Peroxisome Proliferator-activated Receptor γ Activation Modulates Cyclin D1 Transcription via β-Catenin-independent and cAMP-response Element-binding Protein-dependent Pathways in Mouse Hepatocytes

Chandan Sharma‡, Anamika Pradeep‡, Richard G. Pestell§, and Basabi Rana‡¶

From the ‡Division of Molecular Cardiology, The Texas A&M University System Health Science Center, College of Medicine, Temple, Texas 76504 and the ¶Lombardi Cancer Center, Georgetown University Medical Center, Washington, D. C. 20007

Activation of peroxisome proliferator-activated receptor γ (PPARγ) following exposure to PPARγ-specific ligands resulted in growth inhibition in various carcinoma cell lines. Our aim was to elucidate the pathway of PPARγ activation-mediated modulation of cyclin D1 transcription in mouse hepatocytes. To address this we utilized stable control and PPARγ hepatocyte cell lines created via retroviral overexpression utilizing AML-12 hepatocytes. Addition of PPARγ ligand troglitazone (TZD) activated PPARγ2 in proliferating hepatocytes and resulted in growth arrest accompanied by a down-regulation of proliferating cell nuclear antigen, cyclin D1, and β-catenin expression. Furthermore, activation of PPARγ2 attenuated cyclin D1 promoter activity indicating a transcriptional regulation of cyclin D1. Since β-catenin plays a pivotal role in regulating cyclin D1 transcription, we studied whether PPARγ2-mediated inhibition of cyclin D1 transcription involved β-catenin. Interestingly overexpression of either wild-type or S37A mutant β-catenin was unable to rescue PPARγ2-mediated suppression of cyclin D1 transcription, whereas overexpression of cAMP-response element-binding protein (CREB) was capable of antagonizing this inhibitory effect of PPARγ2. Additionally, pretreatment with okadaic acid antagonized PPARγ2-mediated inhibition of cyclin D1 transcription without any effect on β-catenin expression. These studies also showed that a TZD-mediated inhibition of total and phospho-CREBSer133 levels, CREB promoter activity, and cAMP-response element-mediated transcription in PPARγ hepatocytes. Pretreatment of PPARγ hepatocytes with okadaic acid, however, maintained higher total and phospho-CREBSer133 levels in the presence of TZD. These results indicated that PPARγ2 activation inhibited cyclin D1 transcription in hepatocytes via CREB-dependent and β-catenin-independent pathways.

Peroxisome proliferator-activated receptors (PPARs)1 belong to the nuclear receptor superfamily and are involved in regulating various cellular processes. The three known members of the PPAR family, PPARα, PPARγ, and PPARβ/δ, can regulate transcription of genes in response to specific ligands (1–3). The PPARγ subfamily consists of two protein isoforms, PPARγ1 and PPARγ2, with the former being expressed in a wide variety of tissues and the latter being preferentially expressed in adipose tissue (4). PPAR-mediated transactivation of target genes involves dimerization of PPARs with the retinoid X receptor and binding of the resulting heterodimer to specific peroxisome proliferator-activated receptor response elements (PPREs) located within the target gene promoters/enhancers (5). This process of transcriptional activation also involves the recruitment of different coactivator molecules to specify select target gene activation, which include p300 (6), the SRC-1 class of coactivators (7), PGC-1 and PGC-2 (8, 9), ARA70 (10), and DRIP205 (or TRAP220) (11).

In the preadipocytes PPARγ is known to orchestrate the process of differentiation into adipocytes via regulation of the transcription of target genes, which requires a prior exit from the cell cycle (12). In addition, PPARγ activation can inhibit proliferation in a wide variety of cell types (for a review, see Ref. 13) including those originating from hepatocellular carcinoma (14–16). The mechanism(s) by which PPARγ regulates cell growth is still unclear, although it mostly involves a G1/S-specific arrest (14, 17). In fibroblasts PPARγ-mediated growth arrest was associated with reduced transcriptional activity of E2F-DP, which was a consequence of the down-regulation of serine-threonine phosphatase PP2A expression (18). Similarly, in fibroblastic cell lines, activation of PPARγ resulted in induction of the cyclin-dependent kinase inhibitors p21(CIP1) and p18(INK4c) (19) accompanied by differentiation into adipocytes. Additionally, PPARγ activation in vascular smooth muscle cells resulted in G1/S arrest associated with reduced Retinoblastoma phosphorylation and modulation of cyclin-dependent kinase inhibitor levels (17). PPARγ activation in the MCF-7 breast cancer cells attenuated cyclin D1 transcription due to a decreased availability of p300 (20).

