Original Article

Inhibition of p38MAPK Increases Adipogenesis From Embryonic to Adult Stages

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Formation of new adipocytes from precursor cells contributes to adipose tissue expansion and obesity. In this study, we asked whether p38 mitogen-activated protein kinase (MAPK) pathway regulates normal and pathological adipogenesis. In both dietary and genetically (*ob/ob*) obese mice, adipose tissues displayed a marked decrease in p38MAPK activity compared with the same tissues from lean mice. Furthermore, p38MAPK activity was significantly higher in preadipocytes than in adipocytes, suggesting that p38MAPK activity decreases during adipocyte differentiation. In agreement with an inhibitory role of p38MAPK in this process, we found that in vitro inhibition of p38MAPK, with the specific inhibitor PD169316, increased the expression of adipocyte markers in several cellular models, from embryonic to adult stages. Importantly, the expression of adipocyte markers was higher in p38MAPKα knockout cells than in their wild-type counterparts. Phosphorylation of C/EBPβ, which enhances its transcriptional activity, is increased after p38MAPK inhibition. Finally, either inhibition or disruption of p38MAPK increased peroxisome proliferator–activated receptor (PPAR)γ expression and transactivation. Rescue of p38MAPK in knockout cells reduced PPARγ activity to the low basal level of wild-type cells. We demonstrate here, by using multipronged approaches involving p38 chemical inhibitor and p38MAPKα knockout cells, that p38MAPK plays a negative role in adipogenesis via inhibition of C/EBPβ and PPARγ transcriptional activities. *Diabetes* 55:281–289, 2006

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C/EBPβ, CCAAT/enhancer-binding protein β; ERK, extracellular signal–regulated kinase; JNK, c-Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; NFAT, nuclear factor of activated T-cell; PPAR, peroxisome proliferator–activated receptor; PPRE, PPARγ responsive element; PPRE-luc, PPRE-luciferase; TZD, thiazolidinedione.

Intracellular mitogen-activated protein kinase (MAPK) signaling pathways play a pivotal role in many essential cellular processes, such as proliferation, inflammation, and differentiation. The MAPK family comprises three groups: extracellular signal–regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs), and p38MAPK. These serine/threonine kinases are regulated by phosphorylation cascades organized in specific modules and activated by a wide panel of various stimuli. Schematically, ERK is preferentially activated by mitogens such as serum or growth factors, and, accordingly, this pathway is an important regulator of cell cycle and cell proliferation. p38MAPK and JNK are more responsive to various stress stimuli from UV to cytokines, and they have been involved in apoptosis and/or in the response to cellular stresses. Regarding cellular differentiation, the role of MAPKs is extremely complex and depends on multiple parameters, but, clearly, numerous works demonstrate that they can modulate very efficiently this process (1).

Formation of new adipocytes from precursor cells in white adipose tissue contributes to adipose tissue expansion (2). Because of its essential role in cell proliferation and the fact that adipogenic stimuli activate the ERK pathway, the role of this pathway in normal and pathological adipogenesis has been intensively investigated. Numerous works demonstrate that the ERK pathway is able to regulate adipogenesis at each step of the process, from stem cells to adipocytes (rev. in 3). Some of these effects are likely to be due to the direct ERK regulation of adipocyte differentiation effectors, such as the transcription factors CCAAT/enhancer-binding protein β (C/EBPβ) and/or peroxisome proliferator–activated receptor (PPAR)γ (4–9).

Whereas the JNK pathway does not seem to be directly implicated in adipocyte differentiation, the role of p38MAPK in adipocyte differentiation remains unclear. Analysis of preadipocyte cell lines with p38MAPK inhibitors such as SB203580 gave controversial results, showing apparent opposite roles for p38MAPK in adipogenesis. On one hand, addition of the inhibitors early in the differentiation process of 3T3-L1 preadipocytes decreases adipocyte formation and C/EBPβ phosphorylation (10,11). On the other hand, other reports demonstrate that p38MAPK activity can, indirectly, inhibit adipocyte differentiation. CHOP10 is a dominant-negative regulator of C/EBP and is phosphorylated by p38MAPK on Ser-78 and Ser-81 (12). Interestingly, overexpression of wild-type CHOP10 blocks adipogenic differentiation of 3T3-L1 cells (13), whereas the nonphosphorylatable form of CHOP10 (CHOPAla^{78,81}) is a poor

inhibitor of adipogenesis, suggesting that p38MAPK activity blocks adipocyte differentiation. A similar conclusion was made for the transcription factor nuclear factor of activated T-cells NFATc4 (14), which is implicated in adipocyte differentiation (15). Dephosphorylation of NFAT promotes its nuclear localization. Once dephosphorylated and translocated into the nucleus, NFATc4 increases PPARγ expression and therefore promotes adipogenesis. NFATc4 is phosphorylated by p38MAPK, and this phosphorylation inhibits adipose cell formation in NIH3T3 (14).

To better understand the implication of p38MAPK in adipogenesis, we determined whether this pathway regulated adipocyte differentiation in various cell models by biochemical and genetic approaches. We found that inhibition of this pathway or knockout of the p38MAPKα gene increased adipogenesis in several cellular models via the regulation of the adipogenic transcription factors C/EBPβ and PPARγ.

RESEARCH DESIGN AND METHODS

Mice were housed on a 12-h light/dark schedule and had free access to water and food. High-fat (45% fat, 35% carbohydrates, and 20% proteins) and standard diets (10% fat, 70% carbohydrates, and 20% proteins) were purchased from SAFE (Epinay/Orge, France). The experiments were conducted following the standard ethical guidelines (European Union guidelines on animal laboratory care) and approved by the ethical committee of the Nice School of Medicine.

Cells and adipocyte differentiation. Mouse embryonic fibroblasts (MEFs) were prepared on day 14 postcoitum as described previously (16). Preadipocytes from adult tissue were isolated from inguinal and axillary fat pads of 8-week-old mice (17). Two days postconfluent, cells were treated for 48 h with the adipocyte differentiation cocktail containing 85 nmol/l insulin, 0.25 μmol/l dexamethasone, and 0.5 mmol/l 3-isobutyl-1-methylxanthine. The medium was then replaced for 6 days by Dulbecco's modified Eagle's medium with insulin only. Oil red O staining was carried out as described by Dani et al. (18). To perform the quantification of triglyceride accumulation, oil red O was extracted with isopropanol, and the optical density was measured at 490 nm.

The MEFs knockout for p38α isoform gene and their wild-type counterparts were obtained from Dr. M. Karin (San Diego, CA). Knockout MEFs and 3T3-L1 preadipocyte cells were induced to differentiate as described for MEFs.

