Cytokines Promote Wnt Signaling and Inflammation and Impair the Normal Differentiation and Lipid Accumulation in 3T3-L1 Preadipocytes

Received for publication, November 9, 2005; in revised form, January 9, 2006. Published, JBC Papers in Press, February 7, 2006, DOI 10.1074/jbc.M512077200

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Obesity with enlarged fat cells is associated with high local concentrations of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) in the adipose tissue. We examined the effects of this inflammatory state on 3T3-L1 preadipocyte development and differentiation to mature adipose cells. Both IL-6 and TNFα impaired the normal differentiation pattern and lipid accumulation. However, IL-6 allowed a normal early induction of differentiation with inhibition of Wnt10b and Pref-1, whereas expression of C/EBP enhancer-binding protein α, in contrast to peroxisome proliferator-activated receptor-activated receptor γ, was markedly reduced. TNFα also allowed a normal early induction of differentiation, whereas the terminal differentiation to adipose cells was completely prevented. However, both cytokines induced an inflammatory phenotype of the cells but with different profiles. Remarkably, both IL-6 and TNFα maintained and augmented the canonical Wnt signaling associated with low axin and high low density lipoprotein receptor-related protein (LRD), Dishevelled, and β-catenin levels. TNFα, but not IL-6, activated Wnt10b expression, whereas IL-6 increased the apparent phosphorylation of Dishevelled. Thus, both IL-6 and TNFα prevent the normal development of preadipocytes to fully differentiated adipose cells and, instead, promote an inflammatory phenotype of the adipocytes. These results provide an explanation as to why obesity and diabetes are associated with both local and systemic inflammation, insulin resistance, and ectopic lipid accumulation.

Adipose tissue is the major organ for storing and releasing surplus energy. Inability of the adipose cells to take up and store lipids, as seen in lipoatrophic and lipodystrophic conditions, is associated with the accumulation of ectopic triglycerides in the liver and skeletal muscles, insulin resistance, and diabetes (1). Transplantation of fat to animal models of lipoprotein reverses these conditions (2). Interestingly, obesity and insulin resistance are also associated with ectopic lipid accumulation suggesting an insufficient uptake and storage of lipids in the adipose cells in these conditions, as well. The adipose tissue also plays an important role as an endocrine organ secreting different hormones and cytokines that can augment or impair whole-body insulin sensitivity (3).

Accumulation of body fat in adults is initially characterized by an increase in fat cell size followed by an increased cell number and, thus, recruitment of preadipocytes (4–6). Very little is known about factors that regulate the commitment of pluripotent stem cells into the adipose lineage (7, 8). Once committed, the preadipocytes undergo an adipogenic program, which requires a coordinate activation of several pathways initiated by the down-regulation of the inhibitory preadipocyte factor-1 (Pref-1)2 and Wnt proteins (9–11). Induction of the nuclear peroxisome proliferator-activated receptorγ2 (PPARγ2) also plays a crucial role in the early differentiation of the preadipocytes into lipid-accumulating cells (8).

Other key transcription factors are the family of CCAAT/enhancer-binding proteins: C/EBPα, C/EBPβ, and C/EBPδ. Several loss-of-function studies with cells either lacking PPARγ or C/EBPα have shown an absolute requirement for PPARγ for the induction of differentiation, whereas cells lacking C/EBPβ, but expressing PPARγ, can still be induced to accumulate lipids. However, these cells are not the typical insulin-responsive and hormone-secreting adipose cells, because C/EBPα is required for the normal induction of many key adipogenic genes and proteins such as GLUT4, adiponectin, and IRS-1 (12, 13).

During preadipocyte differentiation, a carefully coordinated chain of events occurs where one of the earliest transcription factors to be expressed is C/EBPβ. However, due to its association with CHOP-10, C/EBPβ lacks early DNA-binding capacity (14). Subsequently, C/EBPβ undergoes phosphorylation, achieves DNA-binding capacity, and induces both PPARγ2 and C/EBPα as well as the terminal clonal mitotic expansion (14–16). Transcriptional activation of the C/EBPα gene occurs exceptionally late during differentiation, because repressors prevent premature expression of C/EBPα (17, 18). Once expressed, C/EBPα activates its own gene through C/EBP binding sites in the promoter. This auto-activation is important for the terminal differentiation and maintained C/EBPα expression in the adipocytes (17). C/EBPα is also anti-mitotic, and its expression ends clonal mitotic expansion. At the same time, genes related to the fully differentiated adipocyte are induced.

