Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) and cyclooxygenase inhibitor that is frequently used as a research tool to study the process of adipocyte differentiation. Treatment of various preadipocyte cell lines with micromolar concentrations of indomethacin in the presence of insulin promotes their terminal differentiation. However, the molecular basis for the adipogenic actions of indomethacin had remained unclear. In this report, we show that indomethacin binds and activates peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated transcription factor known to play a pivotal role in adipogenesis. The concentration of indomethacin required to activate PPARγ is in good agreement with that required to induce the differentiation of C3H10T1/2 cells to adipocytes. We demonstrate that several other NSAIDs, including fenoprofen, ibuprofen, and flufenamic acid, are also PPARγ ligands and induce adipocyte differentiation of C3H10T1/2 cells. Finally, we show that the same NSAIDs that activate PPARγ are also efficacious activators of PPARα, a liver-enriched PPAR subtype that plays a key role in peroxisome proliferation. Interestingly, several NSAIDs have been reported to induce peroxisomal activity in hepatocytes both in vitro and in vivo. Our findings define a novel group of PPARγ ligands and provide a molecular basis for the biological effects of these drugs on adipogenesis and peroxisome activity.

Indomethacin and other NSAIDs\(^1\) are used clinically for their anti-inflammatory, anti-pyretic, and analgesic properties (1). The molecular basis for the therapeutic actions of NSAIDs is believed to be their ability to inhibit cyclooxygenase (COX) activity and thereby block the production of prostaglandins (PGs). Two COX enzymes have been identified. COX-1 is constitutively expressed, and the PGs produced by this enzyme are thought to function in the so-called housekeeping functions of the cell; in contrast, the COX-2 isozyme is an inducible enzyme that is normally absent from cells but is expressed in response to growth factors, tumor promoters, and cytokines (2). Most of the NSAIDs inhibit both COX-1 and COX-2, although they vary in their relative potencies against the two COX isozymes (3).

Indomethacin is also widely used as a research tool to study the process of adipocyte differentiation. While there is at least one report of indomethacin blocking adipocyte differentiation (4), treatment of several preadipocyte cell lines with this drug resulted in their terminal differentiation (5–7). Early reports suggested that indomethacin might function as an adipogenic agent through its inhibition of COX activity. However, two lines of evidence indicate that the adipogenic activity of indomethacin cannot simply be ascribed to the inhibition of COX. First, the concentration of drug required to induce differentiation is 2–3 orders of magnitude higher than that required to inhibit COX activity, and second, several NSAIDs that inhibit COX activity fail to induce adipocyte differentiation (7). Thus, the mechanism underlying the adipogenic activity of indomethacin has remained obscure.

Insight into the molecular mechanisms responsible for adipocyte differentiation was recently provided by the identification of a ligand-activated transcription factor, termed PPARγ, as a key regulator of adipogenesis (8). PPARγ, a member of the nuclear receptor superfamily, is selectively expressed in adipocytes and induced early during the course of differentiation of several preadipocyte cell lines (9, 10). Forced expression of PPARγ in fibroblast and myoblast cell lines results in efficient adipocyte differentiation in a PPARγ-dependent fashion (8, 11). Thus, PPARγ functions as a master regulator of adipocyte differentiation. Two other PPAR subtypes, termed PPARα and PPARδ, have been identified in addition to PPARγ (12, 13). PPARα is the predominant PPAR subtype expressed in liver and is activated by a group of chemicals that induce the proliferation of peroxisomes in rodents (14). Gene disruption experiments have demonstrated that PPARα is required for the pleiotropic hepatic response to peroxisome proliferators in rodents (15).

Work from several laboratories had shown that PGs have marked effects, both positive and negative, on adipocyte differentiation (4, 16–19). Interestingly, PPARγ is activated by PGs and PG-like molecules (20–23). Recently, the arachidonic acid metabolite 15-deoxy-\(\Delta^{12,14}\)-PGJ\(_2\) was shown to bind directly to PPARγ and to promote the efficient conversion of fibroblast and mesenchymal stem cell lines to adipocytes (24, 25). The finding that a PG functions as a PPARγ ligand and promotes adipocyte differentiation provided additional evidence that products of the COX pathway play an important role in modulating adipogenesis.

