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EFFECTS OF THE HIV-1 PROTEASE INHIBITOR RITONAVIR ON PREADIPOCYTE DIFFERENTIATION

by

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

HIV-1 protease inhibitor therapy is associated with a novel lipodystrophy syndrome characterized by truncal adiposity, peripheral fat atrophy, dyslipidemia, and type 2 diabetes. The increase in truncal fat may be due to increase in adipocyte number as a result of enhanced preadipocyte differentiation. We show that addition of 10 μg/ml ritonavir to standard differentiation medium enhanced 3T3-L1 preadipocyte differentiation as measured by a 30% increase in triacylglycerol (TG) accumulation and a 50% increase in glycerol 3-phosphate dehydrogenase (GPDH) activity. Although ritonavir partially inhibited protein expression of peroxisome proliferator activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα), and the aP2 gene (which encodes the adipocyte lipid binding protein), it resulted in higher levels of the active form of adipocyte determination and differentiation-dependent factor-1 (ADD-1), also known as sterol regulatory element binding protein 1 (SREBP-1). The enhancing effects of ritonavir on late events of 3T3-L1 preadipocyte differentiation may be mediated by ADD-1/SREBP-1 which has been shown to directly activate transcription of several genes encoding lipogenic enzymes. Preliminary results suggest that ritonavir preferentially enhances differentiation of human preadipocytes derived from abdominal omental but not subcutaneous adipose tissue in primary culture.

DEDICATION

For MyTho

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I. INTRODUCTION

A. HIV-1 Protease Inhibitor-associated Lipodystrophy Syndrome

The use of protease inhibitors in the treatment of HIV-infected patients resulted in dramatic and significant improvements in morbidity and mortality by causing profound and sustained suppression of viral replication (1). However, protease inhibitor therapy was unexpectedly associated with a novel lipodystrophy syndrome, characterized by truncal adiposity, dorsocervical fat accumulation, breast hypertrophy in women, peripheral lipoatrophy, type 2 diabetes, and dyslipidemia (2). Subsequently, the syndrome was detected in a low percentage of HIV-1 infected patients who had never received protease inhibitor therapy (3,4). This suggests that protease inhibitors are not necessarily the sole cause of the syndrome and that they may aggravate an underlying predisposition to develop lipodystrophy perhaps due to the HIV infection. The development of the syndrome has led to avoidance or termination of protease inhibitor therapy, potentially compromising the care of patients with HIV infection.

Fat accumulation is normally due to an increase in adipocyte number as a result of preadipocyte differentiation, as well as an increase in size of existing adipocytes (5,6). The fat increase in the truncal areas described in the lipodystrophy syndrome may be due to enhancement of these processes. Although the underlying mechanism of the syndrome is not yet known, hypotheses have been proposed to explain these deleterious changes in adipose tissue distribution and metabolism.

Carr *et al.* reasoned that the active site of HIV-1 protease may have homology to regions present in mammalian proteins. HIV-1 protease inhibitors were designed to bind to this catalytic site with high affinity to inhibit protease activity (7). Their hypothesis is that the lipodystrophy syndrome may be due to protease inhibitors binding and modulating human protein(s) involved in lipid metabolism (2). Database searches that they conducted revealed that the 12 amino acid sequence located in the catalytic region of the HIV-1 protease shares 63% and 58% similarity to a region incorporating a lipid-binding domain in the low density lipoprotein-receptor-related protein (LRP) and the C-terminal region of the cytoplasmic retinoic-acid binding protein type 1 (CRABP-1), respectively (2).

CRABP-1 binds almost all intracellular retinoic acid and presents retinoic acid to cytochrome P450 isoforms that catalyze the conversion of retinoic acid to cis-9-retinoic acid (8,9). Cis-9-retinoic acid is the only ligand of the retinoid X receptor α (RXRα) which heterodimerizes with peroxisome proliferator-activated receptor γ (PPARγ), an adipogenic transcription factor (10,11). Ligand binding to these receptors has been shown to increase preadipocyte differentiation (12,13,14,15). Moreover, it has been shown that such agonists improve abnormal insulin sensitivity and hyperlipidemia, both features of the lipodystrophy syndrome (15,16).

Binding of CRABP-1 to retinoic acid may be mediated by up to 19 amino acid residues (17). Some of the binding residues are located within or immediately adjacent to the sequence that shares homology with the active site of the HIV-1 protease (2). These residues play an important role in providing the proper conformation for CRABP-1 to bind retinoic acid (10). It is hypothesized that binding of HIV-1 protease inhibitors to

CRABP-1 within the homologous region inhibits binding of retinoic acid. This would lead to a decrease in the amount of retinoic acid being presented to cytochrome P450 3A (P450 3A) by CRABP-1, and subsequently a decrease in cis-9-retinoic acid production by P450 3A. In addition, it is known that HIV-1 protease inhibitors are potent inhibitors of P450 3A (18,19,20,21). Lower levels of cis-9-retinoic acid as a result of impaired CRABP-1 function and P450 3A inhibition would cause decreased RXRα activity, leading to reduced differentiation. The decrease in differentiation and increase in apoptosis would give rise to hyperlipidemia as a result of reduced triacylglycerol (TG) storage and high levels of lipid released into circulation (2). The changes in fat metabolism would be partially proportional to the degree of P450 3A inhibition by protease inhibitors. In fact, lipodystrophy appears worst in patients receiving ritonavir, the most potent P450 3A inhibitor (2).

The other mammalian protein identified by Carr *et al.* that contains a region with homology to the HIV-1 protease catalytic site is low density lipoprotein-receptor-related protein (LRP), a hepatic receptor important for post-prandial chylomycron clearance (22,23). LRP is also co-expressed with lipoprotein lipase (LPL), an enzyme responsible for the uptake of circulating lipoproteins, on capillary endothelium of adipose tissue. The LRP-LPL complex cleaves fatty acids from circulating TG, permitting entrance of free fatty acids into adipocytes for storage as triacylglycerol (24). The short sequence of amino acids present in LRP which shares homology with the active site of HIV-1 protease is a lipid binding domain. Binding of protease inhibitors to hepatic and endothelial LRP would exacerbate hyperlipidemia by preventing uptake of circulating lipoproteins (2).

In short, Carr *et al.* proposed that peripheral fat loss and hyperlipidemia are due to HIV-1 protease inhibitors possibly binding to and modulating CRABP-1, cytochrome P450 3A, and LRP function. Hyperlipidemia may also lead to insulin resistance by interfering with post-receptor insulin signalling, competition between glucose and lipid oxidation pathways in skeletal muscle, or inhibition of glycogen synthase (25,26,27,28). To explain truncal adiposity in the syndrome, Carr *et al.* suggested that abdominal fat expansion may occur by default in the presence of peripheral fat atrophy and hyperlipidemia caused by CRABP-1 inhibition, and breast fat gain may be a result of lipid accumulation in the presence of estrogen. Carr *et al.* further reasoned that abdominal and breast fat deposition may occur independently of LRP-LPL or LRP-LPL inhibition by protease inhibitors may be incomplete. Although the proposed hypothesis works well as a first attempt to explain the protease inhibitor-associated lipodystrophy, there are no published data to support it.