Interleukin (IL)-6 and TNFα are major inflammatory cytokines that have been linked to the development of insulin resistance and Type 2 diabetes (19). IL-6 is secreted by both adipose cells and inflammatory cells, and high circulating IL-6 levels are related to the future risk of developing Type 2 diabetes in humans (20). In contrast, TNFα is only

2 The abbreviations used are: Pref-1, preadipocyte factor-1; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; Dvl, Dishevelled; PPRE, PPAR response element; GSK, glycogen synthase kinase; MCP-1, monocyte chemoattractant protein-1; Fz, Frizzled; IL-6, interleukin-6; TNFα, tumor necrosis factor α; PCNA, proliferation cell nuclear antigen; MAPK, mitogen-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; sFRP2, secreted Frizzled-related protein 2.

This work was supported by the Swedish Research Council (Grant 2004-72X-03506-33A), the European Community’s FP6 EUGENE2 (Grant LSHM-CT-2004-512013), the Swedish Diabetes Association, the Novo Nordisk Foundation, the Sonya Hedenbratt Memorial Fund, and the Torsten and Ragnar Söderberg Foundations. The authors declare that they have no competing financial interests. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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secreted by inflammatory cells, because the prohormone is not processed and cleaved in the adipocytes by the converting enzymes (21).

Recent studies have shown that the adipose tissue production of IL-6 in vivo is positively related to the size of the fat cells and that the interstitial concentrations are considerably higher than the circulating plasma levels (22). The additional recruitment of macrophages from the blood to the adipose tissue in obesity further creates an inflamed condition (23, 24). The high interstitial concentrations make it likely that IL-6 is an important paracrine/autocrine regulator of the preadipocytes (22). We, therefore, examined the effects of IL-6 at concentrations found to be present in the adipose tissue in obesity (22), as well as TNFα, on the preadipocyte differentiation process using the well characterized 3T3-L1 cells.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of high quality and obtained from commercial sources. Immunoblots were performed with the following antibodies: p18 (N-20), p107 (C-18), p130 (C-20), proliferation cell nuclear antigen (PCNA) (PC10), p21-activated kinase (N-20), C/EBPα (14AA), C/EBPβ (C-19), activator protein (AP2α) (C-18), Sp1 (E-3), p-αp21-activated kinase (Thr 423), PPARγ (H-100), Axin (H-98), dishevelled (Dvl-1) (3F12) (all from Santa Cruz, Biotechnology, Inc., Santa Cruz, CA), MAPK (06-182) (Upstate Biotechnology, Lake Placid, NY), Perilipin (PROGP29), and LRp6 (AF1505) (R&D Systems, Inc.), Phospho-p44/p42 MAPK (Cell Signaling, Beverly, MA and New England Biolabs Ltd., UK), and Bcl-2 and β-catenin (BD Transduction).

Cell Culture Conditions—3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (v/v), 2 mM glutamine, and antibiotics. After 2 days of incubation for 1 h with 5-bromo-2′-deoxyuridine prior to detection, and

Nuclear Extracts—Nuclear extracts were prepared as described previously (25). Briefly, 3T3-L1 cells from one 75-cm² flask were washed, scraped in phosphate-buffered saline buffer, and centrifuged for 10 min at 2,000 × g. The pelleted cells were dissolved in buffer A (25 mM Tris-HCl (pH 7.5), 250 mM KCl, 2 mM MgCl₂, 1 mM EDTA, and 5 mM dithiothreitol), and homogenized in a tight homogenizer. The homogenate was pelleted by centrifugation at 3,300 × g for 10 min and washed twice with buffer. High salt extraction of nuclear proteins was performed by incubation of nuclei with buffer B (25 mM Tris-HCl (pH 7.5), 0.42 M NaCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA, 25% sucrose) for 30 min on ice. The nuclei were pelleted at 20,000 × g for 30 min, and the supernatant was frozen at −80 °C prior to analyses. Protein content was determined using the Bradford protein assay kit (Bio-Rad).

Real-time PCR—RNA was isolated from the cultured cells with RNasea (Qiagen GmbH, Hilden, Germany). Gene expression was analyzed with the ABI PRISM 7900 sequence detection system (TaQMan, Applied Biosystems, Foster City, CA). Gene-specific primers and probes were designed using Primer Express software (Applied Biosystems), and the sequences used are available on request. The real-time PCR reaction was essentially performed as recommended using 100 nM probe, 200 nM of both forward and reverse primers, and 10 ng of total RNA in a final volume of 20 μl. The standard curve method or relative quantification of mRNA levels was plotted as -fold change, generally compared with day 0 (initiation of adipogenesis) when additions were made. 18 S ribosomal RNA was used as endogenous control (Applied Biosystems). Analyses were performed in duplicates, and all experiments were repeated at least three times.

