Journal of Cellular Biochemistry 100:161–173 (2007)

Prostaglandin F2α Inhibits Adipocyte Differentiation Via a Gαq-Calcium-Calcineurin-Dependent Signaling Pathway

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Abstract Prostaglandin F2α (PGF2α) is a potent physiological inhibitor of adipocyte differentiation, however the specific signaling pathways and molecular mechanisms involved in mediating its anti-adipogenic effects are not well understood. In the current study, we now provide evidence that PGF2α inhibits adipocyte differentiation via a signaling pathway that requires heterotrimeric G-protein Gαq subunits, the elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$), and the activation of the Ca^{2+}/calmodulin-regulated serine/threonine phosphatase calcineurin. We show that while this pathway acts to inhibit an early step in the adipogenic cascade, it does not interfere with the initial mitotic clonal expansion phase of adipogenesis, nor does it affect either the expression, DNA binding activity or differentiation-induced phosphorylation of the early transcription factor C/EBPβ. Instead, we find that PGF2α inhibits adipocyte differentiation via a calcineurin-dependent mechanism that acts to prevent the expression of the critical pro-adipogenic transcription factors PPARγ and C/EBPα. Furthermore, we demonstrate that the inhibitory effects of PGF2α on both the expression of PPARγ and C/EBPα and subsequent adipogenesis can be attenuated by treatment of preadipocytes with the histone deacetylase (HDAC) inhibitor trichostatin A. Taken together, these results indicate that PGF2α inhibits adipocyte differentiation via a Gαq-Ca^{2+}-calcineurin-dependent signaling pathway that acts to block expression of PPARγ and C/EBPα by a mechanism that appears to involves an HDAC-sensitive step. J. Cell. Biochem. 100: 161–173, 2007.

Key words: PGF2α; calcium; calcineurin; 3T3-L1 preadipocytes; adipogenesis; adipocyte differentiation; histone deacetylase

Adipocytes are specialized cells that play an essential role in the regulation of energy homeostasis [Spiegelman and Flier, 2001; Kershaw and Flier, 2004]. However, the excessive accumulation or increased size of adipocytes can result in obesity and its associated disease sequelae [Visscher and Seidell, 2001]. Hence, understanding the molecular mechanisms that underlie the regulation of adipocyte growth and differentiation are of immense scientific and clinical interest.

Much of what we know regarding the molecular mechanisms that control adipocyte differentiation comes from studies using the 3T3-L1 preadipocyte model cell system [Green and Kehinde, 1975]. These fibroblast-like cells are committed to the adipocyte lineage and can be induced to efficiently differentiate into morphologically distinct, lipid-ladened, mature adipocytes following exposure to a adipogenic hormonal cocktail comprising of methylisobu-

Abbreviations used: PGF2α, prostaglandin F2α; PG, prostaglandin; C/EBP, CCAAT/enhancer-binding protein; PPARγ, peroxisome proliferators-activated receptor γ; HDAC, histone deacetylase; TSA, trichostatin A; Mix, methylisobutylxanthine; Dex, dexamethasone; MDI, methylisobutylxanthine dexamethasone and insulin; MCE, mitotic clonal expansion; $[Ca^{2+}]_i$, intracellular calcium concentration; CsA, Cyclosporin A; MAPK, mitogen-activated protein kinase; MSCV, murine stem cell virus; GFP, green fluorescent protein; NFAT, nuclear factor of activated T cells; ChIP, chromatin immunoprecipitation.

Grant sponsor: National Institutes of Health; Grant number: DK-63298.

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Received 20 December 2005; Accepted 30 May 2006

DOI 10.1002/jcb.21044

tylxanthine (Mix: a phosphodiesterase inhibitor that elevates intracellular cAMP levels), the glucocorticoid dexamesthasone (Dex) and insulin (collectively known as MDI). As a result of extensive studies largely using this in vitro model system, it is now established that the pathway of terminal adipocyte differentiation is a highly coordinated process that proceeds via the sequential induction of a cascade of transcription factors, including the peroxisome proliferator-activated receptor γ (PPARγ) and members of the CCAAT/enhancer-binding protein (C/EBP) family [Rosen and Spiegelman, 2000; Otto et al., 2005]. When exposed to MDI, growth-arrested preadipocytes first re-enter the cell cycle and undergo several rounds of cell division known as mitotic clonal expansion (MCE) [Tang et al., 2003], during which time they upregulate the expression of the early transcription factors C/EBPβ and C/EBPδ [Yeh et al., 1995; Wu et al., 1996]. C/EBPβ and C/EBPδ then bind directly to cognate-binding sites in the proximal promoters of the PPARγ and C/EBPα genes and promote the expression of these two proadipogenic transcription factors [Rosen and Spiegelman, 2000; Otto et al., 2005]. Once expressed, PPARγ and C/EBPα re-enforce each other's expression in an autoregulatory loop and then act coordinately to direct the expression of a panel of adipocyte-specific genes that result in the specification of the terminally differentiated mature adipocyte phenotype [Rosen and Spiegelman, 2000; Otto et al., 2005].

The efficiency of adipocyte differentiation both in vitro and in vivo is strongly influenced by the presence or absence of a number of opposing hormones and other extracellular factors [MacDougald and Mandrup, 2002]. As indicated above, insulin, insulin-like growth factors, glucocorticoids, and agents that elevate intracellular cAMP levels have long been known to promote adipocyte differentiation. In contrast, a wide array of growth factors, cytokines, and other endocrine products, including epidermal growth factor, transforming growth factor-β, Wnt-10b, PREF-1, resistin, and the pro-inflammatory cytokines tumor necrosis factor-α, interleukin-6 and interleukin-1, have all been shown to potently inhibit adipogenesis [MacDougald and Mandrup, 2002]. Another class of products known to influence adipocyte differentiation is the prostaglandins [Serrero et al., 1992; Lepak and Serrero, 1993; Forman et al., 1995; Casimir et al., 1996; Reginato et al., 1998; Aubert et al., 2000]. The prostaglandins are a diverse class of structurally distinct metabolic products of arachidonic acid that are generated via the concerted actions of cyclooxygenases and other subsequent synthetic enzymes [Vane et al., 1998]. Specific prostaglandins have been shown to play distinct and opposing roles in the regulation of adipocyte differentiation. Thus, prostaglandins such as prostacyclin (PGI2) and PGJ2 are known to positively influence adipogenesis [Forman et al., 1995; Reginato et al., 1998; Aubert et al., 2000], whereas other prostaglandins such as PGF2α have been shown to be potent inhibitors of adipocyte differentiation [Serrero et al., 1992; Lepak and Serrero, 1993; Casimir et al., 1996].