Contrary to Carr's hypothesis, our laboratory has previously reported that addition of the HIV-1 protease inhibitor ritonavir to differentiation medium enhances preadipocyte differentiation (29). The 3T3-L1 cell line model was used in this study, and differentiation was assessed by the morphologic appearance of adipocytes staining positive for Oil Red O (neutral lipid stain).

B. 3T3-L1 Preadipocyte Differentiation – A Cell Culture Model

1. Overview

The 3T3-L1 preadipocyte cell line, originally selected from disaggregated mouse embryo cells for their ability to accumulate cytoplasmic triacylglycerol (TG) droplets (30), is an established and widely used model of preadipocyte differentiation. Preadipocytes are flat in shape and resemble fibroblasts in appearance. The mature adipocytes express adipocyte-specific genes and a rounded-up morphology with TG droplets staining positive for Oil Red O. Detailed electron micrographs show that mature 3T3-L1 adipocytes display virtually all of the ultrastructural features of adipocytes *in situ* (31). Evidence suggests that the 3T3-L1 model is a faithful representation of *in vivo* preadipocyte differentiation (32). Subcutaneous implantation of the related 3T3-F442A preadipocytes in mice, at a site normally lacking adipose tissue, leads to the development of mature fat pads that are histologically indistinguishable from white adipose tissue (33,34,35).

3T3-L1 preadipocyte differentiation is induced at confluence with insulin, dexamethasone, and isobutylmethylxanthine (IBMX). Insulin is maintained throughout the entire differentiation protocol of eight days, whereas dexamethasone and IBMX are added only in the first forty-eight hours. Dexamethasone, a synthetic glucocorticoid analog, has been shown to promote preadiocyte differentiation by repressing transcription of pref-1 (preadipocyte factor-1) (36). Pref-1, a recently described preadipocyte protein, has been hypothesized to be involved in the maintenance of the preadipocyte phenotype.

Its protein expression is completely downregulated during preadipocyte differentiation (37,38,39). Dexamethasone also activates CCAAT/enhancer binding protein (C/EBP) δ, C/EBPδ, an important adipogenic transcription factor of the C/EBP leucine zipper protein family (40). Likewise, the cAMP phosphodiesterase inhibitor IBMX which raises intracellular levels of cAMP, is a direct inducer of C/EBPβ (40). Induced early in the differentiation process, expression of C/EBPβ and C/EBPδ diminishes during the terminal phase of differentiation (30). These transcription factors play early catalytic roles in the differentiation pathway, relaying the effects of dexamethasone and IBMX in a cascade-like fashion, leading to activation of C/EBPα expression, discussed below (40).

An early sign of preadipocyte differentiation is the expression of lipoprotein lipoase (LPL) mRNA (41). LPL expression occurs spontaneously at confluence and is independent of the addition of differentiation inducers. This suggests that LPL expression may reflect the growth-arrest stage rather than being an early differentiation phase. LPL as a definitive early marker of preadipocyte differentiation still raises questions because its expression is not dependent on differentiation inducers.

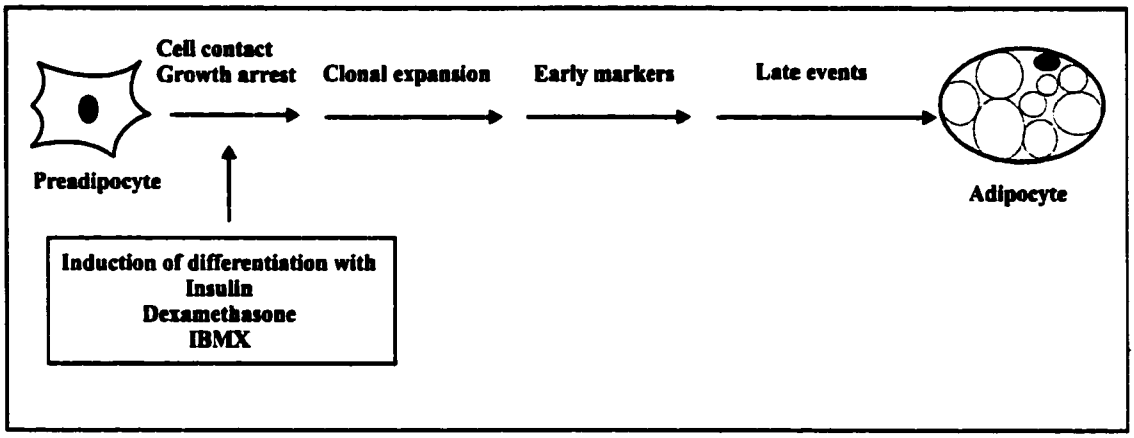
Wnt, a signalling protein that regulates developmental processes, has recently been shown to be a molecular switch that governs preadipocyte differentiation. *Wnt* signalling maintains preadipocyte phenotype by inhibiting expression of the adipogenic transcription factors C/EBPα and the peroxisome proliferator activated receptor γ (PPARγ). Downregulation of *wnt* expression is essential in the induction of preadipocyte differentiation (35).

The early stage of differentiation also shows changes in gene expression of cytoskeletal and extracellular matrix (ECM) proteins (42). These changes include a

decrease in actin and tubulin expression, a switch in collagen gene expression, and increased production of soluble and cell-associated chondroitin sulfate proteoglycan-I (41). Pericular fibronectin and cellular synthesis of fibronectin decreases four- to five-fold during 3T3-F442A preadipocyte differentiation (43). Specific interactions between the cell and the ECM as a result of changes in gene expression may be necessary for cytoskeletal network rearrangement and intracellular cascade of signal transduction that influences differentiation (44). A secondary effect of the ECM is its ability, through altering cell spreading, to expose the cell surface to various growth factors required for the differentiation process (45,46).

During the late phase of differentiation, 3T3-L1 adipocytes acquire adipocyte-specific genes, enzymes and regulatory proteins necessary for de novo lipogenesis as well as TG accumulation (41). There is a marked increase in insulin receptor number, insulin sensitivity, and induction of the adipocyte-specific glucose transporter (GLUT) 4 (47). Leptin secretion is increased during *in vitro* preadipocyte differentiation although its level is much lower than that detected in adipose tissue (48). Differentiation of 3T3-L1 preadipocytes in the presence of indomethacin and insulin yields higher levels of leptin when compared to the traditional differentiation protocol using insulin, dexamethasone, and IBMX (49).

Figure 1. Overview of the 3T3-L1 preadipocyte differentiation model.


Cell contact
Growth arrest
Clonal expansion
Early markers
Late events
Preadipocyte
Adipocyte
Induction of differentiation with
Insulin
Dexamethasone
IBMX

2. Clonal expansion

At confluence, 3T3-L1 preadipocytes temporarily exit the cell cycle (41). Growth arrest at confluent cell density appears to be a prerequisite for subsequent preadipocyte differentiation (41). Upon induction of differentiation, preadipocytes undergo several rounds of mitoses termed clonal expansion which lasts approximately 2 days (30). It has been suggested that mitosis during clonal expansion leads to clonal amplification of committed cells (50). As well, evidence suggests that DNA replication alters the accessibility of promoter control elements to *trans*-acting factors that transcriptionally activate or de-repress regulatory genes that initiate differentiation (30).

