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Toxicology and Applied Pharmacology 199 (2004) 275–284
Toxicology and Applied Pharmacology
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Simultaneous, bidirectional inhibitory crosstalk between PPAR and STAT5b

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Received 3 September 2003; accepted 10 December 2003
Available online 8 March 2004

Abstract

The transcription factors peroxisome proliferator-activated receptor (PPAR) and signal transducer and activator of transcription (STAT5) activate genes involved in fatty acid metabolism (PPARα) and adipogenesis (PPARγ) and mediate hormonal responses important for body growth, liver gene expression, and mammary gland development (STAT5a and STAT5b). These seemingly disparate pathways are subject to mutually inhibitory crosstalk, with growth hormone (GH)-activated STAT5 able to inhibit PPAR-regulated gene transcription by approximately 80%, and conversely, ligand-activated PPAR able to inhibit STAT5-regulated transcription to a similar degree. Given the co-expression of PPAR and STAT5 in multiple tissues, we investigated whether one of the factors dominates the inhibitory crosstalk. A PPAR-responsive *Renilla* luciferase reporter was constructed and used to monitor PPAR transcriptional activity in COS-1 cells co-transfected with a STAT5 firefly luciferase reporter. In cells co-stimulated with GH and a PPAR agonist, STAT5b inhibited expression of the PPAR-regulated *Renilla* luciferase reporter, whereas PPARα and PPARγ inhibited transcription of the STAT5b-regulated firefly luciferase reporter. The extent of the inhibitory crosstalk was dependent on the relative levels of expression of each transcription factor and on the relative concentrations of GH and PPAR agonist. Dose-response studies revealed that STAT5b was inhibited at an approximately 7-fold lower concentration of the PPARγ ligand troglitazone than was required for activation of PPARγ, indicating that only a portion of cellular PPARγ is needed for STAT5b inhibition. Similarly, mono-(2-ethylhexyl)phthalate (MEHP), a reproductive toxicant and primary metabolite of the environmental chemical di-(2-ethylhexyl)phthalate (DEHP), inhibited STAT5b transcriptional activity with an EC_{50} value of 1.1 μM, corresponding to an approximately 10-fold lower concentration than required for activation of PPARγ-dependent transcription. We conclude that the cross-inhibition between PPAR and STAT5 proceeds in a simultaneous, bidirectional manner. Exposure to phthalates and other environmental chemical activators of PPARs may thus lead to alteration of hormone-induced, STAT5-regulated gene expression in tissues such as liver, fat and breast, where both transcription factors are expressed. Conversely, STAT5-activating hormones and cytokines may modulate the responsiveness of PPARs to their foreign chemical ligands.

Keywords: STAT5; PPAR; Crosstalk; Mono-(2-ethylhexyl)phthalate

Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and are activated by a variety of natural and synthetic ligands (Willson et al., 2000), leading to the transcription of target genes involved in fatty acid metabolism (PPARα) (Schoonjans et al., 1995; Zhang et al., 1993), fat cell differentiation (PPARγ) (Spiegelman and Flier, 1996), and cholesterol homeostasis (PPARδ) (Berger et al., 1999). Ligand-activated PPAR binds as a heterodimer with the retinoid X receptor to peroxisome proliferator response elements in the regulatory regions of target genes. The three PPAR subtypes exhibit tissue specificity, with PPARα being highly expressed in liver, PPARγ in adipose tissue, and PPARδ more ubiquitously expressed. PPARα agonists include hypolipidemic fibrate drugs and related chemicals, such as Wy-14,643 (Issemann and Green, 1990) and a variety of naturally occurring saturated and unsaturated fatty acids (Gottlicher et al., 1992). PPARγ is activated by antidiabetic thiazolidinedione drugs (Lehmann et al., 1995) including troglita-

Abbreviations: DEHP, di-(2-ethylhexyl)phthalate; GH, growth hormone; GHR, GH receptor; MEHP, mono-(2-ethylhexyl)-phthalate; PPAR, peroxisome proliferator-activated receptor; PP, peroxisome proliferator; STAT, signal transducer and activator of transcription.

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doi:10.1016/j.taap.2003.12.020

zone, and by natural ligands such as 15-deoxy-Δ12,14 prostaglandin J2 (Kliewer et al., 1995). The phthalate monoesters mono-(2-ethylhexyl)-phthalate (MEHP) and monobenzyl phthalate (MBzP), which are widespread environmental chemical contaminants derived from phthalate diester plasticizers, have also been identified as PPAR agonists (Hurst and Waxman, 2003; Maloney and Waxman, 1999) and peroxisome proliferator chemicals (Lake et al., 1975; Lhuguenot et al., 1988).

PPARs are co-expressed with the transcription factor STAT5[1] in several tissues, including liver, adipose, and breast. Signal transducer and activator of transcription 5 (STAT5) is a latent cytoplasmic transcription factor that is activated by multiple cytokines and hormones via cell surface receptor-associated JAK family tyrosine kinases. Tyrosine phosphorylated STAT5 undergoes homo-dimerization via its Src homology 2 domain, followed by translocation to the nucleus, binding to specific DNA enhancer elements and activation of target gene expression (Bromberg and Darnell, 2000; Darnell, 1997). STAT5 can inhibit PPARα- and PPARγ-regulated transcription by a mechanism that involves PPAR's AF-1 ligand-independent trans-activation domain (Zhou and Waxman, 1999a, 1999b). Crosstalk between STAT5 and PPAR can also occur in the opposite direction, with ligand-activated PPARα and PPARγ inhibiting STAT5-regulated gene transcription in growth hormone (GH)-stimulated cells (Shipley and Waxman, 2003).