PGF2α is known to signal via a specific cell surface G-protein coupled receptor termed the FP prostanoid receptor [Breyer et al., 2001] and has been reported to activate a plethora of downstream signaling pathways, including activation of the Gq heterotrimeric G-protein, stimulation of phospholipase C leading to increases in the intracellular calcium concentration and activation of protein kinase C, activation of phosphatidylinositol-3 kinase, mitogen-activated protein kinases (MAPK) and tyrosine kinases, as well as stimulation of the Rho small G-protein and activation of the beta-catentin/T-cell factor signaling pathway [Watanabe et al., 1994, 1995; Pierce et al., 1999; Breyer et al., 2001; Fujino and Regan, 2001]. However, the PGF2α-induced signaling pathways that are involved in mediating the inhibitory effect of PGF2α on adipogenesis are not currently well defined. Accordingly, the purpose of the current study was to investigate the signaling pathways and molecular mechanisms underlying the inhibitory effect of PGF2α on adipocyte differentiation.

MATERIALS AND METHODS

Cell Culture and Adipocyte Differentiation

3T3-L1 preadipocytes (ATCC) were cultured in growth media: DMEM containing high glucose (Invitrogen) supplemented with 10% (v/v) FCS (Hyclone), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen). To induce adipocyte differentiation, cells were grown in 6-well plates until 2 days post-confluence (day 0), then treated for 2 days with growth media plus MDI (0.5 mM Methylisobutylxanthine, 1 μM

Dexamethasone, and 10 μg/ml Insulin; all from Sigma), then re-fed with growth media containing 10 μg/ml insulin at day 2 and every 2 days thereafter with growth media alone. Where indicated, cells were also treated with the following drugs: 100 nM PGF2α, 5 ng/ml FK506, 1 μg/ml CsA, 20 μM SKF-96365, 5 μM BAPTA/AM, (all from Calbiochem) or vehicle control (ethanol). Ten days after stimulation, cells were fixed with formalin and stained with the lipophilic dye Oil Red O (Sigma). Stained cells were either photographed or counterstained with Giemsa and visualized by bright field microscopy.

Retroviral Expression Constructs and Infection of 3T3-L1 Preadipocytes

The pMSCV-GFP, pMSCV-H2K, pMSCV-PPARγ, pMSCV-VIVIT-GFP retroviral expression vectors were created as previously described [Neal and Clipstone, 2002]. pMSCV-GFP-RGS2 was generated by introducing the GFP-RGS2 fusion sequence from pEGFP-C1-mRGS2 [Reif and Cyster, 2000] into pMSCV-H2K. pMSCV-CD8-βARK was constructed by inserting the CD8-βARK fusion sequence from the pcDNAIII T8 bARK [Crespo et al., 1995] into pMSCV-GFP. Retroviral expression vectors were cotransfected together with pVSV-G (Clontech) into the GP293 pantropic packaging cell line (Clontech) using Lipofectamine Plus (Invitrogen). Media was replaced after 24 h and viral supernatants were harvested 2 days post-transfection and stored at −80°C. For infections, 5×10^4 3T3-L1 cells were plated per well of a 6-well plate. The next day, media was replaced with 2 ml of viral supernatant containing 8 μg/ml polybrene (Sigma) and plates were centrifuged at 2,000 rpm for 1.5 h at room temperature. After removal of viral supernatant, normal growth media was added and cells were expanded for subsequent analysis.

Immunoblot Analysis

At the indicated times following induction of differentiation, 3T3-L1 cells were lysed by RIPA buffer (0.1% SDS, 1% Deoxycholate, 1 mM EDTA, 1% TX-100, 50 mM Tris, PH 8.0, 500 mM NaCl), and centrifuged at 100,000 rpm for 20 min at 4°C (Beckman TL-100 ultracentrifuge). The whole cell extracts were resolved by SDS–PAGE and subjected to immunoblot analysis with the relevant primary antibodies: antibodies against PPARγ (H-100), C/EBPα (14AA), C/EBPβ (H-7), Erk-1 (C-16), p18 (N-20), p27 (C-19), and actin were from Santa Cruz Biotechnology, anti-p21 antibody was from BD Pharmingen (556431), anti-phospho-Thr188-C/EBPβ was from Cell Signaling Technology (3084S), anti-aP2/FABP4 (10004944) was from Cayman Chemicals, and appropriate horseradish peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse) were purchased from Amersham Biosciences. Immunoblots were visualized by enhanced chemiluminescence using ECL reagents (Amersham Biosciences).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from 3T3-L1 cells by RNeasy kit (Qiagen, Valencia, CA) at the indicated times following induction of differentiation and cDNA was synthesized with an oligo-dT primer and reverse transcriptase (Promega). PCR amplification was performed by using specific primers for aP2 (forward primer, 5′-GAACCTGGAAGCT-TGCTTGTCGCC-3′; reverse primer, 5′-ACCAGCTTGTCAC-CATCTCG-3′). HPRT (forward primer, 5′-GTTGGATACAGGCCAGACTT-TGTTG-3′; reverse primer, 5′-GAGGGTAGGCTGGCCTA-TAGGCT-3′) serves as a loading control. PCR products were resolved by agarose gel electrophoresis, and visualized with ethidium bromide.

Chromatin Immunoprecipitation (ChIP)

3T3-L1 cells were treated as indicated for 2 days and then fixed by adding 37% formaldehyde to a final concentration of 1% and incubated at 37°C for 10 min. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 M, cells were washed twice with cold PBS and then collected in 1 ml of PBS containing protease inhibitors. Cell lysis, chromatin sonication, and ChIP was performed using a commercially available kit (Upstate) following procedures recommended by the manufacturer. DNA fragments specifically immunoprecipitated with either 10 μg anti-C/EBPβ antibody or 10 μg normal mouse IgG as a negative control, were purified using a QIAquick PCR purification kit (Qiagen) and analyzed by PCR and subsequent agarose gel electrophoresis. The PCR primers used for ChIP

were: PPARγ2 promoter forward 5′-TACGTTTATCTGGTGTTTCAT-3′, PPARγ2 promoter reverse 5′-TCTCGCCAGTGACCCAC-3′, C/EBPα promoter forward 5′-CTAGTGTTGGCT GGAAGTG-3′, and C/EBPα promoter reverse 5′-CTTCTCCTGTGACTTTCCAAG-3′.

