Peroxisome proliferator-activated receptor activators modulate the osteoblastic maturation of MC3T3-E1 preosteoblasts

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Abstract The reduced bone mineral density (BMD) observed in osteoporosis results, in part, from reduced activity of bone-forming osteoblasts. We examined the effect of peroxisome proliferator-activated receptor (PPAR) activators on MC3T3-E1 preosteoblast maturation. Activators of PPARα, δ and γ induced alkaline phosphatase activity, matrix calcification and the expression of osteoblast genes as determined by reverse transcriptase-polymerase chain reaction. However, at relatively high concentrations of the specific PPARγ ligands, ciglitazone and troglitazone, maturation was inhibited. PPARα, δ and γ1 were expressed in MC3T3-E1 cells. PPARγ1 mRNA and protein levels were induced early during osteoblastic maturation. We speculate that endogenous and pharmacological PPAR activators may affect BMD by modulating osteoblastic maturation.
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Key words: Peroxisome proliferator-activated receptor activation; Osteoblast maturation; Osteoporosis; Thiazolidinedione

1. Introduction

Osteoporosis affects an estimated 25 million people in the USA with fractures as the most prominent clinical consequence [1,2]. The reduction in bone mineral density (BMD) results, in part, from reduced activity of bone-forming osteoblasts [3]. Osteoblasts are derived from mesenchymal precursor cells. The mechanisms directing the cells along an osteoblastic lineage and the processes driving maturation are not established, although a multitude of biological factors and several key transcription factors have been identified [4–7]. Novel members of the nuclear hormone receptor superfamily, the peroxisome proliferator-activated receptors (PPARs), were recently implicated in the modulation of the relative proportion of bone cell production by the marrow stroma [7,8].

Three PPARs are currently known, PPARα, PPARδ and PPARγ. Each has a specific tissue distribution [9], although all types have been shown to be expressed in rat bone tissue [10]. PPARs are ligand-activated transcription factors that heterodimerize with the retinoid X receptor α and bind to specific sequences in the promoters of target genes [9]. Ligands are semi-selective for the various PPARs depending on the concentration and cell type [11]. Fatty acids and certain prostaglandin metabolites, particularly of the A and J series [12–14], are potential endogenous ligands for the PPARs whereas the insulin-sensitizing thiazolidinediones, including ciglitazone (CIG), troglitazone (TRO) and rosiglitazone (ROS), are highly specific ligands for PPARγ [15].

Diascro et al. [16], Nuttall et al. [17] and Lecka-Czernik et al. [8] have recently shown an inhibitory effect of PPARδ and/or PPARγ on the osteoblastic differentiation of various precursor cells or cell lines, concomitant with the promotion of adipocytic differentiation using relatively high concentrations of PPAR activators or PPARγ overexpression. However, prostaglandin metabolites of the J and A series, at concentrations appropriate for PPAR activation (10 μM), were shown to induce osteoblast mineralization in vitro and bone formation in vivo suggesting that PPARs may have a positive effect on osteoblastic maturation [18–21]. The precise role of PPARs in osteoblastic differentiation and maturation is therefore undefined. We present data from a study examining the effect of a variety of PPAR ligands over a range of concentrations on the osteoblastic maturation of the mouse preosteoblast cell line, MC3T3-E1. PPARδ mRNA was expressed at the highest level in MC3T3-E1, PPARγ1 and PPARα at relatively lower levels, and PPARγ2 was not expressed. Activators of PPARα, δ and γ induced the osteoblastic maturation of MC3T3-E1 cells with increased alkaline phosphatase (AP) activity and matrix calcification. However, at relatively high concentrations of PPARγ activating ligands in particular, maturation was inhibited. Since PPAR activators are used clinically in the treatment of type 2 diabetes and hyperlipidemia, it will be of interest to monitor the BMD of these patients.