STAT5 (primarily STAT5a) plays a role in the terminal differentiation of mouse mammary epithelial cells during pregnancy and lactogenesis (Liu et al., 1997; Teglund et al., 1998) and is also activated in normal nonpregnant mouse and human breast epithelial cells (Nevalainen et al., 2002). In addition to their central role in hormone- and cytokine-induced cell signaling, STATs, including STAT5, are activated by diverse oncoproteins. The constitutively activated STAT signaling that results contributes to oncogenesis (Bowman et al., 2000), in part by down-regulation of apoptosis-related genes, leading to increased cell survival (Nevalainen et al., 2002). In mouse mammary gland, STAT5 prevents apoptosis of terminally differentiated epithelial cells (Humphreys and Hennighausen, 1999). Human mammary carcinoma (MCF-7) cells stably transfected with a human GH cDNA display elevated STAT5 transcriptional activity and exhibit increased proliferation compared with wild-type MCF-7 cells, demonstrating a role for GH, and potentially STAT5, in mitogenesis (Kaulsay et al., 1999). Other data suggest that PPARγ may behave as a tumor suppressor gene, although several mouse models, paradoxically, indicate that under certain circumstances, PPARγ ligands may induce tumorigenesis (Koeffler, 2003). The role of PPARγ in fat cell differentiation is well established (Tontonoz et al., 1994), and the possibility that PPARγ ligands may act as pro-differentiation/antiproliferative agents has been well discussed (Mueller et al., 1998; Sarraf et al., 1998; Yoshida et al., 2003). Given the co-expression of STAT5 and PPAR in multiple tissues (Escher and Wahli, 2000; Groner and Hennighausen, 2000) and the above evidence for their potentially opposing roles in cell survival and cancer, we sought to determine whether STAT5 or PPAR dominates the inhibitory crosstalk when both signaling pathways are active.

In the present study, we developed a dual luciferase reporter assay to simultaneously, and independently, monitor STAT5b and PPAR transcriptional activity. Each factor is shown to inhibit the transcriptional activity of the other factor in cells co-stimulated with a STAT5 activator and a PPAR agonist. We evaluate the impact of changes in ligand concentration and the relative levels of STAT5 and PPAR expression on the direction and the magnitude of the inhibitory crosstalk. Finally, we demonstrate that the environmental phthalate monoester MEHP not only serves as an agonist of PPARγ, but can inhibit hormone-stimulated STAT5b transcriptional activity in a PPARγ-dependent manner.

Materials and methods

Plasmids. The PPAR-activated firefly luciferase reporter pHDx3luc, obtained from Dr. J. Capone (McMaster University, Toronto, ON, Canada), contains three tandem copies of the PP response element from the rat enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene upstream of a minimal promoter cloned into the plasmid pCPS-luc. The reporter plasmid pZZ1, provided by Dr. B. Groner (Institute for Experimental Cancer Research, Freiburg, Germany), contains the β-casein milk protein gene promoter cloned upstream of the firefly luciferase gene. Mouse PPARα cloned into the expression plasmid pCMV5 was obtained from Dr. E. Johnson (Scripps Research Institute, La Jolla, CA). Mouse PPARγ expression plasmid pSV-Sport-mPPARγ and pSV-Sport empty vector were obtained from Dr. J. Reddy (Northwestern University, Chicago). Rat GH receptor (GHR) cloned into the expression plasmid pcDNAI was provided by Dr. N. Billestrup (Hagedorn Research Institute, Denmark). pME18S expression plasmid encoding mouse STAT5b and pME18S empty vector were obtained from Dr. A. Mui (DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto, CA). β-Galactosidase expression plasmid pSV-βgal was purchased from Promega (Madison, WI).

Construction of the PPAR Renilla luciferase reporter pHDx3rluc. phRL-CMV (Promega) was digested with *Xba*I and *Nhe*I to give a 947-bp fragment containing the *Renilla* luciferase gene. This fragment was ligated to pHDx3luc after digestion of the latter plasmid with *Xba*I

[1] 'STAT5' and 'PPAR' are used to refer generically to STAT5a and STAT5b, and to PPARα and PPARγ, respectively. The individual terms STAT5a, STAT5b, PPARα, and PPARγ are used to refer to the indicated specific transcription factors.

to remove the firefly luciferase gene. *Nhe*I and *Xba*I generate compatible sticky-ends, allowing for ligation of the excised *Renilla* luciferase gene into the *Xba*I-digested pHDx3luc. The resultant *Renilla* luciferase reporter plasmid, pHDx3rluc, was characterized and verified with respect to its orientation and functionality as a PPAR-activated *Renilla* luciferase reporter in a COS-1 transient transfection assay (see below).

Cell culture and transfection studies. COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cells were plated in 48-well tissue culture plates at a density of 2.5×10^4 cells/well in 500-μl culture medium. Twenty-four hours later, the medium was replaced with 250 μl DMEM containing 10% serum. The cells were then transfected using 0.3 μl FuGENE 6 transfection reagent (Roche Molecular Biochemicals) and