Statistical Analyses—Conventional statistical methods were used to calculate means ± S.E. Student’s t test was used to compare differential gene expression and transcription factor binding between untreated and cytokine-treated samples. A value of p < 0.05 was considered statistically significant.

RESULTS

IL-6, Like TNFα, Reduces Lipid Accumulation and Supports a Proliferative State—In the presence of IL-6, there was a marked inhibition in the early lipid accumulation (4 days) (Fig. 1, A and C) and a clear reduction (20–30%, p < 0.05) in the Oil Red O accumulation was also seen after 8 days (Fig. 1, B and D) as well as after 11 days (data not shown). In addition, many of the preadipocytes maintained a fibroblast-like appearance in the presence of IL-6 (Fig. 1, E and F). Also these fibroblast-like cells accumulated lipids, albeit to a much lesser extent. In the presence of IL-6, the cells were significantly larger (19.4%), although they contained less lipids, were less adherent to the surface, and exhibited a higher migration rate that resulted in cluster formations (see supplemental Fig. S1, A and B), which were obvious 4–6 days after induction of differentiation. Addition of the PPARγ ligand, pioglit-
morphology and size as well as the migration pattern during the adipocyte differentiation process.

We also examined the effect of TNFα on the differentiation pattern. This cytokine was more potent than IL-6 and virtually no lipid accumulation (<5% of the cells) was seen in the presence of as low a concentration as 1.5 ng/ml (Fig. S1, G and J). Thus, early preadipocytes are highly sensitive to the inhibitory effect of cytokines on differentiation.

IL-6 and TNFα Impair the Normal Differentiation Pattern—We then examined the effects of IL-6 on the expression of genes related to the differentiation and development of a normal adipocyte phenotype. Most genes related to adipose cell differentiation and function, such as adiponectin, resistin, perilipin, and Foxc2 were reduced, whereas aP2, which is mainly regulated by PPARγ, was not reduced (Fig. 2, A–E). Also a number of other important genes induced during differentiation and related to insulin action, lipid synthesis, and uptake, such as glucose transfer 4 (GLUT4), insulin receptor substrate (IRS)-1, c-CBL-associated protein (CAP), fatty acid synthase, and lipoprotein lipase (LPL) were markedly reduced in the presence of IL-6 (Fig. 2, F–J). In contrast, IL-6 increased the expression of IL-6 itself as well as MCP-1 and plasminogen activator inhibitor (PAI)-1 (Fig. 3, A–C). In general, the addition of the PPARγ-ligand abrogated the IL-6 effect and normalized CAP and perilipin; this was also verified at the protein level (see supplemental Fig. S3).

We also examined the effect of TNFα which, in addition to the ablated lipid accumulation, also completely prevented the normal differentiation (discussed later). However, the expression of chemo- and cytokines like IL-6 and MCP-1 was dramatically increased (Fig. 3, A–C) and considerably more than for IL-6, whereas specific macrophage markers like F4/80 and MAC-1 were not induced (data not shown). TNFα, in contrast to IL-6, did not show an increase but actually decreased PAI-1 expression (Fig. 3C).

The Early Induction of Adipocyte Differentiation Is Not Altered by IL-6—Because IL-6 (like TNFα) exerts such profound effects on the growth and differentiation of the preadipocytes, we set out to systematically explore the molecular mechanisms involved.

Down-regulation of Pref-1 is required for preadipocytes to undergo adipose conversion. However, Pref-1 mRNA was decreased within hours after induction of differentiation whether or not IL-6 was present (Fig. 4A). Wnt signaling and secretion also play a key role for maintaining the cells in an undifferentiated state (11). However, Wnt10b mRNA, previously shown to inhibit adipocyte differentiation (26), declined rapidly upon induction of differentiation whether or not IL-6 was present (Fig. 4B). We also measured Wnt1 and Wnt3a, but these ligands were not expressed under any conditions used (data not shown).