2. Materials and methods

PPAR activators were purchased from Caymen Chemical (Ann Arbor, MI, USA) or Biomol (Plymouth Meeting, PA, USA) and the anti-PPARγ polyclonal antibody (SA-206), capable of recognizing both PPARγ1 and γ2, was also from Biomol. PPAR activators were dissolved in dimethylsulfoxide (DMSO) as 1000-fold stock solutions and diluted 1000-fold into culture medium. Control cells received an equivalent amount of DMSO. TRO was kindly supplied by Parke Davis (Ann Arbor, MI, USA) and ROS was kindly provided by Smith Kline Beecham (King of Prussia, PA, USA). Radiolabelled nucleotide triphosphates were obtained from Amersham Life Sciences (Arlington Heights, IL, USA).

2.1. Cell culture

The MC3T3-E1 mouse preosteoblast cell line (Riken Cell Bank, Japan) was grown in αMEM (Fisher Scientific, Pittsburgh, PA, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone Labs, Logan, UT, USA) supplemented with sodium pyruvate (1 mM), penicillin (100 U/ml) and streptomycin (100 U/ml) at 5% CO_2 and 37°C. During osteoblastic maturation studies, αMEM containing

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5% FBS was added to confluent cells supplemented with 25 μg/ml ascorbate and 3 mM β-glycerophosphate (osteogenic factors). PPAR activators were added at confluence and with subsequent medium changes (every 3 days). The 3T3-L1 mouse preadipocyte cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM containing 10% FBS. Adipocyte differentiation was induced by adding 1 μg/ml insulin, 0.5 mM isobutylmethylxanthine and 0.25 μM dexamethasone at confluence for 2 days followed by insulin-containing medium every subsequent 2 days.

2.2. AP activity

Cells cultured in 24 well dishes, were washed twice with phosphate-buffered saline (PBS) and lysed with scraping and sonication in 200 μl of lysis buffer (0.2% NP40, 1 mM $MgCl_2$). Reaction buffer (1 ml, 1:1 v/v 221 alkaline buffer: stock substrate solution, Sigma, St. Louis, MO, USA) was added to an aliquot of cell lysate and the reaction was incubated at 37°C for 10–60 min depending on the rate of product formation. The reaction was stopped by addition of 1 N NaOH (12 μl). The substrate was cleaved by AP to yield a yellow colored product that was quantified spectrophotometrically at a wavelength of 405 nm. Results were normalized to total protein determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Matrix calcification–calcium determination

Cells were washed thoroughly with PBS and incubated overnight at room temperature in 0.1 N HCl (100 μl) to dissolve calcium mineral from the calcified matrix. Supernatants were collected and the calcium level determined using the *o*-cresolphthalein complexone method (Sigma kit, Procedure #587, Sigma). The cells were washed again with PBS and protein dissolved in 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS) (200 μl) overnight at room temperature. Protein content was determined by the Bradford assay and was used to normalize the calcium data.

2.4. Western blot

Cell nuclear extracts were prepared as described by Osborn et al. [22]. Protein (25 μg) was fractionated by SDS–polyacrylamide gel electrophoresis on a mini-gel apparatus (Novex, San Diego, CA, USA) and then transferred to nitrocellulose. Blots were blocked with 5% non-fat skimmed milk in PBS at room temperature for 2 h and probed for 1 h at room temperature with anti-PPARγ polyclonal antibody (1:2000 dilution). Blots were washed six times for 5 min in PBS containing 0.3% Tween 20 and incubated for 1 h with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. Blots were extensively washed and developed using the ECL system (Amersham).

