Cross-talk between Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) and Peroxisome Proliferator-activated Receptor-α (PPARα) Signaling Pathways

GROWTH HORMONE INHIBITION OF PPARα TRANSCRIPTIONAL ACTIVITY MEDIATED BY STAT5b

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Hepatic peroxisome proliferation induced by structurally diverse non-genotoxic carcinogens is mediated by the nuclear receptor peroxisome proliferator-activated receptor (PPARα) and can be inhibited by growth hormone (GH). GH-stimulated Janus kinase-signal transducer and activator of transcription 5b (JAK2/STAT5b) signaling and the PPAR activation pathway were reconstituted in COS-1 cells to investigate the mechanism for this GH inhibitory effect. Activation of STAT5b signaling by either GH or prolactin inhibited, by up to 80–85%, ligand-induced, PPARα-dependent reporter gene transcription. GH failed to inhibit 15-deoxy-Δ12,14-prostaglandin-J2-stimulated gene transcription mediated by an endogenous COS-1 PPAR-related receptor. GH inhibition of PPARα activity required GH receptor and STAT5b and was not observed using GH-activated STAT1 in place of STAT5b. GH inhibition was not blocked by the mitogen-activated protein kinase pathway inhibitor PD98059. STAT5b-PPARα protein-protein interactions could not be detected by anti-STAT5b supershift analysis of PPARα-DNA complexes. The GH inhibitory effect required the tyrosine phosphorylation site (Tyr-699) of STAT5b, an intact STAT5b DNA binding domain, and the presence of a COOH-terminal trans-activation domain. Moreover, GH inhibition was reversed by a COOH-terminal-truncated, dominant-negative STAT5b mutant. STAT5b must thus be nuclear and transcriptionally active to mediate GH inhibition of PPARα activity, suggesting an indirect inhibition mechanism, such as competition for an essential PPARα co-activator or STAT5b-dependent synthesis of a more proximal PPARα inhibitor. The cross-talk between STAT5b and PPARα signaling pathways established by these findings provides new insight into the mechanisms of hormonal and cytokine regulation of hepatic peroxisome proliferation.

The peroxisome proliferator-activated receptors (PPARs)1

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1 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; GH, growth hormone; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element; GHR, GH receptor; JAK2, Janus kinase 2; STAT, signal transducer and activator of transcription; CYP, cytochrome P450; EMSA, electrophoretic mobility shift assay; comprise a family of three nuclear receptors characterized by unique functions, ligand specificities, and tissue distributions (1–3). Ligands for PPARα include fibrate hypolipidemic drugs, specific fatty acids and eicosanoids, and leukotriene B4 (4–7), whereas anti-diabetic thiazolidinediones (8) and 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) (9, 10) are high affinity ligands for PPARγ. Like many other nuclear receptors, PPARs are transcription factors that bind to specific DNA response elements (PPREs) upstream of target genes in heterodimeric complexes with the 9-cis-retinoid acid receptor RXR, leading to the activation of target gene transcription (11).

PPARα and PPARγ play a key role in lipid homeostasis, adipocyte differentiation, and inflammatory responses (3, 12). PPARα target genes include liver-expressed enzymes involved in fatty acid β-oxidation and microsomal ω-hydroxylation (13–15). PPARα thus plays a direct role in liver homeostasis by regulating lipid storage and by modulating the metabolism of important lipid signaling molecules, including prostaglandins and leukotrienes. PPARα gene knock-out mice do not exhibit hepatic (16) or renal (17) peroxisome proliferative responses induced by fibrate drugs and other PPAR activators, demonstrating the key role played by PPARα in these processes. PPARα also mediates the carcinogenicity of foreign peroxisome proliferators (18), which are non-genotoxic hepatocarcinogens when administered chronically to rodents (19).

PPARα gene expression and PPARα-stimulated transcriptional activity are tightly controlled by a variety of hormones that act at multiple levels and via different mechanisms. Glucocorticoids induce PPARα protein expression at the transcriptional level (20), which may account for the expression of PPARα diurnally and in a stress-inducible manner (21), whereas insulin treatment decreases PPARα mRNA levels (22). Peroxisome proliferative responses in rodents are suppressed by the thyroid hormone triiodothyronine (23), whose receptor may compete with PPAR for heterodimerization with the retinoid X receptor RXR and for trans-activation of PPAR DNA response elements (PPREs) (24). Hepatic peroxisome proliferation can also be modulated by sex hormones, with female rats being less responsive than males to clofibrate and other peroxisome proliferators (25) and testosterone treatment abolishing this sex difference (26).

The observation that hypophysectomy enhances peroxisome proliferation in female rats (26) suggests that a pituitary factor(s) serves as a negative regulator of peroxisome proliferation. In rats, the continuous plasma growth hormone (GH) profile characteristic of adult females fully suppresses liver
expression of the clofibrate-inducible P450 4A2 fatty acid \( \omega \)-hydroxylase (27). The same suppressive effect is observed in primary rat hepatocyte cultures, where GH inhibits peroxisosomal \( \beta \)-oxidation induced by clofibrate (28, 29). GH has diverse effects on metabolism and growth, some of which are indirectly mediated by insulin-like growth factor-1 but many of which reflect the direct effects that GH has on gene expression. In particular, GH, like many cytokines and growth factors, directly activates JAK-STAT signaling pathways. GH binds to and thereby dimerizes its plasma membrane receptor (GHR) in a process that leads to JAK2 kinase-catalyzed tyrosine phosphorylation of STAT proteins, which are latent, cytoplasmic transcription factors (30). The tyrosine-phosphorylated STAT proteins dimerize and translocate into the nucleus, where they bind to specific DNA response element and thereby activate target gene transcription (31). Among the seven mammalian STATs, four forms (STATs 1, 3, 5a, and 5b) can be activated by GH (32–35).