The mitotic rounds during clonal expansion appear necessary for preadipocytes to enter a unique growth-arrested stage that is required for subsequent differentiation (30). However, DNA replication during clonal expansion is not sufficient for preadipocytes to progress to the next phase of differentiation, as certain mitogens such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) stimulate post-confluent mitosis but do not induce differentiation (30). Preadipocytes that have undergone post-confluent mitosis appear to be at a crossroads in the differentiation program. Mitogenic stimulation or replating cells at low density induces cell division and ceases differentiation, while under appropriate culture conditions, preadipocytes enter terminal differentiation (51). *c-Myc*, known to be important for promoting cell growth (52), has been implicated in the mitogenic response of preadipocytes during clonal expansion (53). 3T3-L1 preadipocytes constitutively expressing *c-myc*, when treated with differentiation-inducing agents, undergo mitotic clonal expansion followed by growth arrest but do not

differentiate. Expression of antisense *c-myc* in these cells reverses the inhibitory effect of *c-myc* and induces differentiation upon treatment with differentiation medium. Thus, *c-myc* may act as a molecular switch instructing 3T3-L1 preadipocytes to enter either a pathway of proliferation or terminal differentiation.

Recent data indicate that proliferation during cell growth and clonal expansion is differentially regulated. Messenger RNA and protein levels of the retinoblastoma (Rb) proteins p130 and p107 have been shown to be tightly regulated during preadipocyte differentiation (54). In preadipocytes, expression of p130 is detected but not p107. As clonal expansion occurs, p107 mRNA and protein levels increase whereas p130 protein expression decreases. The p130:p107 switch correlates with the ability of 3T3-L1 preadipocytes to undergo hormonally induced mitotic clonal expansion rather than differentiation, as similar changes in expression of these proteins occur during clonal expansion of 3T3-C2 cells which do not differentiate in response to hormonal stimulation (55). Furthermore, the change in p107 and p130 expression appears specific to clonal expansion since this change is not detected during serum-stimulated cell growth. As preadipocytes permanently exit the cell cycle and enter the terminal differentiation phase, the p130:p107 switch is inactivated and once again p130 is predominantly expressed (54).

Distinct expression pattern of other genes are also observed during clonal expansion. Growth arrest specific (*gas*) 6 is preferentially expressed during post-confluent clonal expansion, whereas *gas*3 and *gas*1 are expressed in serum-starved preadipocytes (56). FKBP51, an intracellular receptor for the immunosuppressive drug FK506, transiently increases during clonal expansion. FK506, which does not exert

effect on clonal expansion and preadipocyte differentiation, reverses the inhibitory effect of rapamycin, another immunosuppressant that shares intracellular receptors (FKBPs) with FK506 (57,58).

C/EBPβ and C/EBPδ, induced by dexamethasone and IBMX early in the differentiation program during clonal expansion, play a role in the induction of differentiation (40). Studies have shown that ectopic expression of a truncated dominant-negative isoform of C/EBPβ inhibits differentiation. Furthermore, ectopic expression of C/EBPβ in NIH-3T3 cells induces capability of these cells to differentiate into adipocytes upon hormonal stimulation.

Induction of C/EBPβ and C/EBPδ leads to expression of the adipogenic transcription factor PPARγ (peroxisome proliferator activated receptor γ). Co-expression of C/EBPβ and C/EBPδ gives rise to higher levels of PPARγ mRNA, suggesting that C/EBPβ and C/EBPδ heterodimerize and directly regulate PPARγ expression (59). In fact, two binding sites for C/EBP have been identified in the PPARγ2 promoter (60). In addition, C/EBPβ expression in preadipocytes, alone or in combination with C/EBPδ, induces differentiation when these cells are treated with a PPARγ activator (59,61). These data suggest that there is a regulatory loop between C/EBPs and PPARγ.

The decreased expression of C/EBPβ and C/EBPδ at the end of clonal expansion is concomitant with the induction of another member of the C/EBP family, C/EBPα (40). Delayed expression of C/EBPα may be due to the slow acquisition of C/EBPβ/δ binding ability (62). Although expressed early in the differentiation program, C/EBPβ and C/EBPδ are not immediately active. These transcription factors acquire the ability to

bind to the C/EBP regulatory element in the C/EBPα gene promoter 40 hours after exposure to adipogenic inducers. As they become binding-competent, C/EBPβ and C/EBPδ are localized to centromeres. This localization occurs through C/EBP concensus-binding sites in centromeric satellite DNA.

C/EBPα, a DNA-binding protein, has been shown to play a role in the co-ordinate transcriptional activation of adipocyte-specific genes. C/EBPα, which is antimitogenic, becomes centromeric-associated as clonal expansion ceases and the cells become terminally differentiated (62). The presence of C/EBPα may allow continued expression of PPARγ, which together with C/EBPα, activate expression of genes that give rise to the mature adipocyte phenotype.

In addition to their role in transcriptionally activating adipocyte-specific genes, PPARγ and C/EBPα have been shown to promote preadipocyte differentiation by suppressing cell growth (63). 3T3-L1 preadipocyte differentiation induced by ectopic expression of either PPARγ, or C/EBPα, or both, is blocked by retinoic acid. However, in systems overexpressing PPARγ and C/EBPα, retinoic acid is only inhibitory when added prior to clonal expansion. These results suggest that co-expression of PPARγ and C/EBPα, together with other factors regulating the cell cycle, induces a permanent exit from the cell cycle and establishes irreversible commitment to differentiation.

3. *Adipogenic transcription factors*

a. *PPARγ*

PPARγ, a nuclear hormone receptor, was identified as a component of the adipocyte regulatory factor 6 which binds to the enhancer of the adipocyte-specific gene aP2 (64,65). PPARγ, with its heterodimerization partner retinoid X receptor α (RXRα), binds to DR-1-like (direct repeat of the hormone response element separated by one base) sequences in this enhancer. The binding of ligands to nuclear hormone receptors has been shown to significantly augment their transcriptional activity. Both natural and synthetic ligands of PPARγ have been identified. A possible endogenous ligand for this receptor is the compound 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15-d-PGJ2) (66,67). Synthetic ligands of PPARγ belong to a class of drugs termed thiazolidinediones (TZD) which increase insulin sensitivity (66,68).

PPARγ1 and PPARγ2 differ in their amino termini as a result of alternative splicing (60). PPARγ1 is expressed at low levels in several tissues whereas PPARγ2 is abundantly and more specifically expressed in adipocytes (60). Studies suggest that PPARγ2 is more robust in regulating gene expression due to the amino terminus's ability to increase activity of the ligand-independent transactivation domain (69). PPARγ3 mRNA, recently identified, encodes a protein identical to PPARγ1 although it is transcribed from an independent promoter region (70). Currently, tissue distribution of PPARγ3 is not known. PPARγ has been shown to play an important role in preadipocyte differentiation. Its ectopic expression and activation causes a high percentage of

preadipocyte differentiation, and its deletion in mice leads to total absence of adipose tissue (71).

b. C/EBPα

C/EBPα has been shown to play an important role in adipogenesis. Deletion of C/EBPα in mice results in decreased fat deposition (72). Forced expression of C/EBPα in 3T3-L1 fibroblasts leads to preadipocyte differentiation in the absence of hormonal stimulation (73,74), and expression of antisense C/EBPα inhibits 3T3-L1 preadipocyte differentiation (75).