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA (2 μg) was reverse-transcribed using a standard protocol as described previously [23]. PCR was performed using 1 μl of cDNA, 25 ng of each primer (synthesized by Gibco BRL), 17.5 μM dNTPs, 1.5 μCi [α-^{32}P]dCTP and 0.40 U of *Pfu* polymerase (Stratagene) in a 10 μl reaction volume containing 1×*Pfu* buffer (Stratagene) with a Perkin-Elmer Gene Amp PCR System 2400. The sense and antisense primers 5′-CGACAAGTGTGATCGGAGCTGCAAG-3′ and 5′-GTTGAAGTTCTTCAGGTAGGCTTC-3′; 5′-GGCCAACGGCAGTGGCTTCGTC-3′ and 5′-GGCTGCGGCCTTAGTACATGTCCT-3′; 5′-TTCTGACAGGACTGTGTGACAG-3′ and 5′-ATAAGGTGGAGATGCAGGTTC-3′; 5′-GCTGTTATGGGTGAAACTCTG-3′ and 5′-ATAAGGTGGAGATGCAGGTTC-3′ were used for amplification of PPARα, δ, γ1 and γ2 producing bands of 226, 478, 354 and 350 bp, respectively. The sense and antisense primers 5′-CCTAAGGCCAACCGTGAAAAG-3′ and 5′-TCTTCATGGTGCTAGGAGCCA-3′; 5′-CTTGCTGGTGGAAGGAGGCAGG-3′ and 5′-CACGTCTTCTCCACCGTGGGTC-3′; 5′-CTCGGGTGTAACAGCTAGCTAC-3′ and 5′-CGTTCAGAAGGACAGCTGTCTG-3′; 5′-CTCTGTCTCTCTGACCTCACAG-3′ and 5′-GGAGCTGCTGTGACATCCATAC-3′ were used for amplification of β-actin, AP, bone sialoprotein (BSP) and osteocalcin (OC) producing bands of 645, 393, 383 and 359 bp, respectively. Each cycle consisted of 96°C 45 s, 62°C 45 s, 72°C 2 min, for various cycle numbers depending on the gene abundance, terminating in 72°C for 5 min. The PCR reaction products were electrophoresed on 6% polyacrylamide non-denaturing gels that were subsequently dried, and exposed to X-ray film. For semi-quantitative PCR, the linear range of amplification was first established and PCR cycles chosen to be within that range. Genes of interest were compared to β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, amplified as previously described [23]) which served as normalizing controls.

2.6. Transient transfection

Cells were plated in 12 well dishes at 50% confluence and transiently transfected with a total of 5 μg of plasmid DNA/well using the MBS transfection kit (Stratagene). Transfections contained 2 μg of the reporter plasmid, 2 μg of CMV-β-galactosidase plasmid and 1 μg of carrier DNA. The heterologous promoter–reporter gene construct consisted of 110 bp (−471 to −581) of the acyl CoA oxidase promoter. This region contains a single PPAR response element (PPRE), capable of interaction with all of the PPARs. The PPRE is upstream of the rabbit β-globin minimal promoter (bp −109 to +10), which provides the necessary elements for basal transcription, and is hooked up to a luciferase reporter gene (kind gift of Dr. Jonathon Tugwood, Zeneca Central Toxicology Laboratory, Cheshire, UK) [24]. Following transfection, the cells were incubated in αMEM containing 5% charcoal-stripped FBS (which removes potential PPAR activators) in the absence or presence of ROS (2.5 μM), carbaprostacyclin (CPC,