The normal cell growth cycle is tightly regulated via a complex interplay of multiple regulatory proteins. The transition

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1 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; TCF, T cell factor; TZD, troglitazone; PCNA, proliferating cell nuclear antigen; PP, protein phosphatase; HA, hemagglutinin; wt, wild-type; luc, luciferase; CD1, cyclin D1; Py, PPARγ; βcat, β-catenin.
from G1 to S phase is regulated by the controlled expression of cyclins, particularly cyclins D1 and E, which can bind and activate the corresponding cyclin-dependent kinases (21). In hepatocytes, ectopic expression of cyclin D1 can drive cells through G1 to S phase in a growth factor-independent manner (22), and chronic expression of cyclin D1 is sufficient to initiate hepatocellular carcinoma (23). Therapeutic interventions targeted toward regulation of cyclin D1 expression might thus be an effective means of controlling growth. The availability of the PPARγ ligands to regulate cyclin D1 expression indicates the possibility of utilizing these ligands as chemotherapeutic drugs. Detailed studies of the mechanism(s) by which PPARγ attenuates cyclin D1 expression are required to achieve this goal. Apart from the NFκB and AP-1 (24, 25) family of transcription factors, transcription from the cyclin D1 gene can also be induced following activation of the Wnt signaling pathway via a β-catenin- and TCF-mediated transactivation (26, 27). The cyclic AMP-response element (CRE) is another key site in the cyclin D1 promoter regulating cyclin D1 expression (28) particularly in hepatocytes (29).

PPARα and PPARγ are expressed together in several tissues, and their relative abundance changes in response to physiological effectors and disease (30, 31). Although expressed at lower levels in normal liver, hepatic expression of PPARγ2 is elevated in obese mice compared with lean littermates (32, 33). Additionally, PPARγ levels are elevated in hepatocellular carcinoma cell lines (15, 16); activation of PPARγ in hepatocellular carcinoma cell lines inhibits growth (14). In a recent study, adenovirus-mediated overexpression of PPARγ1 resulted in hepatic steatosis in the liver of PPARγ−/− mice (34). In other studies expression of PPARγ and its downstream target genes were increased following a homozygous deletion of cyclin D1 gene (35). These cyclin D1−/− mice developed hepatic steatosis with increased neutral lipid accumulation in the liver indicative of increased PPARγ activity. These results clearly indicate a distinct involvement of PPARγ in regulating hepatocyte physiology, although the mechanism and effectors involved are largely unclear.

To understand the role of PPARγ in regulating hepatocyte growth and to elucidate the pathway involved, we designed studies with control and PPARγ overexpression. The capability of the PPARγ agonist rosiglitazone (TZD) to inhibit growth (14). In a recent study, adenovirus-mediated overexpression of PPARγ resulted in hepatic steatosis in the liver of PPARγ−/− mice (34). In other studies expression of PPARγ and its downstream target genes were increased following a homozygous deletion of cyclin D1 gene (35). These cyclin D1−/− mice developed hepatic steatosis with increased neutral lipid accumulation in the liver indicative of increased PPARγ activity. These results clearly indicate a distinct involvement of PPARγ in regulating hepatocyte physiology, although the mechanism and effectors involved are largely unclear.

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PPARγ Modulates Cyclin D1 Transcription in Hepatocytes

A. subconfluent populations of control and PPARγ hepatocytes were incubated in the absence (−) or presence (+) of 10 μM TZD for the indicated periods of time. At the end of incubation, cells were harvested, and equal amounts of total protein from each sample were analyzed by Western blot using antibody against PPARγ. The same samples blotted with an actin antibody served as control. The same samples blotted with an actin antibody served as control.