RESULTS

Differentiating 3T3-L1 Preadipocytes are Sensitive to the Inhibitory Effects of PGF2α for the First 2 Days of MDI-Induced Adipogenesis

As a prelude to investigating the molecular mechanisms underlying the inhibitory effects of PGF2α on adipocyte differentiation, we first determined the temporal window of sensitivity of differentiating 3T3-L1 preadipocytes to the anti-adipogenic effects of PGF2α. As shown in Figure 1, we found that the presence of PGF2α during the first 48 h of MDI-induced differentiation potently inhibited adipogenesis, as measured by staining of accumulated cellular triglyceride stores with mineral Oil Red O. The presence of PGF2α for only the first 24 h of differentiation also had a significant inhibitory effect on adipogenesis, as did the presence of PGF2α for the 24–48 h time period post-MDI induced stimulation (data not shown). In contrast, we found that if PGF2α was added to differentiating 3T3-L1 preadipocyte cultures after the initial first 48-h time period, there was little inhibitory effect on the differentiation of 3T3-L1 cells into lipid-laden, Oil Red O-positive mature adipocytes. These results indicate that differentiating 3T3-L1 preadipocytes are only sensitive to the anti-adipogenic effects of PGF2α for the first 48 h of the differentiation program and that maximal inhibition requires the continual presence of PGF2α throughout this entire time period.

PGF2α Inhibits Adipogenesis Through a Gαq-Calcium-Dependent Signaling Pathway

PGF2α is known to mediate its biological effects through the FP prostanoid receptor, a member of the seven-transmembrane family of GPCR that is coupled to the heterotrimeric G protein Gq [Ito et al., 1994; Watanabe et al., 1995]. In order to begin delineating the specific signaling pathway(s) responsible for the PGF2α-mediated inhibition of adipocyte differentiation, we first sought to determine the requirement for the individual Gαq and Gβγ G protein subunits in the anti-adipogenic effects of PGF2α. For these studies we took advantage of two well-characterized specific inhibitors of Gαq and Gβγ G protein subunits: the regulator of G protein signaling-2 (RGS2) and CD8-βARK, respectively. RGS2 is a GTPase activating protein that is a selective and potent inhibitor of Gαq but not other Gα G protein subunits [Heximer et al., 1997], while CD8-βARK is a chimeric molecule comprised of the Gβγ-binding domain of the β-adrenergic receptor kinase fused to the extracellular and transmembrane domains of the CD8 molecule, and works by scavenging free Gβγ subunits and preventing

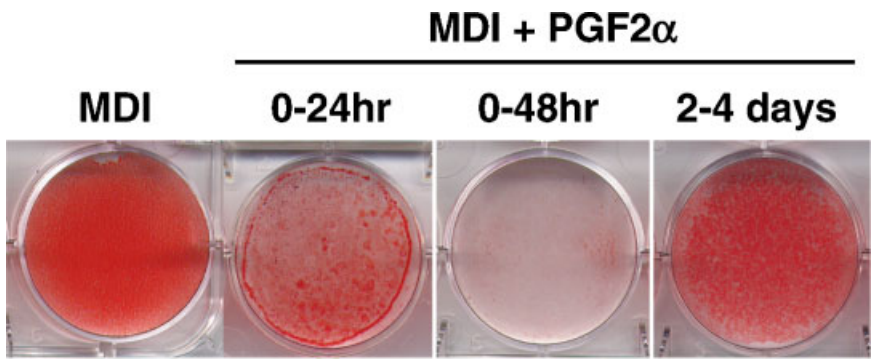

Fig. 1. PGF2α inhibits adipocyte differentiation at an early time point. Two-day post-confluent 3T3-L1 preadipocytes were induced to differentiate by treatment with the hormonal cocktail MDI in either the presence or absence of PGF2α (100 nM) for the indicated time period. After 10 days, the extent of adipocyte differentiation in each well was assessed by Oil Red O staining. Results are representative of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

them from interacting with their downstream effectors [Crespo et al., 1995]. When expressed in 3T3-L1 preadipocytes by retroviral-mediated gene transfer, we found that expression of RGS2, but not CD8-βARK, was able to significantly attenuate the inhibitory effects of PGF2α on adipocyte differentiation, thereby indicating that PGF2α inhibits adipocyte differentiation via a signaling pathway that is dependent upon the specific activation of Gαq, but not Gβγ, G protein subunits.

The best characterized downstream effector of Gαq is phospholipase Cβ [Rhee, 2001], which catalyses the hydrolysis of phosphatidylinositol 4, 5-bisphosphate to Ins (1,4,5) P_3 and diacylglycerol, leading to increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) and activation of protein kinase C, respectively. Since treatment of 3T3-L1 preadipocytes with PGF2α has previously been shown to elicit an increase in $[Ca^{2+}]_i$ [Miller et al., 1996], and elevated $[Ca^{2+}]_i$ is known to inhibit adipocyte differentiation [Ntambi and Takova, 1996; Neal and Clipstone, 2002], we next evaluated whether the anti-adipogenic effects of PGF2α were mediated by changes in $[Ca^{2+}]_i$ and the subsequent activation of downstream Ca^{2+}-dependent signaling events. Thus, 3T3-L1 preadipocytes were induced to undergo MDI-induced differentiation in the presence of PGF2α, together with either SKF96365, an inhibitor of receptor-mediated plasma membrane Ca^{2+}-influx, or BAPTA/AM, a membrane-permeable Ca^{2+}-chelator. As shown in Figure 2B, both inhibitors were able to rescue adipocyte differentiation under these conditions. Similar results were also obtained with 2-aminorthoxydiphenyl borate, an Ins (1,4,5) P_3 antagonist, (data not shown).