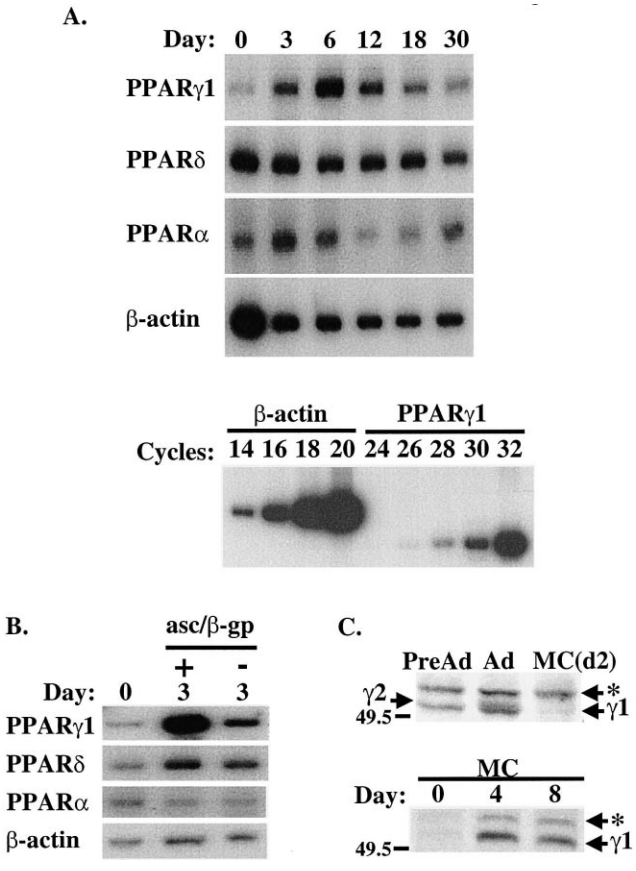

Fig. 1. PPAR expression in MC3T3-E1 cells. (A) RT-PCR of total RNA from MC3T3-E1 cells at different days post confluence (day 0) cultured in medium supplemented with 25 μg/ml ascorbate (asc) and 3 mM β-glycerophosphate (β-gp) (osteogenic medium). The lower panel shows the level of expression of β-actin and PPARγ1 as a function of PCR cycle number for a single sample. (B) Asc and β-gp induced PPARγ1 mRNA in MC3T3-E1 cells. (C) Western blot analysis for PPARγ in nuclear extracts isolated from 3T3-L1 preadipocytes (PreAd), mature adipocytes (a positive control for PPARγ1 and γ2 expression, Ad) and MC3T3-E1 cells at day 2 post confluence (upper panel) and days 0, 4 and 8 post confluence (lower panel) cultured in osteogenic medium (asc+β-gp). PPARγ2 was detected in the mature adipocytes but not the preadipocytes as expected. A third band of approximately 65 kDa was also detected (*). The migration of the molecular weight marker of 49.5 kDa is indicated.

2 μM) or Wyeth 14643 (WY, 10 μM) for 40 h. Cells were lysed and luciferase and β-galactosidase activities determined as previously described [25]. Luciferase activity was normalized for transfection efficiency using the β-galactosidase activity.

2.7. Statistics

Computer-assisted statistical analyses were performed using the ANOVA program, and P values calculated using Fisher's PLSD significance test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. PPAR expression in MC3T3-E1 cells

PPARα, δ and γ are ligand-activated transcription factors of the nuclear hormone receptor superfamily. PPARγ has two isoforms, a result of different promoter usage and alternative splicing, that are termed γ1 and γ2. The expression of the latter is predominantly restricted to adipose tissue. RT-PCR showed expression of PPARα, δ and γ1 mRNA in MC3T3-E1 utilizing 30, 18 and 28 cycles, respectively (Fig. 1A, upper panel). PPARγ1 mRNA was first detectable at 26 cycles by RT-PCR (Fig. 1A, lower panel), however, PPARγ2 mRNA was not detected even up to 35 cycles (data not shown). The level of expression of PPARγ1 mRNA consistently increased from day 0 to 6 and declined from day 6 to 30 in culture, whereas the expression levels of PPARδ and β-actin (15 cycles) mRNA remained relatively unchanged throughout the culture period (Fig. 1A, upper panel). Expression levels of PPARα were more variable, in some experiments expression increased early in culture (Fig. 1A, upper panel), whereas in others expression was unchanged with culture time (Fig. 1B). Interestingly, PPARγ1 mRNA, but not PPARδ (or PPARα), was induced by the presence of osteogenic factors, ascorbate and β-glycerophosphate, in the medium (Fig. 1B), that was similarly observed at the protein level (Fig. 1C). A

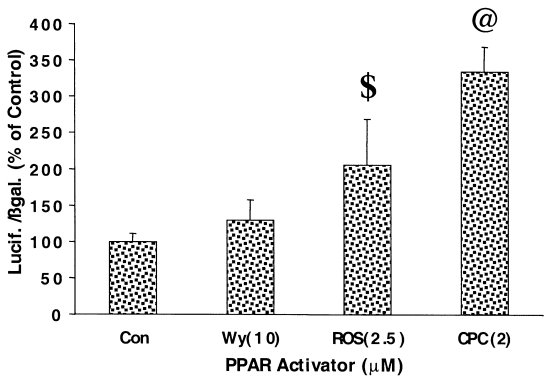

Fig. 2. MC3T3-E1 cells express functional PPARs. MC3T3-E1 cells were transiently co-transfected with a PPRE–luciferase construct and a CMV-β-galactosidase plasmid and treated with the PPAR activators WY (10 μM), ROS (2.5 μM) and CPC (2 μM) for 40 h. Cells were lysed and luciferase activity determined and normalized to β-galactosidase activity. The luciferase/β-galactosidase (Lucif./βgal.) value for control cells (Con, DMSO-treated) was set at 100%. Values are presented as mean ± S.D., $: $P < 0.02$, @: $P < 0.001$, $n = 3$. The results are representative of three experiments.