Two isoforms of C/EBPα, the full-length 42-kDa and the shorter 30-kDa protein, are generated as a result of a ribosomal scanning mechanism (76,77). The full-length protein contains three trans-activation domains (78,79,80). The 30-kDa isoform is generated as a result of a fraction of ribosomes ignoring the first two AUG codons and initiating translation at the third AUG (76,77). The shorter protein retains its dimerization and DNA-binding domains; however, it has an altered trans-activation potential compared with the full-length protein. Studies have shown that the full-length protein is a stronger transcriptional activator than the 30-kDa isoform and the p42/p30 ratio increases during preadipocyte differentiation (76). Indeed, proper translational regulation of the isoform ratio is a prerequisite for proliferation arrest and terminal differentiation (81). Sustained ectopic expression of the truncated isoform in 3T3-L1 preadipocytes induces a conversion to small and spindle-shaped cells that continue to multiply during differentiation and thus are poorly differentiated.

c. ADD-1/SREBP-1

The adipocyte determination and differentiation-dependent factor-1 (ADD-1) is a member of the basic helix-loop-helix (bHLH) leucine zipper family of transcription factors (82,83). ADD-1 was originally isolated from a rat adipocyte cDNA expression library based on its ability to bind to an E-box motif (CANNTG), the DNA sequence recognized by bHLH proteins (84). The human homologue of this protein was independently cloned as sterol regulatory element binding protein 1 (SREBP-1) from HeLa cell cDNA library (82). The dual DNA-binding specificity of ADD-1/SREBP-1 to both E boxes and sterol regulatory element 1 (SRE-1) is conferred by a critical tyrosine residue in the DNA binding region of the protein, which replaces the canonical arginine found in other bHLH family members (85). Another member of the SREBP family is SREBP-2 which is encoded by a separate gene (83,86). Both SREBP-1 and SREBP-2 bind to SRE-1 with a nucleotide specificity that precisely matches the requirement for sterol-regulated transcription (87). Currently, only one transcript of the SREBP-2 gene has been detected in humans, hamsters, and mice (83).

Cloning of SREBP-1 yielded two alternatively spliced isoforms, designated SREBP-1a and SREBP-1c (82). The two isoforms are derived from different promoters that give rise to two different 5' exons (1a and 1c), both of which are spliced to a common exon 2. The 5' exon of SREBP-1a encodes an acidic sequence of 29 amino acids, 8 of which are negatively charged. This sequence functions in concert with four negatively charged amino acids from exon 2 to give a transcriptional activator of 42 amino acids. SREBP-1a lacking the transcriptional activator sequence retains its binding

ability to SRE-1 but loses its transcriptional activation activity, and thus is a dominant negative regulator (88). SREBP-1c contains a 5' exon that encodes only 5 amino acids, one of which is negatively charged (82). The SREBP-1 transcript also undergoes alternative splicing at the 3' end, giving rise to exons 18a and 19a for SREBP-1a, and 18c and 19c for SREBP-1c (89).

Studies using transgenic mice show that SREBP-1c is much less potent than SREBP-1a in stimulating transcription of SREBP-responsive genes (90). Consistently, results from transfection experiments also indicate that SREBP-1c is a weaker activator of transcription when expressed at physiologic levels in human embryonic kidney 293 cells. SREBP1-c, expressed at supraphysiologic levels, becomes a robust transcriptional activator. In addition, SREBP-1c has been shown to possess a greater ability to activate transcription of genes involved in fatty acid metabolism whereas SREBP-1a preferentially regulates cholesterol metabolism (90).

ADD-1, cloned from a rat cDNA library, contained the SREBP-1c sequence at the amino terminus (91). The carboxyl terminus of ADD-1 has been shown to correspond to the carboxyl terminus of SREBP-1a at the DNA level, using computer alignment. At the protein level, ADD-1 may have five single nucleotide additions or deletions which change the reading frame and introduce a premature terminator codon. If these apparent aberrations are corrected, then the carboxyl-terminal protein sequence of rat ADD-1 corresponds to human SREBP-1a (91).

The membrane-bound form. The precursor form of ADD-1/SREBP-1 is a 125-kD tripartite protein consisting of the N-terminal transcription factor domain, a middle hydrophobic region containing 2 hydrophobic trans-membrane segments, and a C-

terminal regulatory domain (92). The N-terminal domain is a transcription factor of the basic helix-loop-helix leucine zipper family. The domain begins with an acidic segment that resembles the "acid blobs" of many transcriptional activators. Without affecting the ability of the protein to bind to the relevant DNA sequence, deletion of this acidic segment in transfection studies renders ADD-1/SREBP-1 incapable of transcription activation. Truncated form therefore acts as inhibitor of transcription (88). The basic segment of the N-terminal domain binds to specific DNA sequences, and the adjacent helix-loop-helix leucine zipper segment mediates homo- or heterodimerization. The hydrophobic trans-membrane segments are flanked by a short hydrophilic lumenal loop. The C-terminal domain has a regulatory role in the sterol-sensitive cleavage of the precursor form to give mature ADD-1/SREBP-1 (93). Immunoflourescence, protease protection, and glycosylation site tagging experiments reveal that ADD-1/SREBP-1 is bound to ER and nuclear membrane in a hairpin fashion, with the amino and carboxyl termini facing the cytoplasm and the hydrophilic lumenal loop projecting into the lumen of the organelle (94,95).

Activation. The mechanism of activation of ADD-1/SREBP-1 has been extensively studied in liver cells. Currently, it is not known if the same pathway that regulates the formation of the active form of the protein in hepatocytes also exists in the adipogenic and/or lipogenic program in adipocytes. In response to cholesterol depletion, the 125-kDa membrane-bound precursor form is proteolytically cleaved to give the 68-kDa active form. The active form of ADD-1/SREBP-1 migrates as a doublet on sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). This banding pattern may be due to phosphorylation or other post-translational covalent modification (96).

Generation of the active form of ADD-1/SREBP-1 begins with the cleavage of the precursor protein at a leucine-serine bond in the lumenal loop by site-1 protease (S1P), a serine protease belonging to the subtilisin family. This cleavage step splits the protein into halves, each with a single trans-membrane segment. The intermediate form corresponds to the N-terminal segment (95,97). Cleavage by S1P must occur in order for the next protease in the regulatory pathway to act (92). The active form of ADD-1/SREBP-1 is released via cleavage of the intermediate form at a leucine-cysteine bond located within the first trans-membrane domain by site-2 protease (S2P), which may be a metalloprotease with a high degree of hydrophobicity (98,99). The 68-kDa active form then enters the nucleus and activates gene transcription.

