Inhibition of adipocyte differentiation by insulin-like growth factor-binding protein-3

Sophie S. Y. Chan, Lynette J. Schedlich, Stephen M. Twigg, and Robert C. Baxter

1Kolling Institute of Medical Research, Royal North Shore Hospital, and 2Discipline of Medicine, University of Sydney, New South Wales, Australia

Submitted 16 October 2008; accepted in final form 7 January 2009

Chan SSY, Schedlich LJ, Twigg SM, Baxter RC. Inhibition of adipocyte differentiation by insulin-like growth factor-binding protein-3. Am J Physiol Endocrinol Metab 296: E654–E663, 2009. First published January 13, 2009; doi:10.1152/ajpendo.90846.2008.—Insulin-like growth factor-binding protein-3 (IGFBP-3) interacts with the type II nuclear receptors retinoid X receptor (RXR) and retinoic acid receptor-α and modulates their transcriptional activity. Peroxisome proliferator-activated receptor (PPAR)γ, a related nuclear receptor that dimerizes with RXRα, plays an important role in adipocyte differentiation. IGFBP-3 is regulated during adipocyte differentiation, but its role in this process is unknown. We demonstrate that IGFBP-3 interferes with the PPARγ-dependent processes of adipocyte differentiation and maintenance of the gene expression characteristic of mature adipocytes. Treatment of adipocytes with exogenous IGFBP-3, but not an IGFBP-3 mutant that does not bind RXRα or PPARγ, decreased markers of adipocyte differentiation, PPARγ, and resistin but increased the preadipocyte marker plasminogen activator inhibitor-1. Furthermore, expression of human IGFBP-3, but not the IGFBP-3 mutant, by preadipocytes inhibited preadipocyte differentiation as determined by gene markers and lipid accumulation. IGFBP-3 interacted with PPARγ in vitro and in 3T3-L1 adipocyte lysates and inhibited PPARγ heterodimerization with RXRα in vitro. Wild-type IGFBP-3, but not mutant IGFBP-3, blocked ligand-induced transactivation of PPAR response element in 3T3-L1 cells. The observation that IGFBP-3 inhibits adipocyte differentiation and impacts on the PPARγ system suggests a role for IGFBP-3 in the pathogenesis of obesity and insulin resistance.

3T3-L1 cells; peroxisome proliferator-activated receptor-γ; insulin-like growth factor-binding protein-3

INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN (IGFBP)-3 is a high-affinity binding protein for the insulin-like growth factors IGF-I and IGF-II. IGFBP-3 can modulate IGF activity and also has IGF-independent actions on cellular functions (13). The mechanism of some of the IGF-independent effects of IGFBP-3 may depend on its ability to translocate to the nucleus in a variety of cells via a nuclear localization sequence-dependent, importin-β-mediated process (24, 29, 39). We have previously shown that IGFBP-3 is detectable in the nucleus of 3T3-L1 adipocytes (7). IGFBP-3 interacts directly with the DNA-binding domain of the nuclear transcription factor retinoid X receptor (RXR) (38) and modulates RXRα-mediated signaling (30). IGFBP-3 also binds to the RXR dimerization partners vitamin D receptor (VDR) (40) and retinoic acid receptor (RAR) and inhibits RXR:RAR heterodimerization and the ligand-induced transactivation of the RAR response element (41).

Peroxisome proliferator activated receptor (PPAR)γ is a class II nuclear receptor with structural homology to RXR, RAR, and VDR (31), that heterodimerizes with RXRα to become transcriptionally active (6). It has been termed a master regulator of adipocyte differentiation and is the target of thiazolidinediones, PPARγ agonists used for the treatment of type 2 diabetes mellitus (36). PPARγ is also important for the maintenance of the characteristics of mature 3T3-L1 adipocytes in vitro in terms of gene markers of terminal differentiation and adipocyte function (47). Adipocyte differentiation involves an ordered cascade of molecular events (18). PPARγ gene expression is upregulated early during differentiation of adipocytes (49) and is widely used as a marker of adipocyte differentiation. Resistin is an adipocytokine known to cause insulin resistance, albeit in rodents (46). The level of resistin mRNA is upregulated during differentiation of adipocytes (20). The resistin gene contains a functional PPAR response element (PPRE) and is regulated directly by PPARγ (34). Plasminogen activator inhibitor-1 (PAI-1) mRNA and PAI-1 production are higher in preadipocytes and stromal cells compared with adipocytes (2) and a marker of undifferentiated preadipocytes. IGFBP-3 is upregulated during adipocyte differentiation in primary mouse cells and 3T3-L1 cells (4) and also during in vitro differentiation of human preadipocytes (50). This suggests that IGFBP-3 may be playing a regulatory role in the process of adipocyte differentiation. However, although we and others (7, 25) have demonstrated that IGFBP-3 is inhibitory to insulin-mediated glucose uptake in adipocytes, the role of IGFBP-3 during adipocyte differentiation has not been studied. We now report that IGFBP-3 impacts on PPARγ-dependent processes of terminal differentiation and the differentiation of preadipocytes to mature adipocytes. We also show that IGFBP-3 binds directly to PPARγ and inhibits ligand-dependent transactivation of a PPRE in 3T3-L1 cells, suggesting a possible mechanism for its inhibition of PPARγ-dependent adipocyte differentiation.