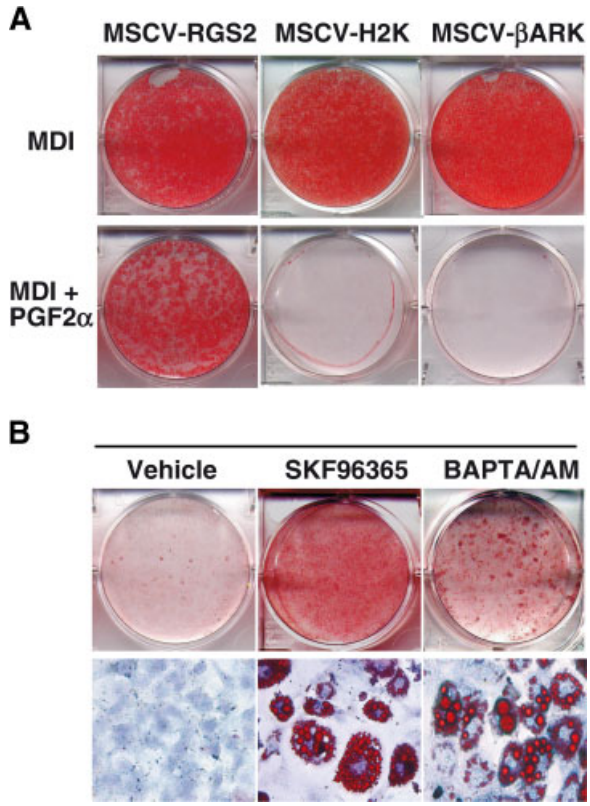

Fig. 2. PGF2α inhibits adipocyte differentiation via a Gαq/calcium-dependent signaling pathway. **A**: 3T3-L1 preadipocytes infected with either MSCV-RGS2, MSCV-βARK, or the MSCV-H2K control retrovirus, were allowed to reach confluence and then induced to undergo differentiation by the standard MDI protocol. PGF2α (100 nM) or vehicle was added for the first 2 days. Ten days later, differentiation was assessed by Oil Red O staining. **B**: Two-day post-confluent 3T3-L1 cells were induced to differentiate with MDI in the presence of 100 nM PGF2α. Where indicated 20μM SKF-96365, 5μM BAPTA/AM, or vehicle control was present for the first 2 days of the differentiation process. Ten days after the induction of differentiation, cells in each well were stained with Oil Red O and photographed, then counterstained with Giemsa and visualized by bright field microscopy (Final magnification, 400×). Results are representative of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The Calcineurin Phosphatase Plays a Critical Role in Mediating the Inhibitory Effect of PGF2α on Adipocyte Differentiation

Since it appears that the PGF2α-mediated inhibition of adipocyte differentiation is, at least in part, dependent upon changes in the $[Ca^{2+}]_i$, and we have recently identified the calcium-regulated serine/threonine phosphatase, calcineurin as a critical component of a Ca^{2+}-dependent signaling pathway involved in the negative regulation of adipocyte differentiation [Neal and Clipstone, 2002], we next evaluated whether calcineurin might play a role in the inhibitory effects of PGF2α on adipocyte differentiation. As shown in Figure 3A, we found that treatment of cells with either FK506 or cyclosporin A, two structurally unrelated, highly specific calcineurin inhibitors, both effectively attenuated the inhibitory effects of PGF2α on adipocyte differentiation, as determined by Oil Red O staining. Similarly, we found that the retroviral-mediated expression of VIVIT-GFP, a previously characterized specific calcineurin

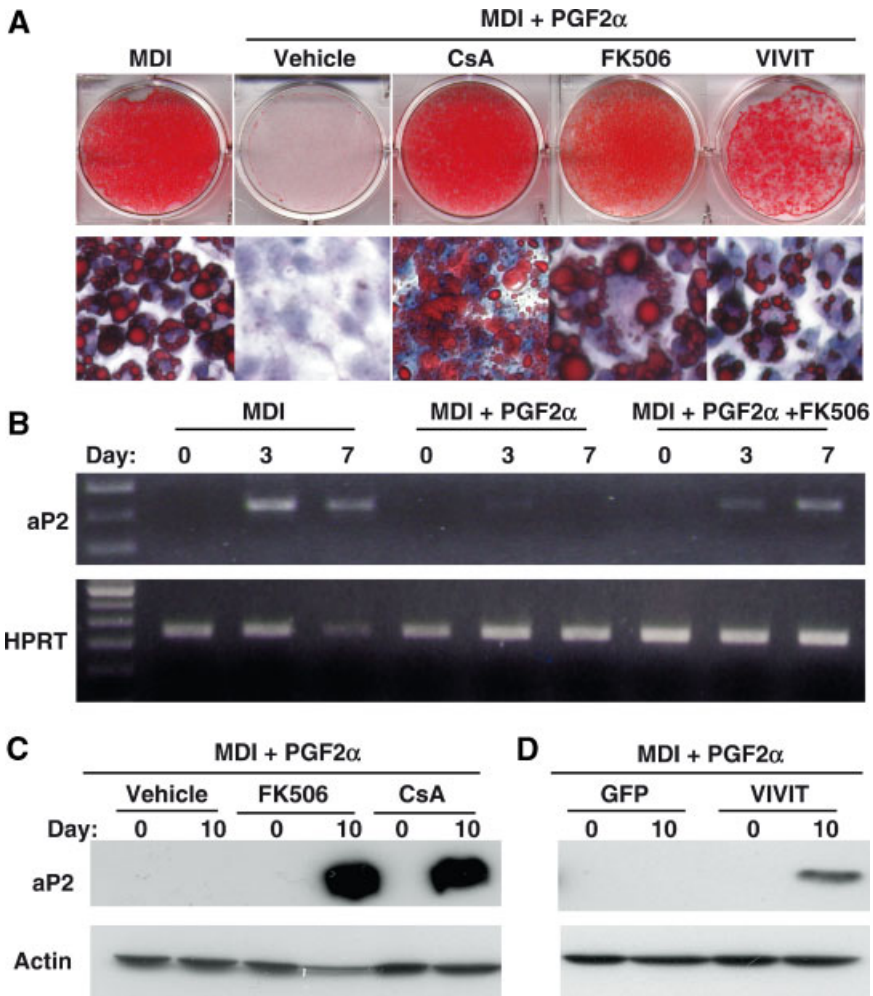

Fig. 3. PGF2α inhibits adipogenesis through a calcineurin-dependent pathway. **A**: At 2 days post-confluence, 3T3-L1 preadipocytes were induced to differentiate with MDI by the standard protocol in the presence of either vehicle, PGF2α (100 nM) alone, PGF2α plus CsA (1 μg/ml) or PGF2α plus FK506 (5 ng/ml) as indicated for the first 2 days. 3T3-L1 preadipocytes transduced with the MSCV-VIVIT virus were also induced to differentiate with MDI in the presence of 100 nM PGF2α. After 10 days, the extent of differentiation was assessed by Oil red O staining, and either directly photographed or counterstained with Giemsa and visualized by bright field microscopy (Final magnification, 400×). **B**: RT-PCR analysis of *aP2* gene expression in 3T3-L1 preadipocytes treated with either MDI alone, MDI plus 100 nM PGF2α, or MDI plus 100 nM PGF2α and 5 ng/ml FK506 for the indicated number of days (upper panel). HGPRT expression was examined as a loading control (lower panel). **C**: Immunoblot analysis of aP2 protein expression in 3T3-L1 preadipocytes induced to differentiate with MDI in the presence of 100 nM PGF2α and either vehicle, 5 ng/ml FK506 or 1μg/ml CsA. **D**: Immunoblot analysis of aP2 protein expression in either MSCV-GFP or MSCV-VIVIT-transduced 3T3-L1 cells induced to differentiate with MDI in the presence of 100 nM PGF2α. As a control for protein loading immunoblots were stripped and re-probed with an anti-actin antibody. Results in Figure 3A,B are representative of more than three independent experiments, while those of Figure 3C,D are representative of two independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inhibitory peptide [Aramburu et al., 1999], also significantly rescued the inhibitory effects of PGF2α on adipocyte differentiation. The role of calcineurin in mediating the inhibitory effects of PGF2α on adipocyte differentiation was further confirmed by analyzing the expression of the late, mature adipocyte-specific marker gene, *aP2*. As shown in Figure 3B, we found that PGF2α inhibited the MDI-induced expression of the aP2 mRNA, and that this inhibitory effect was reversed in the presence of FK506. Similarly, we found that FK506, CsA, and VIVIT-GFP also all reversed the inhibitory effects of PGF2α on the expression of the aP2 protein (Fig. 3C and D). Collectively, therefore, these data provide strong evidence that PGF2α