In the present study, we investigate the mechanism that underlies the inhibitory effect of GH on peroxisome proliferation using COS-1 cells transfected to express both the GHR/JAK/STAT signaling pathway and the peroxisome proliferator-activated PPAR pathway. We find that GH inhibits PPARα-stimulated reporter gene transcription in a process that is mediated by STAT5b but not by STAT1. We further demonstrate that STAT5b tyrosine phosphorylation, DNA binding, and transcriptional activation are each essential for GH to mediate its inhibitory effects on PPARα activity. These findings are discussed in the context of the implications of this cross-talk between STAT5b and PPARα for the regulation of PPARα-dependent responses by hormones and cytokines.

**MATERIALS AND METHODS**

**Plasmids—**PPRE-firefly luciferase reporter plasmid derived from the rabbit CYP4A6 gene, pLUC46–880, and mouse PPARα cloned into the expression plasmid pCMV5 (pCMV-mPPARα) were provided by Dr. E. Johnson (Scripps Research Institute, La Jolla, CA) (33). The PPRE-firefly luciferase reporter plasmid pHDX3/Luc, obtained from Dr. J. Capone (McMaster University, Ontario, Canada), contains three tandem copies of the PPRE from the rat enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene upstream of a minimal promoter (15) cloned into the plasmid pCP5-luc (36). The PPAPα expression plasmid pSV-Sport-mPPARα was obtained from Dr. J. Reddy (Northwestern University, Chicago) (37). Mouse RXXR expression plasmid pCMX-mRXXRα was obtained from Dr. R. Evans (Salk Institute, San Diego) (38). Rat GHR cloned into the expression plasmid pBl2K5 were provided by Dr. N. Billestrup (Hagedorn Research Institute, Denmark) (39) and Dr. J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN) (40). pME18S expression plasmids encoding mouse STAT1, STAT3, STAT5a, and STAT5b were obtained from Dr. A. Mui (DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto, CA) (41).

Mouse prolactin receptor long form cloned into the expression plasmid pDNA (42), and COOH-terminal truncated forms of mouse STAT5a and STAT5b (designated STAT5aΔ749 and STAT5bΔ754, respectively) and cloned into the expression plasmid pXW were provided by Dr. B. Groner (Institute for Experimental Cancer Research, Freiburg, Germany) (43). Human STAT5a, STAT5b, STAT5aΔ694, and STAT5bΔ699F cloned into the expression plasmid pXK were provided by Dr. W. Leonard (NHLBI, National Institutes of Health) (44). ppCMV-expression plasmids encoding rat STAT5b and the DNA-binding region mutants STAT5b-VVVI(466–469) and STAT5b-EE-(437–438), where the indicated residues are replaced by alanine, were provided by Dr. L. Yu-Lee (Baylor College of Medicine) (45).

**Cell Culture and Transfections—**COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Transfections of COS cells grown in culture plates in 12-well culture plates, carried out by calcium phosphate precipitation (46). At 9 h after addition of the DNA–calcium phosphate precipitate, cells were washed and incubated in Dulbecco’s modified Eagle’s medium without serum for 12 h. Peroxisome proliferators were then added to the culture medium in combination with GH at concentrations specified in each figure. Cells were lysed 24 h later, and firefly luciferase and β-galactosidase (pCMV-βgal; internal control) reporter activities were measured using a Galecto-light chemiluminescent reporter kit (Tripos, Bedford, MA). In some experiments, Renilla luciferase expression plasmid (pRL-TK, Promega, Madison, WI) was used in place of the pCMV-βgal internal control plasmid, as indicated in the figure legends. Firefly and Renilla luciferase activities were assayed using a dual-luciferase assay reporter gene (Promega, Madison, WI). Transfections were performed using the following amounts of plasmid DNA/well (3.8 cm²) of a 12-well tissue culture plate: 0.36 µg of reporter construct (pHDX3/Luc or pLUC46–880), 14 ng of pCMV-mPPARα, 0.2 µg of GHR expression plasmid, 0.12 µg of JAK2 expression plasmid, and 0.2 µg of STAT expression plasmid. pCMV-βgal (0.16 µg) or pRL-TK (30 ng) were included as internal controls in each cell transfection. The total amount of DNA was adjusted to 0.96 µg/well using salmon sperm DNA (Stratagene, La Jolla, CA). Data shown in each figure are mean values ± S.D. (for n = 3 replicates) or mean values ± half the range (for duplicates) and are representative of at least three such independent experiments.

