Peroxisome Proliferator-activated Receptor α Inhibits Hepatic S14 Gene Transcription

EVIDENCE AGAINST THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR α AS THE MEDIATOR OF POLYUNSATURATED FATTY ACID REGULATION OF S14 GENE TRANSCRIPTION*

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The peroxisome proliferator-activated receptor (PPARα) has been implicated in fatty acid regulation of gene transcription. Lipogenic gene transcription is inhibited by polyunsaturated fatty acids (PUFA). We have used the PUFA-sensitive rat liver S14 gene as a model to examine the role PPARα plays in fatty acid regulation of hepatic lipogenic gene transcription. Both PPARα and the potent peroxisome proliferator, WY14643, inhibit S14CAT activity in transfected primary hepatocytes. WY14643 and PPARα target the S14 T3 regulatory region (TRR, 2.8 to 2.5 kilobases), a region containing 3 T3 response elements (TRE). Transfer of the TRR to the TKCAT gene following WY14643 and PPARα treatment. Gel shift analysis showed that PPARα, either alone or with RXRα, did not bind the S14TRR. However, PPARα interfered with TRβ/RXRα binding to a TRE (DR+4). Functional studies showed that co-transfected RXRα, but not T3 receptor β1 (TRβ1), abrogated the inhibitory effect of PPARα on S14 gene transcription. These results suggest that WY14643 and PPARα functionally interfere with T3 regulation of S14 gene transcription by inhibiting TRβ/RXR binding to S14 TREs. Previous studies had established that the cis-regulatory targets of PUFA control were located within the proximal promoter region of the S14 gene, i.e. between –220 and –80 bp. Finding that the cis-regulatory elements for WY14643/PPARα and PUFA are functionally and spatially distinct argues against PPARα as the mediator of PUFA suppression of S14 gene transcription.

Dietary polyunsaturated fatty acids (PUFA), particularly highly unsaturated n-3 PUFA, have a strong suppressive effect on hepatic lipogenesis and triglyceride synthesis leading to a decline in plasma triglycerides (1–7). The suppression of hepatic lipogenesis is due, at least in part, to an inhibition of transcription of several genes encoding proteins involved in both lipogenesis and glycolysis, including fatty acid synthase, stearoyl-CoA desaturase, L-type pyruvate kinase, and the S14 protein (1–9).

The S14 gene has been used as a model for PUFA control of lipogenic gene transcription because the developmental, tissue-specific, hormonal, and nutritional control of S14 is similar to that found for several lipogenic enzymes (2, 3). For example, transcription of both fatty acid synthase (FAS) and S14 genes is induced during postnatal development and by insulin, high carbohydrate feeding, and triiodothyrinone (T3) and suppressed by diabetes, starvation, and hormones that elevate hepatic cAMP levels. Feeding rats diets enriched in n-3 PUFA rapidly and coordinately inhibits transcription of both hepatic genes (1, 3). PUFA administration to primary hepatocytes suppresses mRNAs5 and mRNAs14 levels (ED50 ≤ 100 μM), as well as inhibiting transcription of S14CAT fusion genes. Transfection analysis has localized PUFA-response elements (PUFA-RE) to the proximal promoter region of the S14 gene, i.e. between –220 and –80 bp upstream from the transcription start site. The targets for PUFA control of S14 are distinct from the cis-regulatory elements involved in T3 between –2.8 and 2.5 kb and insulin/glucose (between –1.6 and –1.4 kb) control of S14 gene transcription (3).

Since our goal is to define the molecular basis of PUFA-mediated control of gene transcription, we became interested in the peroxisome proliferator-activated receptor (PPAR) as a prospective candidate for a PUFA-regulated factor (PPAR-RE) affecting lipogenic gene transcription. PPAR belongs to the steroid/thyroid supergene family and is involved in lipid metabolism (10–12). PPAR activates gene transcription by binding peroxisome proliferator response elements (PPRE) in association with retinoid X receptor (RXR) (10–19). Several PPAR subtypes have been identified, some showing tissue-specific distribution and ligand-dependent activation (10–12, 19–26). PPARα appears to be the predominant PPAR subtype in liver (27), and PPARα can be activated by fatty acids as well as peroxisome proliferators to induce enzymes involved in peroxisomal and mitochondrial β-oxidation and cholesterol metabolism (12, 20, 21, 24, 29–41).