If prostanoids can function as PPARγ ligands and induce adipogenesis, how then does a COX inhibitor such as indomethacin, which blocks PG synthesis, promote adipocyte differentiation? In this report, we show that indomethacin and...
several other NSAIDs function as PPARγ ligands, suggesting a mechanism for the adipogenic actions of these compounds. Furthermore, we demonstrate that these same NSAIDs also activate PPARα, providing a basis for the reported effects of NSAIDs on peroxisome activity in liver.

EXPERIMENTAL PROCEDURES

Chemicals—Indomethacin, flufenamic acid, fenoprofen, ibuprofen, piroxicam, acetaminophen, and salicylic acid were purchased from Sigma. The peroxisome proliferator Wy14,643 was purchased from Biomol (Plymouth Meeting, PA) and 15-deoxy-D_{12,14}-PGJ_{2} from Cayman Chemical Company (Ann Arbor, MI).

Cotransfection Assay—To generate the pSG5-GAL4-PPARγ-LBD and pSG5-GAL4-PPARγ-LBD chimeric receptor expression plasmids, cDNAs encoding the ligand binding domains (LBDs) of the human PPARα (amino acids 167–468) (26) and the human PPARγ (amino acids 176–477) (27) were amplified by polymerase chain reaction and subcloned into the pSG5-GAL4 expression plasmid (28). The pCMV-PPARγ expression plasmid has been described (27). The pCMV-PPARγ expression plasmid has been described (27).

Ligand Binding Assays—The LBD of human PPARγ (amino acids 176–477) (27) was overexpressed in Escherichia coli as a histidine-tagged fusion protein and bacterial lysates were prepared as described previously (25). For competition binding assays, bacterial extracts (approximately 100 μg of protein) containing the PPARγ-ligand binding domain were incubated at 4 °C for 2–3 h with 40 nM [3H]BRL49653 (specific activity, 40 Ci/mmol) in the absence or presence of unlabeled competitor in buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 10 mM dithiothreitol. Bound was separated from free radioactivity by elution through 1.5 ml Sephadex G-25 desalting columns (Boehringer Mannheim). Bound radioactivity eluted in the column void volume and was quantitated by liquid scintillation counting. Data shown are the result of binding assays performed in duplicate, and each experiment was repeated at least twice with similar results.

Adipocyte Differentiation Assays and Northern Analysis—C3H10T1/2 clone 8 murine fibroblasts (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and 10 μg/ml penicillin and streptomycin. One day after reaching confluence, the cells were treated with BRL49653 or the various NSAIDs in the presence of 200 nm insulin. Fresh media and test compounds were added every 2 days. Lipogenesis was measured in cells at 9 days post-confluence as described previously (29). For Northern analysis, total RNA was prepared from vehicle- and compound-treated cells using the RNeasy Total RNA Kit (Qiagen, Chatsworth, CA). Fifteen μg of total RNA was electrophoresed on a formaldehyde gel and transferred to nitrocellulose. The blot was probed with mouse aP2 and GAPDH probes labeled via the random priming technique with [α-32P]dCTP. The results of Northern assays were quantitated using a Molecular Dynamics Computing Densitometer and Image Quant software.

RESULTS

Indomethacin Activates PPARγ—The PPARs are activated by a large number of structurally diverse compounds including prostanooids, long-chain fatty acids, the fibrate class of hypolipidemic drugs, leukotriene antagonists, and anti-diabetic thiazolidinediones (13). While chemically diverse, these compounds share certain structural characteristics including a lipophilic backbone and an acid moiety, usually a carboxylate. Indomethacin and many of the other NSAIDs are amphipathic carboxylates that share these broad structural features (Fig. 1). This suggested to us that indomethacin might exert its adipogenic effects through direct activation of PPARγ.