Activity of S1P requires the involvement of a membrane-bound regulatory protein designated SREBP cleavage-activating protein (SCAP) (100). Studies have shown that SCAP comprises two distinct domains. The N-terminal polytopic domain consists of eight membrane-spanning segments separated by hydrophilic loops of varying length (100). The carboxyl-terminal hydrophilic domain contains five WD (Tryptophan-Aspartate) repeats which are thought to mediate protein-protein interactions (100,101). The carboxyl terminus of SCAP projects into the cytosol and forms a tight complex with the C-terminal regulatory domain of ADD-1/SREBP-1 (93,102). Disruption of the SCAP-ADD-1/SREBP-1 complex by over-expression of truncated dominant negative forms of either SCAP or ADD-1/SREBP-1 blocks S1P cleavage of ADD-1/SREBP-1. Furthermore, truncated forms of ADD-1/SREBP-1 which lack the carboxyl terminus fail to form complexes with SCAP and fail to undergo S1P cleavage (102). These studies

suggest that SCAP-ADD-1/SREBP-1 complex is necessary for S1P cleavage of ADD-1/SREBP-1.

Mature 68-kDa ADD-1/SREBP-1 is rapidly degraded by proteolysis. This process can be blocked by N-acetyl-leucyl-leucyl-norleucinal (ALLN) (96), an inhibitor of neutral cysteine proteases. Addition of ALLN to HeLa cells increases the amount of mature ADD-1/SREBP-1, suggesting that ALLN inhibits activity of the protease responsible for its degradation.

Role in preadipocyte differentiation. ADD-1/SREBP-1 has been shown to play an important role in preadipocyte differentiation. Increased levels of ADD-1/SREBP-1 mRNA are observed in 3T3-L1 preadipocytes undergoing differentiation. Ectopic expression of a dominant-negative form of the protein in 3T3-L1 preadipocytes inhibits their differentiation and over-expression of wild-type ADD-1/SREBP-1 in NIH-3T3 cells induces preadipocyte differentiation (103,104). Studies have shown that the adipogenic function of ADD-1/SREBP-1 may be linked to PPARγ. Co-expression of ADD-1/SREBP-1 with PPARγ in a PPARγ reporter system results in greater transcriptional activity, whereas transfection of ADD-1/SREBP-1 alone has little effect (103). This suggests that ADD-1/SREBP-1 is responsible for the generation of a factor that enhances PPARγ activity. In fact, inhibition of preadipocyte differentiation mediated by dominant-negative ADD-1/SREBP-1 is reversed by addition of a TZD ligand for PPARγ (104). Furthermore, media from fibroblasts transfected with ADD-1/SREBP-1 was shown to contain a factor that could both bind to and activate a PPARγ ligand binding domain. This body of evidence suggests that ADD-1/SREBP-1 is involved in the production of an endogenous PPARγ ligand. Recently, it has been shown that PPARγ is a direct target

gene of ADD-1/SREBP-1 (105). These results, derived from overexpression systems, suggest that ADD-1/SREBP-1 and PPARγ may act together to regulate preadipocyte differentiation.

ADD-1/SREBP-1 has been shown to be a direct positive regulator of lipogenic enzymes. Over-expression of wild-type ADD-1/SREBP-1 in NIH-3T3 cells increases mRNA levels of fatty acid synthase (FAS) and LPL (103). As well, it has been shown that ADD-1/SREBP-1 positively regulates stearoyl–CoA desaturase 1 (SCD1) and SCD2 which are involved in the synthesis of unsaturated fatty acids (106). Recently, microarray studies reveal that glycerol 3-phosphate dehydrogenase, a late marker of preadipocyte differentiation, may be under the positive regulation of ADD-1/SREBP-1 (107).

Overexpressing a constitutively active form of ADD-1/SREBP-1 from the adipose-specific aP2 promoter in transgenic experiments was expected to cause obesity in mice. Surprisingly, these transgenic animals displayed lipodystrophy (108). The mice were also diabetic and had fatty livers, phenotypes present in humans with lipodystrophy. The unexpected lipodystrophy may be due to the high transgene expression produced by the aP2 promoter (aP2 accounts for several percent of total adipocyte protein). The high levels of truncated constitutively active form of ADD-1/SREBP-1 used in these experiments may give rise to a dysregulated adipogenic program, possibly causing non-specific promoter interactions. Indeed, effects on adipose tissue from low level of ADD-1/SREBP-1 expression are apparently undetectable (90). Disruption of the endogenous gene shows little effect on gene expression or adipose tissue mass, although embryonic lethality is more common (109).

4. Adipocyte-specific genes

a. aP2

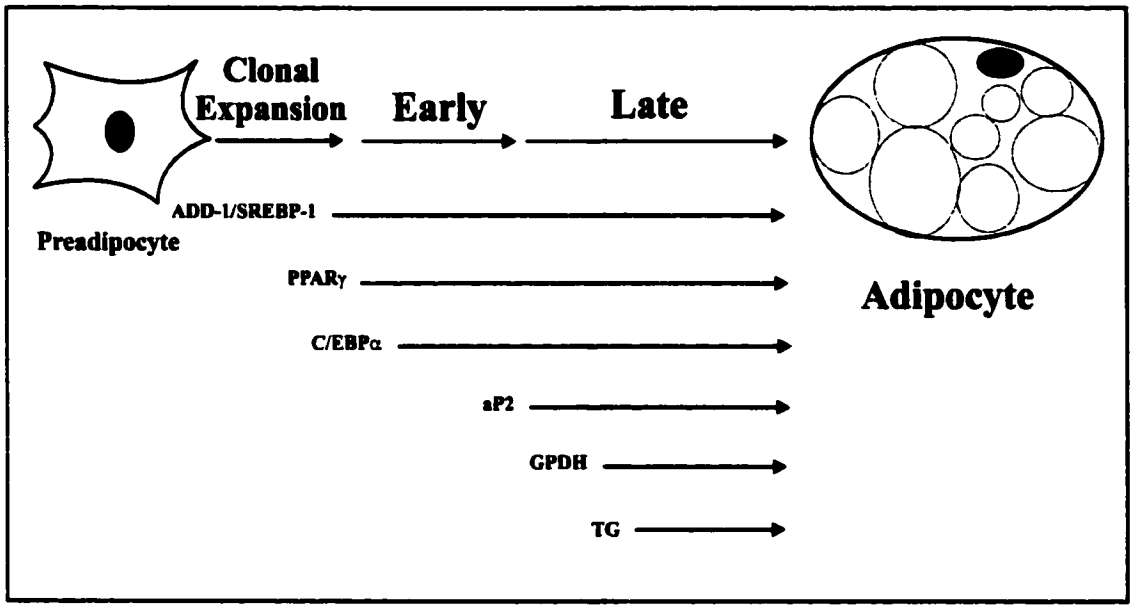
The aP2 gene encodes the adipocyte lipid-binding protein (ALBP). ALBP is a member of a family of small monomeric soluble proteins that bind fatty acids, retinoids, and other lipids. Thus, it is one of the key players in intracellular fatty acid trafficking in adipocytes (110).