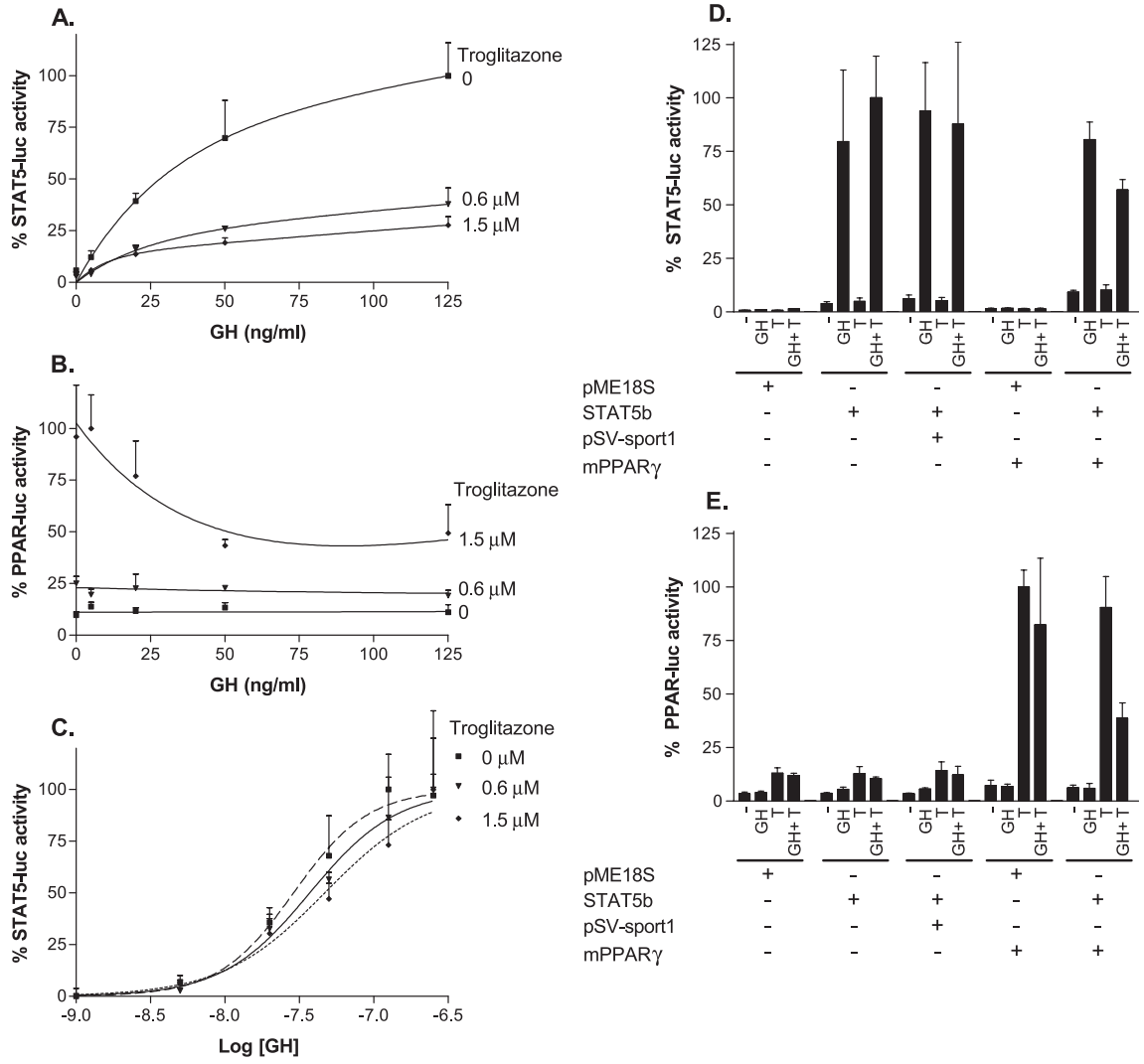

Fig. 1. Troglitazone-activated PPARγ inhibits a STAT5b-activated firefly luciferase reporter (A) at the same time that GH-activated STAT5b inhibits a PPARγ-activated *Renilla* luciferase reporter (B). COS-1 cells were transfected for 24 h with the STAT5b reporter plasmid pZZ1 (70 ng) and the PPAR reporter plasmid pHDx3rluc (70 ng) together with pSV-βgal (20 ng) as an internal control. Cells were co-transfected with expression plasmids encoding GHR, STAT5b, and mPPARγ (20, 5, and 5 ng, respectively). Twenty-four hours after transfection, cells were co-stimulated for a further 24 h with increasing concentrations of GH and the PPARγ ligand troglitazone (0, 0.6, or 1.5 μM). Cell lysates from triplicate wells were then prepared and assayed for firefly luciferase, *Renilla* luciferase, and β-galactosidase activity. Activities are expressed as firefly or *Renilla* luciferase normalized by the reporter activity of the β-galactosidase internal standard, mean ± SD values, as a percentage of the observed maximum reporter activity. STAT5-luc and PPAR-luc refer to the STAT5-responsive pZZ1 firefly luciferase reporter and the PPAR-responsive pHDx3rluc *Renilla* luciferase reporter, respectively. (Panel C) The inhibition by PPARγ did not alter the EC_{50} for GH-stimulated activation of the pZZ1 firefly luciferase reporter, pZZ1. pZZ1 firefly luciferase activity is presented as a percentage of the maximum activity at each of the indicated troglitazone concentrations. The concentration of GH required to elicit 50% of maximal STAT5b transcriptional activity (EC_{50}) was 29 ng/ml in the absence of troglitazone (■), 37 ng/ml with 0.6 μM troglitazone (▼), and 47 ng/ml with 1.5 μM troglitazone (♦). This apparent troglitazone-dependent increase in EC_{50} was not statistically significant (*F* test, Graph Pad Prism, v4.0). The *x*-axis represents the logarithm of GH concentration (ng/ml). (Panels D and E) Empty plasmid vectors did not activate or inhibit the transcriptional activity of the STAT5-luc (D) or PPAR-luc reporters (E). Conditions were as described for A and B, but using fixed concentrations of GH (250 ng/ml) and troglitazone (T) (3 μM) and substituting mPPARγ and STAT5b with the relevant empty vector control, pSV-Sport1 and pME18S, respectively.

250 ng total DNA per well of a 48-well plate. Individual wells received 70 ng of reporter plasmid (pHDx3rluc or pZZ1), 5 ng of PPARα or PPARγ expression plasmid or pSV-Sport1 empty vector, 20 ng of GHR expression plasmid, 5 ng of STAT5b expression plasmid or pME18S empty vector, and 20 ng pSV-βgal. Salmon sperm DNA was used as a carrier to adjust the total to 250 ng DNA per well. Twenty-four hours after addition of the DNA–Fugene 6 mixture to the cells, the culture medium was changed to serum-free DMEM containing Wy-14,643, troglitazone, MEHP and/or GH at the concentrations indicated in the figure legends. Cells were lysed 24 h later in 250 μl passive lysis buffer (Promega) and firefly and *Renilla* luciferase activities were measured in the same cell extract using a dual luciferase assay kit (Promega). Firefly luciferase and *Renilla* luciferase are dissimilar in structure and substrate requirements, making it possible to assay each enzyme in an independent manner. The dual luciferase assay kit uses two different substrates, beetle luciferin and coelenterate luciferin, to monitor firefly luciferase and *Renilla* luciferase activity, respectively. β-Galactosidase activity was measured as an internal control using the Galacto-light Plus kit (Tropix). Firefly and *Renilla* luciferase activities were normalized to the β-galactosidase activity determined for the same cell lysate. Data are presented as means ± SD luciferase activities for $n = 3$ separate determinations. Results shown in each figure are representative of at least three independent sets of experiments.

