15-Deoxy-$\Delta^{12,14}$ Prostaglandin J2: A Putative Endogenous Promoter of Adipogenesis Suppresses the *ob* Gene

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Leptin is considered a key factor in the regulation of appetite and energy expenditure, but little is known about the control of its synthesis and release. Thiazolidinediones (TZDs) have recently been shown to downregulate leptin expression, and it has been speculated that downregulation of the *ob* gene occurs through activation of the transcription factor, peroxisome proliferator–activated receptor γ (PPARγ). However, there are no studies using an endogenous PPARγ ligand. We examined the effect of 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2), a putative natural ligand of PPARγ, on *ob* gene expression in fully differentiated 3T3-L1 adipocytes and compared its effect with that of two other PPARγ activators, the TZD troglitazone (Trog) and indomethacin (Indo). 15d-PGJ_2, Trog, and Indo all inhibited leptin expression at concentrations at which they activate PPARγ. The inhibition of leptin expression of PPARγ activators was surprising, since PPARγ is known to induce adipogenesis during which the *ob* gene is expressed. To address the possibility that PPARγ plays different roles before and after the induction of adipogenesis, we examined the effects of the three PPARγ ligands on the expression of leptin and the glucose transporter protein GLUT4, both of which are expressed during differentiation of 3T3-L1 preadipocytes to adipocytes. In the absence of PPARγ ligands, leptin and GLUT4 synthesis increased from day 3 to day 9 or 10 during differentiation. However, in the presence of any of the three PPARγ ligands, GLUT4 expression was unaffected, while *ob* gene expression was inhibited. We hypothesize that PPARγ may be essential for induction of adipocyte differentiation but then needs to be inactivated to allow expression of the *ob* gene.

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LEPTIN, the *ob* gene product, has been implicated as a major factor in energy homeostasis,[1] yet little is known about the mechanisms that control its synthesis or release. It has recently been reported that thiazolidinediones (TZDs), a new class of antidiabetic agents, downregulate leptin mRNA and protein in vitro and in vivo.[2,3] TZDs bind to and activate the nuclear receptor peroxisome proliferator–activated receptor γ (PPARγ). Activated PPARγ is a transcription factor that has been shown to play an important role in the differentiation of preadipocytes to adipocytes.[4,5] The finding that TZDs bind to PPARγ and downregulate leptin expression at similar concentrations suggested that TZDs inhibit leptin expression by activating PPARγ.[6,7] Therefore, it seemed possible that other synthetic PPARγ ligands, for instance, indomethacin (Indo),[8] may likewise affect *ob* gene expression. Recently, it has been demonstrated that the arachidonic acid metabolite 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2), which is present in human urine, binds strongly and specifically to PPARγ and promotes adipocyte differentiation.[7,9] For these reasons, 15d-PGJ_2 is currently considered a strong candidate for the role of a natural PPARγ ligand. Whether 15d-PGJ_2 affects *ob* gene expression is not known.

To address these questions, we have compared the effects of 15d-PGJ_2, Indo, a nonsteroidal anti-inflammatory agent that binds to PPARγ, and troglitazone (Trog), a TZD, on *ob* gene expression in differentiating and fully differentiated 3T3-L1 adipocytes. In addition, these three PPARγ ligands were tested for their effects on the expression of the glucose transporter protein 4 (GLUT4) gene, which, like leptin, is expressed during adipocyte differentiation.

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MATERIALS AND METHODS

Cell Culture

3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD) were maintained in basal medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin 50 U/mL, and streptomycin 50 μg/mL) with a medium change every alternate day. Two days after reaching confluence, the cells were maintained for 48 hours in differentiation medium (basal medium supplemented with 500 nmol/L insulin, 250 nmol/L dexamethasone, and 250 mmol/L isobutylmethylxanthine). The differentiation medium was then replaced with basal medium containing 250 nmol/L insulin and the cells were cultured for another 48 hours. Thereafter, the cells were maintained in basal medium. Cell treatment with Trog, Indo, and 15d-PGJ_2 entailed supplementing the medium with various concentrations of the test compounds in appropriate solvents (dimethyl sulfoxide for Trog, ethanol for Indo, and methylacetate for 15d-PGJ_2). Control cells were treated with the respective vehicles.