B. exponentially growing control and PPARγ hepatocytes were transfected with tk-PPRE3-Luc and β-galactosidase vectors and cultured in growth medium in the presence (+) or absence (V) of 10 μM TZD for the indicated periods of time. Luciferase and β-galactosidase assays were performed, and the results obtained were expressed as percentage of control considering the values in TZD-untreated samples (V) as 100%. Each transfection was performed in triplicate, and each experiment was repeated at least three times. The data represent the mean ± S.D. of three independent experiments.

containing retroviral vector (pBabe-PPARγ-puro), respectively, were utilized (48). Western analysis at different time intervals with a PPARγ-specific antibody showed the specific expression of PPARγ2 protein in the PPARγ hepatocytes and not in control cells (Fig. 1A). Luciferase assays designed with the PPARγ-responsive reporter tk-PPRE3-Luc (containing PPRE sites attached to a luciferase vector) (38) showed a low PPRE luciferase activity in the control cells, irrespective of the presence of TZD, that was highly activated in the PPARγ cells following exposure to TZD (Fig. 1B). Comparison of the basal level of activity of this promoter also showed a significantly higher activity in the PPARγ cells compared with the control cells in the absence of TZD (data not shown). These results indicated that the PPARγ hepatocytes express a functional PPARγ2 protein.

Ectopic Expression and Activation of PPARγ2 in Proliferating Hepatocytes Arrests Growth and Inhibits Expression of PCNA—To determine whether the ectopic expression and activation of PPARγ2 in AML-12 hepatocytes can suppress growth, we analyzed the cell proliferation rate in the two cell types. These results showed that activation of PPARγ2 following addition of TZD resulted in complete inhibition of growth in the PPARγ cells (Fig. 2A) and was associated with an overall decrease in PCNA expression (Fig. 2B). Since PPARγ activation inhibits growth at the G1/S boundary in other cell types (14, 17) and inhibited PCNA expression in AML-12 hepatocytes, we examined the effect of PPARγ2 on cell cycle distribution. These studies indicated a major population of the PPARγ hepatocytes in G0-G1 phase that was further increased following addition of TZD (data not shown). The control hepatocytes, however, multiplied exponentially with a major population in the S phase but not in the G0-G1 phase. This clearly indicated a PPARγ2-mediated inhibition of hepatocyte growth at G0-G1 phase of the cell cycle.

Activation of PPARγ2 in Proliferating Hepatocytes Inhibits Cyclin D1 Expression—Since PPARγ2 activation in hepatocytes resulted in a G1/S arrest, we determined whether it inhibited expression of cyclin D1. TZD-mediated activation of PPARγ2 inhibited expression of cyclin D1 protein in the PPARγ hepatocytes without affecting its expression in the control hepatocytes (Fig. 3A). To determine whether PPARγ2 activation in the hepatocytes attenuated cyclin D1 expression, we transfected the full-length 1745 cyclin D1 promoter luciferase construct along with the β-galactosidase plasmid (for correction of transfection efficiency) in both PPARγ and control cells and performed luciferase and β-galactosidase assays following treatment with TZD over a period of 72 h. While addition of TZD to the control cells showed no change in the cyclin D1 promoter activity (Fig. 3B, compare − and + TZD lanes in control cells), it significantly attenuated cyclin D1 promoter activity in the PPARγ cells during the 72-h culture period (compare − and + lanes in PPARγ cells). Cyclin D1 luciferase assays carried out to determine the dose of TZD required for optimal inhibition of cyclin D1 transcription indicated that 1 μM TZD significantly inhibited cyclin D1 transcription in PPARγ cells (Fig. 3C).