This gene is of particular interest in the investigation of the effect of the HIV-1 protease inhibitor ritonavir on 3T3-L1 preadipocyte differentiation. It has been suggested that aP2 is repressed by the endogenous adipocyte enhancer-binding protein 1 (AEBP1) (111). AEBP1, like CRABP-1 and LRP, also shares homology with the active site of the HIV-1 protease to which ritonavir binds (29). Studies have shown that AEBP1 represses aP2 expression by binding to the AE-1 sequence in the promoter region. Carboxypeptidase activity of AEBP1 is required for the protein to function as a transcriptional repressor and recombinant AEBP1 mutants lacking carboxypeptidase activity showed no repressor function (111). HIV-1 protease inhibitors may possibly disturb AEBP1 function by binding to the homologous region of the protein and modulating its carboxypeptidase activity.

b. GPDH

The enzyme glycerol 3-phosphate dehydrogenase (GPDH) is a late marker of 3T3-L1 preadipocyte differentiation (112). It catalyzes the rate-limiting step reaction in the triacylglycerol (TG) synthesis pathway, converting dihydroxyacetone phosphate to glycerol 3-phosphate, the predominant substrate for TG synthesis in adipose tissue. During the late phase of differentiation, adipocytes exhibit marked increases in mRNA levels and enzymic activity of GPDH.

Figure 2. Adipogenic markers during 3T3-L1 preadipocyte differentiation.


Clonal Expansion
Early
Late
ADD-1/SREBP-1
Preadipocyte
PPARγ
C/EBPα
aP2
GPDH
TG
Adipocyte

C. Other Models of Preadipocyte Differentiation

The use of established clonal cell lines such as the 3T3-L1 model has proved to be successful and readily available. However, one disadvantage of immortalized cell lines is their aneuploid nature which gives rise to characteristics that may differ from those of tissue preadipocytes (30). Thus, studies utilizing primary culture of diploid human preadipocytes are one step closer to the *in vivo* situation. Primary culture of human preadipocytes from different anatomic sites (intra-abdominal versus subcutaneous preadipocytes) is also advantageous in providing depot-specific insights of preadipocyte differentiation.

It has been established that differentiation of human preadipocytes in culture requires insulin, glucocorticoid, and cAMP-elevating agents (113). Within eighteen days of terminal differentiation, late markers of preadipocyte differentiation are observed. The cells display cytosolic lipid accumulation along with expression of the lipogenic enzymes LPL and GPDH (113). One major difference between primary human preadipocyte differentiation and 3T3-L1 preadipocyte differentiation is that human preadipocytes *in vitro* do not require cell division prior to entering terminal differentiation (114). This does not rule out, however, the possibly critical role of DNA synthesis as a prerequisite of terminal differentiation. Human preadipocytes in culture may have already undergone the critical mitoses *in vivo* in response to the stimulation of local hormones. These cells may need only a few hormonal signals that allow them to acquire morphological and functional characteristics of mature adipocytes without requiring the additional

replication steps for the initiation of terminal differentiation. Preadipocytes isolated from human adipose tissue therefore may be in a late stage of adipocyte differentiation.

II. OBJECTIVE

Ritonavir, when added to the standard differentiation medium, was found to enhance 3T3-L1 preadipocyte differentiation as assessed by lipid droplets staining positive for Oil Red O (29). The aim of this project was to characterize the enhancing effects of ritonavir on 3T3-L1 preadipocyte differentiation. 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of ritonavir. Measurement of TG accumulation and GPDH activity was performed to study the effects of ritonavir on late events of differentiation. To elucidate a potential mechanism through which ritonavir enhances differentiation, protein expression of the adipogenic transcription factors PPARγ, C/EBPα, ADD-1/SREBP-1 as well as the adipocyte-specific gene aP2 was examined. Effects of ritonavir on human intra-abdominal and subcutaneous preadipocyte differentiation in primary culture were also investigated.

III. MATERIALS AND METHODS

A. 3T3-L1 Preadipocyte Differentiation

3T3-L1 murine preadipocytes were obtained from American Type Culture Collection and maintained at low passage. 3T3-L1 preadipocytes were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Two days post-confluence, differentiation was induced by the addition of insulin in 10% (v/v) calf serum. Concentrations of insulin varied from 10–100 nM (for each experiment, only one concentration of insulin was used) in order to achieve approximately 50% preadipocyte differentiation. These conditions allow for the detection of the enhancing effects of ritonavir. Cells were incubated with differentiation medium for up to 8 days. Dexamethasone (0.25 μM) and IBMX (0.5 mM) were added for the first 2 days only. Ritonavir at a concentration of 10 μg/ml, or 0.1% (v/v) vehicle, dimethyl sulfoxide (DMSO), was present throughout the 8 day differentiation period. The concentration of ritonavir used is comparable to that found in plasma from patients treated with this HIV-1 protease inhibitor (115). Medium was changed every 2 days.

B. Human Preadipocyte Differentiation in Primary Culture

For some experiments, preadipocytes were obtained from Zen-Bio Inc. For others, preadipocytes were derived from adipose tissue samples obtained from consenting patients undergoing elective abdominal surgery (approved by the Loeb Health Research Institute Ethics Committee) and differentiated according to protocol supplied by the company. Patients did not have any systemic illness and were weight-stable.

Adipose tissue samples were obtained on the day of surgery and transported to the laboratory in DMEM:Ham's F12 (F12) (1:1 v/v) supplemented with 2XPSN which was 200 units/ml penicillin (P), 0.2 mg/ml streptomycin (S), and 100 units/ml nystatin (N). To remove fibrous tissue and blood vessels, adipose tissue was immersed in sterile phosphate buffer saline (PBS) supplemented with 2XPSN. Tissue was then cut into small pieces and weighed. Collagenase digestion of the sample was carried out for 45 minutes at 37°C in 3 ml DMEM:F12:PSN / g tissue supplemented with 33 μM biotin (B), 17 μM pantothenate (P), 6 mg collagenase A / g tissue, and 60 mg bovine serum albumin (BSA) / g tissue. The collagenase reaction was stopped by the addition of 3 ml DMEM:F12:PSN(100 units/ml P, 0.1 mg/ml S, and 50 units/ml N):BP / g tissue. This suspension was then filtered through a 200 μm nylon filter. The filtrate was spun at room temperature at 1 000 x g for 20 minutes using a Megafuge 1.0 Heraeus centrifuge. The resulting cell pellet was resuspended in DMEM:F12 PSN BP and subsequently filtered through 100, 50, and 25 μm nylon filters to yield the preadipocyte fraction. The preadipocyte fraction was then spun as described above. Cell pellet was reconstituted and cells were counted.

Preadipocytes were then seeded at a density of 30 000 cells/cm^2 (this specific density ensures that cells become confluent either the next day or 2 days after). When cells were confluent, differentiation was induced with a cocktail consisting of DMEM:Ham's F10 (1:1 v/v), 3% (v/v) fetal bovine serum (FBS), 33 μM biotin, 17 μM pantothenate, 100 nM insulin, 1 μM dexamethasone, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 units/ml nystatin. Cells were incubated with differentiation cocktail for 16 days. IBMX (0.2 mM) and rosiglitazone (1 μM) were present in the first 3 days only. Ritonavir at a concentration of 10 μg/ml, or 0.1% (v/v) DMSO, was present throughout the 16 day differentiation period. Cells were fed with fresh differentiation medium every 3 days. When feeding, only half of the medium in a well was removed and equal volume of fresh medium was added.