Mouse enbryonic stem cells CGR8 (19) were grown on gelatin-coated plates and maintained in Dulbecco's modified Eagle's medium (Cambrex, Verviers, Belgium), supplemented with nonessential amino acids, 2 mmol/l L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 1 mmol/l sodium pyruvate, 10% (vol/vol) fetal bovine serum (Invitrogen, Grand Island, NY) and 143 μmol/l β-mercaptoethanol (Sigma, St. Louis, MO), and 100 units/ml of leukemia inhibitory factor. For differentiation, cells were maintained in culture media without leukemia inhibitory factor, as described previously (20). Briefly, this protocol included two key steps: *1*) between days 0 and 7, embryoid bodies were formed in suspension and treated daily between days 2 and 5 with 0.1 μmol/l all-trans retinoic acid (Sigma) and *2*) from day 7 to the end, cells were seeded onto Petri dishes and treated with 85 nmol/l insulin (Lilly France SAS, Suresnes, France) and 2 nmol/l 3,3′,5-tri-iodotyronine (Sigma). For p38MAPK inhibition, 10 μmol/l PD169316 (Calbiochem, Darmstadt, Germany) were added to the culture media, which was renewed everyday from day 7 to the end of the differentiation protocol. Adipocyte colonies were analyzed 21 days after the formation of embryoid bodies. Area of adipocyte outgrowths was measured and normalized to the area of each embryoid body using IP Lab software (Image J 1.29x; National Institutes of Health, Bethesda, MD). The data were expressed as a histogram showing the percentage of embryoid bodies with 0–10%, 0–20%, 20–40%, 40–60%, 60–80%, or 80–100% of their area forming adipocytes.

The DBAp38C69 embryoid body cell line (obtained from Dr C.A. Gabel, Pfizer Laboratory, Groton, CT) knockout for the p38α isoform gene and the DBA-252 wild-type cells were induced to differentiate according to the same protocol. Teratocarcinomas were induced by subcutaneous injections of 5×10^6 cells of each cell line in DBA1/LacJ mice (21).

Western blotting analysis. Tissue extracts were prepared using lysis buffer (20) and were analyzed by Western blotting using antibodies against p38MAPK phosphorylated on Thr180/Tyr182, p38MAPK, JNK phosphorylated on Thr183/Tyr185, C/EBPβ phosphorylated on Thr 188, and C/EBPβ (Cell Signaling Technology, Danvers, MA).

Real-time quantitative RT-PCR. Gene expression analysis was performed with the ABI Prism 7000 (Applied Biosystem) and SYBR green reagents (Eurogentec, Seraing, Belgium). cDNAs were synthesized from 2 μg total RNA using Superscript II reverse transcriptase (Invitrogen). Primers set were designed according to the manufacturer software. Samples contained 1 × SYBR green master mix, 0.5 μmol/l primers, and synthesized cDNA in a 25-μl volume. The PCR conditions were as follows: 10 min at 95°C, then 40 cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 72°C. We used 36B4 as an internal control. Primer sequences are as follows: CEBP/α forward 5′ GACCATTAGC CTTGTGTGTACTGTATG 3′, reverse 5′ TGGATCGATTGTGCTTCAAGTT 3′; aP2: forward 5′ TTCGATGAAATCACCGCAGA 3′, reverse 5′ GGTCGACTTTC CATCCCACT 3′; PPARγ: forward 5′ CTGTTTTATGCTGTTATGGGTGAAA 3′, reverse 5′ CTGTTTTATGCTGTTATGGGTGAAA 3′; leptin: forward 5′ GACA CCAAAACCCTCAT 3′, reverse 5′ CAGTGTCTGGTCCATCT 3′; adiponectin: forward 5′ TCATGCCGAAGATGACGTTACT 3′, reverse 5′ CCATCCAACCTG CACAAGTTC 3′; 36B4: forward 5′ TCCAGGCTTTGGGCATCA 3′, reverse 5′ CTTTATCAGCTGCACATCACTCAGA 3′.

Northern blot analysis. Total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA). For Northern blot analysis, 10–15 μg total RNA per lane were blotted onto Hybond-N nylon membrane (Amersham Biosciences, Buckinghamshire, U.K.), cross-linked with UV, and hybridized with radiolabeled DNA probes. Hybridization signals were quantified using a Molecular Dynamics radioimager and normalized to S26 signal.

Transactivation assay. MEFs were transfected using Superfect reagent (Qiagen, Valencia, CA) with 1 μg of each of the various plasmids, according to the manufacturer's instructions. Forty-eight hours after transfection, in presence or absence of thiazolidinedione, cells were lysed and luciferase activity was assessed as previously described (16). The promoter driving luciferase is composed of the minimal thymidine kinase promoter and of three copies of the PPARγ responsive element (PPRE)-binding site; therefore, the luciferase activity of this construct is a reliable marker of PPARγ activity. PPARγ cDNA was under the control of β-actin gene promoter (CAG-PPARγ construct) (22), and PD169316 did not interfere with its expression.

RESULTS

In vivo p38MAPK activity decreases along adipocyte differentiation. To determine whether p38MAPK activity is altered during development of white adipose tissue, we examined its activity in different fat depots of dietary (high-fat diet [HFD]) and genetically *ob/ob* models of obesity compared with their respective lean control mice (Fig. 1*A*). p38MAPK protein level was not significantly different in the four groups of mice. However p38MAPK activity depended on the fat depot location, being from 2.5- to 10-fold higher in inguinal and axillary (I + A) depots than in the epididymal and perirenal depots (Fig. 1*A*, *white bars*). Strikingly, whereas obesity did not significantly affect p38MAPK activity in epididymal and perirenal tissues, we observed an 80% decrease in p38MAPK activity in inguinal and axillary depots in HFD (obese-HFD) and *ob/ob* obese mice compared with lean animals (Fig. 1*A*, *black bars*). By contrast, in the same tissues, we observed a significant increase in JNK activity in response to HFD (online appendix [available at http://diabetes.diabetesjournals.org]), as previously described (23). These results suggest that p38MAPK activity specifically decreased in white adipose tissue of obese mice.