inhibits adipocyte differentiation through a signaling pathway that is dependent upon calcineurin.

The PGF2α-Calcineurin Signaling Pathway does not Interfere With the MCE Phase of Adipocyte Differentiation

Given that differentiating preadipocytes are only sensitive to the anti-adipogenic effects of PGF2α during the first 48 h of differentiation, a time period that corresponds to the onset of the MCE phase of adipocyte differentiation, which is thought by many to play a critical role in the regulation of adipogenesis, we examined the possibility that PGF2α inhibits adipocyte differentiation by interfering with MCE and subsequent cell cycle exit. As shown in Figure 4A, in response to MDI stimulation, control, PGF2α-treated, and PGF2α plus FK506-treated cells all undergo an approximately fourfold increase in mean cell number over the course of a 4-day period, corresponding to the two rounds of cell division that are typically observed during MCE.

Once preadipocytes have undergone MCE, they next exit the cell cycle and undergo terminal adipocyte differentiation, a step believed to be controlled by changes in the expression of a variety of cell cycle regulatory proteins including cyclin-dependent kinase inhibitors and members of the retinoblastoma-related family of proteins [Richon et al., 1997; Cowherd et al., 1999; Morrison and Farmer, 1999]. As shown in Figure 4B, MDI treatment of 3T3-L1 preadipocytes resulted in the expected time-dependent increase in expression of both p21 and p27, whose expression has previously been shown to correlate with the commitment of differentiating preadipocytes to terminal differentiation [Morrison and Farmer, 1999]. Notably, the presence of PGF2α did not appear to affect the expression of either of these cell cycle regulatory proteins. Similar results were obtained when we analyzed the expression of p18 and the retinoblastoma-related proteins p107 and p130, which are also known to undergo characteristic changes in expression during the initial MCE phase of adipogenesis (data not

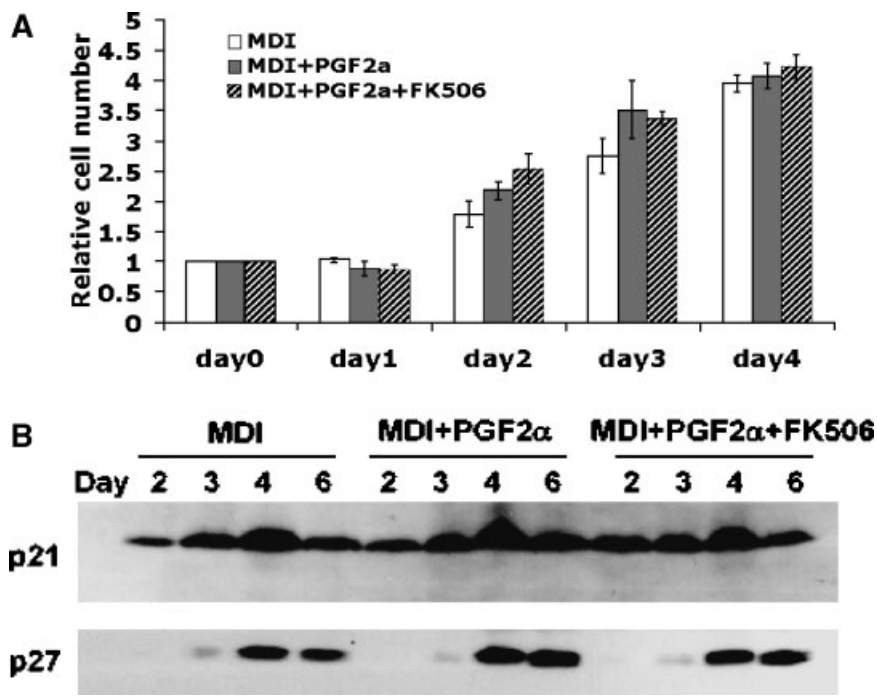

Fig. 4. PGF2α treatment does not interfere with the MCE phase of adipogenesis. **A**: 3T3-L1 preadipocyte were induced to differentiate with MDI in the presence or absence of 100 nM PGF2α and 5 ng/ml FK506 as indicated. At daily intervals total numbers of viable cells in the cultures were determined in triplicate by Coulter counter analysis. The standard deviations are indicated. **B**: Whole cell extracts from 3T3-L1 cell treated as above were collected at the indicated time points and analyzed by SDS–PAGE followed by immunoblotting with either an anti-p21 (upper panel) or anti-p27 antibody (lower panel). Results are representative of at least three independent experiments.

shown). Taken together, these data demonstrate that PGF2α does not appear to inhibit adipocyte differentiation by interfering with either the MCE phase of adipogenesis or the subsequent exit of differentiating 3T3-L1 preadipocytes from the cell cycle.