C. TG Assay

At the indicated time points in the differentiation protocol, 3T3-L1 cells were washed twice with cold PBS. TG was extracted in a mixture of isopropanol:heptane (2:3 v/v) in 2 fractions (first fraction 30 minutes and second fraction 15 minutes). The 2 fractions were combined and dried in a Savant SpeedVac SC200. The TG pellet was reconstituted in 300 µl of isopropanol. TG was then saponified with 150 µl saponification reagent containing 1.8 M potassium hydroxide. Following saponification, the reaction mixture was allowed to react with 300 µl of 3 mM sodium metaperiodate and 300 µl of 1:250 acetyl acetone solution to give a color product which was quantified spectrophotometrically at 410 nm using a Pharmacia Biotech Ultrospec 3000. The standard curve was obtained using triolein. Total TG was calculated by multiplying TG concentration (µg/ml) by 0.3 ml, the volume of isopropanol used to reconstitute TG pellet after drying. Cellular protein was scraped in 0.1 N NaOH and assayed by the BioRad protein assay kit, using bovine serum albumin as a standard. The BioRad protein assay makes use of the binding of Coomassie Brilliant Blue G-250 to proteins producing a color product which was quantified at 595 nm using Pharmacia Biotech Ultrospec 3000. Total amount of TG per well (µg) was then divided by total amount of protein (mg) per well to give µg TG per mg protein.

D. GPDH Assay

Confluent 3T3-L1 preadipocytes, either maintained in control medium or subjected to differentiation for 8 days, were washed twice with cold PBS on ice and lysed in lysis buffer (25 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.5). Cell lysates were then sonicated for 5 seconds at 4°C with Branson Sonifier 450. Homogenized samples were spun at 436 000 x g for 10 minutes at 4°C, using TLA 100.1 rotor and Beckman's Optima TL ultracentrifuge. Microsomal components of lysates were pelleted and discarded. The cytosolic fraction (supernatant) was kept to measure GPDH activity using program 6 Kindata on the Soft Pac Module Kinetics cartridge on Beckman DU-50 Spectrophotometer. To measure GPDH activity, 40 µl 4 mM dihydroxyacetone phosphate (DHAP) as substrate was added to 20 µl of sample, 80 µl lysis buffer, and 60 µl cocktail (0.67 mM β–NADH, 342 mM triethanolamine pH 7.7, 168 mM β-mercaptoethanol, 0.842 mM EDTA pH 8.0). In this reaction mixture, NADH acts as a co-factor of GPDH as the enzyme converts DHAP to L-glycerol-3-phosphate, and is reduced to NAD in the process. Absorbance readings of reduced NAD at 340 nm were taken every 15 seconds for 2 minutes. GPDH activity (units / ml / min) was calculated according to the following formula:

[(absorbance at time 0 – absorbance at time 2 min) / (2 min) x (32.134)] / [(volume of sample used µl) x (1000)].

The same cytosolic fraction was used to determine protein concentration, using the BioRad assay as described above. Specific GPDH activity (units/mg/min) was determined by dividing GPDH activity (units/ml/min) by protein concentration (mg/protein/ml).

E. Western Analysis of Proteins

At indicated time points in the differentiation protocol, 3T3-L1 cells were washed twice with cold PBS and lysed in Laemmli buffer (116) consisting of 60 mM Tris pH 6.8, 2% (v/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 0.002% (v/v) bromophenol blue, 1 mM sodium orthovanadate, 710 mM β-mercaptoethanol. Lysates were then pushed through a 26.5 gauge needle to shear DNA and boiled for 5 minutes. Solubilized protein was measured by the Sigma protein assay kit, using bovine serum albumin as a standard. The principle of the Sigma protein assay is based on a color reaction in which an alkaline cupric tartrate reagent complexes with the peptide bonds of the proteins and forms a purple color when the phenol reagent is added. Proteins (75 – 150 μg) were separated using SDS-PAGE. The resolving gel consisted of 10-15% (v/v) acrylamide:bis (as indicated - depending on the size of the protein of interest), 375 mM Tris HCl pH 9.0, 0.1% (v/v) TEMED, 0.1% (v/v) SDS, and 0.1% (v/v) ammonium persulfate. The stacking gel consisted of 3.9% (v/v) acrylamide:bis, 126 mM Tris HCl pH 6.8, 0.01% (v/v) TEMED, 0.01% (v/v) SDS, and 0.05% (v/v) ammonium persulfate. The running buffer was made up of 16.8 mM Tris, 192 mM glycine, 3.5 mM SDS, pH 8.6. Proteins were then transferred to a nitrocellulose membrane in transfer buffer consisting of 16.8 mM Tris, 192 mM glycine, 3.5 mM SDS, 20% (v/v) methanol, pH 8.6, at 70 V. The membranes were blocked with 5% (w/v) skim milk in 0.1% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20) in PBS. The membranes were then probed with either mouse monoclonal PPARγ antibody (1 μg/ml; Santa Cruz), rabbit polyclonal C/EBPα antibody (1 μg/ml; Santa Cruz), rabbit polyclonal aP2 antibody

(1:250, a kind gift from Dr. D. Bernlhor, University of Minnesota), or rabbit polyclonal ADD1 antibody (1 μg/ml; Santa Cruz) in 3% bovine serum albumin (BSA), 0.02% (w/v) sodium azide in PBS at 4°C overnight. Blots were subsequently incubated with the appropriate secondary horseradish-peroxidase conjugated antibody (Amersham) in 5% (w/v) skim milk in PBS. Specific bands were detected by enhanced chemiluminescence kit (DuPont NEN) and visualized on Kodak X-AR film. The relative intensity of the bands was assessed using Molecular Analyst (Bio-Rad) imaging software, expressed in integrated optical density units.

F. Northern Analysis

At appropriate time points in the differentiation protocol, 3T3-L1 cells were washed with cold PBS and incubated for 5 minutes on ice in lysis buffer consisting of 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% (w/v) N-lauryl sarcosine, and 0.1 M β-mercaptoethanol. Cell lysates were then pushed through 23.5 gauge needle 5 times on ice. 60 µl sodium acetate (2 M, pH 4), 600 µl water-saturated phenol, then 120 µl chloroform : isoamyl alcohol were sequentially added to lysates. This mixture was spun in a centrifuge at 10 000xg 4°C for 30 minutes. The upper aqueous layer which contained RNA was immediately transferred to a fresh micro-centrifuge tube. 600 µl cold isopropanol was added and the mixture was precipitated overnight at –20°C. Mixture was then spun in a centrifuge at 18 186xg 4°C for 30 minutes. Supernatant was discarded and the RNA pellet was resuspended in 100 µl lysis buffer, followed by the addition of 300 µl ethanol. This mixture was also precipitated overnight at –20°C, then spun in a centrifuge at 18 000xg 4°C for 30 minutes. Supernatant was discarded and pellet was washed with 300 µl 70% (v/v) ethanol. Mixture was then spun in a centrifuge at 18 186xg 4°C for 30 minutes. RNA pellet was dissolved in 15–20 µl diethyl pyrocarbonate (DEPC) water. Concentration and purity of RNA was measured spectrophotometrically at 260 and 280 nm using a Pharmacia Biotech Ultrospec.