Little is known concerning signaling pathways implicated in vivo in the recruitment of new adipocytes from precursor cells (preadipocytes). To determine whether the p38MAPK pathway is involved in recruitment of new adipocytes, we studied p38MAPK activity in adipocytes and preadipocytes isolated from white adipose tissue of lean and obese animals. Tissues were dissected and dissociated with collagenase. After centrifugation, floating adipocytes were collected, and the pellet containing the stroma vascular cells was resuspended and filtrated to separate and isolate the preadipocytes from the other cells (17). Following isolation, cells were incubated for 24 h in medium with 10% serum in order to overcome the immediate stress response due to the collagenase treatment

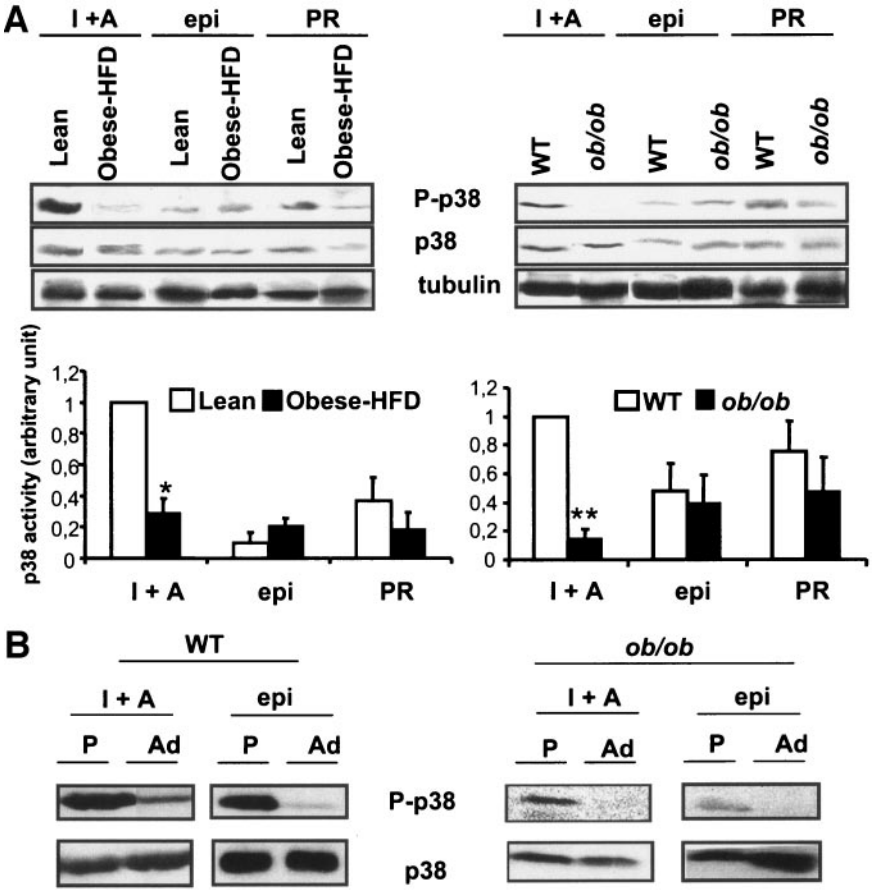

FIG. 1. In vivo p38MAPK activity is decreased in obese mice and from preadipocytes to adipocytes. *A*: Representative immunoblots of p38MAPK activity in inguinal and axillary (I + A), epididymal (epi), and perirenal (PR) fat depots from standard diet (Lean) and high-fat diet (HFD) (*left panels*) or wild-type and *ob/ob* mice (*right panels*). Each lane represents an individual mouse. Graph shows the mean of quantifications ± SE of the immunoblot signals (from five mice). Statistical analysis was performed using the Student's *t* test. **P* < 0.05 and ***P* < 0.005. *B*: p38MAPK activity in adipocytes (Ad) and preadipocytes (P) isolated from inguinal and axillary (I + A) or epididymal (epi) fat depots from wild-type and *ob/ob* mice.

(24), and cell lysates were prepared. While we noticed no difference in p38 protein expression level, p38MAPK activity was threefold lower in adipocytes than in preadipocytes isolated from both lean and obese inguinal fat pads (Fig. 1*B*). Interestingly, this decrease in p38MAPK activity is also observed in adipocytes isolated from the epididymal fat depot of lean and obese animals, suggesting that decreased p38MAPK activity correlates not only to pathological adipogenesis but also more generally to in vivo adipocyte differentiation.

In vitro inhibition of p38MAPK increases adipogenesis in preadipocytes and mouse embryonic fibroblats. We then determined whether there is a causal relationship between p38MAPK activity decrease and adipocyte maturation during in vitro adipocyte differentiation. Preadipocytes from inguinal and axillary depots were isolated and induced to differentiate into adipocytes for 10 days in the presence or absence of a specific inhibitor of p38MAPK, PD169316. This inihibtor, used at 10 μmol/l, is highly specific for p38MAPK and has no inhibitory activity on the other MAPKs, ERK, or JNK (online appendix). Adipocyte differentiation was evaluated after oil red O staining of triglycerides and quantified by measurement of optical density at 490 nm. Inhibition of p38MAPK by PD169316 increased triglyceride accumulation by 1.5 ± 0.3-fold compared with nontreated cells (Fig. 2*A*). Moreover, the expression of specific adipocyte markers such as aP2, PPARγ, adiponectin, leptin, and C/EBPα was two- to threefold higher in the presence of p38MAPK inhibitor (Fig. 2*B*). We investigated the effect of PD169316 in another cellular model of adipocyte precursor cells: the primary cultures of MEFs, which can be differentiated in adipocytes following the same protocol. As in adult preadipocytes, the expression of the aP2, PPARγ, adiponectin, and leptin adipocyte markers was increased in MEFs by up to twofold after the inhibition of p38MAPK by PD169316 (online appendix).

In vitro inhibition of p38MAPK increases adipogenesis in embryonic stem cells. We then asked whether p38MAPK plays a role during the early stages of adipocyte differentiation by analyzing the effect of p38MAPK inhibition on adipogenesis in the CGR8 embryonic stem cell line. Adipocyte differentiation of embryonic stem cells can be achieved after a 3-week period, including the formation of cell aggregates during 7 days (embryoid bodies), a critical early treatment with retinoic acid followed by the application of classical adipogenic inducers from day 7 of the differentiation (18,20).

To determine whether p38MAPK activity varies during adipocyte differentiation in embryonic stem cells, we performed a Western blot analysis using anti-phospho p38MAPK antibodies. Two peaks of activation were detected, between days 4 and 5 and between days 13 and 16 (Fig. 3), while no change in p38 expression was observed.