Quantitation of Leptin in the Culture Media

Adipocytes were incubated with known concentrations of the test compounds or the respective vehicles for a specified period, and leptin secreted in the media was determined using a mouse leptin radioimmunoassay (RIA) kit (Linco Research, St Charles, MO).

Measurement of GLUT4 by Western Blot

Cells on the tissue culture dish after incubation with either the vehicle or the compound to be tested were washed with phosphate-buffered saline and then disrupted using 2% sodium dodecyl sulfate (SDS). After centrifugation, proteins in the supernatant were determined, and equivalent amounts of protein per sample (20 μg) were then analyzed on a denaturing SDS-polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane. After blocking by incubation for 24 hours with 5% bovine serum albumin in Tris-saline buffer (pH 7.4) containing 0.05% Tween 20, the membrane was incubated with

GLUT4-specific antibody (a monoclonal antibody against the insulin-sensitive glucose transporter GLUT4; Genzyme, Cambridge, MA). This antibody recognizes a C-terminal epitope in the cytoplasmic domain of GLUT4, which is immunologically distinct from other glucose transporters.[10] The membrane was then incubated with horseradish peroxidase–conjugated goat anti–mouse immunoglobulin G. The blot was visualized by enhanced chemiluminescence. The major band corresponding to a molecular weight of approximately 47 kd was identified as GLUT4.

Levels of ob *and* GLUT4 *mRNAs*

Total RNA from adipocytes was isolated using Trizol (GIBCO-BRL, Gaithersburg, MD) following the protocol provided by the manufacturer. The average yield was 10 to 20 μg/10^6 cells. The 260/280 nm absorption ratio of the RNA preparations varied between 1.7 and 1.8, and their integrity was verified on agarose gel stained with ethidium bromide. Reverse transcription polymerase chain reaction (RT-PCR) was performed using the Superscript preamplification system (GIBCO-BRL). In brief, 1 μg total RNA and 0.5 μg oligo$(dT)_{12\text{-}18}$ primer were incubated in a total volume of 12 μL at 70°C for 10 minutes and then chilled in ice. The RNA/primer mixture was then diluted to 20 μL with buffer containing $MgCl_2$, dNTPs, and dithiothreitol for a final concentration of 2.5 mmol/L, 0.5 mmol/L, and 10 mmol/L, respectively. After incubation at 42°C for 5 minutes, 1 μL Superscript II (200 U/mL) was added, followed by another incubation for 50 minutes at 45°C. The reaction was then terminated by incubation for 15 minutes at 70°C. A volume of 2 μL from the RT reaction was then amplified in a total volume of 50 μL containing $MgCl_2$ (1.5 mmol/L), dNTPs (0.2 mmol/L), specific sense and antisense primers (0.2 μmol/L each), and Taq polymerase (2.5 U) in a thermal cycler (model 2400; Perkin Elmer, Norwalk, CT) using 25 to 30 cycles (denaturation at 94°C for 2 minutes, annealing at 60°C for 1 minute, and elongation at 72°C for 3 minutes per cycle). The number of cycles was optimized to ensure that the amplification was within the exponential phase. The linearity of total RNA (per reaction) and mRNA band density was obtained up to 1.5 to 2 μg total RNA. The amplified DNA (10 to 20 μL) was analyzed on a 2% agarose gel stained with ethidium bromide. The leptin primer pair consisted of oligonucleotides spanning nucleotides 115 to 134 (sense primer, 5′-AATGTGCTGGAGACCCCTGT-3′) and 599 to 618 (antisense primer, 5′-CAGCATTCAGGGCTAACATC-3′), which generated a fragment of 504 base pairs (bp) when amplified. The GLUT4 primer pair consisted of a nucleotide sequence spanning 584 to 605 (sense primer, 5′-GGGAGAAATCGCCCCCACTCAT-3′) and 1323 to 1343 (antisense primer, 5′-CTGGCTGAAGAGCTCGGCCAC-3′), which generated a fragment of 760 bp. The actin primer pair consisted of a nucleotide sequence spanning 886 to 910 (sense primer, 5′-TGGA-ATCCTGTGGCATCCATGAAAC-3′) and 1210 to 1234 (antisense primer, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′) generating a fragment of 349 bp when amplified.