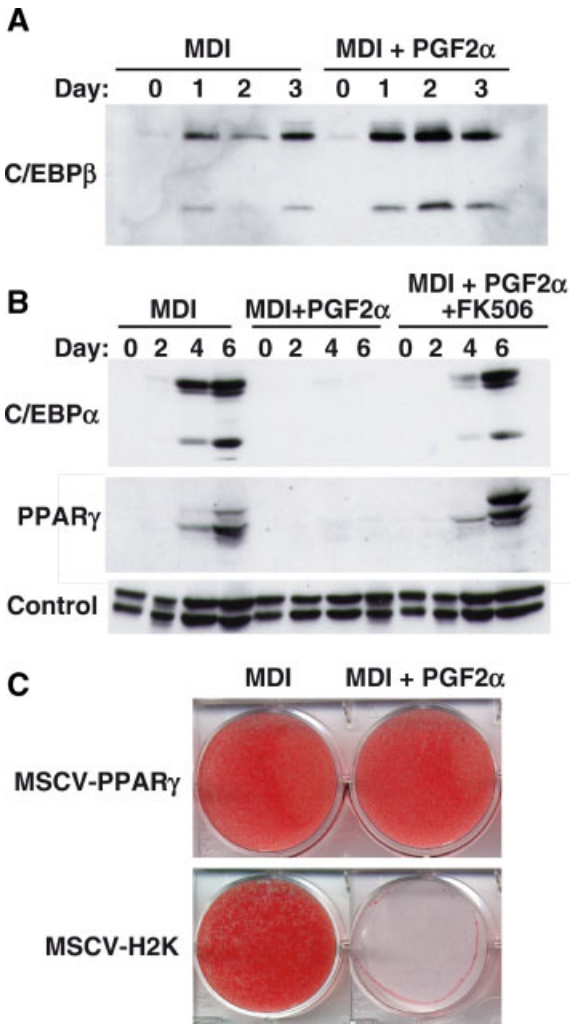

Fig. 5. PGF2α inhibits the expression of the PPARγ and C/EBPα transcription factors via a calcineurin-dependent pathway. 3T3-L1 preadipocyte cells were induced to differentiate with MDI using the standard protocol in the presence of either vehicle, 100 nM PGF2α, or 100 nM PGF2α plus 5 ng/ml FK506. Whole cell extracts were prepared at the indicated time points and analyzed by SDS–PAGE followed by immunoblotting with either (**A**) anti-C/EBPβ, or (**B**) anti-C/EBPα (upper panel) and anti-PPARγ (lower panel). Immunoblots were stripped and re-probed with a pan anti-Erk antibody as a control to ensure consistent loading. **C**: Ectopic expression of PPARγ rescues the PGF2α-induced inhibition of adipocyte differentiation. 3T3-L1 preadipocyte cells infected with either the MSCV-H2K control or MSCV-PPARγ retrovirus were allowed to reach confluence and then induced to differentiate with MDI in the presence or absence of 100 nM PGF2α. After 10 days the extent of adipocyte differentiation in each well was assessed by Oil red O staining. Results are representative of more than three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PGF2α Acts Via a Calcineurin-Dependent Signaling Pathway to Inhibit the Expression of the Proadipogenic Transcription Factors PPARγ and C/EBPα

To further investigate the molecular mechanisms underlying the inhibitory effect of PGF2α on adipocyte differentiation, we next examined the effects of PGF2α on the expression of the C/EBPβ, PPARγ, and C/EBPα transcription factors that are known to play a critical role in the regulation of adipogenesis [Rosen and Spiegelman, 2000; Otto et al., 2005]. As shown in Figure 5A, treatment of cells with PGF2α alone had no effect on the MDI-induced expression of the early transcription factor C/EBPβ. In marked contrast, however, we found that PGF2α treatment had a significant inhibitory effect on the expression of both PPARγ and C/EBPα (Fig. 5B). Thus, while the MDI-induced increased expression of PPARγ and C/EBPα was readily detectable in control cells, neither PPARγ nor C/EBPα were detectable in cells treated with PGF2α. Significantly, however, we found that this inhibitory effect of PGF2α on the expression of PPARγ and C/EBPα was attenuated in the presence of FK506 (Fig. 5B). These results therefore suggest that PGF2α inhibits adipocyte differentiation via a calcineurin-dependent signaling pathway that acts to block the expression of the proadipogenic transcription factors C/EBPα and PPARγ.

In order to confirm that the inhibitory effect of PGF2α on adipocyte differentiation was indeed simply caused by a block in the expression of proadipogenic transcription factors, we determined whether the anti-adipogenic effects of PGF2α could be overcome by the ectopic expression of PPARγ. Thus, 3T3-L1 preadipocytes were transduced with either a control retrovirus or a recombinant retrovirus encoding PPARγ1, and then induced with MDI to undergo differentiation in the presence of inhibitory concentrations of PGF2α. As expected, the differentiation of control virus-transduced cells was potently inhibited in the presence of PGF2α. However, we found that ectopic expression of PPARγ1 was able to overcome this inhibitory effect of PGF2α and efficiently rescue the adipocyte differentiation of MDI-stimulated 3T3-L1 preadipocytes treated with PGF2α (Fig. 5C). Collectively, these results are there-

fore consistent with a model in which PGF2α acts via a calcineurin-dependent signaling pathway to inhibit adipocyte differentiation by preventing the normal MDI-induced expression of the critical proadipogenic transcription factors PPARγ and C/EBPα.

The PGF2α-Calcineurin Signaling Pathway does not Affect Either the Differentiation-Induced Phosphorylation of C/EBPβ on Threonine 188 or its Recruitment to the PPARγ2 and C/EBPα Promoters

During adipocyte differentiation, the expression of PPARγ and C/EBPα are known to be dependent upon the activity of the early transcription factors C/EBPδ and C/EBPβ, with C/EBPβ in particular being thought to play a critical role in this process [Rosen and Spiegelman, 2000; Otto et al., 2005]. Previous studies have demonstrated that the DNA-binding activity of C/EBPβ is regulated in a phosphorylation-sensitive manner [Tang and Lane, 1999; Otto et al., 2005]. In addition, other studies have shown that phosphorylation of C/EBPβ specifically on threonine 188 is critical for the activation of C/EBPβ-dependent transcriptional activity, especially the ability of C/EBPβ to promote the expression of C/EBPα [Park et al., 2004]. Given the apparent phosphorylation-dependent regulation of C/EBPβ activity, together with the critical role of C/EBPβ in the regulation of PPARγ and C/EBPα expression, we considered the possibility that the PGF2α-induced activation of calcineurin phosphatase activity might act to inhibit adipogenesis by directly influencing the phosphorylation status of C/EBP3β? To test this hypothesis we first performed a chromatin immunoprecipitation experiment to examine the effects of PGF2α on the recruitment of endogenous C/EBPβ to both the PPARγ2 and C/EBPα promoters during adipocyte differentiation. As shown in Figure 6A, C/EBPβ is specifically recruited to both the endogenous PPARγ2 and C/EBPα promoters in response to stimulation with MDI. Importantly, this MDI-induced recruitment of C/EBPβ is not affected by treatment with PGF2α. Next, we examined the effects of PGF2α treatment on the differentiation-induced phosphorylation of C/EBPβ on threonine 188. As shown in Figure 6B, stimulation of cells with MDI led to the expected inducible phosphorylation of C/EBPβ on threonine 188, which was unaffected by treatment of cells with

