hepatocytes treated with 1 mM Wy-14,643. The level of ERK phosphorylation in hepatocytes was lower than seen in ML457 cells; however, ML457 cells contain higher levels of ERK protein (data not shown), accounting for some of this discrepancy. It is not surprising that differentiated hepatocytes would respond less strongly to mitogen-activated signaling pathways than an immortalized cell line. The preneoplastic liver cells promoted by PPs in vivo are probably more similar to the growth-competent immortalized liver cells than to mature differentiated hepatocytes.

Consistent with the induction of ERK phosphorylation, an induction of junB expression is seen in hepatocytes after a 1-h treatment with 1 mM Wy-14,643. The 100 μM dose of Wy-14,643 had no effect on junB expression but did induce the expression of two genes involved in peroxisome proliferation, FACO and CYP4A1. However, FACO and CYP4A1 expression were not seen with the 1 mM dose of Wy-14,643. This is consistent with the idea that the induction of peroxisome proliferation occurs at low doses of PPs whereas induction of immediate-early gene expression and cell proliferation requires higher doses. This inverse relationship may suggest an antagonism between induction of peroxisome proliferation and activation of immediate-early signaling pathways.

The induction of peroxisome proliferation in differentiated hepatocytes is mediated by the activation of the peroxisome proliferator-activated receptor α (PPARα) (36, 37). The PPARs (α, γ, δ) are members of the steroid receptor superfamily and function as transcription factors. PPARα is transcriptionally activated by the PPs, fatty acids, and other ligands (37, 38). The antagonism between peroxisome proliferation and immediate-early gene expression discussed above may be due to interaction between growth-regulatory signaling pathways and PPARα. For example, many nuclear receptors including PPARα (39) are mutually antagonistic with the transcription factor AP-1 (fos-jun heterodimer), apparently via competition for common cofactors such as CREB-binding protein (40). Interestingly, preneoplastic foci and tumors promoted by PPs often have reduced peroxisomal gene expression relative to the surrounding normal hepatocytes (41), possibly due to increased AP-1 activity and hence decreased PPARα activity within the proliferating cells.

A role for PPARα in PP-induced hepatocarcinogenesis is hypothesized but unconfirmed, yet PPAR-independent mechanisms could be responsible for, or at least contribute to, PP-induced hepatocarcinogenesis. Activation of the ERK signaling pathway leading to enhanced cell proliferation is one potential mechanism. Alternatively, activation of the ERKs may contribute to PP-induced hepatocarcinogenesis by modulating PPARα activity. Recently, studies have shown that the ERKs phosphorylate PPARα in response to insulin, epidermal growth factor, or 12-O-tetradecanoylphorbol-13-acetate and that this phosphorylation affects PPARα transcriptional activity (42, 43) and inhibits adipocyte differentiation (43). Similar interaction between the ERKs and PPARα is likely since insulin stimulates PPARα phosphorylation in rat adipocytes (44) and 12-O-tetradecanoylphorbol-13-acetate enhances fatty-acid-induced PPARα transcriptional activity in rat hepatoma cells (45). However, the potential cross-talk between ERK and PPARα may have synergistic or antagonistic characteristics and does not exclude a contribution of ERK alone to hepatocarcinogenesis. ERK activation by the PPs is independent of PPARα since ML457 cells do not undergo peroxisome proliferation nor contain detectable levels of PPARα.3

Activation of the ERKs by the PPs could have profound effects on cell proliferation and may be an integral part of their tumor-promoting capabilities. Tumor promotion involves selective effects on the growth of preneoplastic cells over the surrounding normal tissue. These preneoplastic cells could preferentially respond to the activation of the ERK signaling pathway that plays an important role in growth regulation. A role for ERK in tumor promotion is supported by one study which showed that constitutively active mutants of MEK1 in fibroblasts cause an elevated basal level of ERK activity leading to a reduced growth factor requirement for DNA synthesis and rapid tumor formation in nude mice (46). In a separate study, a correlation was seen between tumor growth rate and the degree of ERK activation in NIH 3T3 cell clones overexpressing insulin receptor subunit 1 (47). Further support is provided by the activity of the skin tumor promoter BHTOOH. Activation of ERK2 by BHTOOH and its metabolites in cultured keratinocytes correlated with their tumor-promoting activities in vivo. In addition, BHTOOH-induced ERK2 activation provided some protection from the cytotoxicity induced by the BHTOOH metabolites (48). In this model, activation of ERK2 in initiated cells could provide a selective growth advantage along with a protective effect against toxicity, whereas the inhibitory effects of toxicity on non-initiated cells could provide further selective pressure (48). Similarly, the PPs have inhibitory effects on cell growth (17, 32) in addition to their induction of ERK activity, both of which may contribute to their tumor-promoting activity.

In summary, we have shown that the PPs Wy-14,643, MEHP, ciprofibrate ethyl ester, and clofibrate stimulate ERK1 and ERK2 activity in a MEK-dependent manner and that this activity is necessary for the complete induction of immediate-early genes by the PPs in ML457 cells. In addition, induction of ERK phosphorylation by Wy-14,643 was evident in primary rat hepatocytes. The mechanism by which the PPs are activating the ERK/MEK pathway is currently under investigation. These results place the PPs in the growing class of tumor promoters that modulate growth-regulatory pathways in the cell.

Acknowledgments—We thank Gary Dysart and John DeLuca for preparing the rat hepatocytes. We also thank John Balliet and Sujata Manam for critical reading of the manuscript and helpful discussions.

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