Western blot analysis. COS-1 cells in 6-well plates were transfected as in the 48-well plate experiments described above. Cell number, plasmid amount, and Fugene transfection reagent amount were increased by a factor of 12.25 to account for the larger surface area of the 6-well plates. Whole cell lysates were prepared and 20 μg was analyzed by 7.5% SDS-polyacrylamide gel electrophoresis followed by Western blotting using anti-hPPARγ antibody (Santa Cruz Biotechnology, sc-7196; dilution 1/200) as described elsewhere (Zhou and Waxman, 1999a).

Results

PPAR and STAT5b transcriptional activity can be monitored simultaneously using a dual luciferase reporter system

GH-activated STAT5b inhibits PPAR transcriptional activity, as demonstrated in transfection studies using the firefly luciferase reporter pHDx3luc (Zhou and Waxman,

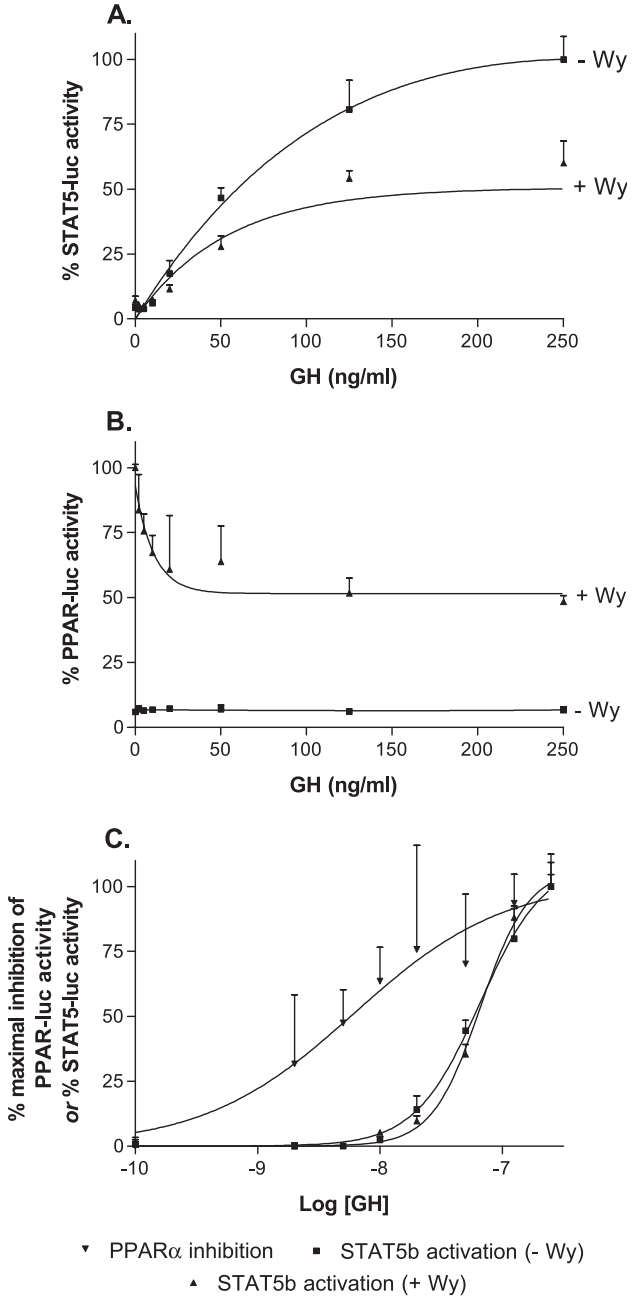

Fig. 2. Wy-14,643-activated PPARα inhibits a STAT5b-activated firefly luciferase reporter (A) at the same time that GH-activated STAT5b inhibits a PPARα-activated *Renilla* luciferase reporter (B). COS-1 cells were transfected for 24 h with the STAT5b reporter plasmid pZZ1 and the PPAR reporter plasmid pHDx3rluc together with pSV-βgal as an internal control and expression plasmids encoding GHR, STAT5b, and mPPARα as outlined in Fig. 1. Twenty-four hours after transfection, cells were co-stimulated for a further 24 h with increasing concentrations of GH and the PPARα ligand Wy-14,643 (5 μM). Cell lysates from triplicate wells were then prepared and assayed for luciferase and β-galactosidase activity. Activities are expressed as luciferase normalized by the reporter activity of the β-galactosidase internal standard, mean ± SD values, as a percentage of the maximum reporter activity. STAT5-luc and PPAR-luc refer to the STAT5-responsive pZZ1 firefly luciferase reporter and the PPAR-responsive pHDx3rluc *Renilla* luciferase reporter, respectively. (Panel C) Inhibition of PPAR reporter activity by STAT5b occurred at a lower concentration of GH than was required to activate a STAT5b reporter. pZZ1 firefly luciferase activity is presented as a percentage of the maximal activity in the absence (■ EC_{50} = 66 ng GH/ml) or presence of Wy-14,643 (▲ EC_{50} = 66 ng GH/ml). pHDx3rluc *Renilla* luciferase activity is presented as a percentage of maximal observed inhibition of Wy-14,643-activated PPARα by GH-activated STAT5b (▼ EC_{50} = 6 ng GH/ml). The *x*-axis represents the logarithm of GH concentration (ng/ml).