RESULTS

Effects of 15d-PGJ_2, Indo, and Trog on Leptin Protein and mRNA

Incubation of fully differentiated 3T3-L1 adipocytes in basal medium supplemented with 15d-PGJ_2 (3 μmol/L), Indo (100 μmol/L), and Trog (5 μmol/L) reduced the level of leptin in the incubation medium by 78% ± 5.8%, 83.2% ± 4.2% and 83.5% ± 8.5%, respectively, after 24 hours, and by 85% ± 2.5%, 97.7% ± 1.3%, and 93.4% ± 0.4% after 48 hours (Fig 1). Thus, 15d-PGJ_2 and Indo, like Trog, were potent inhibitors of leptin secretion by 3T3-L1 cells.

Consistent with the reduction in secreted leptin protein, leptin mRNA levels were also significantly lower in the presence

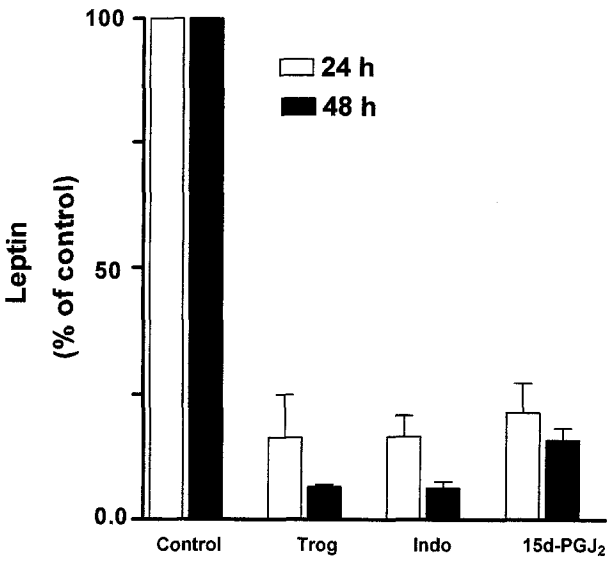

Fig 1. Effects of Trog, Indo, and 15d-PGJ_2 on leptin secretion by 3T3-L1 adipocytes. 3T3-L1 cells were differentiated. Fully differentiated cells (usually on day 10-12 from the day of differentiation) were treated with basal medium supplemented with Trog (5 μmol/L), Indo (100 μmol/L), or 15d-PGJ_2 (3 μmol/L) and leptin secreted in the medium was measured using a leptin RIA kit at the specified times. Control cells were treated with the respective vehicle alone. Three dishes were treated for each compound. Results are the mean ± SE. Fully differentiated 3T3-L1 cells secreted 0.5-3 ng leptin/10^6 cells/mL/24 h.

versus the absence of 15d-PGJ_2, Indo, and Trog. The inhibition of leptin transcription was 40%, 71%, and 88%, respectively, for 15d-PGJ_2, Indo, and Trog after 24 hours of incubation. These results indicate that for all three compounds tested, the downregulation of leptin was at the level of transcription. The discrepancy in the percent inhibition of leptin protein (Fig 1) and leptin mRNA (Fig 2) in the case of 15d-PGJ_2 was due to the short half-life of 15d-PGJ_2.

Dose-Response of PPARγ Ligands for Inhibition of Leptin Expression

To assess whether the downregulation of leptin by 15d-PGJ_2, Indo, or Trog was concentration-dependent, fully differentiated 3T3-L1 adipocytes were incubated with different concentrations of the ligands for 24 hours and leptin secreted into the medium was measured. The 50% effective dose (ED_{50}) for Indo-induced downregulation of leptin varied between 5 and 10 μmol/L, whereas the ED_{50} for Trog-induced downregulation of leptin varied between 100 and 200 nmol/L. Inhibition of leptin secretion by 15d-PGJ_2 was also dose-dependent and the ED_{50} varied between 0.5 and 1 μmol/L (Fig 3).