Three lines of evidence suggest that PPARα might be involved in PUFA control of lipogenic gene expression: (a) feeding rats high-fat diets induces peroxisomal enzymes (37–40), (b) in vitro transcription studies show that fatty acids activate PPARα (12, 20, 24, 26, 28, 34–37), and (c) the potent peroxisome proliferator, WY14643, suppressed both mRNAs5 and S14CAT activity in cultured primary rat hepatocytes (42). In this report, we characterize the peroxisome proliferator regulation of S14 gene transcription. In contrast to our expectation, the cis-regulatory targets for WY14643 and PPARα action
mapped to a region of the S14 promoter containing TREs and not to the proximal promoter region containing the negative PUFA-response elements (nPUFA-RE). Based on these studies, PPARα may not be the mediator of PUFA regulation of hepatic S14 gene expression.

EXPERIMENTAL PROCEDURES

Plasmids—Construction of all S14CAT reporter genes, the thymidine kinase (TK) fusion gene TKCAT222, which contains the S14TRR (−2.9 to −2.5 kb), RSVCAT, and the rat liver thyroid hormone receptor β1 (TRβ1) (MLVTRβ1) have been described previously (3). An expression vector encoding the mouse PPARα (pSGS-PPARα) was generously provided by Stephen Green (Cheshire, UK) (10). A TK CAT reporter gene (TKCAT223) containing the rat acyl-CoA oxidase (AOX)-PPRE was constructed by polymerase chain reaction amplification of the region between −119 and −463 bp upstream from the rat AOX gene (43) using rat genomic DNA as template and oligonucleotide primers (sense: 5'-ATATAGGATCCAGGTAACCTTGTCAGG and antisense: 5'-ATATAGGATCCAGGTAACCTTGTCAGG) (synthesized at the MSU Macrostructure Facility). After amplification, the 735-bp fragment was gel-purified and inserted upstream from the TK promoter.

Transfections—Primary rat hepatocytes were prepared by the collagenase perfusion method, cultured on Primaria tissue culture plates, and transfected with Lipofectin (Life Technologies) as described previously (3). Hepatocytes were treated with 100 μM WY14643 (Chemsys Science Laboratories, Lenexa, KS). WY14643 was dissolved in Me2SO, and Me2SO was used as a control for WY14643 treatment. Media were changed after 24 h, and cells were harvested for protein assay and CAT activity assay after 48 h of treatment (3). CAT activity: CAT units = counts/ min of 14C-butylated chloramphenicol/h/100 μg of protein.

Gel Shift Analysis—Mouse PPARα and RXRα were synthesized in vitro by programming the TNT transcription/translation system (Promega) with 1 μg of pSGS-PPARα, pSGS-RXRα, or prec-erbAβ, which contains the full-length coding region for PPARα, RXRα, and TRβ1, respectively (43). The AOX-PPRE was synthesized using oligonucleotides: 5'-GATCCTTCCGAGCTGACTTCCTGGTCCA and 5'-AGCTTGAAACAGGTTAAGCAGTCGTTGGG, annealed, end-labeled with 32P using T4-polydeoxyribonucleotide kinase, and used in gel shift assays (42, 44). DNA-protein complexes were formed by incubating end-labeled DNA (1-10 fmol) for 20 min at room temperature in a reaction mixture containing 4 μl of unprogrammed cell lysate or 2 μl of RXRα- and 2 μl of pMPPARα-programmed cell lysate in buffer (25 mM Tris/HCl (pH 7.5), 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM MgCl2, and 2 μg of poly(dI-dC) (Boehringer Mannheim)) (44). Previous studies showed the requirement of both RXRα and PPAR to bind AOX-PPRE (13, 19, 42). Unlabeled AOX-PPRE was present in the reaction mixture at a concentration of 10 μg/ml, approximately 10 times higher than labeled AOX-PPRE, to assure complete competitors and were added prior to the addition of labeled probe. After the binding reaction, 5 μl of buffer containing 0.16% bromophenol blue and 0.16% xylene cyanol was added to the DNA-protein complex just prior to loading on an 8% polyacrylamide gel (acrylamide:bisacrylamide, 75:1, w/w) in 3:1 TBE (1 × TBE = 89 mM Tris, 89 mM borate, 2.5 mM EDTA, pH 8.3) as electrophoresis buffer. After electrophoresis at 350 V for 90 min, the gels were dried, exposed to x-ray film at 80°C with intensifying screens, and quantified with AMBIS radioanalytic imaging detector (AMBIS Inc, San Diego, CA).