Activation of PPARγ2 by TZD Inhibits Expression of β-Catenin—Since β-catenin is known to regulate cyclin D1 transcription (27), we determined whether PPARγ2-mediated attenua-
**Fig. 3. Activation of PPARγ2 in the hepatocytes inhibits cyclin D1 transcription.** A, Western blot analysis of total protein extracts from control and PPARγ hepatocytes treated as indicated for different periods of time with antibodies against cyclin D1 and actin. B, exponentially growing control and PPARγ hepatocytes were transiently transfected with the full-length cyclin D1 promoter luciferase construct (−1745 CD1-luc) along with β-galactosidase (as control) and treated with (+) or without (−) 10 μM TZD for the indicated periods of time. At the end of incubation cells were harvested, and luciferase and β-galactosidase assays were performed. The results obtained were calculated as the ratio of relative light units to the β-galactosidase values and expressed as percentage of control considering those obtained from the corresponding TZD-untreated (−) or without (−) 10 μM TZD for 72 h. The ratio of relative light units to the β-galactosidase values obtained from the TZD-treated (+) samples for each set of transfection were represented as percentage of control considering those obtained from the corresponding TZD-untreated (−) or with (+) 10 μM TZD for 72 h. The ratio of relative light units to the β-galactosidase values obtained from the TZD-treated (+) samples for each set of transfection were represented as percentage of control considering those obtained from the corresponding TZD-untreated (−) or without (−) 10 μM TZD for 72 h. The ratio of relative light units to the β-galactosidase values obtained from the TZD-treated (+) samples for each set of transfection were represented as percentage of control considering those obtained from the corresponding TZD-untreated (−) or without (−) 10 μM TZD for 72 h. The ratio of relative light units to the β-galactosidase values obtained from the TZD-treated (+) samples for each set of transfection were represented as percentage of control considering those obtained from the corresponding TZD-untreated (−) or with (+) 10 μM TZD for 72 h. The ratio of relative light units to the β-galactosidase values obtained from the TZD-treated (+) samples for each set of transfection were represented as percentage of control considering those obtained from the corresponding TZD-untreated (−) or without (−) 10 μM TZD for 72 h.

**Fig. 4.** Activation of PPARγ2 inhibits expression of β-catenin and attenuates β-catenin/TCF-responsive reporter activity. A, Western analysis of total protein extracts isolated from subconfluent control and PPARγ hepatocytes treated with an antibody against β-catenin. The same samples probed with actin served as control. B, exponentially growing control and PPARγ hepatocytes were transiently transfected with the β-catenin-responsive reporter constructs pGL3OT and pGL3OF along with β-galactosidase. Luciferase and β-galactosidase assays were performed following treatment without (−) or with (+) 10 μM TZD for 72 h. The ratio of relative light units to the β-galactosidase values obtained from the TZD-treated (+) samples for each set of transfection were represented as percentage of control considering those obtained from the corresponding TZD-untreated (−) controls as 100%. Each transfection was performed in triplicate, and the data represent the mean ± S.D. of three independent experiments. *, p < 0.0001 compared with the untreated control.

**β-Catenin Overexpression Is Unable to Restore PPARγ2 Activation-mediated Down-regulation of Cyclin D1 Transcription and Growth**—To determine whether PPARγ2 activation reduced cyclin D1 transcription via attenuating β-catenin levels, we measured cyclin D1 promoter activity in the PPARγ cells (Fig. 4B, compare lanes 1 and 2 with lanes 5 and 6) without any change in the mutant pGL3OF activity (compare lanes 3 and 4 with lanes 7 and 8). These observations suggested that activation of PPARγ2 might attenuate cyclin D1 transcription and thus arrest hepatocyte growth in part by attenuating the β-catenin-TCF/lymphoid enhancer factor-1 signaling pathway.
FIG. 5. Transient or stable overexpression of β-catenin is unable to restore cyclin D1 transcription following activation of PPARγ. A, subconfluent populations of PPARγ hepatocytes were transiently transfected with −1745 CD1-luc and β-galactosidase constructs in the presence of increasing concentrations of either empty vector (lanes 1–4), wild-type β-catenin (lanes 5–8), or mutant S37A-β-catenin (lanes 9–12). Luciferase and β-galactosidase assays were performed following incubation in the absence (−) or presence (+) of TZD for 72 h. B, subconfluent PPARγ hepatocytes were transfected with either pGL3OT (OT lanes) or pGL3OF (OF lanes) reporter constructs in the presence of either empty vector (lanes 1–4), wild-type β-catenin (lanes 5–8), or S37A-β-catenin (lanes 9–12) and harvested at 72 h. The data (both A and B) represent the mean ± S.D. of three independent experiments performed in triplicate. C, equal amounts of total protein isolated from individual clones of Pγ/WT-β-catenin, Pγ/S37A-β-catenin, or Pγ cells were subjected to Western analysis using an antibody against HA-11 to detect the expression of ectopic β-catenin. The same samples blotted with actin served as control. D, luciferase assays were performed with PPARγ, γ/WT-β-catenin, and γ/S37A-β-catenin hepatocytes transiently transfected with either pGL3OT or pGL3OF. Each transfection was performed in triplicate, and the data represent the mean ± S.D. of two independent experiments. E, subconfluent populations of control, PPARγ, γ/WT-β-catenin (γ-WT-bc), or γ/S37A-β-catenin (γ/S37A-bc) cells were transiently transfected with −1745 CD1-luc and β-galactosidase constructs, and luciferase and β-galactosidase assays were performed following incubation with (+) or without (−) 10 µM TZD for 72 h. The data represent the mean ± S.D. of three independent experiments performed in triplicate. F, equal amounts of nuclear protein extracts isolated from γ/WT-β-catenin or γ/S37A-β-catenin cells treated as indicated were subjected to Western blot analysis using antibodies against PCNA and actin (as control).
demonstrated that transient overexpression of varying concentrations of TZD using the indicated antibodies. Each transfection was performed in triplicate, and the data were expressed as mean ± S.D. of two independent experiments. Western analysis similar to that described in A with cell extracts isolated from HT-29 colon cancer cells treated with the indicated concentrations of TZD for 48 and 72 h.