**EMSA Analysis—**Whole cell extracts were prepared by lysing transfected COS-1 cells in lysis buffer (Tropix, Inc.) containing 1% dithiothreitol added prior to use. 10 µg of cell extract was added to 2 µl of 5× gel-shift buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM dithiothreitol, 50 mM Tris-HCl), plus 1 µl of 2 µg/µl poly(dioxynucleosinic-dioxycyttidylic) acid (Boehringer Mannheim), with water added to adjust the total volume to 15 µl. Samples were incubated for 10 min at room temperature, 10 min on ice, and then 20 min at room temperature and then 10 min on ice. Loading dye (2 µl of 30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was then added before the mixture was loaded onto an acrylamide gel (5.5% acrylamide, 20% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol) was then added before the mixture was loaded onto an acrylamide gel (5.5% acrylamide, 0.7% bisacrylamide in 0.5× TBE). The gel was electrophoresed at 4 °C for 40 min at 100 V before loading. The gel was first electrophoresed for 20 min at 100 V at 4 °C at which point the dye entered the gel; electrophoresis was then continued at room temperature for 5 h. This procedure minimizes formation of nonspecific protein-DNA complexes (47). For STAT5b supershift assays, 3 µl of anti-STAT5b antibody (Santa Cruz Biotechnology, Santa Cruz, CA; antibody sc-835) was added 10 min after the labeled DNA probe, followed by a 10-min incubation at room temperature and 10 min on ice before loading on the gel. The STAT5 binding site of the rat β-casein promoter (5′-GGA-CTT-CTT-GAA-ATT-AAG-GGA-3′) was used as gel-shift probe for GH-activated STAT5a and STAT5b, and the sis-inducible element (SEI)-binding site (5′-gct-ga-ATT-TCC-CTG-AAA-TGc-ga-3′) was used as gel-shift probe for analyzing GH-activated STAT1 and STAT3 (34). 32P-Labeled DNA probe corresponding to the Z element of the CYP4A6 promoter (5′-CCC-AAA-CAT-TCA-CTT-AGG-GCA-3′) was used as probe for PPARα binding (48).

**Western Blotting—**Whole cell extracts from COS-1 cells (15 µg; see above) were resolved on 10% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose, and then probed with anti-STAT antibodies. STAT5b-specific antibody sc-835 is a rabbit polyclonal antibody raised to mouse STAT5b amino acid 711–727 (Santa Cruz Biotechnology). Mouse anti-STAT antibodies (Transduction Laboratories, Lexington, KY) were used as follows: anti-STAT5 (antibody S21520) was raised to sheep STAT5a amino acids 451–649; anti-STAT3 (antibody S21320) was raised to rat STAT3 amino acids 1–175; anti-STAT1 (antibody S21120) was raised to human STAT1 amino acids 592–731.

**RESULTS**

**GH Activation of STAT5b Inhibits PPARα Transcriptional Activity—**To investigate the mechanism by which GH inhibits PPARα-dependent liver peroxisome proliferative responses, we reconstituted GH signaling and PPARα-dependent peroxisome proliferation pathways by cotransfection of key components into COS-1 cells. GH signal transduction was reconstituted by cotransfection of expression plasmids encoding GHR, JAK2 kinase, and STAT5b, the major GH-responsive STAT form in liver (34). The PPARα pathway was reconstituted by cotransfection of a mouse PPARα expression plasmid, together with a reporter plasmid containing 880 µg of 5′-flanking DNA of the rabbit CYP4A6 gene (13) fused to a firefly luciferase reporter gene. Transfected cells were stimulated with the peroxisome proliferator Wy-14,643 (14) at 20 µM for 24 h in the presence or absence of GH (200 ng/ml). Fig. 1A shows that Wy-14,643 activation of the CYP4A6 promoter is significantly decreased in the presence of GH. Wy-14,643 activation of the
CYP4A6 promoter is mediated by PPARα, which binds to one strong and two weaker PPREs within the 5’-flanking 880 nucleotides (13, 49). This GH inhibitory effect may be due to GH suppression of PPARα-dependent transcription via the CYP4A6 PPREs; alternatively, GH may interfere with other transcription factors required for either basal or Wy-14,643-inducible CYP4A6 promoter transcription, independent of PPRE. To distinguish these possibilities, we examined the effect of GH on PPARα-activation of the reporter construct pHD(X3)Luc, which contains three tandem repeats of a PPRE from the gene that encodes enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, cloned upstream of a minimal promoter and luciferase reporter gene (15). Fig. 1B shows that GH inhibits, by up to 80–85%, PPARα induction mediated by this isolated PPRE. Maximal GH inhibition was achieved at 25 ng/ml, well within the physiological range of GH concentrations and consistent with the reported K₈ of GHR for GH of ~2 ng/ml (50). The inhibitory effects of GH on PPARα activation were also apparent with several other PPARα activators, including both foreign chemicals (nafenopin, a fibrate hypolipidemic drug) and the endogenous PPARα activators (8S)-hydroxyeicosatetraenoic acid and leukotriene B₄ (Fig. 1C).

Requirement of GHR, JAK2, and STAT5b for GH Suppression of PPARα Activity—Since some, but not all GH intracellular events involve JAK/STAT signaling pathways (30), we examined whether GHR, JAK2, and STAT5b are each required for the inhibitory effect of GH on PPARα activity. Fig. 2A shows that inhibition of PPARα activity by 25 ng/ml GH (lanes 3 versus lane 2) was not observed in the absence of cotransfected GHR (lane 4) or STAT5b (lane 5). A similar dependence on GHR and STAT5b was observed at higher GH concentrations (100–500 ng/ml GH; data not shown). In the absence of transfected JAK2, GH could still activate STAT5b and inhibit PPARα activity using the low levels of JAK2 expressed endogenously in COS-1 cells, although a higher GH concentration (500 ng/ml) was required for maximal PPARα inhibition (data not shown). Interestingly, cotransfection of GHR, JAK2, and STAT5b significantly reduced the moderate PPARα-dependent but Wy-14,643-independent basal luciferase reporter activity, even in the absence of GH (Fig. 2A, lanes 6 versus 1). This Wy-14,643-independent activity has been associated with the presence of endogenous PPARα activators, such as fatty acids (4, 5). Inhibition of this basal PPARα activity likely results from the constitutive activation of STAT5b upon overexpression of JAK2, as shown in the gel-shift studies described below (Fig. 2B). GH stimulation further decreased the Wy-14,643-independent PPARα activity up to 4-fold (data not shown).