RESULTS

Effect of WY14643 and mPPARα on S14 Gene Promoter Activity—In primary rat hepatocytes, the potent peroxisome proliferator WY14643 suppressed hepatic mRNA514 and S14CAT activity in a dose-dependent manner with ED50 ≤ 50 μM (42). In the current study, the effects of WY14643 and PPARα on S14 gene transcription were examined using the S14CAT124 fusion gene containing a 5'-flanking region extending from −4315 to +19 bp of the S14 gene. Primary hepatocytes were co-transfected with S14CAT124 and a rat TRβ1 expression vector (MLVTRβ1) in the presence and absence of co-transfected mPPARα. Hepatocytes were treated with T3 to induce (∼50-fold) CAT activity (3). WY14643 was added to cells without and with co-transfected PPARα. WY14643 treatment and mPPARα co-transfection alone suppressed T3-induced CAT activity by 45% and 60%, respectively (Fig. 1). In the presence of WY14643/PPARα cis-Regulatory Elements Localize to the
The cis-regulatory elements targeted by PPARα and WY14643 were localized by deletion analysis using a strategy similar to the one used to localize the nPUFA-RE within the S14 proximal promoter region (3). The S14 gene has three major functional cis-regulatory regions. The proximal promoter (−290/+19 bp) contains the basal elements required to initiate gene transcription and to confer tissue specificity. This region also contains targets for PUFA-mediated suppression of transcription (3, 44). The pluriplastic response region (PRR, −1.6 to −1.4 kb) contains cis-acting regulatory elements required for tissue-specific, insulin/carbohydrate, and glucocorticoid control of transcription (2). The thyroid hormone response region (TRR, −2.9 to −2.5 kb) contains three functional thyroid hormone response elements (TRE) that bind TRβ1/RXR heterodimers and are required for the T3-mediated transactivation of the S14 gene (45).

A series of promoter deletions was prepared in which the S14TRR region was retained to ensure high transcriptional activity and to allow for an examination of an inhibitory effect of WY14643 and PPARα on S14CAT activity (Fig. 2B). As
shown in Fig. 1, both WY14643 and PPARα inhibited S14CAT124 by ~50%, and the combination inhibited S14CAT124 activity by 85%. This same pattern of control was seen with all S14 deletion constructs suggesting that the PRR (at −1.6/−1.4 kb) or the PUFA-RE (at −220/−80 bp) was not involved in WY14643/PPARα-mediated control of transcription.

The minimal elements required for the S14 gene to be responsive to WY14643 and PPARα were the elements within the proximal promoter region (−80/−19 bp) containing a TATA box and an NF-1 site and the upstream TRR. The S14TRR was tested as the prospective target of WY14643/PPARα control by fusing this element (−2.9/−2.5 kb) to the heterologous thymidine kinase promoter (TKCAT222). The specificity of WY1463/PPARα effects on transcription was examined by comparing the CAT activity of TKCAT222 with that of: 1) an enhancer-less TKCAT fusion gene (TKCAT202); 2) a S14CAT fusion gene containing the S14TRR fused upstream from the −290/−19-bp region of the S14 promoter (S14CAT149); and 3) a TKCAT fusion gene containing the AOX-PPRE, TKCAT223 (Fig. 3A). TKCAT202 was not significantly affected by WY14643 or co-transfected PPARα (Fig. 3B). Inserting the S14TRR upstream from the TK promoter conferred high levels of T3-induction (~50-fold) of CAT activity (see legend for Fig. 3 and Ref. 3). Both WY14643 and PPARα and the combination of these treatments inhibited TKCAT222 by ~50% and ~90%, respectively. This pattern of control is identical with that seen with S14CAT149 and suggests that the S14TRR is the target of WY14643/PPARα action.