PPARγ Modulates Cyclin D1 Transcription in Hepatocytes

To further examine whether expression of β-catenin could rescue PPARγ2-mediated attenuation of cyclin D1 promoter activity, stable cell lines were created utilizing PPARγ cells overexpressing either HA-tagged wild-type β-catenin or S37A-β-catenin named Pγ/S37A-β and Pγ/S37A-β cat cells, respectively. Following selection of the cells with the appropriate antibiotic, clone 22 of Pγ/S37A-β and clone 15 of Pγ/S37A-β cat showed maximal HA expression (Fig. 5C) and were chosen for further studies. Luciferase assays with pGL3OT and pGL3OF reporters showed an activation of TCF reporter activity (pGL3OT) in the Pγ/S37A-β cat cells compared with the PPARγ cells (Fig. 5D), compare lane 1 with lanes 3 and 5, suggesting that the overexpressed β-catenin proteins were functional. Cyclin D1 luciferase assays carried out in these cells showed that stable overexpression of either wild-type or S37A mutant β-catenin in the PPARγ cells was also unable to antagonize the inhibitory effect of PPARγ2 on cyclin D1 promoter (Fig. 5E, compare lanes 3 and 4 with lanes 5 and 6 and lanes 7 and 8) as observed earlier with transient β-catenin expression (Fig. 5A). Additionally expression of PCNA in both Pγ/S37A-β and Pγ/S37A-β cat cells showed a TZD-mediated reduction (Fig. 5F, compare D and + TZD lanes) as in the PPARγ cells (Fig. 2B). A comparison of cell number showed a greater reduction following addition of TZD in the Pγ/S37A-β cat cells compared with the PPARγ cells (data not shown). These results clearly demonstrated that overexpression of β-catenin activated TCF-responsive reporter activity in these cells but was unable to rescue cyclin D1 transcription, PCNA expression, and cell proliferation from the inhibitory effects of TZD indicating that the growth pathway (particularly cyclin D1 transcription) in these cells might be regulated via β-catenin-independent mechanisms.

PPARγ Activation Can Modulate Cyclin D1 Levels without Affecting β-Catenin Steady State Levels in Other Cell Types—Since the gastric cancer cell line (MKN-45) and the colon cancer cell line (HT-29) are known to express functional PPARγ protein and show growth inhibition following activation of PPARγ (49, 50), we determined the changes in steady state levels of cyclin D1 and β-catenin in these cell lines following addition of TZD. Western analysis of MKN-45 (Fig. 6A, cyclin D1 panel, compare 0 and 50 μM TZD lanes at 72 h) and HT-29 (Fig. 6C, cyclin D1 panel, compare 0 and 25 μM TZD lanes at 48 and 72 h) cells indicated a TZD-mediated reduction of cyclin D1 expression in both cell types without any effect on β-catenin expression. Cyclin D1 luciferase assays carried out in MKN-45 cells also showed a dramatic reduction of promoter activity with 25 and 50 μM TZD (Fig. 6B), which corresponded to the low cyclin D1 expression at 72 h with similar TZD concentrations (Fig. 6A). These results indicated that PPARγ activation could modulate cyclin D1 expression without affecting β-catenin expression.