Effects of PPARγ Ligands on Leptin Expression During Differentiation

The observation that PPARγ ligands inhibit leptin expression seemed to conflict with the established role of PPARγ as an inducer of adipocyte differentiation and the fact that leptin expression develops during adipocyte differentiation. To further study this apparent paradox, we investigated the effects of the PPARγ ligands on leptin expression in differentiating 3T3-L1

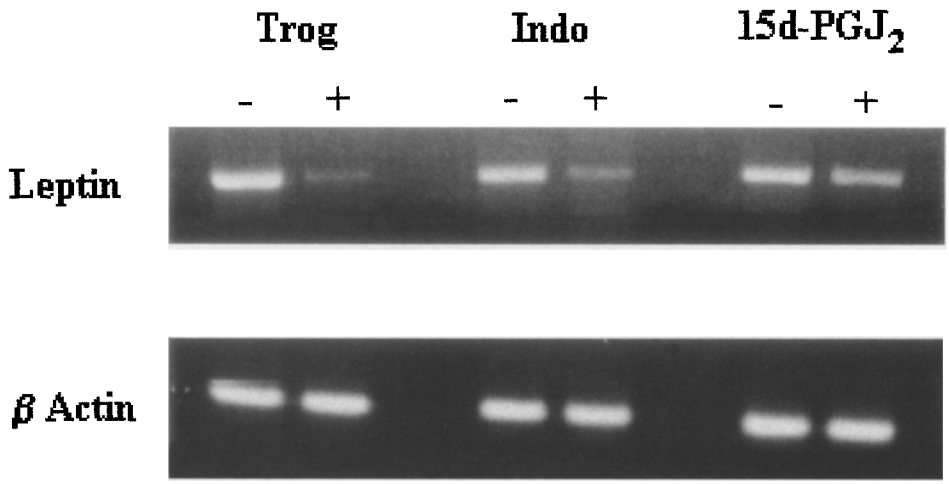

Fig 2. Effects of Trog, Indo, and 15d-PGJ_2 on leptin mRNA in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with the compounds as described in Fig 1, and at the end of 24 hours, cells were washed 3 times with phosphate-buffered saline. RNA from cells on the dish was isolated using Trizol reagent. Leptin and β-actin mRNAs in the samples were then measured using the RT-PCR method.

cells. In the absence of PPARγ ligands, leptin levels increased progressively, whereas leptin remained inhibited in the presence of any one of the three ligands. No significant difference in adipocyte differentiation, as assessed by cell morphology and lipid accumulation, could be detected in cells incubated with or without the PPARγ ligands. Thus, 15d-PGJ_2, Indo, and Trog suppressed the expression of the *ob* gene but did not interfere with adipocyte differentiation (Fig 4).

Effects of PPARγ Ligands on GLUT4 Expression

The glucose transporter protein GLUT4, like leptin, is undetectable in preadipocytes, is expressed late during differentiation, and is abundantly present in fully differentiated adipocytes. The inhibitory effect of PPARγ ligands on leptin expression prompted us to examine whether the three PPARγ ligands interfered also with the expression of GLUT4. There was no effect by any of the three PPARγ ligands on either GLUT4 protein or mRNA (Fig 5). Furthermore, GLUT4 expressed at different stages of differentiation was virtually identical in the presence and absence of 15d-PGJ_2 (Fig 6). Therefore, different mechanisms appear to be involved in the regulation of leptin and GLUT4 transcription during adipocyte differentiation.

DISCUSSION

We have studied the role of PPARγ activation in the regulation of leptin expression by comparing the effects of the putative natural PPARγ ligand, 15d-PGJ_2, and two other PPARγ ligands, Indo and Trog, on leptin expression in differentiating and fully differentiated 3T3-L1 adipocytes. Our results suggest that the level of leptin protein secreted, as well as the level of *ob* mRNA, were downregulated in the presence of the PPARγ activators studied. Since TZDs have been shown to downregulate leptin at the level of transcription,[2] it appears likely that this is also the case with 15d-PGJ_2 or Indo. However,