We tested the possibility that indomethacin activates PPARγ via a transient transfection assay. An established chimera system was used (28) in which the LBD of PPARγ was fused to the DNA binding domain of the yeast transcription factor GAL4. The advantage of the GAL4 chimera assay is that it minimizes background due to the cell’s endogenous receptors. Expression plasmid for the GAL4-PPARγ LBD chimera was transfected into CV-1 cells together with a reporter construct containing five copies of the GAL4 response element driving expression of the reporter chloramphenicol acetyltransferase (UAS₃-tk-CAT). Dose-response analysis revealed that indomethacin is an efficacious activator of PPARγ, inducing PPARγ activity roughly 40-fold at 1 × 10⁻⁸ M (Fig. 2). This activation is comparable with the maximal induction obtained with the PPARγ ligands 15-deoxy-D_{12,14}-PGJ₂ and the anti-diabetic thiazolidinedione BRL49653 (25, 28) (see below). Indomethacin activated PPARγ with an EC₅₀ of approximately 4 × 10⁻⁵ M (Fig. 2). Similar EC₅₀ and fold activation values were obtained for indomethacin in transient transfection assays performed with an expression vector for wild-type PPARγ and a reporter driven by the fatty acid binding protein/aP2 enhancer region which contains two PPARγ response elements (10) (Fig. 2). Thus, indomethacin is an efficacious activator of PPARγ.

Indomethacin Binds PPARγ—We next sought to determine whether indomethacin activates PPARγ through direct interactions with the receptor. We and others (24, 28) have previously shown that the anti-diabetic thiazolidinedione BRL49653 can bind to PPARγ with high affinity. The ability of indomethacin to bind to PPARγ was assessed in a competition binding assay using [3H]BRL49653 and bacterially expressed PPARγ-LBD. As shown in Fig. 3, indomethacin competed efficiently with [3H]BRL49653 for binding to the PPARγ-LBD, with an IC₅₀ of approximately 1 × 10⁻⁷ M. In control experiments, acetaminophen, an NSAID that does not activate PPARγ (see below), failed to compete with [3H]BRL49653 for binding to the PPARγ-LBD (Fig. 3). These data demonstrate that indomethacin can interact directly and specifically with the PPARγ LBD and thus define a novel PPARγ ligand.

Other Classes of NSAIDs Activate PPARα and PPARγ—Several chemically distinct classes of NSAIDs are used clinically including thiazinecarboxamides (e.g. piroxicam) and derivatives of arylacetic acid (e.g. indomethacin), aminoarylcarboxylic acid (e.g. flufenamic acid), arylpropionic acid (e.g. ibuprofen and fenoprofen), and salicylic acid (e.g. aspirin) (Fig. 1) (1). We next tested whether representative compounds from the different classes of NSAIDs could also activate PPARγ. CV-1 cells were transfected with the GAL4-PPARγ-LBD expression plasmid and the UAS₃-tk-CAT reporter and treated with 1 × 10⁻⁴ M of piroxicam, flufenamic acid, ibuprofen, fenoprofen, and salicylic acid. As shown in Fig. 4A, flufenamic acid, fenoprofen, and ibuprofen were efficient activators of PPARγ, activating the receptor to a degree comparable to that obtained with the PPARγ ligands BRL49653 and 15-deoxy-D_{12,14}-PGJ₂, and the peroxisome proliferator Wy14,643. However, in contrast to indomethacin, no activation of PPARγ was observed in transfected cells treated with 1 × 10⁻⁴ M of these compounds (data not shown). Thus, indomethacin is the most potent of the NSAIDs that we tested for PPARγ activation. Treatment of
transfected cells with piroxicam resulted in only a modest activation of PPARγ (approximately 5-fold), whereas treatment with salicylic acid or acetylsalicylic acid resulted in little or no induction of reporter expression (Fig. 4A). We note that the compounds that activated PPARγ efficiently (>6-fold) were all amphipathic acids (Fig. 1) and thus conform in their general structural features to known PPAR activators.

The ability of these compounds to interact with PPARγ was assessed in the competition binding assay using [3H]BRL49653. These studies revealed a good correlation between the compounds that activated PPARγ in the transfection assay and those that interacted directly with the receptor. Although flufenamic acid, fenoprofen, and ibuprofen competed efficiently with [3H]BRL49653 for binding to the PPARγ LBD, little or no competition was seen with piroxicam, salicylic acid, or acetylsalicylic acid (Fig. 4B). Taken together, the transfection and binding analyses demonstrate that some but not all NSAIDs bind and activate PPARγ.