MATERIALS AND METHODS

Recombinant human IGFBP-3 was expressed using an adenoviral expression system in 911 cells (E1-transformed human embryonic retinal cells) and purified as previously described (15). The mutated form of human IGFBP-3 (IGFBP-3mut), which has the amino acid substitutions 228KGRKR→MDGEA, was generated by site-directed mutagenesis as described (14, 53). In this mutant, five critical residues for nuclear localization and nuclear receptor binding have been replaced with the corresponding residues of IGFBP-1, which is known not to translocate to the nucleus or bind to class II nuclear receptors.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Recombinant human IGFBP-2 was provided by Sandoz (now Novartis, Basel, Switzerland). The 3T3-L1 cells (obtained from American Type Culture Collection, Manassas, VA) were cultured and differentiated with standard differentiation medium containing dexamethasone, IBMX, and insulin or the PPARγ agonist 10 μM ciglitazone, as previously described (7).

The expression plasmids pIGFBP-3 and pIGFBP-3mut were generated by subcloning the relevant sequences into pOP13 vector (stratagene, La Jolla, CA) as described (5). Stable transfection of cells with pIGFBP-3 and pIGFBP-3mut was performed as described (11). Transient transfection was performed as described previously (41). IGFBP-3 expression in conditioned media was determined by in-house RIA (3).

Pull-down and immunoprecipitation assays. Full-length hPPARγ1 was cloned into pSET-A to generate His6-PPARγ by use of standard methods (1). Plasmid carrying hRXRα was previously described (41). His6 pull-down assays were performed according to published methods (41). Briefly, His6-PPARγ and vector control sequences were expressed in Escherichia coli and captured from cell lysates with Ni-NTA beads. After incubation with IGFBP-3, the bound proteins were separated by 10% SDS-PAGE prior to membrane transfer and detected by Western immunoblot (IB) using in-house hIGFBP-3 antisera.

In other experiments, GST-RXRα immobilized on 10-μl glutathione beads was preincubated without or with 6 μg of IGFBP-3 for 1 h at 22°C with gentle rotation. His6-PPARγ was expressed in E. coli and purified from cell lysates on a Ni-NTA agarose column. His6-PPARγ (4 μg) was added to each reaction and incubated as above for a further 30 min. This gave a two molar excess of IGFBP-3 with respect to His6-PPARγ in the experiment. Bound His6-PPARγ was analyzed on reducing SDS-PAGE and immunoblotted using a monoclonal anti-polystyrene antibody.

For immunoprecipitation experiments on cell lysates, 3T3-L1 adipocytes were lysed with 300 mM NaCl, 20 mM Tris-HCl, 1% Triton, 1 mM Na vanadate, 10 mM NaF, 4 mM Na pyrophosphate, and protease inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany), pH 7.5. The 3T3-L1 adipocyte lysate was incubated with either mouse PPARγ-specific mouse monoclonal antibody (Santa Cruz Biotechnology), mouse IGFBP-3-specific goat antibody (R&D Systems, Minneapolis, MN), normal goat serum (NGS; Sigma Chemical, St. Louis, MO), or normal mouse serum (NMS; Sigma Chemical). Protein A beads (Amersham Pharmacia Biotech, Uppsala, Sweden) were added to the lysate-antibody mixture and rotated slowly overnight at 4°C. The immunoprecipitated complexes were washed three times with ice-cold buffer (10 mM Tris-HCl, 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Na orthophosphate, 10 mM Na pyrophosphate, 100 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 mM Na orthovanadate, and protease inhibitors), recovered from agarose beads and separated on SDS-PAGE. The membrane was probed with either mPPARγ or mIGFBP-3 antibodies. Analysis of immunoblots was as described (7).

Reporter assays and real-time RT-PCR. Transcriptional reporter assays were performed as described (41). Briefly, the 3T3-L1 cells were transiently cotransfected with 1 μg of reporter plasmid, PPRE-thymidine kinase (TK)-luciferase (16), and 0.15 μg of internal control reporter plasmid (pRL-TK). In addition, they were cotransfected with either 0.85 μg of pOP13 vector (empty vector), 0.85 μg of pIGFBP-3, or 0.85 μg of pIGFBP-3mut. The next day, cells were treated for 48 h with either 10 μM ciglitazone or vehicle (ethanol). Cell lysates were assayed for firefly and Renilla luciferase activity as described (41).

Real-time RT-PCR was performed as previously described (9). Nile Red staining was performed according to published methods (22). For the Nile Red spectrofluorometric assay, cells were trypsinized and 1 μg/ml of Nile Red was added to cell lysates. Yellow/gold fluorescence was measured in a spectrofluorometer (Shimadzu, Kyoto, Japan) at 488 nm excitation/540 nm emission.

Statistics. All experiments were conducted in triplicate at least three times independently. Figures show representative experiments. Statistical analysis was performed with StatView, version 5 (SAS Institute, Cary, NC). Results were analyzed using ANOVA followed by Fisher’s protected least significant difference test. ANOVA with repeated-measures testing was applied where appropriate. Statistical difference applies to differences between experiments. Data shown in graphs are means ± SE.