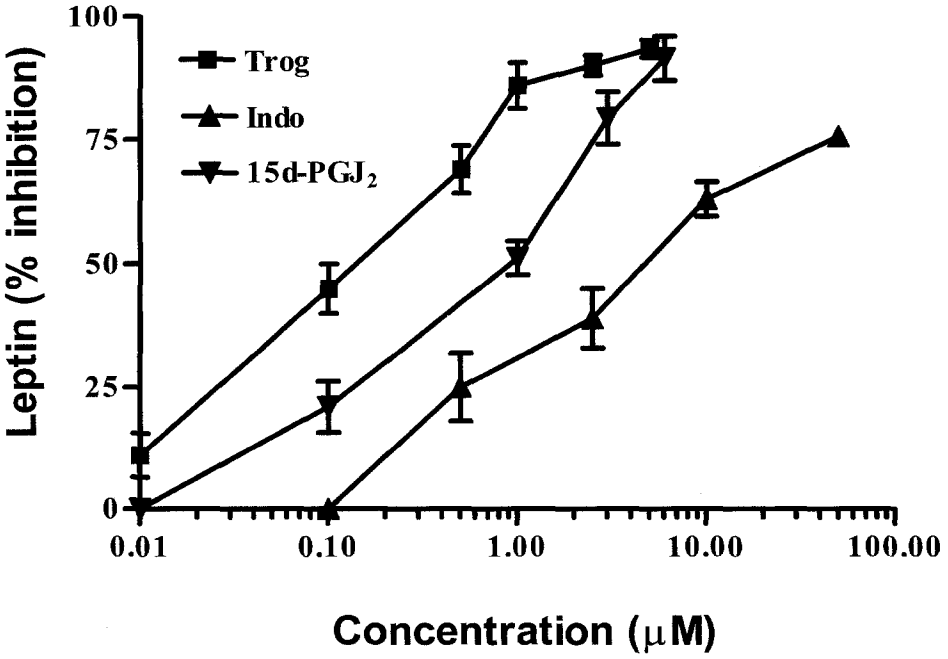

Fig 3. Dose-response of Trog-, Indo-, and 15d-PGJ_2-induced downregulation of leptin in 3T3-L1 cells. Fully differentiated cells were treated with various concentrations of the agents, and at the end of 24 hours, leptin was determined in the media. Triplicate dishes were used for each experiment. Results are the mean ± SE.

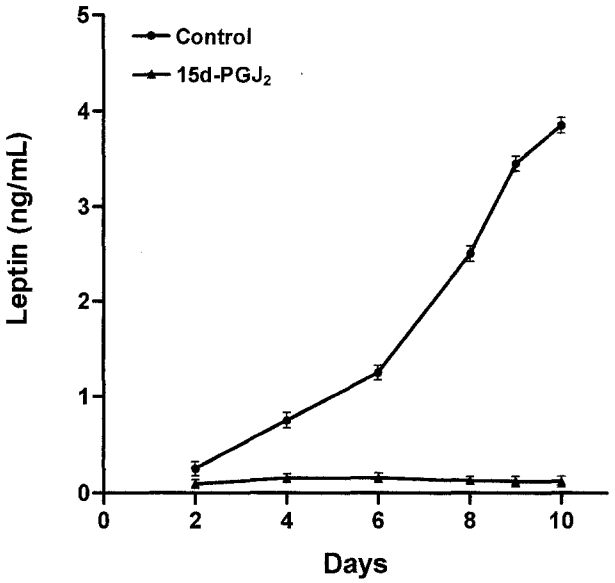

Fig 4. Effects of PPARγ ligands on leptin expression during differentiation. 3T3-L1 cells were differentiated using the normal differentiation protocol in the presence and absence of Trog, Indo, or 15d-PGJ$_2$, and the concentration of leptin in the media was measured on the day shown. In the case of Trog or Indo, the agent was added every 48 hours, whereas for 15d-PGJ$_2$, it was added every 24 hours. Control cells received the respective vehicle. Measurements were made in triplicate. Trog and Indo had the same inhibitory effects (data not shown) as shown for 15d-PGJ$_2$.