1999a, 1999b). This inhibitory crosstalk is mutual, insofar as PPARα and PPARγ can both inhibit STAT5b-dependent transcription assayed using the STAT5-responsive β-casein promoter-firefly luciferase reporter pZZ1 (Shipley and Waxman, 2003). To determine whether the inhibitory effects of STAT5b dominate those of PPAR, or vice versa, studies were carried out in COS-1 cells co-transfected with a *Renilla* luciferase reporter (pHDx3rluc) to monitor PPAR transcriptional activity, and STAT5b in combination with GHR and a firefly luciferase reporter (pZZ1) to monitor STAT5b activity. This enabled us to simultaneously monitor GH-activated STAT5b and troglitazone-induced PPARγ transcriptional activities in a single transfection experiment. Cells were transfected and then stimulated for 24 h with troglitazone in the presence of increasing concentrations of GH. Fig. 1 shows that under conditions where PPARγ inhibited STAT5b-regulated transcription by 75% (panel A), STAT5b inhibited PPARγ-regulated transcription by approximately 50% (panel B). This experiment also demonstrates that STAT5b can be substantially inhibited by PPARγ at a concentration of troglitazone that is lower than that required to activate PPARγ transcriptional activity. Thus, 0.6 μM troglitazone inhibited STAT5b nearly maximally (Fig. 1A) under conditions where it induced only approximately 25% of maximal PPARγ transcriptional activity (Fig. 1B). Further analysis of these data replotted in logarithmic form (Fig. 1C) showed that troglitazone-activated PPARγ did not have a significant effect on the concentration of GH required for STAT5b activation. Thus, the initial signaling events required for STAT5b activation, which involves GH-induced dimerization of GHR leading to activation of the tyrosine kinase JAK2, are not targets for the inhibitory action of PPARγ. To demonstrate that the mutually inhibitory crosstalk between PPARγ and STAT5b is specific, experiments were carried out using the relevant empty vector plasmids as controls. The empty vector plasmids for STAT5b (pME18S) and PPARγ (pSV-sport1) have no transcriptional activity and do not respond to ligand (Fig. 1D, first set of bars, and Fig. 1E, third set of bars). PPARγ inhibited STAT5b-regulated transcription, whereas the control plasmid did not (Fig. 1D, last versus third set of bars). Analysis of *Renilla* luciferase activity in the same

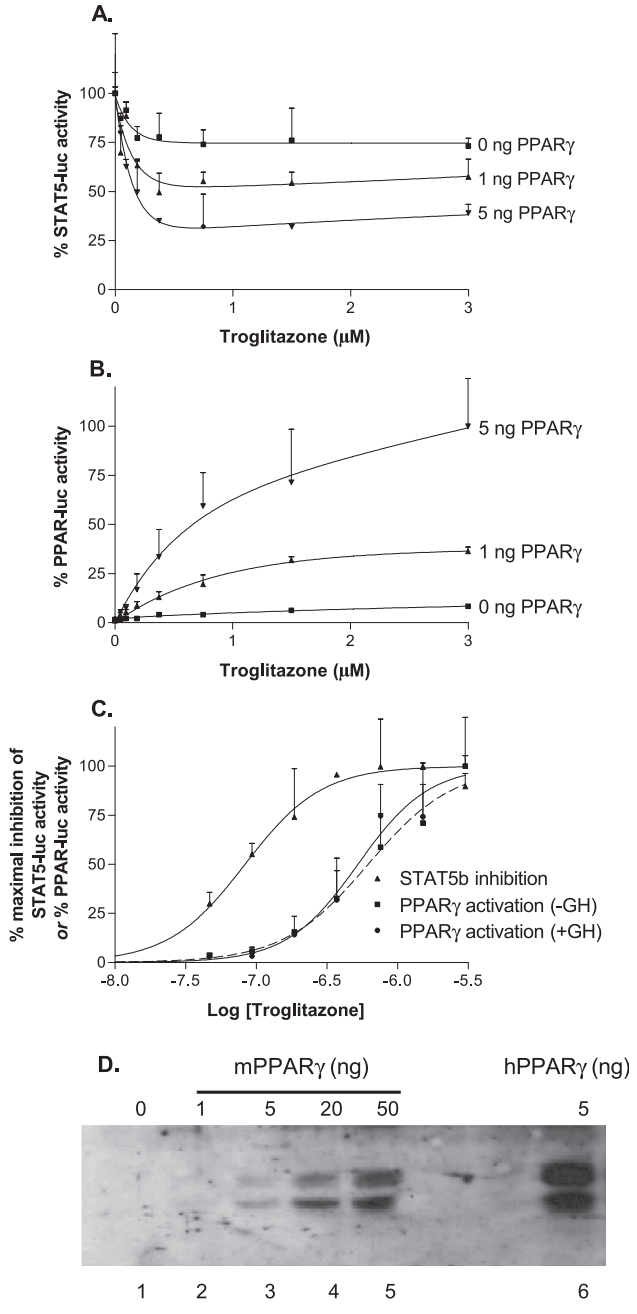

Fig. 3. Inhibition of STAT5b firefly luciferase reporter activity pZZ1 (A) and activation of the PPAR-activated *Renilla* luciferase reporter pHDx3rluc (B) as a function of troglitazone concentration and PPARγ expression level. COS-1 cells were transfected for 24 h with the STAT5b reporter plasmid pZZ1 (70 ng) and the PPAR reporter plasmid pHDx3rluc (70 ng) together with expression plasmids encoding GHR (20 ng), STAT5b (5 ng), and the indicated amounts of mPPARγ. Twenty-four hours after transfection, cells were co-stimulated for a further 24 h with GH (250 ng/ml) and increasing concentrations of troglitazone. Cell lysates from triplicate wells were then prepared and assayed for firefly and *Renilla* luciferase activity. Activities are expressed as luciferase activity, mean ± SD values, as a percentage of the maximum reporter activity. STAT5-luc and PPAR-luc refer to the STAT5-responsive pZZ1 firefly luciferase reporter and the PPAR-responsive pHDx3rluc *Renilla* luciferase reporter, respectively. (Panel C) Maximum inhibition of STAT5b reporter activity by PPARγ occurred at a lower concentration of troglitazone than was required to fully activate the PPAR reporter. pHDx3rluc *Renilla* luciferase activity is presented as a percentage of the maximum activity observed in the absence (● EC_{50} = 0.52 μM troglitazone) or presence of GH (■ EC_{50} = 0.62 μM troglitazone). The concentration of troglitazone that resulted in 50% of the maximal inhibition of STAT5b (EC_{50}) was 0.083 μM (▲). The *x*-axis represents the logarithm of troglitazone concentration (M). (Panel D) Transfection of increasing amounts of PPARγ expression plasmid results in an increase in PPARγ protein expression. COS-1 cells were co-transfected with mPPARγ1 (0–50 ng) or hPPARγ1 (5 ng), STAT5b (5 ng) and GHR expression plasmids (20 ng), the STAT5 reporter plasmid pZZ1 (70 ng) and the PPAR reporter plasmid pHDx3rluc (70 ng) for 24 h. Cell lysates were analyzed on Western blots probed with anti-hPPARγ antibody, which gives a darker band with the hPPARγ positive control sample (lane 6) than with the cross-reactive mPPARγ (lane 3). The double band observed is presumed to be due to alternative splicing of PPARγ (Nicholas et al., 2001). Data shown are representative of three independent experiments.

samples demonstrated that STAT5b inhibited PPARγ transcriptional activity, whereas the empty vector control did not (Fig. 1E, last versus fourth set of bars).