RESULTS

Effect of IGFBP-3 on gene expression in mature adipocytes. In mature 3T3-L1 adipocytes differentiated in the presence of insulin, dexamethasone, and IBMX, exogenous recombinant human IGFBP-3 caused a dose-dependent decrease in PPARγ mRNA expression after 24 h (Fig. 1A). Compared with control, PPARγ mRNA, determined by quantitative real-time RT-PCR, was reduced by 38% (P = 0.003) by IGFBP-3 at 100 ng/ml, 56% (P = 0.003) at 300 ng/ml, and 59% (P = 0.0002) at 1 μg/ml. At the same concentrations of IGFBP-3, resistin mRNA was reduced by 37% (P = 0.08), 56% (P = 0.01), and 54% (P = 0.02) (Fig. 1B). In contrast, exogenous IGFBP-3 increased the preadipocyte marker PAI-1 after 24 h (Fig. 1C). At 300 ng/ml, IGFBP-3 increased PAI-1 mRNA nonsignificantly (4.6-fold, P = 0.12), and when added at 500 ng/ml and 1 μg/ml, IGFBP-3 significantly increased PAI-1 mRNA 9.6-fold (P = 0.008) and 8.4-fold (P = 0.004) respectively.

There was no observable effect of exogenous IGFBP-3 treatment for 24 h on the morphology of adipocytes (data not shown). Thus, IGFBP-3 dose-dependently decreased the adipocyte gene markers PPARγ and resistin but increased the preadipocyte gene marker PAI-1. None of these effects was seen with IGFBP-3mut, which is unable to translocate to the nucleus or bind RXRα. When exposed to the highest dose of 1 μg/ml IGFBP-3mut, the gene expression of PPARγ (Fig. 1D), resistin (Fig. 1E), and PAI-1 (Fig. 1F) were all unchanged under conditions where wild-type IGFBP-3 caused the expected changes. To ensure that cells had equal exposure to exogenous wild-type and mutant IGFBP-3, lysates were prepared from 3T3-L1 adipocytes after 3 days of incubation with 1 μg/ml of each IGFBP-3 preparation. Figure 1G shows that approximately equal amounts of each IGFBP-3 form were present in cell lysates.

In summary, the effect of IGFBP-3 on adipocyte gene expression appears to involve the nuclear translocation of IGFBP-3 and/or binding of IGFBP-3 to RXR:PPARγ, as exposure to exogenous IGFBP-3mut had no effect on adipocyte gene expression in terminally differentiated adipocytes. IGFBP-3 inhibits adipocyte differentiation. The effect of IGFBP-3 on the PPARγ-dependent process of differentiation of preadipocytes to adipocytes was studied. Figure 2A shows that, during differentiation of 3T3-L1 preadipocytes (day −3) to mature adipocytes (day 9) in standard differentiation medium, PPARγ mRNA increased 12.8-fold (P = 0.003). When exogenous IGFBP-3 (500 ng/ml) was added during differentiation (day 0 to day 9), there was no effect at day 3, but by day 9, IGFBP-3 significantly reduced PPARγ mRNA by 72.8% (P = 0.02).

During adipocyte differentiation, there was a marked induction of resistin mRNA from preadipocytes to adipocytes (P = 0.0003; Fig. 2B). There was no change in resistin expression between IGFBP-3-treated and control cells at day 3, but at day 9, IGFBP-3 (500 ng/ml) significantly inhibited resistin mRNA by 78.2% (P = 0.001). The preadipocyte marker PAI-1 was
IGFBP-3 inhibits adipocyte differentiation

Effect of IGFBP-3 on adipocyte gene expression was assessed by treating mature 3T3-L1 adipocytes with increasing doses of IGFBP-3 for 24 h in SF medium. Effect of IGFBP-3 on peroxisome proliferator-activated receptor-γ (PPARγ) mRNA, resistin/18S mRNA, and plasminogen activator inhibitor-1 (PAI-1)/18S mRNA (C) was assessed by real-time PCR. Results are expressed as % change from control samples where no IGFBP-3 was added. Effect of 1 μg/ml IGFBP-3mut and 1 μg/ml wild-type IGFBP-3 in SF medium for 24 h on mature 3T3-L1 adipocytes with respect to adipocyte gene expression was studied. Effects of IGFBP-3 and IGFBP-3mut on PPARγ/18S mRNA (D), resistin/18S mRNA (E), and PAI-1/18S mRNA (F) were assessed by real-time PCR. Statistical significance: *P < 0.05, **P < 0.01, and ***P < 0.001. G: after 3 days' exposure of cells to IGFBP-3 or IGFBP-3mut, 3T3-L1 lysates were prepared and analyzed by immunoblot. Top: IGFBP-3 immunoblot (IB): lane 1, 10 ng recombinant human (rh)IGFBP-3 standard; lanes 2–4, 25 μl of a total of 100 μl of lysates from cells incubated with no IGFBP-3, 1 μg/ml wild-type IGFBP-3, or 1 μg/ml IGFBP-3mut, respectively. Bottom: IB for α-tubulin (as loading control) in the same samples.