We then tested the specificity of the p38MAPK inhibitor in embryonic stem cells (online appendix) and added PD169316 at a different time. The addition of the inhibitor during the early peak (between days 0 and 7) did not affect adipogenesis (20). PD169316 was then concomitantly added to the adipogenic inducers from day 7. Adipocyte differentiation was analyzed by measuring the area of adipocyte outgrowth in each embryoid body. Addition of the p38MAPK inhibitor increased by twofold adipocyte areas within each embryoid body compared with untreated cells (Fig. 4*A*). We then determined the number of embryoid bodies with 0–20%, 20–40%, 40–60%, 60–80%, or

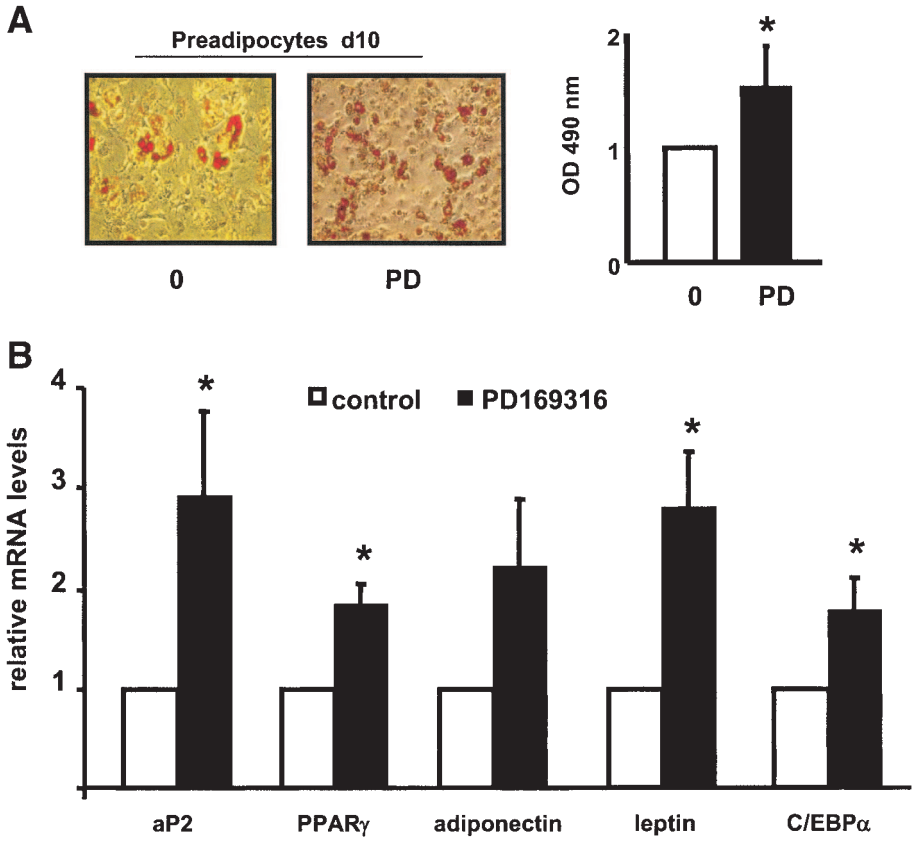

FIG. 2. p38MAPK inhibition stimulates adipogenesis of preadipocytes. Adult preadipocytes were isolated and differentiated into adipocytes in the presence or absence of PD169316 and analyzed 10 days after the induction of differentiation. *A*: Differentiated cells after oil red O staining and triglyceride accumulation measurement as described in RESEARCH DESIGN AND METHODS. *B*: Quantifications of aP2, PPARγ, adiponectin, leptin, and C/EBPα mRNA expression by real-time RT-PCR, normalized to expression of the control gene 36B4. These experiments were performed with preadipocytes from four different mice. Graphs show means ± SE. *$P < 0.05$.

80–100% of their surface covered by adipocytes. All the embryoid bodies treated with the inhibitor had between 40 and 80% of their surface covered by adipocytes, whereas in the untreated conditions the maximum area occupied by the adipocytes was 60% (Fig. 4*B*). Interestingly, inhibition of p38MAPK activity between days 13 and 16 (second peak of activation) increased adipogenesis as well as a treatment from day 7 to 21 (online appendix). We then analyzed the expression of adiponectin, leptin, and C/EBPα by real-time quantitative RT-PCR and aP2 and PPARγ by Northern blot. PD169316 alone did not affect the expression of these markers compared with no retinoic acid–treated cells. Retinoic acid–treated cells presented a specific expression of these markers that was significantly increased by PD169316 treatment (Fig. 5*A* and *B*). Altogether, these data showed that PD169316 potentiates adipogenesis in embryonic stem cells.

p38MAPKα knockout increased adipogenesis of embryonic stem cells in vitro and in vivo. To rule out an effect of the chemical inhibitor unrelated to p38MAPK, we investigate the role of p38MAPK in adipogenesis by a more direct genetic approach. The capacity of p38MAPKα$^{-/-}$ embryonic stem cells (25) to differentiate into adipocyte was analyzed. According to the protocol described for CGR8 cells, the capacity of p38MAPKα$^{-/-}$ DBA-252 embryonic stem cells to differentiate after retinoic acid treatment into adipocytes was evaluated and compared with their p38MAPKα$^{+/+}$ counterparts. Real-time PCR analysis of the expression of adipocyte specific markers showed that deletion of p38MAPKα strongly increased the

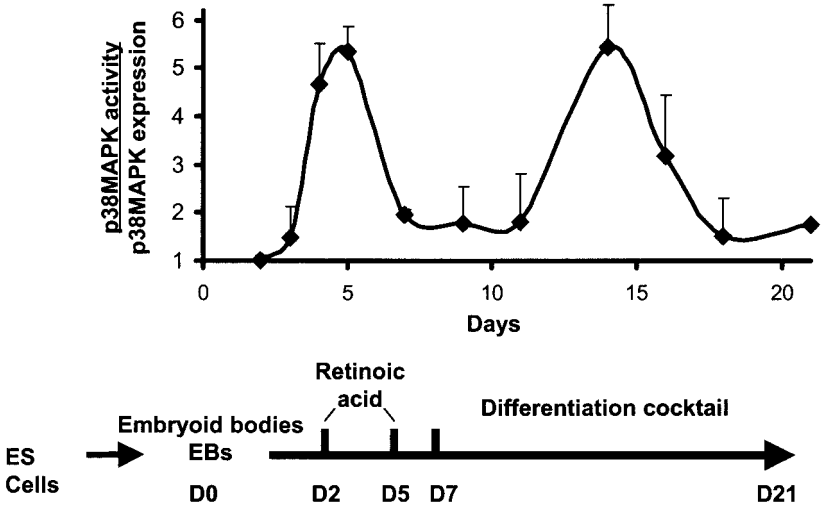

FIG. 3. p38MAPK activity along adipocyte differentiation in embryonic stem (ES) cells. Embryoid bodies (EBs) were formed from ES cells and were treated with retinoic acid between days 2 and 5 of the protocol then induced to differentiate in adipocytes from day 7 to 21 as described in RESEARCH DESIGN AND METHODS (see also schematic representation below the graph). The graph represents the relative amount of phophorylated p38MAPK over p38MAPK protein level during the differentiation of EBs into adipocytes, with means of three experiments ± SE.