Activation of the retinoblastoma family proteins, p130 and p107, is directly correlated with the entry into the clonal mitotic expansion phase. Following induction of differentiation, the expression of these proteins is switched with an increase in p107 and a decrease in p130 (27, 28). Again, IL-6 did not interfere with the normal p130/p107 switch (Fig. 4C). Thus, IL-6 did not interfere with these critical initial steps required for the induction of differentiation.

Clonal Mitotic Expansion—C/EBPβ and C/EBPs are expressed immediately after induction of differentiation. Initially, C/EBPβ is inactivated by binding to CHOP-10, a dominant-negative member of the C/EBP family. However, CHOP-10 decreases rapidly and C/EBPβ is released (29, 30). IL-6, like TNFα (data not shown), did not alter the normal down-regulation of CHOP-10, and the mRNA levels remained low until the cells had entered the stage of terminal differentiation (Fig. 4D).
FIGURE 2. IL-6 interferes with the gene expression of adipocyte-specific genes, and genes of importance for insulin signaling and action during differentiation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were differentiated with 20 ng/ml IL-6 and/or 1 μM pioglitazone. A–E, IL-6 reduces the expression of several genes related to cell differentiation. RNA was extracted at different days as shown, and mRNA levels were determined with real-time PCR. The data were first normalized to 18 S rRNA. Then, all mRNA levels were normalized to expression level in the control sample (without IL-6) at each time point. F–J, IL-6 reduces the expression of genes relevant to insulin signaling and action. Differentiation and RNA extraction performed as above. Data are presented as the mean ± S.E. (n = 3–5). Significance levels were tested after 4 and 8 days. *, p < 0.05; **, p < 0.02; and ***, p < 0.002, compared with untreated.
Binding activity of C/EBPβ and -δ coincides with the entry of the preadipocytes into the S phase of the cell cycle and the onset of clonal mitotic expansion. C/EBPβ gene expression peaked after ~24 h, and was similar whether or not IL-6 was present (Fig. 4E), while C/EBPδ mRNA was slightly, but significantly (p < 0.05), increased in the presence of IL-6 but showed a similar time course as the control cells (Fig. 4E). Neither C/EBPβ activating (LAP) or inhibitory (LIP) proteins in nuclear extracts (Fig. 4F), nor phosphorylation (data not shown), were altered by the presence of IL-6. To further verify that C/EBPβ was not changed by IL-6 we examined the binding to C/EBP-binding sites. However, specific binding by nuclear extracts prepared for up to 72 h after induction of differentiation was also not altered by the presence of IL-6 (data not shown). Similarly, TNFα did not inhibit the early induction of either C/EBPβ or C/EBPδ (data not shown).

PPARγ, C/EBPβ, and C/EBPδ Induction Is Normal, whereas C/EBPα Expression Is Reduced by IL-6—An absolute requirement for adipogenesis and lipid accumulation is expression of the PPARγ isoforms (12, 13), PPARγ1 and PPARγ2; the latter only being expressed in the adipose cells. Both C/EBPβ and C/EBPδ are activators of the PPARγ gene, but, once expressed, there is feedback activation between C/EBPβ and PPARγ through C/EBP regulatory elements in the PPARγ promoter (29). We analyzed the binding of PPARγ in nuclear extracts to PPRE during the initial 72 h of differentiation (Fig. 5A). This was not altered by...
FIGURE 5. Induction of the transcription factors PPARγ and C/EBPα and nuclear binding to response elements during initiation of differentiation of 3T3-L1 cells. 3T3-L1 preadipocytes were differentiated with 20 ng/ml IL-6 and/or 1 μM pioglitazone as indicated. A, binding of PPARγ to PPRE binding sites. Nuclear extracts were prepared during initiation of differentiation and binding analyses were performed with ELISA. The data were normalized to the control sample at time 0 h. B, IL-6 reduces C/EBPα but not PPARγ protein expression in nuclear extracts. Addition of 1 μM pioglitazone restored C/EBPα protein expression. Western blotting of nuclear extracts is shown. C, IL-6 reduces C/EBPα mRNA levels during differentiation both in the absence and presence of 1 μM pioglitazone. The data were normalized to 18S rRNA, and expression at time 0 h was set to 1. D, IL-6 decreased the binding of C/EBPα to C/EBP binding sites. Nuclear extracts were prepared during initiation of differentiation, and binding analyses were performed with ELISA. The data were normalized to the control sample at time 0 h. E, sustained activation of the MAPKs p44/42 (ERK1/2). Activation of the MAPKs p44/p42 was detected with a specific phospho-antibody detecting both tyrosine and threonine phosphorylation. MAPK proteins are shown for equal sample loading. F, the transcription repressors, AP2α and Sp1, were released with a similar time course in the presence or absence of IL-6. Western blotting of nuclear extracts is shown. Each bar represents the mean ± S.E. (n = 2–5). *, p < 0.05; ***, p < 0.002, compared with untreated.
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FIGURE 6. Maintained activation of the canonical Wnt signaling pathway in the presence of cytokines. 3T3-L1 preadipocytes were differentiated in the presence of 20 ng/ml IL-6 or 1.5 ng/ml TNFα, and/or 1 μM pioglitazone, or 200 ng/ml sFRP2. A, sFRP2, an antagonist of the canonical Wnt signaling, could not prevent the effect of IL-6 on β-catenin expression. TNFα also activated the canonical Wnt signaling, and this was also unaffected by the presence of sFRP2. Effect of TNFα on cyclin D1 protein expression is shown below for comparison. Expression of β-catenin was analyzed by Western blotting. B, IL-6 and TNFα sustained the Dishevelled-1 (Dvl-1) protein expression. Dvl-1 was analyzed by Western blotting. Two major bands were recognized by the antibody, the upper band appears to be phosphorylated Dishevelled (pDvl-1). C, normal induction of Axin was prevented by IL-6 and TNFα. Axin was analyzed by Western blotting. D, maintained expression of LRP6 in the presence of IL-6 and TNFα. Addition of sFRP2 did not reduce the protein expression of LRP6. Western blotting of LRP6 is shown.