Cyclin D1 Promoter Activity Can Be Restored following Activation of PPARγ2 in the Absence of β-Catenin—To confirm that repression of cyclin D1 transcription following PPARγ activation was independent of β-catenin expression, we tried to restore cyclin D1 transcription to normal levels following PPARγ2 activation using pharmacological agents and determined the expression levels of β-catenin under the same conditions. Since the phosphatase inhibitor okadaic acid can induce cyclin D1 mRNA expression (51), we performed cyclin D1 luciferase assays in the presence and absence of TZD following a preincubation with okadaic acid. Incubation with okadaic acid not only resulted in a significant induction of basal levels of cyclin D1 transcription (data not shown), it completely...
blocked the inhibitory effects of TZD on cyclin D1 transcription in PPARγ cells (Fig. 7A, compare lanes 5 and 6 with lanes 7 and 8) as well as in PyS37A-β-catenin cells (Fig. 7B), and overexpression of β-catenin (in PyS37A-β-catenin cells) had no synergistic effect. Okadaic acid-mediated inhibition of phosphatase activity was thus capable of completely attenuating the effect of PPARγ on cyclin D1 transcription. Addition of okadaic acid, however, was unable to maintain higher levels of β-catenin expression following addition of TZD in either PPARγ (Fig. 7C) or PyS37A-β-catenin cells (Fig. 7D). In similar experiments, pretreatment of PPARγ cells with lithium chloride (LiCl, an activator of CREB (52)) was also capable of partially reversing the inhibitory effects of TZD on cyclin D1 transcription (Fig. 7E). LiCl, however, was completely unable to block PPARγ-mediated down-regulation of β-catenin expression in these cells (data not shown). Since both okadaic acid and LiCl were capable of maintaining high levels of cyclin D1 transcription despite PPARγ activation and in the absence of β-catenin expression, we conclude that PPARγ-mediated inhibition of cyclin D1 transcription in these cells was mediated through a β-catenin-independent pathway.

Overexpression of CREB Can Overcome PPARγ-mediated Attenuation of Cyclin D1 Promoter Activity in the Absence of β-Catenin—The transcription from the cyclin D1 promoter can be modulated by a variety of transcription factors including CREB (28, 29). To determine any possible participation of CREB in PPARγ-mediated attenuation of cyclin D1 transcription, we performed cyclin D1 luciferase assays in PPARγ cells following cotransfection of increasing concentrations of wild-type CREB and estimated the effect of PPARγ activation on the promoter activity. The results described in Fig. 8A showed that overexpression of as low as 50 ng of wild-type CREB was capable of significantly restoring the promoter activity following stimulation by TZD (compare lanes 1 and 2 with lanes 9 and 10) and completely abolished the inhibitory effect of PPARγ at higher concentration (compare lanes 5 and 6 with lanes 13 and
and compare lanes 7 and 8 with lanes 15 and 16. The cyclin D1 luciferase studies of Fig. 8B showed that only wt-CREB (lanes 3 and 4) and not the empty vector (lanes 1 and 2) or dominant negative CREB (KCREB, lanes 5 and 6) could activate cyclin D1 transcription following PPARγ2 activation, confirming the participation of CREB in these events. Cyclin D1 luciferase assays designed with wild-type CREB and increasing concentrations of KCREB showed that addition of KCREB significantly inhibited the capability of wt-CREB to reactivate cyclin D1 promoter following addition of TZD (Fig. 8C, compare lane 4 with lanes 6, 7, 8, and 9), indicating that KCREB functioned as a dominant negative vector. Additionally overexpression of S37A-β-catenin in the presence of wt-CREB in the PPARγ cells produced no synergistic effect on cyclin D1 promoter activation (Fig. 8D, lanes 7 and 8) following addition of TZD and was equivalent to the level of activation by wt-CREB alone (lanes 5 and 6). These studies indicated that overexpression of CREB was capable of antagonizing the inhibitory effects of PPARγ2 on cyclin D1 transcription in the absence of β-catenin and overexpression of β-catenin had no additional effect.