To confirm the reconstitution of STAT5b activity in the GH-stimulated COS-1 cells, EMSA assays were carried out using a β-casein promoter probe, which contains a single STAT5b-binding site. No STAT5b DNA binding was detected upon GH stimulation of untransfected COS-1 cells (Fig. 2B, lanes 1 and 2). This indicates that COS-1 cells have at most low levels of GHR and/or STAT5b and are thus suitable for use as recipient cells in these transfection studies. In cells cotransfected with GHR, JAK2, and STAT5b, a low basal STAT5b DNA binding was observed in the absence of GH; this STAT5b activity was not affected by Wy-14,643 treatment (Fig. 2B, lanes 3 versus 5). Thus, overexpression of JAK2 and STAT5b results in some constitutive activation of STAT5b DNA binding activity. Stimulation with GH yielded a much higher level of STAT5b DNA binding activity (lane 7 versus 5). The β-casein DNA-binding complexes obtained in these experiments were confirmed to contain STAT5b, as shown by supershift analysis using anti-STAT5b antibody (lanes 4, 6, and 8).

Inhibition of PPARα Activity by GH-Activated STAT3 but Not STAT1—In addition to STAT5b, GH can activate three other STAT proteins, STATs 1, 3, and 5a (32–35). Fig. 3 shows, however, that in COS-1 cells transfected with STAT1, GH did...
not decrease PPARα-induced luciferase reporter activity, even at a high GH concentration (500 ng/ml). Partial GH inhibition of PPARα activity was seen in COS-1 cells transfected with STAT5 or with the closely related (41, 51) STAT5α. In control experiments, all four STATs were shown by Western blotting to be highly expressed in the transfected COS-1 cells compared with untransfected controls (Fig. 3B; lanes 2–4 versus lane 1). The lower effectiveness of STAT5α compared with STAT5β is not because of its inefficient activation by GH, as shown by EMSA using [32P]-labeled β-casein probe (Fig. 3C, lanes 3 and 4 versus lanes 6 and 7). STAT1 and STAT4 expressed in GH-treated cells were also shown to be functionally active in DNA binding using an SIE ( sis-inducible element) EMSA probe (see “Materials and Methods”) (data not shown).

Prolactin Inhibition of PPARα Activity—Prolactin is a GH-related pituitary polypeptide hormone that also signals via its cell-surface receptor through a JAK2/STAT5 pathway (42). STAT5α, originally identified as a prolactin-activated mammary gland factor, and STAT5β can both confer a prolactin response to the mammary β-casein gene promoter (42, 51). Fig. 4 shows that in the presence of prolactin, STAT5β, but not STAT5α, inhibits PPARα activation in cells cotransfected with prolactin receptor and JAK2. This inhibition by prolactin is less extensive (~40%) than seen for GH-activated STAT5β in the same system (~80%; cf., Fig. 3A) and also required a higher concentration of hormone to achieve its maximum effect (100 ng/ml prolactin versus 25 ng/ml GH). STAT5β activation by prolactin was confirmed by gel-shift assay using a β-casein probe (data not shown).

GH-activated STAT5β Fails to Inhibit PPRE-dependent Transcription Induced by 15-Deoxy-D12,14-PGJ2 (15d-PGJ2)—15d-PGJ2 is a naturally occurring PPAR ligand that activates PPARγ and other PPARs (52). Treatment of COS-1 cells with 5 μM 15d-PGJ2 stimulated PPRE-dependent luciferase reporter activity by ~20-fold, even in the absence of PPARγ transfection (Fig. 5). This induction was not further enhanced by co-transfection of a PPARγ expression plasmid (data not shown), suggesting that the 15d-PGJ2-stimulated PPRE response observed in this experiment involves an endogenous COS-1 PPAR that is distinct from PPARγ or PPARα. GH did not, however, inhibit 15d-PGJ2-induced, PPRE-dependent reporter gene transcription (Fig. 5), demonstrating that the GH suppressive response has specificity for the PPARα activation pathway.