the presence of IL-6, also supported by the finding that induction for both PPARγ1 and -2 proteins was normal (Fig. 5B).

After a delay of ~30 h after the induction of differentiation, C/EBPα is transactivated by C/EBPβ and C/EBPδ through C/EBP regulatory elements in the C/EBPα promoter (31). PPARγ also induces transactivation of C/EBPα through PPRE in the C/EBPα promoter. C/EBPα gene expression peaked at 96 h (Fig. 5C), and this late activation corresponds to induction of terminal differentiation and the anti-mitotic effect of C/EBPα (31–33). The induction of C/EBPα mRNA expression, 24–48 h and later, was markedly reduced by the presence of IL-6 (Fig. 5C). Similarly, C/EBPα protein expression in whole cell lysates (data not shown), as well as in nuclear extracts (Fig. 5B), was reduced by ~50–70%. Addition of pioglitazone did not prevent the inhibitory effect of IL-6 on C/EBPα gene activation (Fig. 5C), whereas the protein expression was clearly increased (Fig. 5B).

The binding of C/EBPα in nuclear extracts to the C/EBP binding sites was markedly decreased 48 h and later during the differentiation process (Fig. 5D). Protein dilution experiments (data not shown) indicated that nuclear extracts from IL-6-exposed cells contained one or more factors that altered the normal binding of C/EBPα, but the nature of this remains to be identified.

These data show that the early process leading to the induction of differentiation and activation of C/EBPβ, C/EBPδ, and PPARγ2 are not altered by IL-6, whereas the binding and activation of C/EBPα is markedly reduced. Although TNFα did not inhibit the early induction of C/EBPβ or C/EBPδ, activation of both PPARγ and C/EBPα was completely inhibited (see below).

MAPK Activation and Cyclin-dependent and p21-activated Kinases—The presence of IL-6 both increased and sustained the phosphorylation of ERK1/2 (p44/p42) for 4–6 days following the induction of differentiation (Fig. 5E). This effect was not related to an altered ERK protein expression (Fig. 5E) and was also seen in the presence of the PPARγ ligand. When preadipocytes exit the cell cycle and proceed into the differentiation step, the post-mitotic growth arrest is characterized by an increase in the cyclin-dependent kinase inhibitors, p18 and p21 (32), which are dependent on C/EBPα protein interactions (34). p21 was not expressed in 2-day confluent preadipocytes, as expected, whereas p21 was still observed at day 8 in the presence of IL-6 when the cells should be fully differentiated adipocytes (see supplemental Fig. S4A). Furthermore, the low expression of p18 in the presence of IL-6 further supports that the 3T3-L1 preadipocytes still are in the proliferating mode (see supplemental Fig. S4B). Similarly, p21-activated kinase-1 protein and phosphorylation remained increased during the differentiation in the presence of IL-6 (see supplemental Fig. S4A).