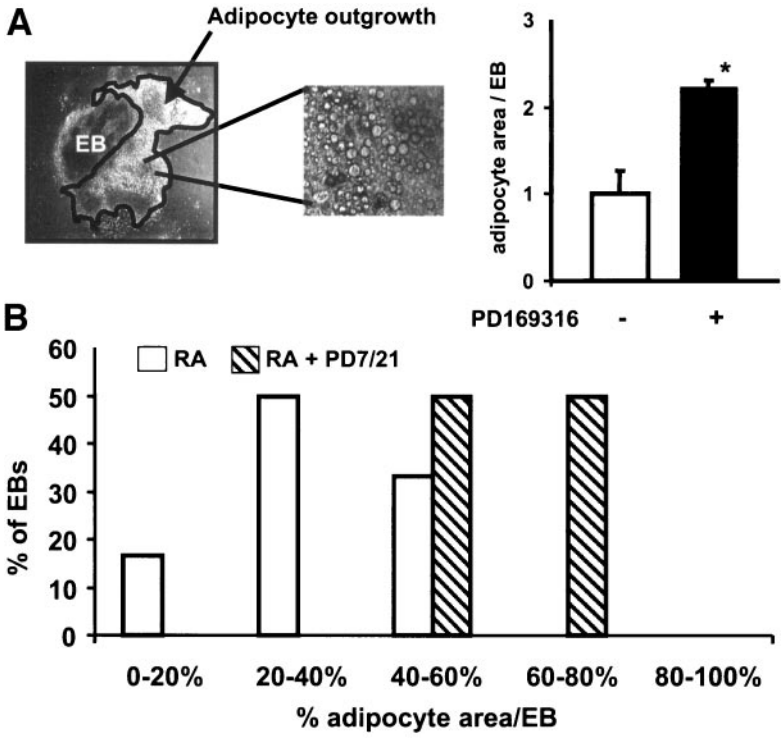

FIG. 4. p38MAPK inhibition increases adipocyte outgrowth area in differentiated embryonic stem cells. Embryonic stem cells were differentiated as described in the *legend* of Fig. 3. Embryoid bodies were treated or not treated with the PD169316 p38MAPK inhibitor between day 7 and day 21 (PD7/21). *A*: Photograph representing an embryoid body (EB) with the adipocyte area outlined. Areas were measured using IP Lab software at magnification ×2.5, and the presence of adipocytes was determined at higher magnification (see *insert*: ×60). The graph represents the quantification of adipocyte outgrowth areas of 20 EBs (for each condition) treated or not treated with PD169316. The graph shows means ± SE of three experiments. *$P < 0.02$. *B*: The histograms represent the percentage of embryoid bodies (EBs) with various percentage of the adipocyte surface. Two independent observers assessed the percent of adipose conversion in a large number of EBs from each condition.

expression of aP2, PPARγ, adiponectin, leptin, and C/EBPα genes (Fig. 6*A*).

Upon subcutaneous injection in animals, embryonic stem cells are tumorigenic, inducing teratocarcinoma containing various differentiated tissues derived from the injected cells. To analyze the capacity of p38MAPKα$^{+/+}$ and p38MAPKα$^{-/-}$ embryonic stem cells to differentiate in vivo after subcutaneous injection, six animals for each cell line were injected in two sites per mouse. After 15–20 days, tumors were removed and analyzed for the expression of adipocyte markers. Teratocarcinomas from p38MAPKα$^{-/-}$ displayed a significantly higher expression of PPARγ, adiponectin, and leptin than tumors from wild-type cells (Fig. 6*B*). By contrast, no change was found in markers of other lineages, such as for example Map2, a neuronal marker (data not shown).

Altogether, our data demonstrated that inhibition or knockout of p38MAPK increases adipogenesis both in vivo, in teratocarcinomas, and in vitro in adipose precursor cells, adult preadipocytes and embryonic fibroblasts, and in embryonic stem cells.

p38MAPK inhibits C/EBPβ and PPARγ activities. To investigate the molecular mechanisms implicated in p38MAPK regulation of adipogenesis, we analyzed the effect of PD169316 on C/EBPβ and PPARγ transcription factor activities in MEFs. Several reports (6,26,27) have demonstrated that expression and transcriptional activity of C/EBPβ are induced and that C/EBPβ proteins are activated by phosphorylation on Thr-188 within the two 1st days of the differentiation protocol. C/EBPβ phosphorylation on Thr-188 on day 2 of differentiation was analyzed in the presence or absence of PD169316. While C/EBPβ protein expression level was not affected by the inhibition of p38MAPK, addition of PD169316 significantly increased its phosphorylation in MEFs and preadipocytes (Fig. 7*A* and *B*). C/EBPβ activation results in an increase in PPARγ expression (28); accordingly, we found that inhibition of p38MAPK increased PPARγ mRNA levels (Fig. 2*B*) and protein expression (Fig. 8*A*). JNK and, to a lesser extent p38MAPK, phosphorylates PPARγ1 on Ser-82 in vitro (29), and this phosphorylation negatively regulates PPARγ1 transactivation. However, no role in adipogenesis has been associated with the phosphorylation of this serine. We have investigated the phosphorylation status of PPARγ on Ser-82 in wild-type and p38MAPKα$^{-/-}$ MEFs treated or not treated with PD169316. Despite their incapacity to undergo terminal adipocyte differentiation due to a defect of contact inhibition (30), we analyzed PPARγ expression 2 days after the induction of differentiation in these cells. PPARγ was higher in p38MAPKα$^{-/-}$ and cells treated with the inhibitor than in p38$^{+/+}$ cells (Fig. 8*B*). Furthermore, we show that the stronger signal obtained with antiphospho PPARγSer-82 is due to the increase of PPARγ expression rather than the increase of PPARγ phosphorylation (Fig. 8*B*). We then determined whether PPARγ transcriptional activity is regulated by p38MAPK. PPARγ binds to the PPRE and activates the transcription of genes implicated in adipogenesis, such as aP2 (31). We analyzed PPARγ activity in MEF cells treated or not treated with PD961396 by transitory transfections of a PPRE-luciferase (PPRE-luc) reporter plasmid (22) and in the presence or absence of thiazolidinedione (TZD), a PPARγ agonist. Consistent with the positive effect of the inhibitor on PPARγ protein expression, inhibition of p38MAPK increased by twofold the endogenous PPARγ activity, with or without TZD (Fig. 8*C*). To know whether inhibition of p38MAPK also increased PPARγ activity itself, we repeated these experiments by cotransfecting the reporter with a PPARγ expression vector. Introducing the PPARγ expression vector increased by twofold the basal PPARγ activity, and, in the presence of TZD, the resulting activity was stimulated fivefold by a PD169316 treatment (Fig. 8*D*). These results strongly suggested that, in addition to its effect on C/EBPβ, inhibition of p38MAPK also directly activates PPARγ proteins.