Exogenous IGFBP-3 (500 ng/ml; Fig. 2D, bottom) inhibited lipid accumulation in adipocytes on day 9 compared with control cells on the same day (Fig. 2D, top). The ability of IGFBP-3 to inhibit triglyceride accumulation was quantified spectrofluorometrically after staining with Nile Red (Fig. 2E). During adipocyte differentiation in control cells, there was a 6.4-fold increase (P < 0.0001) in Nile Red uptake from day 0 to day 9. In contrast, cells differentiated in the presence of exogenous IGFBP-3 at 500 ng/ml had 30% less induction in Nile Red uptake compared with control cells at day 9 (P = 0.03). Therefore, the addition of IGFBP-3 during differentiation altered not only gene markers of adipocyte differentiation but also triglyceride accumulation.

IGFBP-3 inhibits ciglitazone-induced adipocyte differentiation. Ciglitazone (10 μM), a specific PPARγ agonist, was used to differentiate the 3T3-L1 cells as an alternative to the stan-

decreased by 50% as preadipocytes (day −3) differentiated to adipocytes (day 9) (Fig. 2C). IGFBP-3 added at 500 ng/ml during differentiation significantly blocked the decrease in PAI-1 compared with control cells at day 9, giving values twofold higher than those in the absence of exogenous IGFBP-3 (P = 0.03). Thus, IGFBP-3 added from the start of differentiation reduced expression of the adipocyte gene markers PPARγ and resistin and increased the preadipocyte gene marker, PAI-1 at day 9.
dard differentiation medium. When ciglitazone was used, there was a twofold increase in PPARγ mRNA from day −4 (nonconfluent preadipocytes) to day 10 (P = 0.0002; Fig. 3A). This increase was prevented when exogenous IGFBP-3 (500 ng/ml) was added with ciglitazone. There was a trend toward decreased PPARγ gene expression at day 7 (P = 0.05), and by day 10, IGFBP-3 significantly inhibited PPARγ mRNA levels by 49% compared with ciglitazone alone (P = 0.0008). Similarly, the 7,000-fold increase in resistin mRNA (P < 0.0001) from day −4 to day 10 (Fig. 3B) was substantially inhibited at day 7 (68% decrease, P = 0.07) and day 10 (80% decrease, P < 0.0001). The preadipocyte gene marker PAI-1 decreased from day −4 to day 10 by 70% (P = 0.01; Fig. 3C). This effect was significantly reversed by day 10, with a threefold increase (P = 0.02) in PAI-1 mRNA in cells treated with IGFBP-3 together with ciglitazone compared with cells treated with ciglitazone alone.

Figure 3D shows that cells differentiated with ciglitazone acquire multiple small triglyceride droplets by day 10 (Fig. 3D, top). In the cells treated with IGFBP-3 and ciglitazone, there was a lesser change in adipocyte cell morphology, including a relatively marked reduction in the amount of accumulated cellular triglyceride (Fig. 3D, bottom). This effect of added IGFBP-3 on lipid accumulation was confirmed with the Nile Red spectrofluorometric assay (Fig. 3E). During adipocyte differentiation in ciglitazone-treated cells, there was a 7.3-fold increase (P < 0.0001) in Nile Red uptake from day 0 to day 10. Cells differentiated in the presence of ciglitazone and IGFBP-3 showed a 34% decrease (P = 0.007) in Nile Red uptake compared with control cells at day 7 and a 50% (P < 0.0001) decrease in Nile Red uptake at day 10. These data demonstrate that exogenous IGFBP-3 can also inhibit the development of a differentiated phenotype in 3T3-L1 adipocytes when ciglitazone is used as the differentiation agent.

**IGFBP-3 does not affect adipocyte differentiation.** To determine that the effect of IGFBP-3 on adipocyte differentiation was not due to IGFBP-3 binding IGFs and thus inhibiting signaling through the type 1 IGF receptor (IGFR1), the effect of another high-affinity binding protein for IGFs, IGFBP-2, was tested. IGFBP-2 (750 ng/ml) added to 3T3-L1 cells from day 0 of differentiation with standard differentiation medium, had no effect on gene expression of PPARγ (Fig. 4A), resistin (Fig. 4B), or PAI-1 (Fig. 4C). Similarly, IGFBP-2 had no effect on gene expression when cells were differentiated with ciglitazone nor on adipocyte morphology during their differentiation with standard differentiation medium or ciglitazone (data not shown). Thus, it is unlikely that the blocking of IGFR1 signaling accounts for the observed effects of IGFBP-3 on adipocyte differentiation.

**Cellular expression of IGFBP-3, but not IGFBP-3mut, inhibits adipocyte differentiation.** To further examine the effects of IGFBP-3 during adipocyte differentiation, 3T3-L1 preadipocytes were transfected with empty pOPI3 vector alone or vectors containing cDNA encoding IGFBP-3 or IGFBP-3mut. Both IGFBP-3 and IGFBP-3mut transfectants expressed a higher level of IGFBP-3 detected by semiquantitative RT-PCR than vector control cells (Fig. 5A). Figure 5B shows the concentration of immunoreactive IGFBP-3 in conditioned medium from the transfectants at different points during differentiation with standard differentiation medium (days 0, 3, 9). In control vector-transfected cells, there was a fourfold increase in IGFBP-3 from preadipocytes (day 0) to adipocytes (day 9). Medium conditioned by IGFBP-3 and IGFBP-3mut transfectants contained similar levels of IGFBP-3, higher than vector control cells at each time point. Transfection with IGFBP-3 or IGFBP-3mut did not increase cell death as assessed by direct cell counting by hemocytometer with trypan blue exclusion (data not shown).