The carboxyl terminus of p21 contains binding sites for proliferation cell nuclear antigen (PCNA) and cyclins. Binding of p21 to PCNA inhibits DNA replication and, thereby, inhibits proliferation (35). In nuclear extracts from cells during the S phase, PCNA was clearly increased in the presence of IL-6, which is in accordance with a proliferative state (see supplemental Fig. S4B). The addition of the PPARγ ligand reduced PCNA expression but did not normalize it in the presence of IL-6 (see supplemental Fig. S4B).

Taken together, these data show that IL-6 maintained the cells in a proliferative mode, which is an expected consequence of the reduced C/EBPα expression. These findings can explain the increased motility and altered morphology of the cells in the presence of IL-6 (cf. supplemental Fig. S1).

C/EBPα Promoter—The C/EBPα promoter possesses several repressive regulatory elements. Two C/EBP undifferentiated protein-1 elements are located on either side of the C/EBP binding site keeping the promoter in an inactive state prior to differentiation. AP-2α binds to the C/EBP undifferentiated protein-1 sites and represses activation of C/EBPα in preadipocytes until initiation of differentiation when AP-2α
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decreases rapidly (31). The AP-2α protein levels in nuclear extracts
decreased with a similar time course both in the presence and absence of
IL-6 (Fig. 5F).

The transcription factor Sp1 also serves as a repressor of the C/EBPα
promoter. The Sp binding site overlaps the C/EBP binding site and
competitively blocks the activation (17). Following induction of differ-
etiation, Sp1 decreases and the C/EBPα promoter can first be activated by
C/EBPβ/δ and later autoactivated (31). As for AP-2α, Sp1 decreased
with a similar time course in the absence or presence of IL-6 (Fig. 5F).
Thus, IL-6 did not inhibit the activation of the C/EBPα promoter
through an impaired regulation of the repressors AP-2α and Sp1.

Because the C/EBPα promoter also contains at least one CCAAT box
for NF-Y, which can act as a repressor and stabilize the binding of other
proteins to DNA (36), we examined if IL-6 interferes with the binding of
NF-YA to the specific binding sites but no such effect was found (data
not shown).

IL-6, Like TNFα, Sustains the Canonical Wnt Signaling Pathway in
Preadipocytes—Cellular β-catenin remains elevated until after
induction of PPARγ and C/EBPα, when it is degraded in the APC-axin
degradation complex following phosphorylation by GSK3 and casein
kinase-1 (37, 38). Activation of GSK3β is also required for adipogenesis,
because treatment of preadipocytes with inhibitors of GSK3β blocks
differentiation and PPARγ and C/EBPα induction (39).

The presence of IL-6 did not alter the normal down-regulation of
Pref-1 or Wnt10b (Fig. 4, A and B) or increase other Wnt molecules
(Wnt1 and 3a) inferred to play a role in preadipocytes (26). However,
β-catenin remained elevated in the presence of IL-6 both in cytoplasmic
(Fig. 6A) and nuclear extracts (data not shown) throughout the
total differentiation period. The presence of the PPARγ ligand opposed this
effect of IL-6, whereas the addition of the Wnt signaling antagonist (40),
secreted Frizzled-related protein 2 (sFRP2), had no effect (Fig. 6A).

TNFα was even more powerful in increasing the β-catenin expres-
sion, and, again, this was not opposed by the presence of sFRP2 (Fig. 6A).
TNFα also increased the expression of cyclin D1 (Fig. 6A), a marker of
T cell factor/lymphoid enhancer factor (TCF/LEF) activation.

Both IL-6 and TNFα maintained a high expression of the important
Wnt signaling molecule, Dishevelled (Dvl), in the cells throughout the
differentiation period (Fig. 6B). Interestingly, the motility pattern of Dvl
in the presence of IL-6 suggests an increased phosphorylation of this
molecule. Casein kinase-1ε, which has been shown to phosphorylate
Dvl (41), was also found to co-immunoprecipitate with Dvl (data not
shown).

We also examined the expression of axin and the LRP co-receptor during
the differentiation. Axin is normally reduced during activated Wnt
signaling, and this was indeed also the case in the presence of IL-6 or TNFα (Fig.
6C). Axin levels remained low in the presence of IL-6 and were even further
reduced in the presence of TNFα (Fig. 6C). Again, sFRP2 had no effect on
axin expression. The reduced axin protein expression was not due to
any change in mRNA levels (data not shown) supporting that it was caused by
axin protein destabilization. LR6P6 expression showed a similar pattern as
β-catenin (Fig. 6D). Together, these findings show that β-catenin stabiliza-
tion by IL-6 and TNFα was accomplished through both Dvl activation and
axin destabilization.