PPARγ2 Activation Reduces the Levels of Total and Activated Form of CREB—Since overexpression of CREB reactivated cyclin D1 promoter following PPARγ2 activation, it was possible that PPARγ2 activation involved modulation of CREB levels. To determine any effect of PPARγ2 on endogenous CREB, we estimated changes in the levels of phosphorylated CREB (Ser133) or total CREB following activation of PPARγ2 by West-
Fig. 9. PPARγ2 activation modulates CREB levels and attenuates pCRE-luc activity. A, Western analysis of control and PPARγ hepatocytes treated without (−) or with (+) TZD with phospho-CREB, total CREB, and actin antibodies. B, luciferase assays with control and PPARγ cells following transfection of CREB-responsive reporter pCRE-luc and β-galactosidase vectors and treatment in the absence (−) and presence (+) of 10 μM TZD. C, luciferase assays similar to those described in B were carried out following transfection of the CREB promoter (−1264 CREB-luc) and β-galactosidase vectors. D, a functional Ser133 site of CREB is required for restoring cyclin D1 transcription following activation of PPARγ. −1745 CD1-luc assays were performed in PPARγ cells treated with (+) or without (−) TZD following cotransfection of either empty vector (lanes 1 and 2), wt-CREB (lanes 3 and 4), increasing concentrations of CREBM1 (lanes 5 and 6 and lanes 7 and 8), or a combination of wt-CREB and CREBM1 (lanes 9 and 10). E, p300 can synergize with CREB to activate cyclin D1 transcription following activation of PPARγ2. Luciferase assays similar to those described in D were performed in PPARγ cells following cotransfection of either empty vector (lanes 1 and 2), wt-CREB (lanes 3 and 4), or wt-CREB in combination with increasing concentrations of p300 (lanes 5 and 6 and lanes 7 and 8). Each transfection (in B–E) was performed in triplicate, and the data represent the mean ± S.D. of two independent experiments.
Okadaic Acid-mediated Reactivation of Cyclin D1 Transcription Involves CREB—Western blot studies carried out with PPARγ hepatocytes to determine whether okadaic acid-mediated reactivation of cyclin D1 transcription involved CREB indicated that addition of okadaic acid in the presence of TZD could maintain higher levels of total as well as phospho-CREB-Ser133 compared with TZD alone (Fig. 10A), which might be responsible for reactivating cyclin D1 transcription. Cyclin D1 luciferase assays carried out in the presence of okadaic acid as well as KCREB showed that okadaic acid was unable to restore cyclin D1 promoter activity following exposure to TZD in the presence of KCREB (Fig. 10B, compare lanes 5 and 6 with lanes 9 and 10), confirming the participation of CREB in okadaic acid-mediated activation of cyclin D1 transcription. These studies showed that PPARγ activation-related inhibition of cyclin D1 transcription was mediated via inhibition of CREB.

**DISCUSSION**

Several recent studies have demonstrated that activation of PPARγ can suppress growth in proliferating cells (for a review, see Ref. 13) including those originating from hepatocellular carcinoma (14, 16). In this report we demonstrate that activation of PPARγ in proliferating mouse hepatocytes can arrest growth and reduce PCNA expression. Earlier studies with fibroblasts induced to differentiate into adipocytes by PPARγ showed that the corresponding growth arrest involved induction of the cyclin-dependent kinase inhibitors p16INK4a and p21CIP1 (19). In other studies PPARγ-induced cell cycle withdrawal was associated with inhibition of DNA binding and transcriptional activity of the E2F-DP complex due to a down-regulation of the catalytic subunit of the serine-threonine phosphatase PP2A (18). In our studies, PPARγ-mediated suppression of growth in hepatocytes involved a corresponding TZD-dependent inhibition of cyclin D1 and β-catenin production suggesting that the molecular mechanisms responsible for this response might be operating in mid-G1 phase of the cell cycle. In fact, the flow cytometric analysis demonstrated that activation of PPARγ resulted in the accumulation of hepatocytes in G1 phase of the cell cycle (data not shown) as reported in other cell types (14, 17). PPARγ-mediated attenuation of cyclin D1 expression might contribute toward this G1/S arrest in hepatocytes as shown in breast cancer cells (20) since cyclin D1 via stimulating the activity of select cyclin-dependent kinases facilitates G1/S transition.