Like exogenous IGFBP-3, cellular expression of recombinant IGFBP-3, but not IGFBP-3mut, altered gene markers of adipocyte differentiation. In vector control cells, PPARγ mRNA increased 11.9-fold (P < 0.0001) from the start of differentiation (day 0) to the end (day 10) (Fig. 5C). Preadipocytes expressing wild-type IGFBP-3 had reduced PPARγ mRNA on days 4, 7, and 10. In contrast, cells expressing IGFBP-3mut had no significant effect on PPARγ mRNA.
induction during differentiation. When analyzed by ANOVA with repeated measures, IGFBP-3, but not IGFBP-3mut, significantly inhibited the induction of PPARγ/H9253 mRNA (P = 0.02) across the time course of differentiation. A similar effect of endogenously expressed recombinant IGFBP-3 was seen on resistin mRNA, which was induced by five orders of magnitude during the time course of differentiation (P < 0.001; Fig. 5D). This effect was blunted by ~50% in cells expressing wild-type IGFBP-3 (P = 0.02) but not in cells expressing IGFBP-3mut.

The ability of IGFBP-3 to prevent downregulation of PAI-1 mRNA during differentiation was also seen in adipocytes expressing recombinant IGFBP-3. In vector control cells, PAI-1 mRNA decreased during differentiation by 54% (P = 0.0004; Fig. 5E), but in cells expressing wild-type IGFBP-3, there was no significant fall in PAI-1. Cells expressing IGFBP-3mut had a reduction in PAI-1 mRNA similar to that in vector control cells. The effect of IGFBP-3 in preventing the fall in PAI-1 was significant (P = 0.04) by repeated-measures ANOVA testing.

Figure 6A shows that, at day 10 of differentiation, fewer cells attained the adipocyte phenotype when transfected with IGFBP-3 compared with the vector control and IGFBP-3mut transfectants. The effect of stable expression of IGFBP-3 on lipid accumulation was confirmed with the Nile Red spectrofluorometric assay (Fig. 6B). IGFBP-3 transfectants had lower mean fluorescence compared with vector control and

---

**Fig. 4.** Effect of IGFBP-2 on adipocyte gene expression during 3T3-L1 differentiation. Effect of 750 ng/mL IGFBP-2 added during adipocyte differentiation was studied. PPARγ/18S (A), resistin/18S (B), and PAI-1/18S (C) mRNA was measured using real-time PCR. Filled bars, control cells; gray bars, cells differentiated in presence of 750 ng/ml IGFBP-2. Results are expressed relative to expression of IGFBP-2 mRNA by cells at day -3.

**Fig. 5.** Differentiation of 3T3-L1 cells stably transfected with IGFBP-3 or IGFBP-3mut cDNAs. A: IGFBP-3 expression was detected by RT-PCR on lysates of 3T3-L1 preadipocytes stably transfected with pOPI3 (vector), IGFBP-3 (BP-3) cDNA, and IGFBP-3mut (mut) cDNA. Top: PCR products were separated on 1% agarose and visualized by ethidium bromide staining. Bottom: blots were quantified by densitometry, with amount of IGFBP-3 mRNA corrected for housekeeping gene 18S. Results are expressed relative to expression of IGFBP-3 mRNA by cells transfected with wild-type IGFBP-3.

---

**Fig. 5A:**

- **Days:** -3, 0, 3, 9
- **Y-axis:** PPARγ
- **Bars:** Filled bars, cells stably transfected with pOPI3 (control vector); hatched bars, cells transfected with wild-type IGFBP-3 cDNA; open bars, cells transfected with IGFBP-3mut cDNA.

**Fig. 5B:**

- **Days:** 0, 4, 7, 10
- **Y-axis:** Resistin
- **Bars:** Filled bars, cells transfected with wild-type IGFBP-3 cDNA; open bars, cells transfected with IGFBP-3mut cDNA.

**Fig. 5C:**

- **Days:** -3, 0, 3, 9
- **Y-axis:** PAI-1
- **Bars:** Filled bars, cells stably transfected with pOPI3 (control vector); hatched bars, cells transfected with wild-type IGFBP-3 cDNA; open bars, cells transfected with IGFBP-3mut cDNA.

**Fig. 5D:**

- **Days:** 0, 4, 7, 10
- **Y-axis:** IGFBP-3 (%)
- **Bars:** Filled bars, cells transfected with wild-type IGFBP-3 cDNA; open bars, cells transfected with IGFBP-3mut cDNA.

**Fig. 5E:**

- **Days:** 0, 3, 9
- **Y-axis:** PAI-1
- **Bars:** Filled bars, cells stably transfected with pOPI3 (control vector); hatched bars, cells transfected with wild-type IGFBP-3 cDNA; open bars, cells transfected with IGFBP-3mut cDNA.
IGFBP-3 transfectants at day 4 and day 10 of differentiation. Repeated-measures ANOVA showed a significant effect of recombinantly expressed IGFBP-3, but not IGFBP-3mut, across the time course of differentiation, on triglyceride accumulation (P = 0.01).