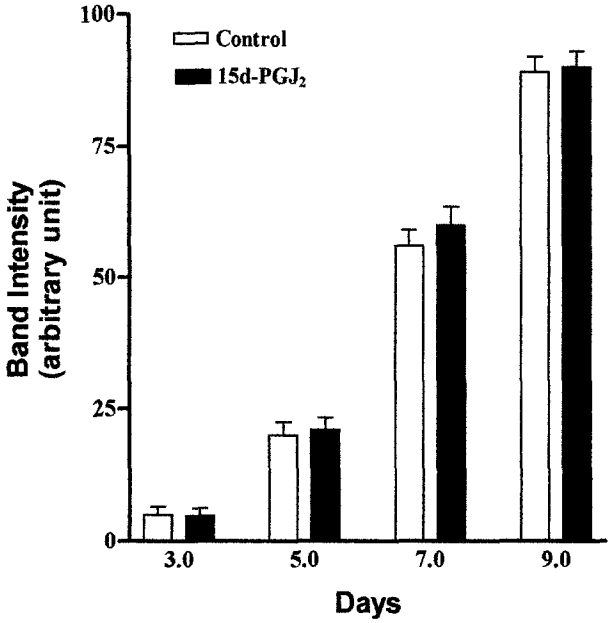

Fig 6. Effects of PPARγ ligands on GLUT4 expression during differentiation. 3T3-L1 cells were differentiated using the normal differentiation protocol in the presence and absence of Trog, Indo, or 15d-PGJ$_2$. At the days specified, cells were washed 3 times with phosphate-buffered saline and RNA was prepared. GLUT4 mRNAs in these preparations were quantified using the RT-PCR technique.

the possibility of mRNA instability cannot be excluded. Moreover, treated adipocytes expressed normal leptin levels 48 hours after removal of the ligands, indicating that the effect of each of the three PPARγ ligands on leptin expression was reversible (data not shown).

Dose-response analysis showed that the ED_{50} for Indo-induced downregulation of leptin was 5 to 10 μmol/L (Fig 3). This compared reasonably well with the reported 50% effective concentration (EC_{50}) for Indo-induced activation of PPARγ of 40 μmol/L.[8] The ED_{50} for Trog-induced downregulation of leptin was 100 to 200 nmol/L, whereas the reported value for

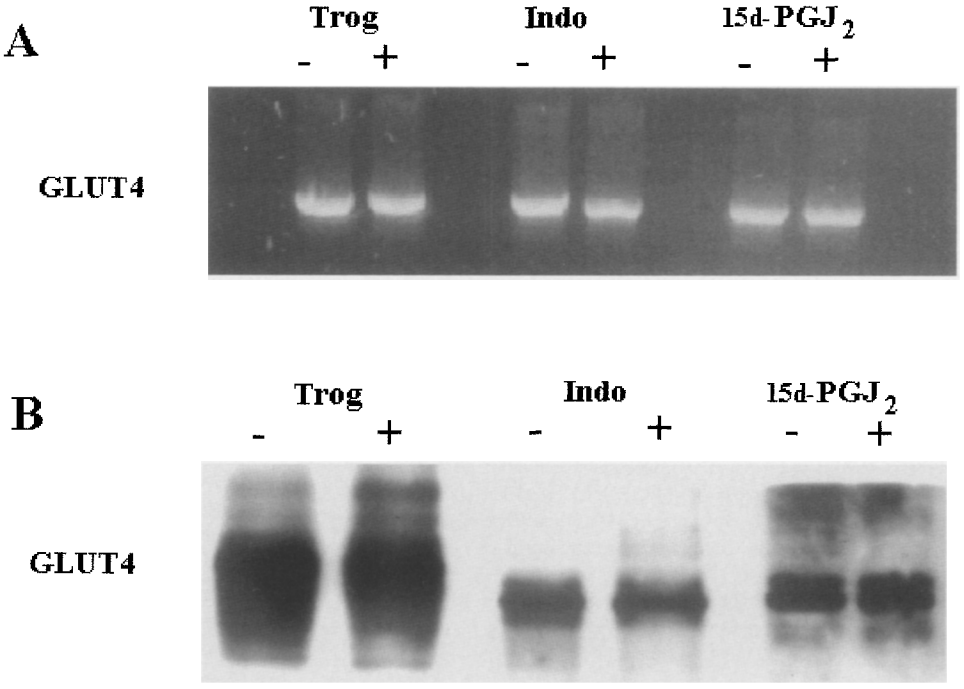

Fig 5. Effects of PPARγ ligands on GLUT4 mRNA and protein in fully differentiated 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes were treated with PPARγ ligands as described in Fig 1. After 24 hours, cells were washed 3 times with phosphate-buffered saline and GLUT4 mRNA quantified as described for leptin mRNA. (B) For measurement of GLUT4 protein, the cells (after washing) were disrupted using 2% SDS. Proteins were separated by SDS-gel electrophoresis, transferred onto nitrocellulose membranes, and blotted using a GLUT4-specific antibody, followed by detection using enhanced chemiluminescence.