We next investigated the inhibitory crosstalk between PPARα and STAT5b. Fig. 2 shows that PPARα inhibited STAT5b-regulated transcription by 50% (panel A), while STAT5b simultaneously inhibited PPARα-regulated transcription to a similar extent (panel B). The inhibition of PPARα-regulated transcription occurred at a substantially lower concentration of GH than was required to fully activate STAT5 reporter activity (Fig. 2C), a finding analogous to that seen with PPARγ (Fig. 1). Taken together, these studies demonstrate that the inhibitory crosstalk between STAT5b and PPARα or PPARγ is mutual, and can take place in both directions simultaneously.

Impact of PPAR ligand concentration and expression level on inhibition of STAT5-regulated transcription

We next investigated the effect of varying troglitazone concentrations and PPARγ plasmid levels on PPARγ-mediated transcription and the inhibition of STAT5b. Increasing inhibition of STAT5b transcriptional activity was observed with increasing troglitazone concentrations and increasing PPARγ plasmid levels (Fig. 3A), with maximal PPAR-dependent luciferase activity and maximal inhibition of STAT5-dependent luciferase activity achieved at 5 ng PPARγ expression plasmid. Analysis of the same cell extracts for PPAR-dependent reporter gene activity verified that PPARγ transcriptional activity increased as a function of troglitazone concentration and amount of transfected PPARγ plasmid (Fig. 3B). Comparison of the troglitazone concentration dependence for the two cellular responses, activation of PPARγ and inhibition of STAT5b, revealed that STAT5b inhibition occurred at an approximately 7-fold lower troglitazone concentration (EC_{50} = 0.083 μM troglitazone) than was required for activation of PPARγ (EC_{50} = 0.5–0.6 μM troglitazone) (Fig. 3C). Thus, STAT5b is maximally inhibited when only a small portion of the cellular PPARγ is activated. Western blot analysis of whole cell extracts prepared from the transfected COS-1 cells demonstrated that transfection of increasing amounts of PPARγ expression plasmid (ng) results in a dose-dependent increase in PPARγ protein (Fig. 3D).

MEHP-activated PPARγ inhibits STAT5b-regulated transcription

MEHP, a bioactive metabolite of the commonly used plasticizer di-(2-ethylhexyl)phthalate (DEHP), activates PPARγ in transfected COS-1 cells (Maloney and Waxman, 1999) and stimulates PPARγ-dependent adipogenesis (Hurst and Waxman, 2003). To investigate whether MEHP-activated PPARγ inhibits STAT5b-regulated transcription, the co-