**IGFBP-3, but not IGFBP-3mut, interacts with PPARγ.** Since IGFBP-3 interfered with PPARγ-dependent processes, the ability of IGFBP-3 to interact directly with PPARγ was investigated using a His6 pull-down assay. His6-PPARγ protein immobilized on Ni-NTA beads, Ni-NTA beads alone (negative control), or His6 alone (negative control) were incubated with 500 ng of IGFBP-3 in a 1-ml incubation volume (Fig. 7A). Compared with Ni-NTA beads alone (lane 1) or His6 alone (lane 2), where weak, nonspecific bands were seen, His6-PPARγ bound IGFBP-3 strongly, visualized as the characteristic 40/43-kDa glycoprotein doublet (lane 3). IGFBP-3 at 500 ng as a positive control is shown in lane 4. Therefore, IGFBP-3 is a binding partner of PPARγ in vitro. In IGFBP-3mut, five critical residues for nuclear localization are replaced with corresponding residues from IGFBP-1, preventing localization to the nucleus (39) or binding to RXRα (38). To investigate the ability of IGFBP-3mut to bind to PPARγ, similar His6 pull-downs were done using IGFBP-3mut (lanes 5–8) rather than wild-type IGFBP-3. No binding of IGFBP-3mut to Ni-NTA beads alone (lane 5) or His6 alone (lane 6) was observed. In contrast to IGFBP-3, IGFBP-3mut did not bind to PPARγ (lane 7). IGFBP-3mut at 500 ng as a positive immunoblot control is shown in lane 8.

**Interaction between IGFBP-3 and PPARγ in 3T3-L1 adipocytes.** Coimmunoprecipitation experiments were used to determine whether the biochemical interaction described between IGFBP-3 and PPARγ occurred in cells. Lysates of 3T3-L1 adipocytes were immunoprecipitated with mIGFBP-3 or mPPARγ antibodies coupled to protein A beads. Samples were separated on 10% SDS-PAGE and subjected to IB analysis for PPARγ. Whole cell lysate from 3T3-L1 adipocytes (200 μg) showed abundant PPARγ protein expression (Fig. 7B, left, lane 1).
which had the effect of obscuring the typical doublet of PPARγ. When PPARγ was immunoprecipitated from lysates (1 mg), it was detected as a characteristic doublet (lane 2). When lysates were immunoprecipitated with mIGFBP-3 antibody (lane 3), PPARγ was detected as a doublet at 60 kDa, indicating that IGFBP-3 interacts with PPARγ in 3T3-L1 adipocytes. Lysates were immunoprecipitated with NGS as a negative control (lane 4).

The reverse and complementary experiment was also performed with samples subjected to IB analysis for mIGFBP-3 (Fig. 7B, right). Whole cell lysate from 3T3-L1 adipocytes (200 μg) revealed IGFBP-3 protein at ~45 kDa (lane 5). When lysates (1 mg) were immunoprecipitated with the mIGFBP-3 antibody, abundant IGFBP-3 was detected (lane 7). When lysates were immunoprecipitated with PPARγ antibody, mIGFBP-3 was detected at ~45 kDa (lane 6), again indicating that IGFBP-3 binds PPARγ derived from 3T3-L1 adipocytes. Lysates were immunoprecipitated with NMS as a negative control (lane 8). In summary, these experiments demonstrate that IGFBP-3 and PPARγ are binding partners in 3T3-L1 adipocytes.

**IGFBP-3 prevents PPARγ heterodimerization with RXRα.** Since IGFBP-3 binds directly to both RXRα (41) and PPARγ, we investigated in vitro whether these interactions could block the formation of RXRα:PPARγ heterodimers. GST-RXRα immobilized on glutathione beads (10 μl) was preincubated with or without IGFBP-3 (6 μg). His6-PPARγ (4 μg) was then added to each reaction. The amount of His6-PPARγ that bound to RXRα was determined by Western immunoblot using a monoclonal anti-polylhistidine antibody (Fig. 7C). In the absence of IGFBP-3, PPARγ was co-precipitated with RXRα (Fig. 7C, top, lanes 2 and 3), indicating that a heterodimer was formed. However, in the presence of IGFBP-3, the amount of PPARγ that bound to RXRα was significantly reduced (Fig. 7C, top, lanes 4 and 5). His6-PPARγ (2 μg), run as a positive control (Fig. 7C, top, lane 1) appeared to have slightly greater mobility than the precipitated samples, possibly due to distortion of the band caused by the presence of 0.1% BSA in the His6-PPARγ standard. Quantitation of data from independent experiments showed that IGFBP-3 reduced the amount of PPARγ coprecipitating with RXRα by 53% (Fig. 7C, bottom). This experiment shows that IGFBP-3, by binding RXRα and/or PPARγ, is able to inhibit formation of the RXRα: PPARγ heterodimer in vitro.

**IGFBP-3, but not IGFBP-3mut, inhibits ligand-induced transactivation of PPRE in 3T3-L1 cells.** The ability of IGFBP-3 to inhibit ligand-induced transactivation of PPRE was examined. The 3T3-L1 preadipocytes were transiently cotransfected with PPRE-TK-luciferase; the internal control reporter plasmid pRL-TK; and pOPI3, pIGFBP-3 (wild-type), or pIGFBP-3mut. Cells were then treated with rosiglitazone (10 μM) or vehicle (EtOH) for 48 h, and the cell lysates were assayed for luciferase activity. Figure 7D shows that rosiglitazone caused a 3.9-fold increase in reporter gene expression (P = 0.0002) in cells transfected with control vector. IGFBP-3 significantly inhibited rosiglitazone-induced transactivation of PPRE by 39% (P = 0.02), whereas IGFBP-3mut had no significant effect on rosiglitazone-induced transactivation of PPRE (P = 0.48).