For comparative purposes, we also tested the various NSAIDs on the PPARα and PPARδ subtypes using the transfection assay. Little or no activation of PPARδ was seen in the presence of 1 × 10^{-4} M of these compounds (data not shown). However, indomethacin, fenoprofen, ibuprofen, and flufenamic acid were efficacious activators of PPARα at this concentration, with fenoprofen activating the receptor to a degree comparable to that obtained with the strong peroxisome proliferator Wy14,643 (Fig. 4A). Thus, the same NSAIDs that activate PPARγ are also efficacious activators of the PPARα subtype.

**NSAIDs Promote Adipocyte Differentiation**—We and others (24, 25, 28, 30–33) have shown that treatment of various fibroblast and mesenchymal stem cell lines with PPARγ ligands, including 15-deoxy-Δ12,14-PGJ2 and the anti-diabetic thiazolidinediones, promotes their efficient conversion to adipocytes. As discussed, indomethacin is used to promote the terminal differentiation of preadipocyte cell lines. We next examined whether the concentration of indomethacin required to activate PPARγ in CV-1 cells was consistent with that required to induce adipocyte differentiation. C3H10T1/2 mouse mesenchymal stem cells were treated with various concentrations of indomethacin and subsequently assayed for lipogenesis, an established measure of adipocyte differentiation (29). Dose-response analysis revealed the EC50 for indomethacin in the lipogenesis assay to be approximately 8 × 10^{-5} M (Fig. 5A). This value is in good agreement with that reported in a previous study (6 × 10^{-5} M) using TA1 cells, a stable adipogenic cell line derived from C3H10T1/2 cells (7), and is also consistent with the EC50 value of indomethacin for PPARγ activation in the transfection assay (Fig. 2). Taken together, these data suggest that PPARγ is the target for the adipogenic actions of indomethacin. Our finding that flufenamic acid, fenoprofen, and ibuprofen also activated PPARγ at micromolar concentrations suggested that these NSAIDs might also promote adipocyte differentiation. We tested this possibility using C3H10T1/2 cells and the
lipogenesis assay. In agreement with previous studies, treatment of the C3H10T1/2 cells with 1 × 10^{-5} M of either flufenamic acid or fenoprofen promoted lipogenesis, albeit less efficiently than indomethacin (Fig. 5B). The results of the lipogenesis assay were confirmed by oil red O staining for lipid accumulation in treated cells (data not shown). The NSAIDs that did not activate PPARγ efficiently in the transfection assay, including piroxicam, salicylic acid, and acetaminophen, failed to induce lipogenesis in the C3H10T1/2 cells (Fig. 5B). We conclude that NSAIDs other than indomethacin can also promote adipocyte differentiation at concentrations at which they activate PPARγ.

We note that ibuprofen, which was a less efficacious activator of PPARγ in CV-1 cells than either flufenamic acid or fenoprofen (Fig. 4A), failed to promote lipogenesis in C3H10T1/2 cells when tested at 1 × 10^{-4} M (Fig. 5B). However, increasing the concentration of indomethacin to 5 × 10^{-4} M resulted in significant lipogenesis (Fig. 5B). In Northern analysis, 1 × 10^{-4} M ibuprofen induced weak expression of the gene encoding aP2, an adipocyte-specific fatty acid binding protein whose expression is directly regulated by PPARγ (Fig. 5C) (10). Consistent with the results of the transfection studies, 1 × 10^{-4} M indomethacin stimulated aP2 gene expression approximately 3-fold more efficiently than ibuprofen (Fig. 5C). Taken together, these data indicate that ibuprofen is less potent than indomethacin, flufenamic acid, or fenoprofen in the activation of PPARγ in both CV-1 and C3H10T1/2 cells.