BRL49653 (another TZD) was 40 nmol/L.[2] The ED_{50} for 15d-PGJ_2–induced downregulation of leptin was about 1 μmol/L. However, since 15d-PGJ_2 has a short half-life (<1 hour), the effective concentration of 15d-PGJ_2 resulting in half-maximal inhibition of leptin is perhaps much lower. Thus, all three chemically diverse classes of PPARγ ligands inhibited leptin expression at the concentrations at which they bind to PPARγ. These findings support the contention that activation of PPARγ will result in inhibition of *ob* gene expression regardless of the nature of the ligand.

Of particular interest is the observation that 15d-PGJ_2, an arachidonic acid metabolite and currently the strongest candidate for the role of a natural PPARγ ligand,[7,9] inhibited leptin expression. This raises the possibility that leptin synthesis in adipocytes may be regulated, at least in part, by fatty acid/prostaglandin metabolites and suggests a connection between endogenous prostaglandins, adipocyte differentiation, and energy metabolism.

Also of interest is the finding that Indo, a PPARγ activator that is chemically unrelated to either Trog or PGJ_2, also inhibited leptin expression. Indo is a widely used medication that exerts a strong anti-inflammatory action by inhibition of cyclooxygenase. Hypoglycemia has been reported as a rare side effect of this agent.[11,12] It is therefore conceivable that Indo, like Trog, may decrease insulin resistance in patients with type 2 diabetes.

PPARγ activation has been demonstrated to be a key event in the induction of adipocyte differentiation.[4,5] Leptin expression, on the other hand, is absent in preadipocytes and only develops during differentiation. How is it then possible that PPARγ activation induced adipocyte differentiation and at the same time inhibited leptin expression? A possible answer would be that PPARγ has different functions at the initial stages compared with the later stages of adipocyte differentiation. To address this issue, we differentiated 3T3-L1 cells in the presence and absence of Trog, Indo, or 15d-PGJ_2 and measured the level of leptin in the medium at different stages of differentiation. The level of leptin increased with progression of differentiation in the absence of 15d-PGJ_2, whereas it remained inhibited when 15d-PGJ_2 was present in the incubation medium (Fig 4). Similar results were obtained when Trog or Indo were used as PPARγ ligands (data not shown). There were no differences in either the morphology or the number of differentiated cells at different stages of differentiation in the presence or absence of the ligands. It should be noted that in these experiments, 3T3-L1 preadipocytes were differentiated using the regular differentiation procedure (described in the Method), and starting at day 0, the ligands were added every 48 hours in the case of Trog or Indo and every 24 hours in the case of 15d-PGJ_2. Under these conditions, the addition of PPARγ ligands had no additional potentiating activities on differentiation.

The glucose transporter protein GLUT4, like leptin, is expressed in adipocytes and is hardly detectable in preadipocytes. We therefore examined the effects of the three PPARγ ligands on the expression of GLUT4 protein and mRNA in fully differentiated 3T3-L1 adipocytes. In addition, we tested 15d-PGJ_2 for its effect on GLUT4 mRNA at different stages during differentiation of 3T3-L1 cells. The results indicate that PPARγ ligands did not interfere with GLUT4 expression during differentiation or in fully differentiated adipocytes. Others have reported that the expression of aP_2, another adipocyte-specific gene induced late during adipogenesis, is also unaffected by TZDs.[2] Thus, the negative effect of PPARγ ligands on transcription appeared to be unique for the *ob* gene. One explanation for this phenomenon would be that PPARγ activation is needed for adipocyte differentiation only at the very beginning (perhaps during the first few hours), and afterward, it needs to be inactivated to allow expression of the *ob* gene to proceed normally.

Lastly, our finding that TZDs did not enhance GLUT4 transcription or protein synthesis confirms a previous report by Tafuri,[13] who showed that the activity of Trog to decrease insulin resistance in 3T3-L1 adipocytes was not associated with an increase in GLUT4, and a report by Park et al,[14] who showed in cultured human muscle cells that Trog stimulated glucose uptake and GLUT1 mRNA but had no effect on GLUT4 mRNA.[14]

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