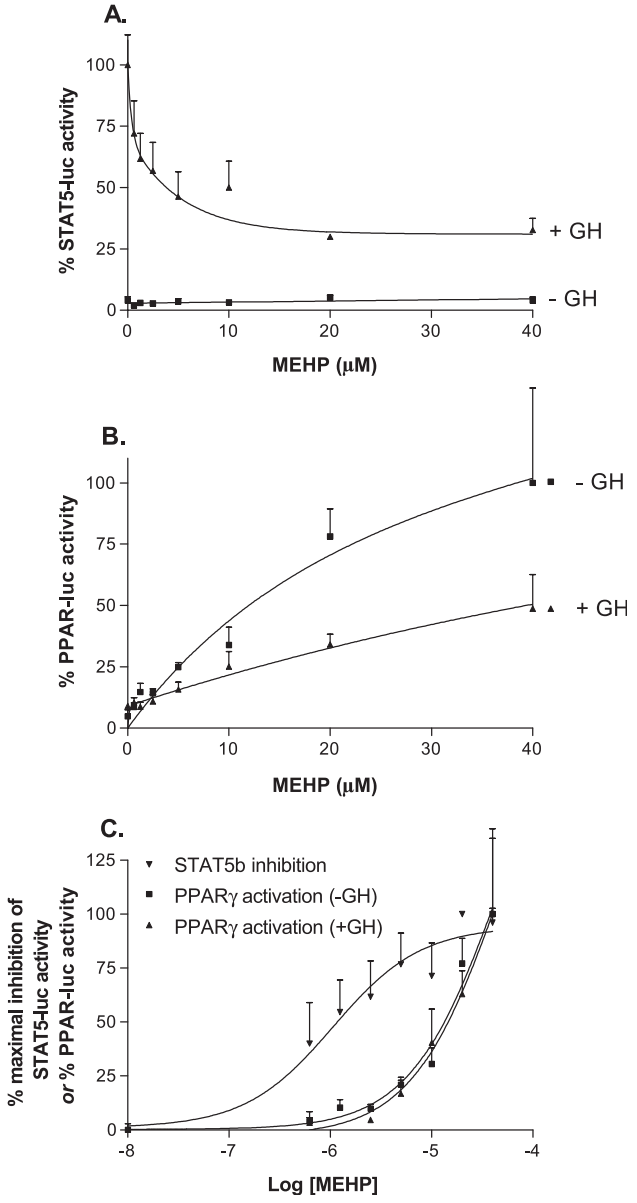

Fig. 4. MEHP-activated PPARγ inhibits a STAT5b-activated firefly luciferase reporter (A) at the same time as GH-activated STAT5b inhibits a MEHP-activated PPAR *Renilla* luciferase reporter (B). COS-1 cells were transfected for 24 h with the STAT5b reporter plasmid pZZ1 and the PPAR reporter plasmid pHDx3rluc together with pSV-βgal as an internal control and expression plasmids encoding GHR, STAT5b, and mPPARγ as in Fig. 1. Twenty-four hours after transfection, the cells were co-stimulated for a further 24 h with GH (250 ng/ml) and increasing concentrations of MEHP. Cell lysates from triplicate wells were then prepared and assayed for firefly luciferase, *Renilla* luciferase, and β-galactosidase activity. Activities are expressed as luciferase normalized by the reporter activity of the β-galactosidase internal standard, mean ± SD values, as a percentage of the maximal reporter activity. STAT5-luc and PPAR-luc refer to the STAT5-responsive pZZ1 firefly luciferase reporter and the PPAR-responsive pHDx3rluc *Renilla* luciferase reporter, respectively. (Panel C) Inhibition by STAT5b does not alter the EC_{50} for MEHP-stimulated activation of the PPAR *Renilla* luciferase reporter, pHDx3rluc. Maximum inhibition of STAT5b reporter activity by PPARγ occurred at a lower concentration of MEHP than was required to fully activate a PPAR reporter. EC_{50} values could not be determined based on the data shown in panel C because PPARγ activation had not reached saturation. The *x*-axis represents the logarithm of MEHP concentration (M).

transfection COS-1 cell model described above was used to evaluate the effect of GH in combination with MEHP treatment on STAT5b and PPAR reporter activities. Fig. 4 shows that MEHP-activated PPARγ inhibited STAT5b-regulated transcription in a dose-dependent manner, with a maximum of 70% inhibition observed (Fig. 4A). At the same time, GH-activated STAT5b inhibited PPARγ-regulated transcription to a similar degree, approximately 50% (Fig. 4B). As was seen for troglitazone, STAT5b was inhibited at a substantially lower concentration of MEHP (EC_{50} for STAT5b inhibition = 1.1 μM) than was required for activation of PPARγ (EC_{50} = 10 μM) (Hurst and Waxman, 2003). Moreover, although GH-activated STAT5b decreased the maximal transcriptional activity of PPARγ (Fig. 4B), it did not alter the concentration at which maximal PPARγ activity was achieved (Fig. 4C).

Discussion

PPARs are activated by structurally diverse chemicals, including MEHP, a metabolite of the widely used plasticizer DEHP, and troglitazone, a member of the thiazoladinedione class of antidiabetic drugs. STAT family members act downstream of the cytokine/GH/prolactin receptor superfamily (Liu et al., 1998) to activate target genes, such as those involved in mammary gland development and lactation (Groner and Hennighausen, 2000). Previous studies have established that STAT5 inhibits PPAR-mediated transcription (Zhou and Waxman, 1999a, 1999b), and vice versa, PPAR inhibits STAT5-mediated transcription (Shipley and Waxman, 2003) by a mechanism that requires the NH_2-terminal, AF-1 trans-activation domain of PPAR. The present studies demonstrate that this inhibitory crosstalk can take place in a simultaneous, bidirectional manner. These findings have implications for tissues such as liver and breast, where PPAR and STAT5 are both expressed and where they regulate processes such as cell growth, differentiation, and apoptosis (Davey et al., 1999; Humphreys and Hennighausen, 1999; Liu et al., 1997). The observation that STAT5a, as well as STAT5b, can inhibit PPAR-regulated transcription (Zhou and Waxman, 1999a) suggests that the findings reported here are applicable to both STAT5 forms and offer insight into the crosstalk between STAT5 and PPAR signaling that may occur in breast tissue, where STAT5a, rather than STAT5b, is the major mediator of mammopoietic and lactogenic signaling (Liu et al., 1997).

Dose-response studies revealed that the relative levels of expressed, and activated, PPARγ and STAT5b are important determinants of the extent of inhibitory crosstalk between the two pathways. STAT5 reporter activity was increasingly inhibited at higher expressed PPARγ protein levels and with an increase in the concentration of the PPARγ agonist troglitazone, both of which were associated with an increase in PPAR reporter activity. Accordingly, the potential for inhibition of STAT5 transcriptional activity is likely to be highest in tissues with high levels of PPAR, and under conditions of exposure to elevated levels of PPAR ligands, including drugs targeted to PPARs, such as hypolipidemic drugs (PPARα) and thiazolidinedione antidiabetics (PPARγ). Conversely, PPAR-mediated transcription is likely to be more susceptible to inhibition by hormones or cytokines that activate STAT5 in tissues that express high STAT5 levels or under physiological conditions associated with elevated STAT5 activity. Examples of the latter include male rodent liver, where STAT5 (primarily STAT5b) activity is elevated compared to female liver in response to pulsatile plasma GH stimulation (Choi and Waxman, 2000; Waxman et al., 1995) and differentiating mammary gland during late stage pregnancy and lactation, where STAT5 (primarily STAT5a) is persistently activated in response to prolactin stimulation (Groner and Hennighausen, 2000).

Previous studies have investigated the underlying mechanism for STAT5b-PPAR inhibitory crosstalk (Shipley and Waxman, 2003; Zhou and Waxman, 1999a). The AF-1 trans-activation domain of PPAR was identified as critical for this crosstalk (Zhou and Waxman, 1999b) and several potential mechanisms, such as the recruitment of histone deacetylases and competition for the coactivators p300 and SRC-1, have been evaluated (Shipley and Waxman, 2003). In the present study, we determined that PPARγ inhibited STAT5b transcriptional activity, but in a way that did not substantially change the concentration of GH required for half-maximal activation of STAT5b (Figs. 1C and 2C). This supports our earlier proposal, based on PPAR inhibition of a constitutively active STAT5b mutant (STAT5b1*6), that the inhibitory action of PPAR does not target the initial signaling events required for STAT5b activation, which involve an apparent GH-induced dimerization of GHR and activation of the receptor-associated tyrosine kinase JAK2 (Shipley and Waxman, 2003). Conversely, the inhibition of PPAR-regulated transcription by GH-activated STAT5b did not affect the concentration of MEHP (Fig. 4C) or troglitazone (Fig. 3C) required for half-maximal activation of PPARγ transcriptional activity.