It is clear, however, that there were important differences between
the effects of IL-6 and TNFα on Wnt signaling. TNFα, in contrast to
IL-6, maintained high Wnt10b and Pref-1 expression in the preadipo-
cytes (Fig. 7, A and B), whereas no effect was seen on Wnt1 or 3a (data
not shown). TNFα, also in contrast to IL-6, completely prevented the
activation of both PPARγ2 and C/EBPα (Fig. 7C).

![Figure 7](image)

FIGURE 7. The presence of TNFα keeps the cells in an unindifferentiated state and prevents activation of both PPARγ2 and C/EBPα. A 3T3-L1 preadipocytes were differentiated in the absence or presence of 1.5 ng/ml TNFα. A, Wnt10b mRNA levels were only transiently reduced in the presence of TNFα. mRNA levels were determined with real-time PCR. The data were first normalized to 18 S rRNA. Then, all mRNA levels were normalized to expression level
in the control sample (without TNFα = 1) at each time point. Compare with control sample in Fig. 3B. B, Pref-1 mRNA levels were also transiently reduced in the presence of TNFα. Real-
time PCR was performed as in A. Compare with control sample results in Fig. 3A. C, TNFα completely prevented the induction of C/EBPs and PPARγ2. Whole cell extracts were analyzed by Western blotting. Each bar represents the mean ± S.E. (n = 3). *, p < 0.05; **, p < 0.02, compared with untreated.

Together, these findings show that preadipocytes are highly sensitive
to cytokines, which can keep the cells in an unindifferentiated, or partly
differentiated, state by maintaining Wnt signaling and preventing a normal
PPARγ and/or C/EBPα induction. Instead, the cells are differenti-
ted toward an inflammatory phenotype. There is a window during
which these cells can activate Wnt signaling in response to cytokines,
because the β-catenin levels were not increased if cytokines were added
4 days or later after induction of differentiation (data not shown).

DISCUSSION

IL-6, like TNFα, is an important pro-inflammatory cytokine and is
considered to play a major role for both the induction of inflammation
as well as insulin resistance in obesity. Addition of IL-6, and even further
with TNFα, induced dramatic effects on the terminal differentiation
program and produced cells with both reduced lipid accumulation and
reduced expression of several genes of importance for normal metabo-
lism, insulin sensitivity and action, like IRS-1, GLUT4, adiponectin,
lipoprotein lipase, fatty acid synthase, and perilipin. In contrast, the cells
assumed an inflammatory phenotype. However, the effect of the cyto-
kines was not overlapping, because TNFα dramatically increased
MCP-1 and IL-6 while IL-6 moderately increased these cytokines but, in
contrast, induced PAI-1. The cytokine-induced impairment in differen-
tiation and lipid accumulation of developing adipocytes combined with
an increased lipolysis; an expected consequence of the reduced perilipin
expression as shown for TNFα (42), would favor whole body insulin
resistance and the accumulation of ectopic lipids in the liver and skeletal
muscles, because lipids cannot be normally taken up and stored in the
adipose cells.

The early program for induction of differentiation, defined as activa-
tion of the C/EBPβ and C/EBPα genes, was not changed by either TNFα
or IL-6. IL-6 also allowed a normal induction of PPARγ1 and PPARγ2,
whereas this was not seen with TNFα. Several markers indicated that
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FIGURE 8. Schematic representation of the cytokine-induced effect on the Wnt signaling pathway and the downstream events leading to adipocyte differentiation. Growth-arrested preadipocytes were induced to differentiate by hormonal stimulation (MDI), TNFα sustains Wnt activation while IL-6 sustains activation of the LRP-Dishevelled complex, and, thereby, both cytokines prevent the normal degradation of β-catenin. At the downstream level, adipocyte differentiation is impaired (IL-6) or prevented (TNFα) through the inhibition of the normal induction of C/EBPα as well as PPARγ (TNF only).

The cells remained in a proliferative state in the presence of IL-6, and the increased expression of Bcl-2 further supports its mitogenic and anti-apoptotic effects in 3T3-L1 cells, like in several neoplastic cells such as myeloma and prostatic cancer cells (43).