We then analyzed PPARγ activity in p38MAPKα$^{-/-}$

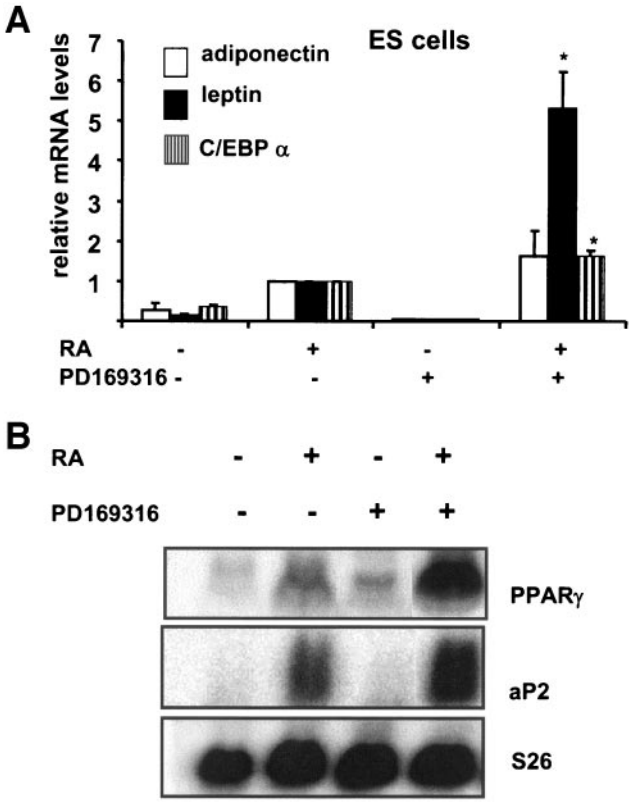

FIG. 5. Inhibition of p38MAPK increases the expression of adipocyte markers in differentiated embryonic stem (ES) cells. Embryoid bodies were treated with or without retinoic acid (RA) and with or without PD169316, as in Fig. 4. After 21 days of differentiation, adiponectin, leptin, and C/EBPα mRNA were quantified by real-time RT-PCR (*A*) and Northern blot analysis (*B*) of aP2 and PPARγ gene expression. Results are the mean of four independent experiments ± SE. Statistical analysis refers to RA-treated conditions. *$P < 0.05$.

MEFs (32) compared with wild-type cells. To normalize for transfection efficiency differences, MEFs were transiently cotransfected with PPRE-luc and RSV-βgalactosidase (RSV-βGal) plasmids and luciferase activities were expressed relatively to βGal activities. Endogenous PPARγ activity was threefold higher in p38MAPKα$^{-/-}$ cells compared with control wild-type cells (Fig. 8*E*), demonstrating that the knockout of p38MAPK is sufficient to activate this transcription factor. MEF cultures were then cotransfected with RSV-βGal, PPRE-luc, and CAG-PPARγ plasmids. In these conditions, PPARγ activity was increased ninefold in p38MAPKα$^{-/-}$ MEFs compared with wild-type cells (Fig. 8*E*). To further demonstrate the specific role of p38MAPK pathway in regulating PPARγ, p38MAPKα$^{-/-}$ MEFs were transiently transfected with a RSV-p38MAPKα expression vector. As shown in Fig. 8*F*, reexpression of p38MAPKα in knockout MEFs significantly decreased PPARγ activity. Interestingly, this effect can be blocked by the treatment of p38MAPKα$^{-/-}$ MEFs with PD169316. Whereas luciferase activity of control cells treated by this compound was not affected, the PD169319 restored the PPARγ transcriptional activity in cells cotransfected with RSV-p38MAPKα (Fig. 8*F*). We demonstrate here that the p38MAPKα isoform is specifically involved in the regulation of PPARγ transcriptional activity.

DISCUSSION

To clarify the role of p38MAPK in normal and pathological adipogenesis, we determined the function of this pathway by complementary biochemical and genetic approaches in various experimental models, from embryonic to adult stages.

In vivo, in dietary and genetic (*ob/ob*) models of obesity, we found a strong decrease of p38MAPK activity in subcutaneous (inguinal and axillary) fat depots compared with the same tissue in lean mice. By contrast to the activations of the JNK and ERK pathways observed in obesity (33), our results suggest an unsuspected negative role of p38MAPK in adipogenesis. No change in p38MAPK activity was detected in epididymal and perirenal fat depots. These differences between fat depots are in agreement with the well-known regional variations in growth, cellularity, metabolism, and gene expression of the white adipose tissue depots in mice and humans (34–36). Interestingly, we found that p38MAPK activity is significantly higher in preadipocyte precursor cells, from both inguinal and epididymal fat depots, than in mature adipocytes, showing an inverse correlation between p38MAPK activation and adipocyte differentiation.

By using the p38MAPK-specific inhibitor PD169316, and analyzing p38MAPKα$^{-/-}$ cells, we then demonstrated that p38MAPK plays a direct negative role in adipocyte differentiation. Inhibition of p38MAPK increased adipogenesis in several primary cell cultures, from the embryonic to the adult stages (MEFs and preadipocytes), and in two different embryonic stem cell lines (CGR8 and DBA 252). We found that this effect is mediated by the regulation of the adipogenic factors C/EBPβ and PPARγ, whose transcriptional activities were activated by PD169316. C/EBPβ has been found to regulate PPARγ expression and is activated by phosphorylation on Thr-188. For example, ERK and GSK3 have been shown to phosphorylate Thr-188 and increase adiponectin expression in 3T3-L1 (7). We found an opposite regulation by p38MAPK; the p38MAPK pathway inhibits C/EBPβ proteins and decreases expression of adipocyte markers. A similar negative role on C/EBPβ function has been described for the phosphatidylinositol 3-kinase pathway (7). These results support the hypothesis that the C/EBPβ phosphorylation site Thr-188 is a crucial convergent target for opposite regulations by several transduction pathways. On one hand, ERK and GSK3 activate C/EBPβ proteins, and, on the other hand, p38MAPK and phosphatidylinositol 3-kinase inhibit them.

We found that the inhibitory role of p38MAPK in adipocyte differentiation is also mediated by the regulation of the crucial adipogenic transcription factor PPARγ. Biochemical inhibition of the p38MAPK pathway induces both PPARγ expression and activity. By transient transfection experiments, this effect was evidenced on transfected PPARγ proteins, independently of the increase in endogenous proteins, suggesting that it is also effective on PPARγ activity itself. Furthermore, PPARγ activity and expression is significantly higher in MEFs p38α$^{-/-}$ compared with wild-type cells, and reexpression of p38MAPKα in knockout cells represses PPARγ activity. Therefore, by both biochemical and genetic approaches, we show that p38MAPK activity negatively regulates PPARγ.