STAT5β Inhibition of PPARα Activity Is Not Mediated by the MAP Kinase ERK1 and ERK2—We next investigated whether MAP kinase plays a role in the GH-induced down-regulation of PPARα activity. GH can activate the MAP kinase kinase MEK1, which phosphorylates and thereby activates the MAP kinases ERK1 and ERK2 (53). MAP kinases are actively involved in growth factor and cytokine receptor signaling (54, 55) and catalyze phosphorylation on serine and/or threonine of several transcription factors, including PPARγ (56). By analogy to the MAP kinase regulation of PPARγ, PPARα, which is also a phosphoprotein (57), might be regulated by MAP kinase-catalyzed serine phosphorylation. To investigate whether MAP kinase activity is required for GH inhibition of PPARα activity, we used the MEK1 and MEK2 inhibitor PD98059 (58) to block MAP kinase activation. Stimulation of transfected COS-1 cells with Wy-14,643 in combination with PD98059 increased PPRE-dependent luciferase activity compared with Wy-14,643 treatment alone (Fig. 6). The enhancement by PD98059 of PPRE luciferase activity varied from 1.5- to 4-fold in different experiments. However, GH treatment inhibited Wy-14,643-induced reporter activity to a similar extent in the presence and in the absence of PD98059 (Fig. 6). Since PD98059 did not block the GH inhibitory effect, the MAP kinases ERK1 and ERK2 are not likely to mediate GH inhibition of PPARα activity. PD98059 treatment also enhanced Wy-14,643-induced PPARα activity in the absence of GHR, JAK2, and STAT5β (data not shown). This suggests that basal MAP kinase activity in these cells exerts a negative effect on PPARα activity but in a manner that is independent of GHR or STAT5β expression or GH treatment.

Tyr-699 Phosphorylation of STAT5β Is Required for GH Inhibition of PPARα Activity—STAT5β is activated by JAK2 kinase-dependent phosphorylation on tyrosine 699. Mutation of this tyrosine to phenylalanine (STAT5b-Y699F) results in a loss of STAT5b dimerization, DNA binding, and transcriptional activation (44). Fig. 7 shows that, when activated by GH, wild-type human STAT5b inhibited PPARα activity to a similar extent as did mouse STAT5b. By contrast, the Y699F substitution abrogated GH inhibition of PPARα activity. Accordingly, STAT5b
tyrosine phosphorylation and/or its downstream activities (STAT5b dimerization, nuclear translocation, DNA binding, or transcriptional activation) are obligatory for STAT5b to mediate GH inhibition of PPARα. When equal amounts of human STAT5a expression plasmid were transfected, it failed to inhibit PPARα activity (Fig. 7). Partial inhibition (40%) was observed, however, at 4-fold higher human STAT5a plasmid levels (data not shown).

**Requirement of STAT5b COOH-terminal trans-Activation Domain and Effects of Dominant-negative STAT5b Mutant**—Deletions of the COOH-terminal trans-activation domain of mouse STAT5a and STAT5b (constructs STAT5aΔ749 and STAT5bΔ754, respectively) result in a loss of transcriptional activity and yield truncated STAT5 proteins that exert dominant-negative effects on wild-type STAT5-induced gene transcription (43). These COOH-terminal truncated STAT5 proteins undergo hormone-induced tyrosine phosphorylation and retain DNA binding activity but show delayed tyrosine dephosphorylation (43). These mutants were used to examine whether the transcriptional activity of STAT5b is necessary for GH inhibitory effects. Fig. 8 shows that, in contrast to wild-type STAT5b, STAT5bΔ754 cannot mediate GH-induced PPARα inhibition, suggesting that the trans-activation domain of STAT5b is required for GH inhibitory effects. In contrast, STAT5aΔ749 inhibited PPARα activity by 40% following GH stimulation.

To test for dominant-negative activity, COS-1 cells were transfected with wild-type STAT5b in the presence of increasing amounts of STAT5aΔ749 or STAT5bΔ754. Fig. 8B shows that STAT5bΔ754 could fully block the STAT5b-dependent GH inhibition of PPARα activity in a dose-dependent manner (lane 2 versus lanes 3 and 4). Interestingly, while STAT5aΔ749 has dominant-negative activity toward wild-type STAT5b and can block its transcriptional activity (43), this mutant had only a modest effect on STAT5b-dependent PPARα inhibition (lane 2 versus lanes 5 and 6). However, interpretation of this result is...
COS-1 cells were cotransfected with expression plasmids encoding GHR, JAK2, STAT5b, and the reporter plasmid pHDX3Luc. Transfected cells were stimulated with 15d-PGJ2 (5 μM) with or without GH (100 ng/ml). Cell extracts were prepared 24 h later and assayed for luciferase activities relative to β-galactosidase transfection controls.

We next investigated whether STAT5b DNA binding activity is required to inhibit PPAR activity. The effects of two rat STAT5b mutants, STAT5b-EE and STAT5b-VVVI (45), were compared with that of wild-type rat STAT5b. In these mutants, STAT5b mutants, STAT5b-EE and STAT5b-VVVI (45), were compared with that of wild-type rat STAT5b. In these mutants, STAT5b-EE and STAT5b-VVVI were site-specific mutants (STAT5b-Y699F and STAT5b-Y694F) were transiently transfected into COS-1 cells together with GHR, JAK2, PPARα and pHDX3Luc reporter plasmid. Transfected cells were treated with Wy-14,643 (Wy) (20 μM) and GH (0, 25 or 100 ng/ml) for 24 h. Cell extracts were then prepared and assayed for luciferase activity relative to a β-galactosidase transfection control.

The level of STAT5b activity was measured in whole cell extracts prepared and assayed for luciferase activity relative to a β-galactosidase transfection control. 