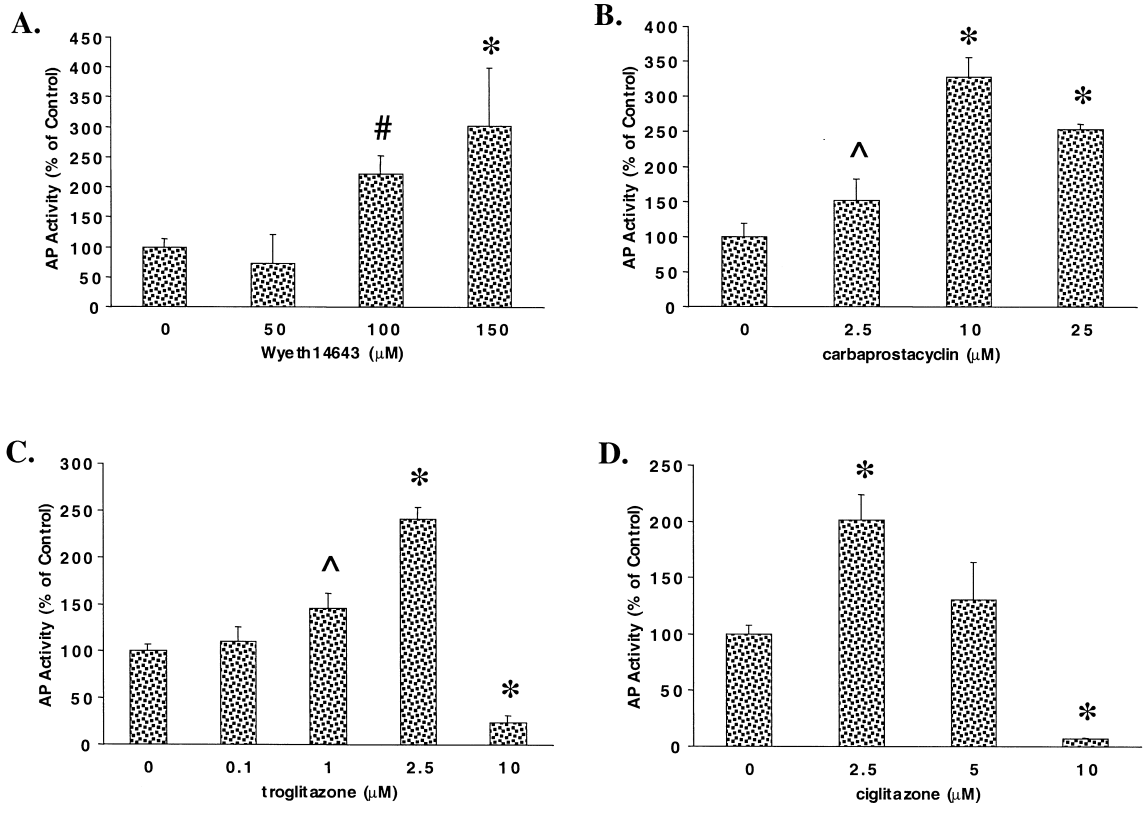

Fig. 3. Effect of PPAR activators on AP activity. AP activity was determined in MC3T3-E1 cells cultured in 24 well plates for 6 days post confluence (day 0) in osteogenic medium in the absence (0 μM) or presence of various doses of the PPAR activators. The AP activity normalized to cell protein obtained from cells without added PPAR activator (0 μM, DMSO-treated) was set at 100%, corresponding to 35–45 U/mg protein depending on the particular experiment. Values are presented as mean ± S.D., ∧: $P < 0.05$, #: $P < 0.002$, *: $P < 0.0001$, $n = 4$. The results are representative of 2–3 experiments each performed in quadruplicate.

protein band of approximately 65 kDa was also detected that was regulated similarly to PPARγ1, however, the identity of the protein is unknown (Fig. 1C). Northern blot analysis of MC3T3-E1 RNA detected the expression of PPARδ mRNA at a similar level to that expressed in mature 3T3-L1 adipocytes, but was not sensitive enough to detect PPARα, PPARγ1 or γ2 (data not shown).