In contrast to our results, a positive role for p38MAPK in adipogenesis has been described in preadipocyte cell lines (10,37,38). They showed that, during the differentiation of 3T3-L1 preadipocyte cells, addition of p38MAPK inhibitors decreased adipocyte formation. They attributed this effect to a lower phosphorylation of C/EBPβ protein in the presence of the inhibitors. By using PD169316 instead of SB20358, we confirmed a positive role of p38MAPK in the

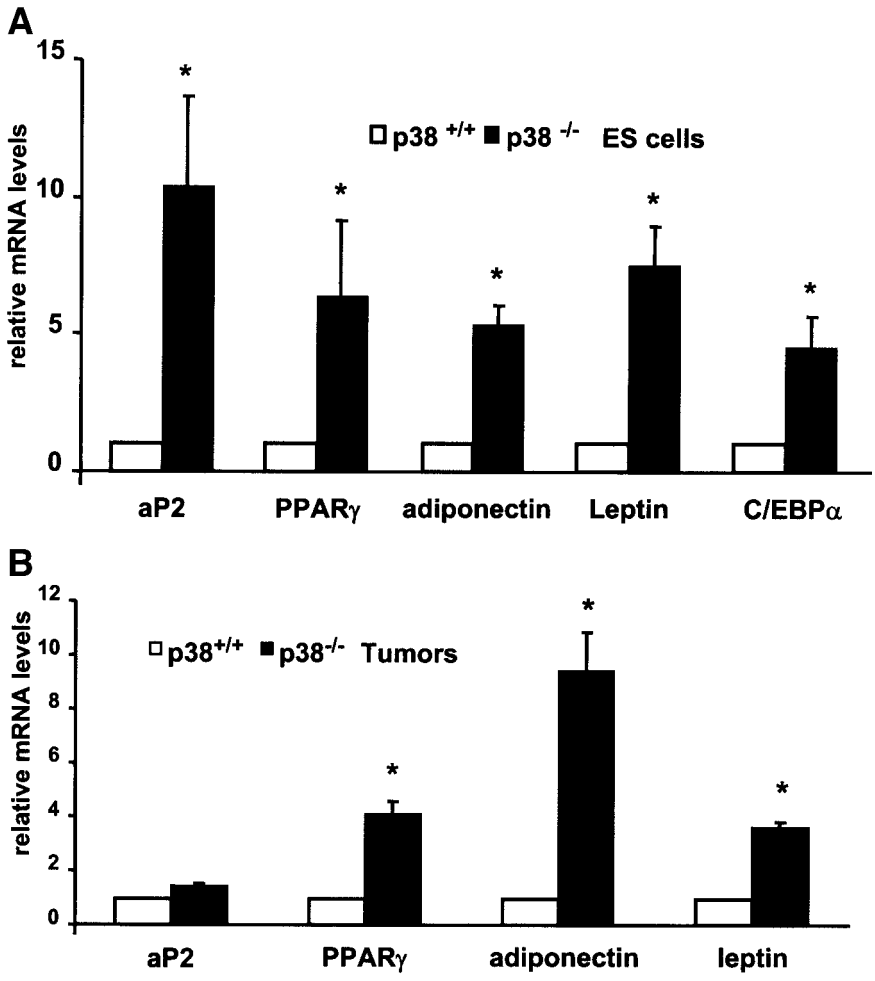

FIG. 6. p38MAPK disruption increased adipogenesis of embryonic stem (ES) cells in vitro and in vivo. *A*: Embryoid bodies from p38MAPKα$^{-/-}$ and p38MAPKα$^{+/+}$ cells were induced to differentiate into adipocytes and analyzed, after 21 days, by real-time RT-PCR for expression of aP2, PPARγ, adiponectin, leptin, and C/EBPα. Graphs are the means of three independent experiments ± SE. *B*: Real-time RT-PCR analysis of adipocyte marker expression in teratocarcinomas induced by injections of either wild-type ES cells (□) or p38MAPKα$^{-/-}$ ES cells (■). Values are the means ± SE of 12 tumors for each cell line. *P < 0.05.

adipogenesis of 3T3-L1. Addition of the p38MAPK inhibitor decreased the formation of adipocytes in 3T3-L1 and downregulated PPARγ transcriptional activity (data not shown). Taken together, these results show that p38MAPK inhibition decreases PPARγ activity in one cellular model, 3T3-L1 cells, and has the opposite effect in the other cellular models analyzed. Because the latter are primary cultures from embryos to adults, they represent more physiological experimental models than the established cell line 3T3-L1.

Altogether, these observations strongly suggest that the regulation of PPARγ by the p38MAPK pathway involves molecular partners that are different in 3T3-L1 and in the other cellular models. One can hypothesize that p38MAPK is acting on PPARγ coactivators. It is known, for example, that one of them, PGC-1, is phosphorylated by p38MAPK. However, PGC-1 is not implicated in white adipocyte differentiation (39,40). Although we do not know whether they are phosphorylated by p38MAPK, other PPARγ coactivators, such as steroid receptor coactivator, CREB-binding protein, or p300, involved in adipogenesis, represent interesting potential targets (41). Alternatively, PPARγ corepressors could also be involved in p38MAPK regulation, especially because they regulate the PPARγ transcriptional activity in adipocytes (42). The comparison between 3T3-L1 and the other cellular models for activity of the various PPARγ partners should help to understand the opposite response of PPARγ transcriptional activity to p38MAPK regulation.

In conclusion, our results demonstrated that p38MAPK inhibition increased adipogenesis via C/EBPβ and PPARγ activations, and further investigations would be of interest to elucidate molecular mechanism involved in this activa-

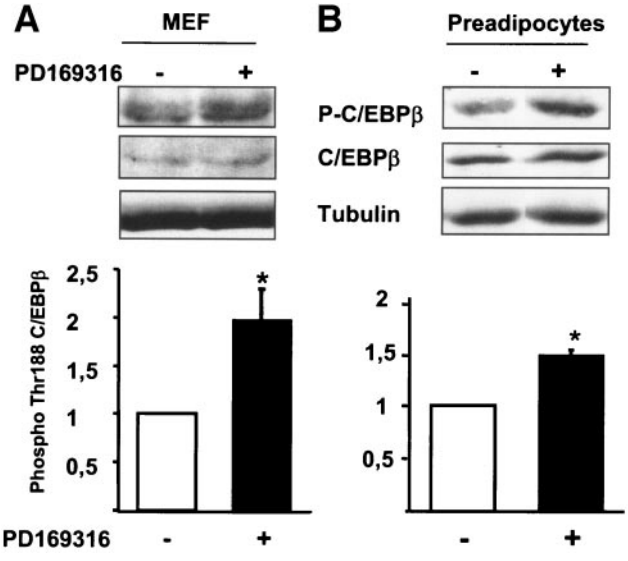

FIG. 7. p38MAPK inhibition increases C/EBPβ activity. MEFs (*A*) and preadipocytes (*B*) were induced to differentiate, in presence or absence of PD169316, and C/EBPβ phosphorylation was analyzed 2 days later. Representative immunoblots of phospho-C/EBPβ, using anti-phosphoThr-188, are shown. Graphs represent the quantification, means ± SE, of immunoblot signals obtained in six different experiments. *P < 0.05.