Wild-type rat STAT5b and its glucocorticoid-induced gene expression (59, 60). STAT5b inhibition of the stimulatory effects of prolactin at the IRF-1 promoter is also proposed to involve direct protein-protein interactions (45). We therefore considered whether a direct interaction between GH-activated STAT5b and PPARα can be detected using an EMSA assay for PPARα-PPRE-protein-DNA complexes. Fig. 9 shows that anti-STAT5b antibody does not alter the mobility of a PPARα-containing DNA complex formed on a PPRE probe from the CYP4A6 gene (lanes 3 versus 2) under conditions where the antibody fully supershifts STAT5b-containing DNA complexes (Figs. 2B and 3C). These data indicate that STAT5b does not bind directly to a PPARα-DNA complex. In control samples, the PPAR-PPRE complex seen in lane 2 was either disrupted or supershifted by various anti-PPARα antibodies (lanes 4 and 5), competed by an excess of unlabeled PPRE probe (lane 6) and was fully dependent on transfected PPARα for its formation (lane 7).

The level of STAT5b activity was measured in whole cell extracts prepared and assayed for luciferase activity relative to a β-galactosidase transfection control. 

Wild-type rat STAT5b and rat STAT5aΔ749 itself confers partial inhibition of PPARα in response to GH stimulation (Fig. 8A). Fig. 8C confirms the expression of STAT5aΔ749, STAT5bΔ754, and wild-type STAT5 proteins in the transfected COS-1 cells, detected with an antibody against the STAT5 SH2 and SH3 domains, which are upstream of the deleted sequences. As expected, the STAT5bΔ754 and STAT5aΔ749 bands seen on this blot were of lower molecular weight (lanes 4 and 7) and could not be detected using an antibody that specifically recognizes the extreme COOH-terminal region of STAT5b (Santa Cruz antibody sc-835; data not shown). When equal amounts of expression plasmid were transfected, STAT5aΔ749 protein was expressed at a level similar to wild-type STAT5b (lane 7 versus lanes 2 and 3). The level of STAT5bΔ754 protein (lane 4) was much lower (Fig. 8C), highlighting the potency of its dominant-negative effects (Fig. 8B). EMSA analysis showed that STAT5aΔ749 and STAT5bΔ754 both bind to a β-casein DNA probe much more efficiently than wild-type STAT5b (Fig. 8D; lanes 2, 3, 8, 9, 11, and 12 versus lanes 5 and 6), in agreement with earlier studies (43). This suggests that these mutants achieve their dominant-negative effect, at least in part, by interfering with wild-type STAT5b DNA binding activity. As expected, antibody specific for the STAT5b COOH-terminal peptide can completely supershift wild-type STAT5b but not the much more intense STAT5bΔ754 binding to the β-casein probe (Fig. 8D, lanes 7 versus 13).

Mutation of STAT5b Tyr-699 abolishes GH inhibition of PPARα activity. Expression plasmids encoding human STAT5a, human STAT5b and their site-specific mutants (STAT5b-Y699F and STAT5b-Y694F) were transiently transfected into COS-1 cells together with GHR, JAK2, PPARα expression plasmids and pHDX3Luc reporter plasmid. Transfected cells were treated with Wy-14,643 (Wy) (20 μM) and GH (0, 25 or 100 ng/ml) for 24 h. Cell extracts were then prepared and assayed for luciferase activity relative to a β-galactosidase transfection control.
JAK/STAT-PPAR Cross-talk

FIG. 8. Dominant-negative STAT5b blocks GH inhibition of PPARα activity by wild-type STAT5b. A. Effects of COOH-terminal truncated STAT5b and STAT5a on PPARα activity. COS-1 cells were cotransfected with expression plasmids encoding GHR, JAK2, and the STAT5b COOH-terminal deletion constructs STAT5bΔ754 (ΔSTAT5b) or STAT5aΔ749 (ΔSTAT5a). Transfected cells were treated with Wy-14,643 (Wy) (20 μM) alone or in the presence of GH (100 ng/ml) for 24 h. Cell extracts were then prepared and assayed for firefly luciferase activity relative to a Renilla luciferase transfection control. ΔSTAT5a, but not ΔSTAT5b, partially inhibited Wy-14,643-induced reporter gene activity in GH-treated cells. B, STAT5bΔ754 blocks wild-type STAT5b-dependent inhibition of PPARα activity. COS-1 cells were transfected with pHD(X3)Luc reporter and PPARα, GHR, JAK2, and mouse STAT5 expression plasmids together with increasing amounts of ΔSTAT5b or ΔSTAT5a expression plasmids (0, 0.5, 1, and 5-fold relative to the amount of cotransfected wild-type STAT5b, calculated on a per microgram plasmid DNA basis). Cells were stimulated with Wy-14,643 (20 μM) and GH (100 ng/ml) for 24 h. Luciferase activities relative to a β-galactosidase transfection control were then assayed in whole cell extracts. C, expression of STAT5b, STAT5a, and their COOH-terminal truncated mutants. The same cell lysates analyzed in B were analyzed for STAT5 protein expression by anti-STAT5 Western blotting (Transduction Laboratories, antibody S21520). The COOH-terminal truncated mutants. The same cell lysates analyzed in B were analyzed for STAT5 DNA binding activity using the β-casein promoter probe, as described under “Materials and Methods.” STAT5b COOH-terminal targeted antibody (Santa Cruz antibody sc-835) was used for supershift (SS) where indicated (anti-5b).