To determine whether the PPARs in MC3T3-E1 cells were functional, a PPRE–luciferase promoter–reporter construct containing 110 bp of the acyl CoA oxidase promoter, harboring a binding site for PPARs [24], was transiently transfected into MC3T3-E1 cells. The cells were then incubated in medium supplemented with PPAR activators. Expression of the luciferase reporter was induced significantly, but only modestly, in multiple experiments in the presence of 2.5 μM ROS (2.05 ± 0.64-fold vs. control, $P < 0.02$, $n = 3$), a specific PPARγ activating ligand, and 2 μM CPC (3.34 ± 0.35-fold, $P < 0.001$, $n = 3$), a PPARα and δ activator, but not by 10 μM WY (1.29 ± 0.28-fold, n.s., $n = 3$), a specific PPARα activator at 10 μM (Fig. 2). Together with the mRNA and protein expression data above, these results indicate that MC3T3-E1 cells expressed functional PPARδ, relatively low levels of functional PPARγ1 and perhaps PPARα, and no PPARγ2.

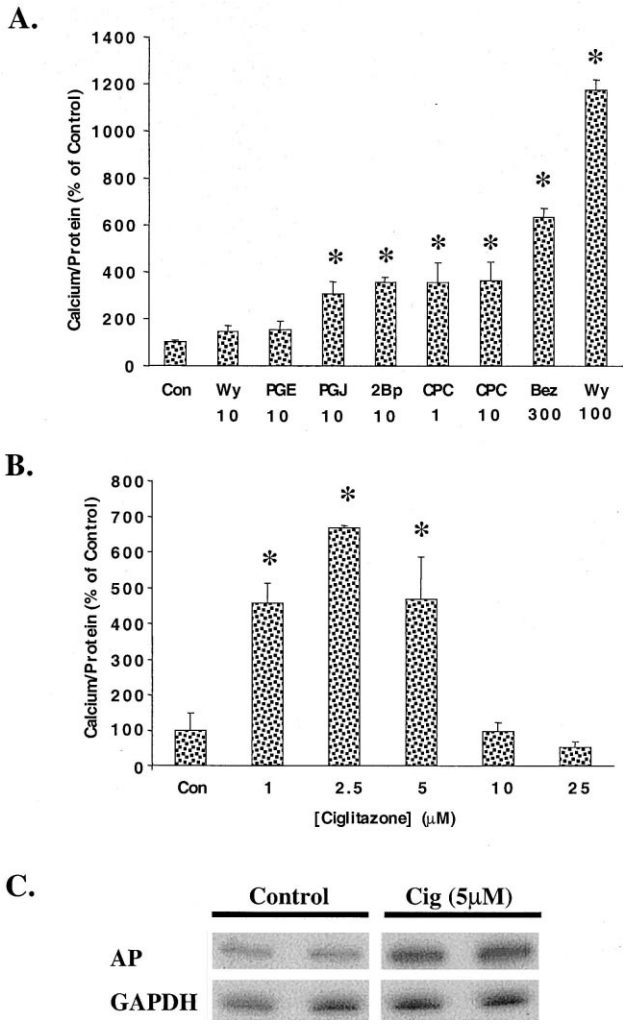

Fig. 4. Effect of PPAR activators on matrix calcification. (A) MC3T3-E1 cells were cultured in 12 or 24 well plates for approximately 30 days post confluence (day 0) in osteogenic medium in the absence (Con) or presence of PPAR activators: WY, PGE, PGJ, 2Bp, CPC and Bez, at the stated concentrations, and (B) increasing doses of CIG. Calcium content of the matrix was determined as described in Section 2 and normalized to protein content. The normalized value obtained from cells incubated under control conditions (Con, DMSO-treated) was set at 100%, corresponding to 4.77 ± 0.41 μg calcium/μg protein. The results are presented as mean ± S.D., *: $P < 0.0001$, $n = 3$. The results are representative of two experiments each performed in triplicate. (C) AP and GAPDH mRNA expression was determined by RT-PCR of total RNA isolated from MC3T3-E1 cells 6 days post confluence cultured in osteogenic medium in the absence (Control) or presence of 5 μM CIG (5 μM).