In contrast to TKCAT222, TKCAT223, which contains the AOX-PPRE upstream from the TK promoter, was only marginally induced by WY14643 (2-fold), but significantly induced by co-transfected PPARα (56-fold). The combination of mPPARα co-transfection and WY14643 enhanced CAT activity by 140-fold (Fig. 3C). These results indicate that the direction of control and sensitivity to WY14643 and PPARα modification is enhancer-dependent. While the S14TRR confers negative control, the AOX-PPRE confers positive control to the TKCAT fusion gene following WY14643/PPARα treatment. These studies confirm and extend the deletion studies (Fig. 2) by showing that the S14TRR is sufficient and necessary for the negative effect of WY14643/PPARα on the S14 or TR promoter activity. We previously established that the PUFA-RE was localized to the −220/−80-bp region (Ref. 3 and Fig. 2A). The current studies show that the cis-regulatory elements for WY14643/PPARα and PUFA control of S14 gene transcription are functionally and spatially distinct.

mPPARα Does Not Bind the S14TRR Directly—Gel shift analysis was used to determine if PPARα-mediated effects on S14 gene transcription were due to direct binding to the S14TRR. Using the AOX-PPRE as a positive control for binding, neither PPARα nor RXRα alone binds the AOX-PPRE (Fig. 4). However, the combination of these receptors bind as a heterodimer. This observation is consistent with previous reports (13, 16, 19, 42). Addition of a 100-fold molar excess of unlabeled AOX-PPRE effectively competes for the formation of the PPARα/RXRα complex. In contrast, a 100-fold molar excess of the S14TRR failed to compete for binding (Fig. 4). No competition was seen with a 500-fold molar excess of TRR (not shown). The S14TRR region contains 3 TREs, which consist of direct repeats of AGGTCAG-related motifs separated by 4 nucleotides (45). These elements, also known as far upstream regulatory elements (FUR 10, 11, and 12) did not compete for PPARα/RXRα binding (data not shown). PPARα/RXRα also did not bind directly to a canonical DR-4 (gatcctAGGTCACag-gAGGTCagag, see Fig. 6). These studies show that PPARα, either alone or with RXRα, does not bind the S14 FUR elements or other DNA elements within the −2.9- to −2.5 kb S14TRR.

PPARα Suppresses Hepatic S14 Gene Expression by Functionally Interfering with TR/RXR Action—Since PPARα did not interact directly with the TRR, we speculated that PPARα might affect T3 action indirectly. A recent report showed that co-transfected PPARα effects on T3-dependent gene transcription were eliminated by elevating cellular RXR levels (46). To determine if PPARα action on S14CAT activity was affected by hepatocellular levels of other receptors, additional TRβ1 (as MLVTRβ1) or RXRα (as pSG5-RXRα) was co-transfected with a constant amount of pSG-PPARα (0.5 μg/well) and TKCAT222.
Dietary polyunsaturated fatty acids (PUFA) suppress transcription of genes encoding hepatic lipogenic enzymes (1-4) and induce expression of genes encoding peroxisomal enzymes (38-41). The fact that PPARs are activated by long chain fatty acids as well as peroxisome proliferators suggests PPAR might function as the common mediator for both PUFA and peroxisome proliferator control of hepatic lipogenic gene expression. Recent homologous recombination studies show that PPARα is the predominant subtype expressed in rodent liver, and this subtype accounts for the peroxisome proliferator regulation of several enzymes involved in lipid metabolism (27). Fatty acids appear to be activators of PPARα only under conditions of lipid overload, which occurs following peroxisome proliferator treatment, high fat feeding, diabetes mellitus, starvation, or pathological states when hepatic mitochondrial β-oxidation is suppressed, i.e. alcoholic liver disease (27, 35, 36). PUFA me-
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