Maximal inhibition of PPAR-regulated transcription was found to take place at a concentration of GH where STAT5b transcriptional activity was only a fraction of its maximum level. This indicates that only a portion of the cellular STAT5b pool needs to be activated to achieve maximal inhibition of PPAR. Although this might suggest that there is an excess of STAT5b protein available for inhibition of PPARγ, it was also observed, in experiments carried out under identical conditions, that STAT5b is inhibited at concentrations of PPAR agonists well below their EC_{50} values for PPAR activation. Taken together, these observations suggest that both signaling pathways are subject to bidirectional inhibition even under conditions where one of the transcription factors (PPAR or STAT5) is expressed at a comparatively low level and under con-

ditions where the concentration of PPAR agonist or STAT5 activator is low. Of particular note is our finding that exposure to approximately 1 μM MEHP is sufficient for half-maximal inhibition of STAT5b transcriptional activity. This concentration of MEHP is substantially lower than the EC_{50} of 10 μM MEHP for activation of PPARγ (Hurst and Waxman, 2003) and is well within the range of peak plasma MEHP concentrations that induce reproductive and developmental toxicities in rodents (Pollack et al., 1985). Given the incomplete nature of the mutual inhibition of STAT5 and PPAR activity seen in these studies (e.g., approximately 70% maximal inhibition of STAT5b by PPARγ, Fig. 3A; and approximately 50% maximal inhibition of PPARα and PPARγ by GH-activated STAT5b, Figs. 1B and 2B), both signaling pathways may proceed at less than maximal levels in cells and tissues where they are co-expressed.

The present findings suggest that the effects of environmental exposure to PPs may in part be dependent on STAT5 signaling and may have either a positive or a negative overall impact, depending on the hormonal status of affected cells and tissues. For example, the activation of PPARγ by environmental PPs could have detrimental effects on physiological processes such as mammary gland development and lactation because of the associated inhibition of STAT5 signaling. This inhibitory crosstalk might not only result in effects of PP exposure during pregnancy on STAT5-dependent mammary gland differentiation; it may also contribute to the male gonad developmental defects induced by DEHP and its primary metabolite MEHP (Li et al., 2000; Sharpe, 2001). Cytokines and growth factors play important roles in reproduction, mediating both proliferation and differentiation of cells in the testis. Multiple STATs are expressed in testes, although their precise roles are unclear. STAT1 and STAT3 are activated by interferon-γ and interleukin-6 in Sertoli cells of rat testis (Jenab and Morris, 1997), and the prolactin receptor has been shown to activate STAT5 in human testes (Hair et al., 2002). Mice lacking PPARα retain susceptibility to DEHP-mediated testicular toxicity (Ward et al., 1998), suggesting that other PPAR isoforms, such as PPARγ (Maloney and Waxman, 1999) may mediate this toxicity. Crosstalk of PPAR with STAT1 (Ricote et al., 1998), STAT3 (Zhou and Waxman, 1999a), and STAT5 (described herein) raises the possibility that PPs such as MEHP exert some of their toxic effects on the developing testes via inhibition of normal STAT signaling. However, it should be pointed out that some phthalate monoesters, such as mono-*n*-butyl phthalate, are not PPAR ligands (Hurst and Waxman, 2003), but still cause reproductive toxicity (Oishi and Hiraga, 1980; Shono and Suita, 2003), suggesting that additional receptors may be involved and other mechanisms may occur.

Treatment with a PPAR ligand may be beneficial in certain cases where modulation of GH signaling alters the balance between cell survival and cell death. Elevated expression of GH in mammary carcinoma cells promotes cell proliferation and STAT5 transcriptional activity (Kaulsay et al., 1999). Consequently, inhibition of this STAT5 activity by treatment with a PPAR ligand may decrease the intrinsic mitogenic properties of STAT5b. This may augment the previously described inhibition of tumorigenesis via PPARγ-induced differentiation (Haydon et al., 2002; Itami et al., 2001; Koeffler, 2003). The activation of PPARγ may have further potential therapeutic benefits by inhibiting the biosynthesis of estrogen, a hormone that has been implicated in breast cancer development (Rubin et al., 2000).

The studies reported here were carried out using transiently transfected COS-1 cells, with all of the advantages, and disadvantages, including potential effects associated with receptor overexpression, that this model entails. Of note, the COS-1 cell model enabled us to vary the individual components of both signaling pathways and to observe changes in crosstalk in response to changes in the relative expression levels of STAT5b and PPAR. These studies were carried out under conditions where the interacting factors were expressed at levels that are apparently very low (e.g., 1–5 ng PPARγ plasmid, which results in PPAR protein levels that are barely detectable or undetectable by Western blot analysis; Fig. 3D). Nevertheless, further investigation will be required to characterize the inhibitory crosstalk under more physiological cellular conditions and expression levels, and to evaluate potential downstream consequences of the crosstalk. One potentially useful model is the 3T3-L1 cell line, a well-established fibroblast model of adipocyte differentiation, where STAT5b and PPARγ are both expressed at significant levels. Other studies, carried out in more complex in vivo systems, have already demonstrated the potential for mutual inhibition of STAT5 and PPAR target gene expression. Thus, protein levels of three PPARα-responsive peroxisomal β-oxidation pathway enzymes are increased up to 2- to 3-fold in STAT5b-deficient mouse liver compared to wild-type mouse liver (Zhou et al., 2002), indicating that STAT5b inhibits basal PPARα-regulated liver gene transcription in vivo. Conversely, ligand activation of PPARα leads to down-regulation of several GH-regulated, sexually dimorphic liver genes (Corton et al., 1998) which are regulated, in part, by STAT5b (Park et al., 1999, 2001; Udy et al., 1997).

In conclusion, we have demonstrated that the bidirectional inhibitory crosstalk between STAT5b and PPAR can take place simultaneously. Inhibition may dominate in one direction or the other depending on the relative expression levels of STAT5b and PPAR and the respective concentrations of PPAR ligands and STAT5 activators. These findings are likely to be particularly relevant to cells and tissues where both factors and signaling pathways are expressed and where their activation and normal physiological function is susceptible to hormonal fluctuations and exposure to environmental PPs.

Acknowledgments

This research was supported in part by NIH Grant 5 P42 ES07381, Superfund Basic Research Center at Boston University (to D.J.W). The study sponsor had no involvement in study design; in the collection, analysis, and interpretation of data; in the writing the report; and in the decision to submit the paper for publication. The authors wish to thank Dr. Christopher Hurst, Boston University, for statistical advice.

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