**DISCUSSION**

This study has demonstrated that IGFBP-3 causes mature 3T3-L1 adipocytes to regress to a less differentiated state in terms of gene marker expression. Consistent with this observation, a previous study found that IGFBP-3 decreased basal and rosiglitazone-stimulated levels of the adipocyte-specific protein adiponectin (21) in 3T3-L1 cell lysates (25). Furthermore, we found that IGFBP-3 can inhibit the PPARγ-dependent process of adipocyte differentiation. IGFBP-3 was shown to be a novel PPARγ binding partner both in vitro and in 3T3-L1 adipocytes. IGFBP-3 can translocate to the nucleus of a number of cell types, but the functions of nuclear IGFBP-3 are not fully understood. IGFBP-3 has now been shown to bind to a number of nuclear hormone receptors, including RXRα (30), RAR (41), VDR (40), and nur77 (26). The structurally related nuclear receptor PPARγ needs to form a heterodimer with RXRα to be transcriptionally active, the structure of which has been recently elucidated (8). Providing a possible mechanism for its effect on adipocyte differentiation, IGFBP-3 inhibited the formation of dimers between RXRα and PPARγ and inhibited ligand-induced transactivation of PPRE. It was observed in immunoprecipitation experiments, that relatively little of the total cell PPARγ could be immunoprecipitated by either PPARγ or IGFBP-3 antibodies. A possible explanation is that the majority of cellular PPARγ is associated with other proteins, such as coactivators or corepressors, that may limit the accessibility of precipitating antibodies (8). Further optimization of immunoprecipitation conditions may also have improved the amount of precipitated complexes. Nevertheless, our data show that IGFBP-3 not only binds to PPARγ but is also able to functionally inhibit PPARγ signaling and PPARγ-dependent adipocyte differentiation.

A mutated form of IGFBP-3, which lacks some key basic residues in the carboxy-terminal domain, had no effect on transactivation of PPRE and was unable to bind PPARγ. Consistent with this, this mutant had no effect either on terminally differentiated adipocytes or on the differentiation of preadipocytes even when expressed at high levels by the cells themselves. This finding supports the hypothesis that IGFBP-3 must be localized to the nucleus and/or bind directly to RXR: PPARγ to have an inhibitory effect on PPRE. Since IGFBP-3 binds to both RXRα and PPARγ, we are unable to conclude whether interaction with both of these receptors is required for IGFBP-3 to inhibit PPARγ function. Our study is consistent with IGFBP-3 directly sequestering RXR or PPARγ or both, blocking RXR:PPAR heterodimerization and inhibiting signaling through the PPRE. This study does not exclude the possibility that IGFBP-3 may also bind directly to the coactivator/corepressor assembly or PPRE itself. Furthermore, since we showed that IGFBP-3 can suppress PPARγ gene expression, it is possible that this, too, might contribute to reduced signaling through PPRE.

PPARγ is crucial in activating the molecular cascade for differentiation but is also important in maintaining the genotypic and functional characteristics of mature 3T3-L1 adipocytes (47). We found that IGFBP-3 reduced gene markers of the mature adipocyte and increased a preadipocyte marker. IGFBP-3 also reduced lipid accumulation as assessed by Nile Red fluorescence, which has previously been used as a quantitative measure of 3T3-L1 adipocyte differentiation (54). Al-
though this method did not show the same degree of change during differentiation as the measures of adipocyte gene expression, it provides a more functional assessment of the same phenomenon. The effects of IGFBP-3 were not seen with the IGFBP-3 mutant that does not translocate to the nucleus or bind to RXRα. This is consistent with the hypothesis that IGFBP-3 may be modulating PPARγ function, which is important in maintaining the mature adipocyte phenotype.

Our study demonstrated that IGFBP-3 inhibits adipocyte differentiation of 3T3-L1 cells whether it is induced by standard differentiation medium or ciglitazone. IGFBP-3 inhibited the induction of the adipocyte gene markers PPARγ and resistin while preventing the downregulation of the preadipocyte gene marker PAI-1 during adipocyte differentiation. These changes were associated with a decrease in lipid accumulation in cells treated with IGFBP-3 during differentiation. The inhibitory effects of IGFBP-3 on adipocyte differentiation were partial, and, as shown in Figs. 2 and 3, they were most marked in the prevention of high-level induction of a later-stage differentiation marker such as resistin across days 7 to 10. Since adipocyte differentiation is thought to be important in mediating fat tissue insulin sensitivity (12) and it is less complete in human states of insulin resistance and obesity (48), the novel effects of IGFBP-3 that we have described in vitro may be of relevance to human disease. One recent report suggests that IGFBP-3 may potentiate the induction of a lipogenic enzyme in human adipocytes (19), although other sensitive markers of late-stage adipocyte differentiation, such as adipocyte-specific gene expression, were not measured in that work. Further studies are thus required to determine the role of IGFBP-3 in human adipose dysregulation.