DISCUSSION

The NSAID indomethacin is frequently included as one of a mixture of compounds used to promote the terminal differentiation of various preadipocyte cell lines in vitro. This differentiation mixture also routinely includes insulin, corticosteroids, and isobutylmethylxanthine. Recent work has indicated that isobutylmethylxanthine and corticosteroids induce the expression of the genes encoding CCAAT/enhancer binding proteins α and δ, respectively, members of the basic region-leucine zipper family of transcription factors (34). These two transcription factors are induced early during the course of 3T3-L1 cell conversion to adipocytes and appear to play key roles in the differentiation cascade (34–37). The mechanism underlying the adipogenic activity of indomethacin, however, has remained unclear. Early speculation focused on the ability of indomethacin to inhibit COX activity. However, Knight et al. (7) showed that the concentration of indomethacin required to promote the differentiation of TA1 cells to adipocytes was 1–2 orders of magnitude greater than the concentrations needed to block prostaglandin synthesis. Furthermore, not all COX inhibitors promoted adipocyte differentiation. Likewise, we have found that several COX inhibitors, including the potent NSAID piroxicam (38), fail to promote adipocyte differentiation. These data provide compelling evidence that the effects of indomethacin are not mediated through the inhibition of prostaglandin production.

In searching for the basis of its adipogenic activity, we have found that indomethacin functions as a micromolar ligand for the adipogenic transcription factor PPARγ. PPARγ is abundantly expressed in adipose tissue where it functions as a key modulator of the adipocyte differentiation program (8–10). PPARγ ligands, including the anti-diabetic thiazolidinediones and the arachidonic acid metabolite 15-deoxy-Δ12,14-PGJ2, are potent inducers of the differentiation of several different fibroblastic cell lines to adipocytes (30–33). Our finding that the concentration of indomethacin required to induce C3H10T1/2 cell differentiation correlates with that required to activate PPARγ in the transfection assay provides strong evidence that the adipogenic actions of this NSAID are mediated through its binding and activation of PPARγ.

While indomethacin is widely used to promote the differen-
have been reported to have marked effects on peroxisome activation in the rodent liver (15). Interestingly, several NSAIDs also induce peroxisomal enzymes and peroxisome proliferation (16–18), further showing that the actions of NSAIDs on peroxisomal and liver function may be mediated via PPARα action. PPARα agonists promote adipocyte differentiation, but also act as a PPARα agonist, promoting adipocyte differentiation. Thus, indomethacin may function to either inhibit or induce adipogenesis depending on the specific conditions and context of drug use in the experiment.

We have shown that the same NSAIDs that activate PPARα also activate PPARγ. PPARγ is the predominant PPAR subtype expressed in the rodent liver (14, 21). Targeted gene disruption experiments have shown that PPARα is essential for the induction of peroxisomal enzymes and peroxisome proliferation in the rodent liver (15). Interestingly, several NSAIDs have been reported to have marked effects on peroxisome activity in hepatocytes when used either in vitro or in vivo. Indomethacin and ibuprofen induced β-oxidation in peroxisomes of cultured hepatocytes (39). Furthermore, treatment of rats with ibuprofen induced peroxisomal β-oxidation, reduced serum triglycerides and cholesterol, and increased liver weight (39). Finally, treatment of rats with benoxaprofen, an NSAID closely related to ibuprofen, at doses comparable to those used clinically induced peroxisomal β-oxidation and increased cytochrome P450A1 apoaprotein and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein levels in liver (40). Expression of both of these genes is known to be induced by PPARα in response to peroxisome proliferators (15, 41, 42). As a whole, the effects of these NSAIDs on liver function are similar to those seen in experiments performed with the fructose class of hypolipidemic drugs (43), agents that are established activators of PPARα (14). Our data strongly suggest that the actions of NSAIDs on peroxisomal and liver functions are mediated through the activation of PPARα. We note, however, that we do not have a binding assay for mammalian PPARα and, as a consequence, have been able to demonstrate direct interactions between the NSAIDs and PPARα. Thus, it remains possible that the NSAIDs modulate PPARα activity through an indirect mechanism. It is interesting that NSAIDs are associated with a variety of detrimental side effects, including hepatotoxicity (1). While there is currently no evidence of a link between these negative effects and PPARα, evaluation of the activities of NSAIDs on PPARα may be useful in minimizing the potential for unwanted side effects as new drugs of this class are developed.

In summary, we have demonstrated that indomethacin and other NSAIDs are efficacious activators of PPARα and PPARγ at micromolar concentrations. These data provide evidence for a common mechanism underlying the seemingly disparate biological effects of these compounds on the induction of adipocyte differentiation in vitro and peroxisome proliferation in vitro.

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