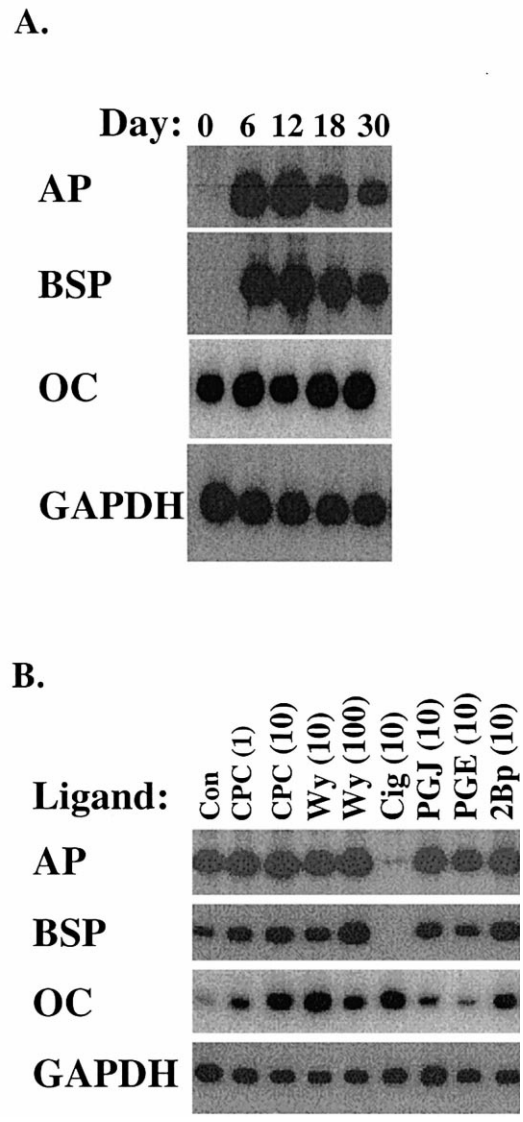

Fig. 5. Effect of PPAR activators on osteoblast gene expression as determined by RT-PCR. (A) Time course of expression. PCR cycles of 24, 22, 30 and 18 were utilized for AP, BSP, OC and GAPDH, respectively. (B) RT-PCR of total RNA isolated from MC3T3-E1 cells cultured for 6 days as above in the absence (Con) or presence of PPAR activators at the indicated concentrations (μM).

3.2. Effect of PPAR activators on osteoblastic maturation

Incubation of MC3T3-E1 cells with the PPAR activators WY, CPC, or the specific γ activators TRO and CIG, dose dependently induced AP activity, an early marker of osteoblastic maturation (Fig. 3A–D). Reduced AP activity was observed however at higher PPAR activator concentrations, par-

ticularly for TRO and CIG (Fig. 3C,D). PPAR activators including WY, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ, an α and γ activator), 2-bromopalmitate (2Bp, an α, δ and γ activator), CPC and bezafibrate (Bez, an α and δ activator) induced matrix calcification, a late marker of osteoblastic maturation (Fig. 4A). Prostaglandin E_2 (PGE, no PPAR activation ability) had no effect on AP activity (data not shown) or matrix calcification (Fig. 4A). CIG dose dependently induced matrix calcification to a maximal effect at 2.5 μM, but inhibited this process at concentrations higher than 10 μM (Fig. 4B). The induced osteoblastic maturation observed with relatively low concentrations of CIG was consistent with an increase in AP steady state mRNA expression in MC3T3-E1 cells treated with 5 μM CIG for 6 days as determined by RT-PCR (Fig. 4C).