3T3-L1 cells are a convenient model for studying the IGF-independent effects of IGFBP-3, as they express negligible amounts of IGF-I before or after differentiation (4). This inhibitory effect of IGFBP-3 on adipocyte differentiation appears to be independent of IGFR1 signaling, as IGFBP-2 had no effect on adipocyte differentiation of 3T3-L1 cells. In the same cell system, we have shown that IGFBP-2 is biologically active in inhibiting IGF-I-induced effects on deoxyglucose uptake into cells (7). In addition, IGFBP-3mut, which binds IGF-1 with an affinity similar to that of native IGFBP-3 (14), also had no effect on adipocyte differentiation. Collectively, these findings indicate that the effect of IGFBP-3 is not due to IGFBP-3 binding endogenous or exogenous IGFs to inhibit IGFR1 activation.

The nuclear effects of IGFBP-3 are increasingly being recognized (27). IGFBP-3 is translocated into the nucleus in association with the importin-β nuclear transport factor (39). When IGFBP-3 and IGFBP-3mut were stably transfected into 3T3-L1 preadipocytes in our study, we found that, unlike wild-type IGFBP-3, IGFBP-3mut had no effect on adipocyte differentiation in terms of gene markers or morphology. Our data clearly show that amino acid residues 228-232 in human IGFBP-3 are required for IGFBP-3 to exert its effect on adipocyte differentiation. However, since IGFBP-3 also interacts with other ligands through this multifunctional domain (14, 23, 28, 39, 43, 51), our data do not exclude the possibility that interaction with some other ligand(s) may be involved in this action of IGFBP-3. Indeed, it is possible that an unknown obligatory role for additional ligands, or competition for IGFBP-3 by other ligands, may explain why the observed inhibitory effects on differentiation in our studies were only partial. Nevertheless, the data in this study remain consistent with the hypothesis that IGFBP-3 modulates the nuclear hormone receptor PPARγ, which by transcriptionally active needs to form an obligate heterodimer with RXRα.

IGFBP-3-transgenic mice have impaired glucose tolerance and insulin resistance (42). In the first publication detailing the phenotypic manifestations of the IGFBP-3-transgenic mice, it was noted that the transgenic mouse strain overexpressing IGFBP-3 under the control of the CMV promoter had more epididymal fat than wild-type mice (32). However, the transgenic mouse strain overexpressing IGFBP-3 driven by the PGK promoter did not have an increase in epididymal fat. It is not clear whether other adipose tissue deposits were affected in the transgenic mice. The morphology of the adipose tissue in these mice was not specifically examined. There was no difference in adipose tissue expression of resistin, RXRα, or PPARγ between the transgenic mice compared with wild-type mice. Although there are discrepancies between these studies and our observation that IGFBP-3 inhibits adipocyte differentiation, it must be considered that the transgenic model represents a very different system from the in vitro mouse 3T3-L1 cell line model. In particular, the IGFBP-3-transgenic mice had increased (~1.5-fold) total and free circulating IGF-I compared with wild-type mice, which may partially overcome the effects of IGFBP-3 in adipose tissue.

It may appear paradoxical that IGFBP-3, an inhibitor of adipocyte differentiation, is induced during the differentiation process (50). Conceivably, as adipogenesis proceeds, increasing IGFBP-3 levels in adipose tissue may eventually serve as a feedback mechanism to limit the differentiation of further preadipocytes. IGFBP-3 has been found to regulate differentiation of several other cell types in various ways. Foulstone et al. (17) found that IGFBP-3 supports myoblast differentiation. Edmondson et al. (10) showed that overexpression of IGFBP-3 downregulates keratinocyte proliferation and modulates early stages of keratinocyte differentiation. Spagnoli et al. (44) showed that IGFBP-3 has differential effects on chondrocytes during their differentiation from chondroprogenitors to mature hypertrophic chondrocytes. They identified the signal transducer and activator of transcription (STAT1) to be an intracellular signaling and transcriptional target of the apoptotic effect of IGFBP-3 in chondrogenesis (45). Recently, O’Rear et al. (33) have shown that there is cross talk between the IGFBP-3-dependent STAT1 signaling and the TGF-β-dependent ERK pathway that regulate mesenchymal chondroprogenitor cell proliferation and differentiation. In adipocytes, TNF-α is well known to inhibit differentiation (35, 52). Of interest, TNF-α treatment of 3T3-L1 adipocytes induced a 10.6-fold increase in IGFBP-3 mRNA on oligonucleotide microarrays (37). This raises the possibility that IGFBP-3 may mediate inhibition of adipocyte differentiation by TNF-α.

In summary, we have shown that IGFBP-3 interferes with the PPARγ-dependent process of adipocyte differentiation and interacts directly with PPARγ. IGFBP-3 may act as a negative feedback signal in the process of adipocyte differentiation. If endogenous IGFBP-3 plays a functional role in regulating adipocyte differentiation, the possibility exists that IGFBP-3 may be a target for intervention in the treatment of obesity and insulin resistance.
ACKNOWLEDGMENTS
The assistance of Hasanthi de Silva is gratefully acknowledged.

GRANTS
This study was supported by an NHMRC Medical Postgraduate Scholarship (S. Y. Chan) and NHMRC Project Grants 302171 (L. J. Scheldich and R. C. Baxter) and 457373 (S. M. Twigg and R. C. Baxter).

REFERENCES