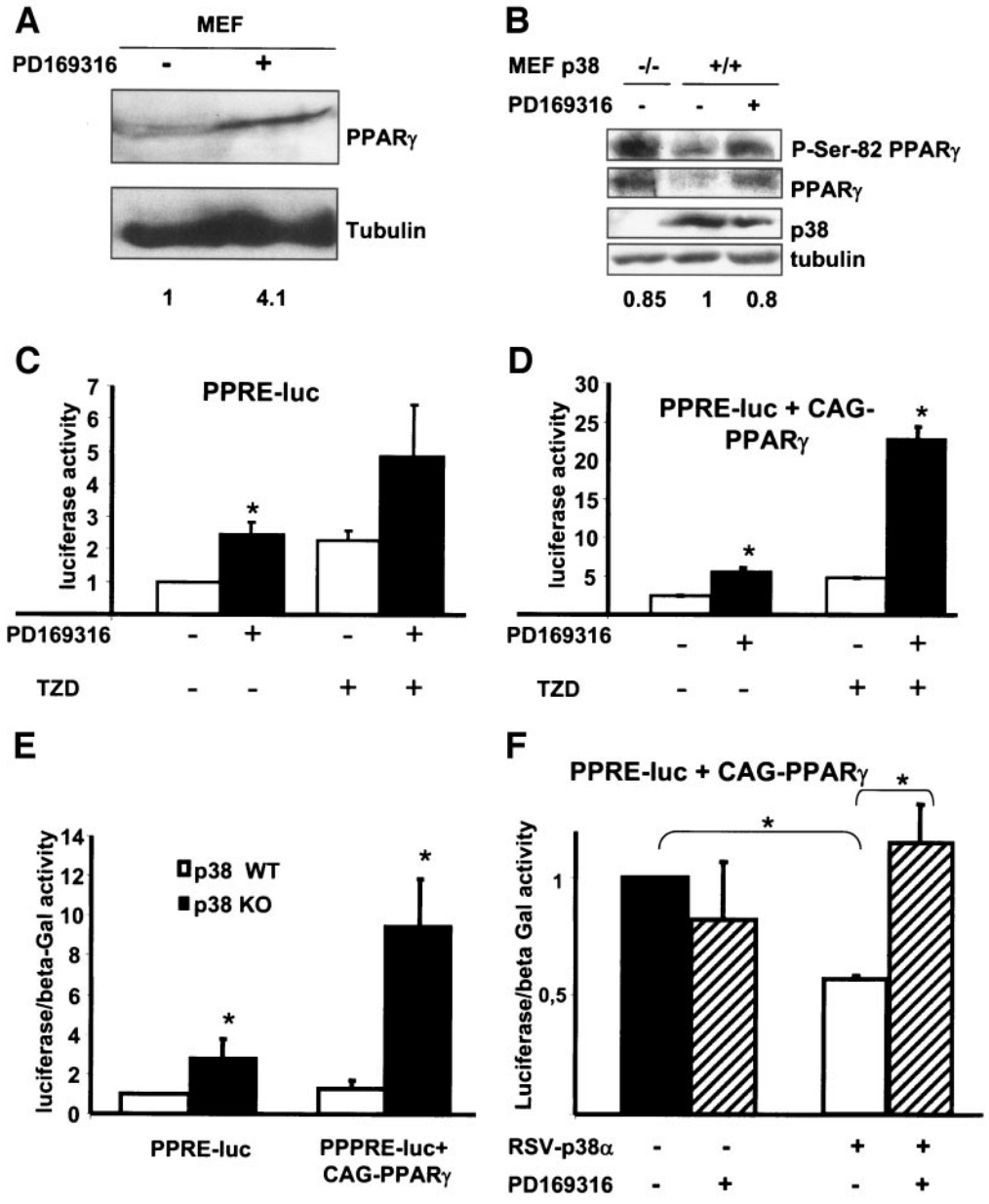

FIG. 8. p38MAPK inhibition increases PPARγ expression and activity. *A*: MEFs were induced to differentiate as described in RESEARCH DESIGN AND METHODS. Representative immunoblot of PPARγ 2 days after the induction of differentiation in presence or absence of PD169316, using anti-PPARγ. *B*: Immunoblot of PPARγ phosphorylation on Ser-82 and PPARγ protein expression in $p38^{-/-}$ and $p38^{+/+}$ MEFs 2 days after the induction of differentiation. The values of the phospho-PPARγ/PPARγ protein level are indicated below the immunoblot. *C*: PPARγ transcriptional activity was evaluated by luciferase assays using the PPRE-luc reporter plasmid. MEFs were transiently transfected with PPRE-luc in absence (□) or presence (■) of PD169316 and with or without TZD, as indicated. Luciferase activity was normalized to 1 for untreated cells. *D*: To test the effect of p38MAPK inhibition on exogenous PPARγ proteins, MEFs were cotransfected with PPRE-luc and a PPARγ expression vector in absence or presence of PD169316 and with or without TZD. *E*: Wild-type (WT, □) and $p38MAPK\alpha^{-/-}$ (■) MEFs were transiently transfected with either PPRE-luc alone or together with CAG-PPARγ in the presence of TZD. For each experiment, cells were also transfected with an RSV-βGal reporter plasmid as internal control for transfection efficiencies. Luciferase activities were corrected for βgalactosidase activities and normalized to 1 for WT cells transfected with PPRE-luc. Each bar represents means ± SE (n = 11). *P < 0.01. *F*: $p38MAPK\alpha^{-/-}$ MEFs were cotransfected with PPRE-luc and CAG-PPARγ and plus (+) or minus (−) RSV-p38MAPKα plasmids in absence or presence of PD169316, as indicated. Luciferase activities were corrected for βgalactosidase activities and normalized to 1 for cells transfected with PPRE-luc + CAG-PPARγ. Each bar represents means ± SE (n = 6). *P < 0.01.

tion. These observations were obtained from in vivo and in vitro experimental models and from embryonic stages to adulthood. Because no activated form of p38MAPK has been described so far, we do not know yet whether specific activation of p38MAPK would lead to inhibition of the development of white adipose tissues and could have a therapeutic effect regarding obesity. Conversely, inhibition of p38MAPK by resulting in the enhancement of both the expression and the activity of PPARγ could represent an interesting approach in the treatment of type 2 diabetes. Analysis of the in vivo effects of p38MAPK inhibitors regarding insulin resistance parameters and obesity should be of great interest.

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