During osteoblastic maturation, specific genes are differentially expressed both temporally and as a result of exposure of the cells to stimulatory or inhibitory physiological factors such as hormones [26]. The temporal expression of AP, BSP and OC mRNA level was determined by RT-PCR for MC3T3-E1 cells cultured from day 0 to 30 in medium supplemented with osteogenic factors (Fig. 5A). Very low levels of expression of AP and BSP were evident at day 0, but levels rose to peak between day 6 and 12 in culture and then declined slightly from day 18 to 30 in culture. OC mRNA levels were relatively constant throughout the culture period (Fig. 5A). On the basis of these results, the level of expression of each osteoblast gene was measured following 6 days in culture in medium supplemented with various PPAR activators. Most dramatically, 10 μM CIG inhibited the expression of AP and BSP mRNA levels (Fig. 5B). A notable induction in expression levels for BSP and OC mRNA was observed with the other PPAR activators, but not PGE (Fig. 5B), indicating that the promotion of osteoblastic maturation by PPAR activators in MC3T3-E1 cells correlated with increased expression of osteoblast genes.

4. Discussion

In this study, we have demonstrated the expression and function of PPARs in the mouse preosteoblastic cell line, MC3T3-E1. PPAR activators induced osteoblastic maturation of the MC3T3-E1 cells, however, at relatively higher concentrations, the PPARγ ligands CIG and TRO significantly inhibited this process.

Inhibition of osteoblastic differentiation by PPAR activators has been observed previously but either utilized relatively high concentrations of PPARγ ligands (5 μM TRO, 5 μM ROS) or required the induced expression of endogenous PPARγ2 or ectopic PPARγ2 overexpression [8,16,17]. In the present experiments, PPARγ2 was not detected in MC3T3-E1 cells, even following treatment with various combinations of adipogenic factors (data not shown). This lack of adipogenic differentiation suggests that MC3T3-E1 cells have reached a level of mesenchymal lineage commitment beyond that of trabecular bone cells and marrow stromal cells, which retain multipotentiality.

Effects of relatively low concentrations of PPARγ activators or effects of PPARα and δ activators on osteoblastic maturation have not been previously reported. We observed enhanced osteoblastic differentiation of MC3T3-E1 cells treated with relatively low concentrations of the PPARγ specific ligand, CIG (<5 μM). Furthermore, activators of PPARα and δ induced osteoblastic maturation with increased AP activity and matrix calcification. Consistent with this, these activators also induced AP, BSP and OC steady state mRNA levels. Consistent with the inhibition of osteoblastic maturation at 10 μM CIG, the mRNA levels of AP and BSP, but not OC, were dramatically reduced. The lack of inhibition of OC mRNA expression indicates that OC is less sensitive to this negative regulation than AP or BSP. Nevertheless, the loss of AP and BSP was sufficient to prevent matrix calcification.

The induction in expression of PPARγl mRNA and protein during osteoblastic maturation of MC3T3-E1 cells and the induction in PPARγl expression by osteogenic factors is preliminary evidence of a role for this PPAR in the maturation process. Identification of a direct interaction between PPAR and the promoters of osteoblast genes adds further preliminary evidence. A putative PPAR binding site was identified in the promoter of the osteopontin gene [27], and co-transfection of BSP–luciferase and OC–luciferase promoter–reporter constructs with a PPARδ expression plasmid in osteoblastic cells regulated luciferase activity [28].

Potential speculative mechanisms for PPAR effects relate to direct PPAR modulation of osteoblast gene transcription and effects on cell cycle regulation [29], and apoptosis [30]. PPAR induces expression of the glucose transporter, GLUT4, during adipocytic differentiation. Recently, GLUT4 was shown to be required for bone growth, perhaps related to increased energy requirements during matrix synthesis [31]. Since PPARs are known to regulate genes involved in lipid biosynthesis and lipid transport in adipocytes [9], they may influence the formation of phospholipid-rich matrix vesicles in osteoblasts that are required for matrix calcification.

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