Most of the identified effects of IL-6 on growth and differentiation of the 3T3-L1 cells can be attributed to the impaired expression of C/EBPα. We examined if IL-6 influenced the normal induction of C/EBPα via the known repressors of activation but found no evidence of this. However, at the critical time of 24–48 h after induction, when C/EBPα should be induced to allow a growth-arrested state, IL-6 maintains signal(s) that preclude the normal binding of C/EBPα to C/EBP binding sites and the activation of the promoter. Remarkably, we found that the canonical Wnt signaling, which keeps the cells undifferentiated, remained activated during the differentiation process in the presence of either IL-6 or TNFα. Direct evidence for an activation of the canonical Wnt pathway by both cytokines was obtained by the maintained expression of Dishevelled, LRP, low axin, and high β-catenin levels.

The secreted Wnt ligands bind to the Frizzled receptors and cross-link these with the co-receptor LRP, which serves as a high affinity docking site for axin (44), preventing β-catenin from being degraded in the axin-adenomatous polyposis coli (APC) degradation complex. Wnt10b, which has been shown to be produced by preadipocytes and to prevent adipocyte differentiation (26, 45), did not show an altered expression profile in the presence of IL-6, whereas it was elevated by TNFα. Wnt1 has also been shown to block adipogenesis (26), but this ligand, like Wnt3a, was not expressed in 3T3-L1 cells. Our results suggest that the major effect of TNFα is to maintain Wnt 10b secretion, thus keeping the cells in an undifferentiated state with regards to the normal adipocyte phenotype while, instead, driving the induction of inflammatory proteins. However, a clear transdifferentiation to macrophages was not seen under these conditions, at least when measured as expression of specific markers like F4/80 and MAC-1.

Activation of PPARγ is important for terminating Wnt signaling by stimulating the proteasomal degradation of β-catenin, and this is dependent on GSK3β activity (39). However, the expression of PPARγ, as well as the binding of nuclear extracts to PPRE, did not show any difference in the presence and absence of IL-6. Thus, it is likely that IL-6 maintains Wnt signaling by directly activating this pathway and Dishevelled and the LRP co-receptors (46) appear likely targets.

Dishevelled plays an important role in Wnt signaling by functioning as a molecular chaperone to deliver axin to the Frizzled-LRP complex (44), and it may also enhance the binding affinity between LRP and axin by promoting phosphorylation of LRP (47). It has also been shown that activated LRP and Dishevelled can recruit Frat/GBP, a strong inhibitor of GSK3 (37, 48). Phosphorylation of Dishevelled, possibly by casein kinase-1ε (37), enhances the binding to Frat-1 and inhibition of GSK3 and, thus, maintains the canonical Wnt signaling cascade. Activation of Wnt signaling also prevents the normal activation of C/EBPα, but not C/EBPβ and C/EBPδ (38), as also found here for both IL-6 and TNFα. However, the surprising finding that PPARγ expression and binding to PPRE were not changed by IL-6 is a sign of an unexpected selectivity in Wnt signaling. Selectivity in Wnt signaling effects, both through the canonical and non-canonical pathways, have also recently been reported in differentiating 3T3-L1 cells (49). Additionally, it was shown (38) that a GSK-selective mutant of β-catenin (S37A) inhibited a select set of adipogenic genes and that aP2 remained unchanged, whereas adiponectin, another marker of differentiation, was markedly inhibited. The complexity of the Wnt signaling cascade should enable the activation and/or repression of many specific pathways and targets.

Addition of the PPARγ ligand, pioglitazone, markedly improved C/EBPα protein expression and the activation of the adipogenic genes in the presence of IL-6. Consistent with this, we also found that the β-catenin protein levels were reduced, probably through an increased proteosomal degradation (50). Thus, one important mechanism for the ability of PPARγ ligands to drive differentiation of preadipocytes is by inhibiting the TCF/LEF transcriptional activation by reducing the cellular β-catenin levels.

In summary, we have shown that IL-6 and TNFα increase the expression of inflammatory genes, whereas the normal adipocyte differentiation and lipid accumulation are inhibited together with a maintained Wnt signaling. A schematic summary of the sites where the cytokines interfere with the normal differentiation process is shown in Fig. 8. In an in vivo setting, these findings can help explain why conditions like obesity and Type 2 diabetes are associated with increased local and systemic inflammation, insulin resistance, and ectopic lipid accumulation. Interestingly, we have recently found that insulin resistance is characterized by an altered expression in the adipose tissue of several genes related to adipose cell differentiation such as GLUT4, IRS-1, adiponectin, and C/EBPα (51). Whether this also is a consequence of an increased Wnt signaling is currently under intense study.

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