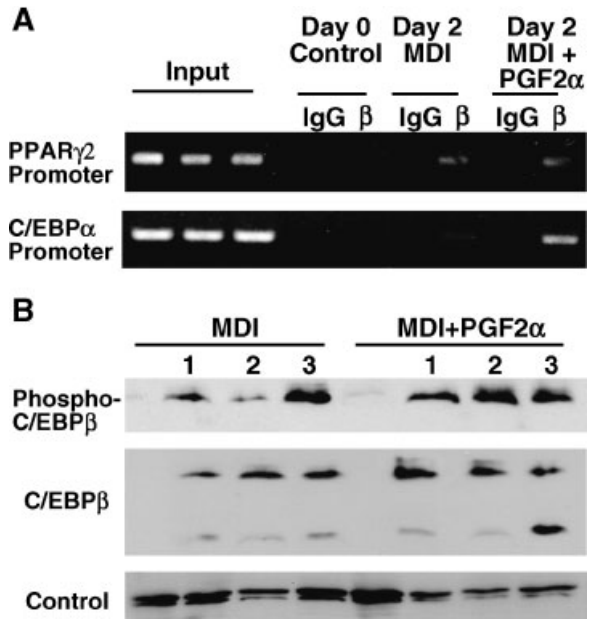

Fig. 6. PGF2α does not affect either the recruitment of C/EBPβ to endogenous PPARγ2 and C/EBPα promoters or its differentiation-specific phosphorylation on theronine-188. **A**: Recruitment of endogenous C/EBPβ to the PPARγ2 and C/EBPα promoters was evaluated by ChIP assay. Two days post-confluent 3T3-L1 preadipocytes cells were treated with either MDI or MDI plus PGF2α, as indicated, and processed for ChIP analysis at the indicated times. Cell lysates were immunoprecipitated with either control IgG or anti-C/EBPβ antibody and specifically associated DNA fragments were identified by PCR with primers specific to either the PPARγ2 promoter (upper panel) or C/EBPα (lower panel). One percent of input is shown as a control. Results are representative of two independent experiments. **B**: Whole cell lysates prepared from 3T3-L1 preadipocyte cells induced to differentiate with MDI in the presence of either vehicle, or 100 nM PGF2α for the indicated times, were analyzed by immunoblotting with an antibody specific for C/EBPβ when phosphorylated on threonine-188 (upper panel), C/EBPβ (middle panel), and a pan anti-Erk antibody to serve as a loading control (lower panel). Results are representative of at least three independent experiments.

PGF2α. Hence, these data indicate that the PGF2α-induced calcineurin-signaling pathway does not appear to prevent adipocyte differentiation by affecting the phosphorylation-sensitive regulation of the C/EBPβ transcription factor.

The Inhibitory Effect of PGF2α on Adipocyte Differentiation is Attenuated by the Specific Histone Deacetylase Inhibitor Trichostatin A (TSA)

Finally, we considered the possibility that PGF2α may inhibit adipocyte differentiation by active repression of the PPARγ and C/EBPα

promoters. Since histone deacetylase (HDAC) enzymes [Ng and Bird, 2000] have been implicated in mediating the effects of many sequence specific transcriptional repressors, we examined the consequences of a specific and potent HDAC inhibitor, TSA, on the anti-adipogenic effects of PGF2α. As shown in Figure 7, the addition of TSA was able to significantly overcome the inhibitory effects of PGF2α on adipocyte differentiation, as measured by both Oil Red O staining (Fig. 7A) and the rescued expression of the pro-adipogenic transcription factors PPARγ and C/EBPα (Fig. 7B). This result therefore suggests that PGF2α likely inhibits adipocyte differentiation and the expression of the proadipogenic transcription factors via a pathway that involves an HDAC-sensitive step.

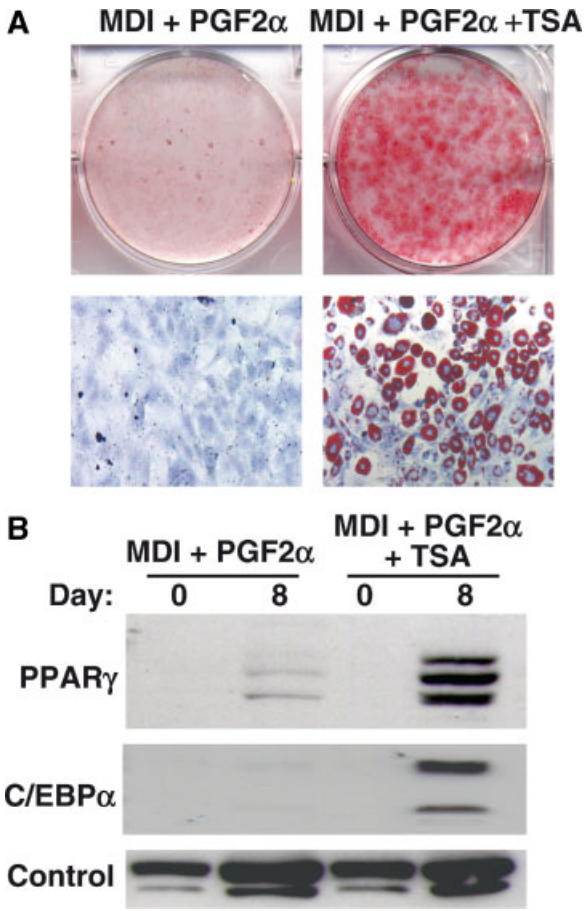

Fig. 7. The specific HDAC inhibitor TSA rescues adipocyte differentiation in the presence of PGF2α. **A**: 3T3-L1 preadipocyte cells were induced to differentiate with MDI in the presence of either 100 nM PGF2α or 100 nM PGF2α plus 100 nM TSA. After 10 days, differentiation was assessed by Oil Red O staining, and either directly photographed or counterstained with Giemsa and visualized by bright field microscopy (final magnification, 200×). **B**: 3T3-L1 preadipocyte cells were treated as described in (A) and whole cell extracts were prepared at the indicated times for immunoblot analysis with either anti-PPARγ (upper panel), or anti-C/EBPα (middle panel). Immunoblots were stripped and re-probed with a pan anti-Erk antibody as a control to ensure consistent loading (lower panel). Results are representative of more than three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

In this study, we have investigated the molecular mechanism underlying the inhibitory effects of PGF2α on adipocyte differentiation. Our principal finding is that PGF2α inhibits adipocyte differentiation via a Gαq G-protein subunit and calcium-dependent signaling pathway involving the calcium/calmodulin-regulated serine/threonine phosphatase calcineurin. This conclusion is consistent with previous reports that PGF2α can act via its specific Gq-coupled FP prostanoid receptor to activate calcineurin in a number of cell types [Horsley and Pavlath, 2003; Liang et al., 2004], as well as with our recent identification of calcineurin as a critical calcium-dependent negative regulator of adipocyte differentiation [Neal and Clipstone, 2002]. We presume that this pathway is likely to operate in vivo to influence adipocyte development under conditions where PGF2α levels are elevated, such as those that occur during chronic inflammatory conditions as a result of increased expression of cyclooxygenase-2.