STAT5b-EE both inhibit PPARα activity in GH-treated cells in a manner similar to the effects of mouse and human STAT5b shown above. By contrast, much less inhibition is seen with STAT5b-VVVI. Thus, the VVVI residues in the DNA binding domain of STAT5b are critical for GH-activated STAT5b to efficiently inhibit PPARα activity, strongly suggesting that this inhibition requires STAT5b DNA binding and transcriptional activation activity.

DISCUSSION

GH and several other hormones, including thyroid hormone (triiodothyronine) and glucocorticoids, modulate the pleiotropic responses of rodent liver to structurally diverse peroxisome proliferators mediated by the nuclear receptor PPARα. The present studies demonstrate that the GH-activated transcription factor STAT5b, and to a lesser extent STAT3, can mediate the inhibitory effects of GH on PPARα transcriptional activity and that these effects occur at a physiological GH level. This specific inhibitory effect on PPARα activity was seen both with rodent and human STAT5b proteins and occurred when STAT5b was activated by either GH or prolactin. Given that STAT5b can also be activated by a large number of cytokines and growth factors, and is widely expressed in mammalian tissues (41, 51), the potential for inhibitory interactions between PPARα and JAK-STAT5b signaling pathways is widespread. Although STAT5a (mammary gland factor) and STAT5b show ~90% amino acid sequence identity, and both by a carboxyl-terminal targeted STAT5b antibody (Santa Cruz antibody sc-835; data not shown). D, EMSA analysis of STAT5b, ΔSTAT5a, and ΔSTAT5b DNA binding activity. The same cell lysates shown in B and C were analyzed for STAT5 DNA binding activity using the β-casein promoter probe, as described under “Materials and Methods.” STAT5b COOH-terminal targeted antibody (Santa Cruz antibody sc-835) was used for supershift (SS) where indicated (anti-5b).
siently transfected with pHD(X3)Luc reporter and expression plasmids
encoding PPARs, GHR, JAK2 and rat STAT5b or either of two rat
STAT5b site-specific mutants localized to STAT5b’s DNA binding do-
main. STAT5b-EE retains DNA-binding activity, while STAT5b-VVVI
is devoid of DNA-binding activity (45). Transfected cells were treated
with Wy-14,643 (20 μM) and GH (25 and 100 ng/ml) for 24 h. Cell
extracts were assayed for firefly luciferase activity relative to renilla
luciferase activity.

bind to and trans-activate target genes via STAT5 response
elements such as that found in the β-casein promoter, they
have distinct tissue-specific functions that cannot be compen-
sated by each other in vivo, as shown in the case of mouse
STAT5 gene knock-out studies (61, 62). The present study
provides further evidence for differences between the two
STAT5 forms, insofar as STAT5a was less effective at inhibiting
PPARα activity, particularly when activated by prolactin
(Fig. 4).

Functional interactions involving the binding of STAT fac-
tors and another nuclear receptor family member, glucocorti-
coid receptor, have been described (59, 60, 63). STAT5a and
glucocorticoid receptor form a molecular complex that en-
hances STAT5a-induced transcription from the β-casein pro-
moter and inhibits glucocorticoid-stimulated transcription
from a glucocorticoid response element. Specific DNA binding
by STAT5a is required for cooperation with glucocorticoid rec-
erator on the β-casein promoter, and although the receptor
does not bind the STAT5a DNA-binding element directly, it
associates with the STAT5a-DNA complex. A potential glu-
ocorticoid receptor-binding site is present within the β-casein
promoter, and this site is required for the synergism between
STAT5a and glucocorticoid receptor (64). These studies estab-
lish a model in which STAT proteins cross-talk with nuclear
receptors by direct protein-protein interactions that modulate
gene expression. By contrast, we were unable to detect mole-
cular complexes involving both STAT5b and PPARα in the pres-
ent study, as judged by EMSA supershift analysis. Transcrip-
tional inhibitory effects of prolactin-activated STAT5b have
also been observed in studies of the full-length IRF-1 (interfer-
on regulatory factor-1) promoter but not with an isolated IRF-1
STAT response element (45). In contrast to our findings with
PPARα, the prolactin inhibitory effects seen on the IRF-1 pro-
moter are conferred equally well by STAT5a or STAT5b. More-
over, unlike our findings in the present study, the effects of
STAT5b on the IRF-1 promoter do not require the DNA binding
activity of STAT and are proposed to involve STAT5b in pro-
tein-protein interactions with other transcription factors (45).

GH activates the MAP kinase pathway in many cell types,
including liver cells (32, 53, 65). MAP kinase can, in turn,
phosphorylate and thereby modulate the activity of a variety of
transcription factors, including STAT proteins, which may un-
dergo MAP kinase-catalyzed serine phosphorylation required
for full transcriptional activity (66, 67). Growth factor-acti-
vated MAP kinase phosphorylates PPARγ, resulting in an in-
hibition of the transcriptional activity of that nuclear receptor
(56, 68). In our experiments, the MAP kinase kinase inhibitor
PD98059 increased the responsiveness of COS-1 cells to
PPARα activators, suggesting that MAP kinase phosphorylates
and thereby inhibits PPARα by a mechanism similar to that
described for PPARγ (56, 68). However, PD98059 did not alter
the extent to which GH-activated STAT5b inhibited PPARα
activity.