PPARγ activation has been shown to regulate cyclin D1 expression at the level of both transcription as well as post-transcription (20, 53). In the studies described here PPARγ activation in hepatocytes resulted in a significant attenuation of cyclin D1 promoter activity suggesting a regulation at the level of transcription. In these studies our aim was to identify the downstream events following PPARγ activation in the hepatocytes responsible for regulating cyclin D1 transcription. A detailed analysis of this pathway will provide useful information regarding the effectors involved in PPARγ-mediated growth arrest and indicate whether this pathway is modulated in a cell type-specific manner. To identify the effectors the initial focus was on β-catenin since β-catenin is a major regulator of cyclin D1 transcription (27) and can activate cyclin D1 gene through a lymphoid enhancer factor-1 binding site in its
promoter. Since PPARγ2 activation in the hepatocytes resulted in a dramatic down-regulation of β-catenin expression, it was conceivable that this event might contribute to the accompanying growth arrest via inhibiting expression of key regulators of G1 progression such as c-Myc and cyclin D1. Interestingly our experiments indicated that activation of PPARγ2 in the presence of ectopically expressed β-catenin was unable to restore cyclin D1 transcription and growth. Our studies also showed that cyclin D1 transcription can be restored in these hepatocytes following activation of PPARγ2 in the absence of β-catenin and in the presence of ectopically expressed CREB. These observations provided a clear indication that PPARγ2-mediated suppression of β-catenin levels and cyclin D1 transcription are independent of each other. This hypothesis is strengthened by other studies that showed that overexpression of an oncogenic mutant form of β-catenin in liver despite inducing hepatomalignancy was unable to induce the levels of β-catenin target genes cyclin D1 and c-myc (54).

The synthesis of cyclin D1 is regulated in the early phase of G1 by extracellular effectors that activate a variety of signaling pathways, which converge on various transcription factors, many of which involve CREB (28). In response to stimulation by cyclic AMP, CREB is activated via phosphorylation at serine residue 133, which enables it to bind to the transcriptional coactivator CREB-binding protein and its homologue p300 (55). This CREB-CREB-binding protein complex then activates transcription of target genes via binding to the CRE site. Additionally CREB can activate CRE-specific activity via binding to other transcription factors (for example Oct-1 and Lim-only factors) ally CREB can activate CRE-specific activity via binding to other transcription factors (for example Oct-1 and Lim-only factors).

These observations provided a clear indication that PPARγ2 activation in the hepatocytes regulated the expression of p300, thus reducing interaction between p300 and c-Fos. The reduction of cyclin D1 transcription was found to be due to an increase in PP1 and PP2A activity, which would also indicate that phosphorylation of CREB and p300 thus reducing interaction between p300 and c-Fos. These observations provided a clear indication that PPARγ2 activation in the hepatocytes regulated the expression of p300, thus reducing interaction between p300 and c-Fos.

Activation of PPARγ in the hepatocytes was unable to induce the levels of β-catenin target genes cyclin D1 and c-myc (54). This CREB-CREB-binding protein complex then activates transcription of target genes via binding to the CRE site. Additionally CREB can activate CRE-specific activity via binding to other transcription factors (for example Oct-1 and Lim-only factors). These studies are required to confirm this hypothesis. Regulation of cyclin D1 expression by therapeutic intervention is an attractive means of treating breast and gastrointestinal tumorigenesis. The ability of PPARγ ligands to effectively attenuate cyclin D1 expression suggests that these ligands have a great potential to be utilized as cancer chemotherapeutic drugs in the future. Elucidation of the pathway and identification of the molecules involved in mediating these effects might contribute toward the enhancement of the potency of these potential chemotherapeutic drugs.
PPARγ Modulates Cyclin D1 Transcription in Hepatocytes

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Peroxisome Proliferator-activated Receptor γ Activation Modulates Cyclin D1 Transcription via β-Catenin-independent and cAMP-response Element-binding Protein-dependent Pathways in Mouse Hepatocytes
Chandan Sharma, Anamika Pradeep, Richard G. Pestell and Basabi Rana

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