While our data clearly highlights the critical role played by the calcium-calcineurin signaling pathway in mediating the inhibitory effects of PGF2α on adipocyte differentiation, it should be noted that PGF2α has also been shown to activate a variety of other downstream signaling pathways, several of which have themselves been previously shown to be capable of inhibiting adipocyte differentiation under certain circumstances (e.g., MAPK and the beta-catenin/T cell factor signaling pathways) [Font de Mora et al., 1997; Ross et al., 2000]. Indeed, a previous report has specifically implicated a role for the MAPK signaling pathway in mediating the anti-adipogenic effects of PGF2α on 3T3-L1 preadipocytes by directly phosphorylating PPARγ and inhibiting its transcriptional activity [Reginato et al., 1998]. However, we could find no evidence that

calcineurin was able to influence PGF2α-induced MAPK activity in 3T3-L1 cells, and furthermore, found that inhibition of the MAPK-signaling pathway was unable to overcome the inhibitory effects of a constitutively active calcineurin mutant on adipocyte differentiation (data not shown). Hence, it does not appear that calcineurin inhibits adipogenesis via the MAPK-signaling pathway. Instead, we believe that the calcineurin and MAPK-signaling pathways most likely act independently to inhibit adipocyte differentiation. In this regard, we do not rule out the possibility that additional PGF2α-induced signaling pathways may also contribute towards the inhibition of adipocyte differentiation. However, based upon our observation that inhibition of calcineurin is able to almost completely attenuate the inhibitory effects of PGF2α on adipogenesis, we feel that the calcineurin-signaling pathway is likely to play the major, if not dominant, role in mediating the anti-adipogenic effects of PGF2α.

Taken as a whole, our data demonstrates that PGF2α inhibits adipocyte differentiation via a calcineurin-dependent signaling pathway that acts to block the normal MDI-induced expression of the critical pro-adipogenic transcription factors PPARγ and C/EBPα. This conclusion is supported by our finding that the inhibitory effects of PGF2α on adipocyte differentiation can be bypassed by the ectopic expression of PPARγ, as well as by our observation that adipocyte differentiation is only sensitive to the inhibitory effects of PGF2α when present during the early stages of differentiation, prior to the expression of PPARγ and C/EBPα, but is largely without effect if added to differentiating preadipocytes at later stages of the differentiation process after the initial expression of PPARγ and C/EBPα. At present we do not currently know precisely how the PGF2α-calcineurin signaling pathway acts to inhibit expression of PPARγ and C/EBPα. However, in this regard, our studies reveal that PGF2α treatment does not affect the initial MDI-induced expression of C/EBPβ (Fig. 5A), nor does it appear to affect either the initial C/EBPβ-dependent clonal expansion phase of adipocyte differentiation, or the subsequent molecular events associated with the permanent exit of preadipocytes from the cell cycle (Fig. 4). We also demonstrate that PGF2α does not inhibit either the recruitment of C/EBPβ to the endogenous PPARγ2 and C/EBPα promoters (Fig. 6A), or affect the critical MDI-induced phosphorylation of C/EBPβ on threonine 188 (Fig. 6B), which is thought to play a critical role in the activation of C/EBPβ-dependent transcription, particularly its ability to induce the expression of C/EBPα [Park et al., 2004]. Hence, based upon these results, it appears that the PGF2α-calcineruin signaling pathway must act to inhibit expression of PPARγ and C/EBPα, at a point in the adipogenic-signaling pathway that is either distal, or parallel, to the recruitment of C/EBPβ to the PPARγ and C/EBPα promoters.

Potential insight into the molecular mechanisms underlying the inhibitory effect of the PGF2α-calcineurin signaling pathway on adipocyte differentiation was gained from our observation that the PGF2α-mediated inhibition of the expression of the pro-adipogenic transcription factors PPARγ and C/EBPα could be reversed by the specific HDAC inhibitor TSA. This result suggests that the inhibitory effect of PGF2α on the expression of PPARγ and C/EBPα is likely to be mediated, at least in part, via an HDAC-dependent step. HDACs are enzymes that play an important role in the regulation of gene expression and are best known for their role in repressing gene transcription via interaction with transcriptional repressors [Ng and Bird, 2000]. Interestingly, both HDACs and transcriptional repressors have previously been implicated in the regulation of adipocyte differentiation: HDAC-1 has been shown to associate with the C/EBPα promoter and play an important role in preventing C/EBPα expression in unstimulated preadipocytes [Wiper-Bergeron et al., 2003], while a number of transcriptional repressors have been implicated in the regulation of adipogenesis via the direct inhibition of both C/EBPα and PPARγ gene expression [Tong et al., 2000; Yun et al., 2002; Banerjee et al., 2003; Shi et al., 2003]. Hence, it is tempting to speculate that one potential mechanism by which the PGF2α-calcineurin signaling pathway may inhibit adipocyte differentiation is by inducing the expression and/or activity of an HDAC-associated transcriptional repressor that is either able to directly inhibit the expression of the PPARγ and C/EBPα genes, or alternatively, acts indirectly, by inhibiting the expression of a gene(s) that is normally required to promote the expression of PPARγ and C/EBPα. Furthermore, although HDACs are best known for their role in repressing transcription, it has recently become apparent that HDACs

can also play a positive role in promoting gene expression [Sakamoto et al., 2004; Nusinzon and Horvath, 2005]. Hence, another alternate possibility to explain the observed TSA-mediated rescue of adipocyte differentiation in PGF2α-treated cells is that TSA may inhibit an HDAC that is involved in promoting the expression of a PGF2α-induced inhibitor of PPARγ and C/EBPα transcription. Current studies are focused on attempting to distinguish between these possibilities.

ACKNOWLEDGMENTS

The authors thank Joel W. Neal for contributing to the early stages of this project and Dr. Jason Cyster and Dr. Marlene Hosey for providing reagents.

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