Several mechanisms may account for the inhibition of
PPARα by GH-activated STAT5b described in the present
study. First, activated STAT5b could inhibit PPARα protein
expression and thereby block PPAR activation of PPRE. How-
ever, this is considered unlikely, since GH-activated STAT5b
had no effect on expression of the internal control β-galactosi-
dase expression plasmid, which utilizes the same cytomegalovirus
promoter as does the PPARα expression plasmid used in
our experiments. Second, STAT5b might compete with PPARα
for binding to the PPAR reporter PPRE elements of plasmid.
This is also unlikely, since the pHDX3/Luc reporter used in
this study does not contain STAT5b-binding sites. Moreover,
gel-shift assays revealed that STAT5b does not bind to an
isolated PPRE (Fig. 9, lane 7). Third, by analogy to the inter-
action of STAT5a with glucocorticoid receptor discussed above,
STAT5b may form a protein-protein complex with PPARα
and thereby prevent PPARα from trans-activating its target genes.
However, the inability of a STAT5b-specific antibody to super-
shift activated PPARα when bound on a PPRE element (Fig. 9)
argues against a direct association of STAT5b with PPARα,
indicating a mechanism distinct from the previously described
STAT5a-glucocorticoid receptor association. We cannot rule out
the possibility, however, that STAT5b might form a complex
with PPARα that is independent of its binding to PPRE and
thus not detected in our experiments.

An alternative possibility is that STAT5b inhibits PPARα
activity by an indirect mechanism. For example, when present
in the nucleus in a transcriptionally active state, STAT5b may
compete for an essential coactivator of PPARα, such as SRC-1,
CBP/p300, or PBP (69–71), or perhaps modulate the binding of
PPAR to other interacting proteins, such as RXRs or RlP140
(72), leading to inhibition of transcriptional activity of PPARα.
Indeed, CBP and/or p300 have been implicated in the antago-
nism between STAT and AP-1 signaling pathways (73). Alter-
natively, given the requirement for intact, functional STAT5b
DNA binding and transcriptional activation domains, STAT5b
may activate transcription of a second gene, leading to produc-
tion of a distinct protein factor that serves as a more proximal
inhibitor of PPARα activity. Both of these possibilities are
consistent with our observation that a dominant-negative
STAT5b mutant (STAT5bΔT54 (43)) blocks wild-type PPARα
inhibitory activity of STAT5b (Fig. 8). Of note, some caution
about the interpretation of the effects of the STAT5b mutants
employed in this study is required. For example, if mutation of
the STAT5b DNA binding domain residues VVVI (45) were to
interfere with STAT5b tyrosine phosphorylation and/or nuclear
translocation, then a failure to translocate into the nucleus
rather than the loss of DNA binding activity per se could
account for the observed lack of PPARα inhibition (Fig. 10).
The precise mechanism underlying the dominant-negative effect
of the COOH-terminal truncated STAT5b also needs to be eluci-
dated. Further study will be required to test and evaluate these
and other possible inhibitory mechanisms.

The inhibition of PPARα activity by activated STAT5b de-
activators, such as clofibrate, are reported to have both induc-
tive (74, 75) and suppressive effects (76) on the enzymes in-
hibitors, including chlorinated hydrocarbons and their metabolites,
molecules are also possible.

Leukotriene B4, a mediator of certain inflammatory and
immunological reactions, has been identified as an endogenous
ligand for PPARα (43). Peroxisome proliferators and PPARα
activators, such as clofibrate, are reported to have both induc-
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54. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843–14846
55. Marshall, C. J. (1995) Cell 80, 179–185
56. Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996) Science 274, 2100–2103
57. Shalev, A., Siegrist-Kaiser, C. A., Yen, P. M., Wahl, W., Burger, A. G., Chin, W. W., and Meier, C. A. (1996) Endocrinology 137, 4499–4502
58. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7686–7689
59. Stocklin, E., Wissler, M., Gouilleux, F., and Groner, B. (1996) Nature 383, 726–728
60. Cella, N., Groner, B., and Hynes, N. E. (1998) Mol. Cell. Biol. 18, 1783–1792
61. Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A., and Hennighausen, L. (1997) Genes Dev. 11, 179–186
62. Udy, G. B., Towers, R. P., Snell, R. G., Wilkins, R. J., Park, S. H., Ram, P. A., Waxman, D. J., and Davey, H. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7239–7244
63. Zhang, Z., Jones, S., Hagood, J. S., Fuentes, N. L., and Fuller, G. M. (1997) J. Biol. Chem. 272, 30607–30610
64. Lechner, J., Welte, T., Tomasi, J. K., Bruno, P., Cairns, C., Gustafsson, J., and Doppler, W. (1997) J. Biol. Chem. 272, 20954–20960
65. Love, D. W., Whatmore, A. J., Clayton, P. E., and Silva, C. M. (1998) Endocrinology 139, 1965–1971
66. Zhu, Y., Qi, C., Calandra, C., and Chen-Kiang, S. (1995) Proc. Soc. Exp. Biol. Med. 198, 513–527
67. Weigent, D. A. (1996) Pharmacol. Ther. 69, 257–257
68. Weigent, D. A., Baxter, J. B., and Blalock, J. E. (1992) Brain Behav. Immun. 6, 365–376
69. Yamada, J., Sugiyama, H., Watanabe, T., and Suga, T. (1995) Res. Commun. Mol. Pathol. Pharmacol. 90, 173–176
70. Ledwith, B., Manam, S., Trulio, P., Jodlyn, D. J., Galloway, S. M., and Nichols, W. W. (1993) Mol. Carcinogen. 8, 20–27