RNA (2 µg), heated at 55°C for 15 minutes, was loaded onto agarose gel consisting of 1% (w/v) agarose, 3% (v/v) formaldehyde, 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), and 1 mM EDTA. The sample loading buffer consisted of 20 mM MOPS, 1 mM EDTA, 6% (v/v) formaldehyde, 50% (v/v) deionized

formamide, and 2 μl RNA loading dye. The running buffer was made up of 20 mM MOPS, 1 mM EDTA, and 3% (v/v) formaldehyde. Prior to transferring, the gel was immersed in 0.9 M NaCl, 90 mM tri-sodium citrate.$2H_2O$, pH 7, for 5 minutes at room temperature. RNA was then transferred overnight onto H-bond nitrocellulose membrane in 3 M NaCl, 0.3 M tri-sodium citrate.$2H_2O$, pH 7, at room temperature. The membrane was baked at 80°C for 4 hours, then prehybridized at 60°C for 4 hours with a solution consisting of 0.6 M NaCl, 60 mM tri-sodium citrate.$2H_2O$, 0.002% (w/v) Ficoll, 0.002% (w/v) polyvinyl pyrrolidone, 0.002% (w/v) BSA, 1 mg/ml salmon sperm DNA, 0.5% (v/v) SDS, pH 7.

Following probe labelling using ^{32}P-CTP and Amersham Multiprime Probe Labelling System, RNA on the membrane was allowed to hybridize overnight at 60°C with ADD-1/SREBP-1 probe, a generous gift from Dr. Spiegelman (Harvard University). Membrane was then washed twice 15 minutes at room temperature with 0.3 M NaCl, 30 mM tri-sodium citrate.$2H_2O$, 0.1% (v/v) SDS, pH 7, then 15 minutes at 60°C with 0.15 M NaCl, 15 mM tri-sodium citrate.$2H_2O$, 0.1% (v/v) SDS, pH 7, then 30 minutes at 60°C with 15 mM NaCl, 1.5 mM tri-sodium citrate.$2H_2O$, 0.1% (v/v) SDS, pH 7. Specific bands were detected by exposing the membrane to Kodak X-AR film.

G. Statistical Analysis

Two-tailed analysis of the Student's t-test was performed on sets of data to obtain significance level (p-value) for the purpose of comparisons of the means.

IV. RESULTS

To study the effect of ritonavir on 3T3-L1 preadipocyte differentiation, TG accumulation (Fig. 3), a late event of differentiation, was measured during the course of differentiation. Confluent 3T3-L1 preadipocytes were differentiated for 8 days in the presence of 10 μg/ml ritonavir or vehicle (0.1% DMSO). TG mass derived from preadipocytes differentiated in the presence of 10 μg/ml ritonavir was compared to TG mass derived from preadipocytes undergoing standard differentiation, at the indicated time points. Under standard differentiation conditions, cells began to develop TG droplets on day 4 and levels of TG continued to increase until day 8. Augmentation of TG accumulation by ritonavir was first apparent on day 6 of the differentiation program, at which point there was 30% more TG. This enhancing effect of ritonavir was still evident by the end of the differentiation protocol, with almost 40% more TG on day 8.

The effect of ritonavir on GPDH (Fig. 4), another late marker of 3T3-L1 preadipocyte differentiation, was then examined. 3T3-L1 preadipocytes were differentiated under standard differentiation conditions with 10 μg/ml ritonavir or vehicle (0.1% DMSO). On day 8 of the differentiation protocol, cells were lysed and total lysates were spun in a centrifuge to obtain cytosolic fractions. An aliquot of the cytosolic fraction was used for the spectrophotometric measurement of GPDH activity, and the remaining portion was used in the determination of concentration of total proteins. GPDH activity of adipocytes was 1226 units/mg/min compared to 50 units/mg/min for control preadipocytes. In the presence of ritonavir, GPDH activity was 50% higher than that of standard differentiation ($p<0.05$).

Figure 3. Effect of ritonavir on TG accumulation during 3T3-L1 preadipocyte differentiation.

3T3-L1 preadipocytes were induced to differentiate (D) as described, in the absence or presence of 10 μg/ml ritonavir (R). TG was extracted from cell cultures at the time points indicated, and TG mass was measured, as described. Data are expressed as means ± range for 2 independent experiments, and represent the percentage increase compared to day 0 (undifferentiated) preadipocytes.

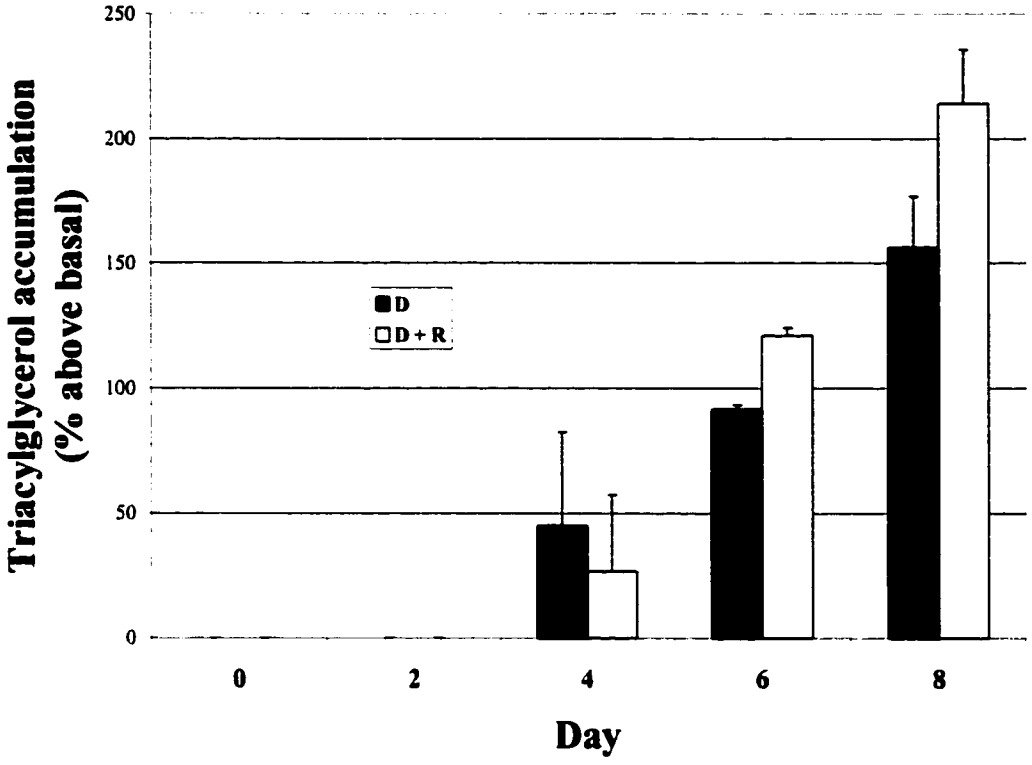
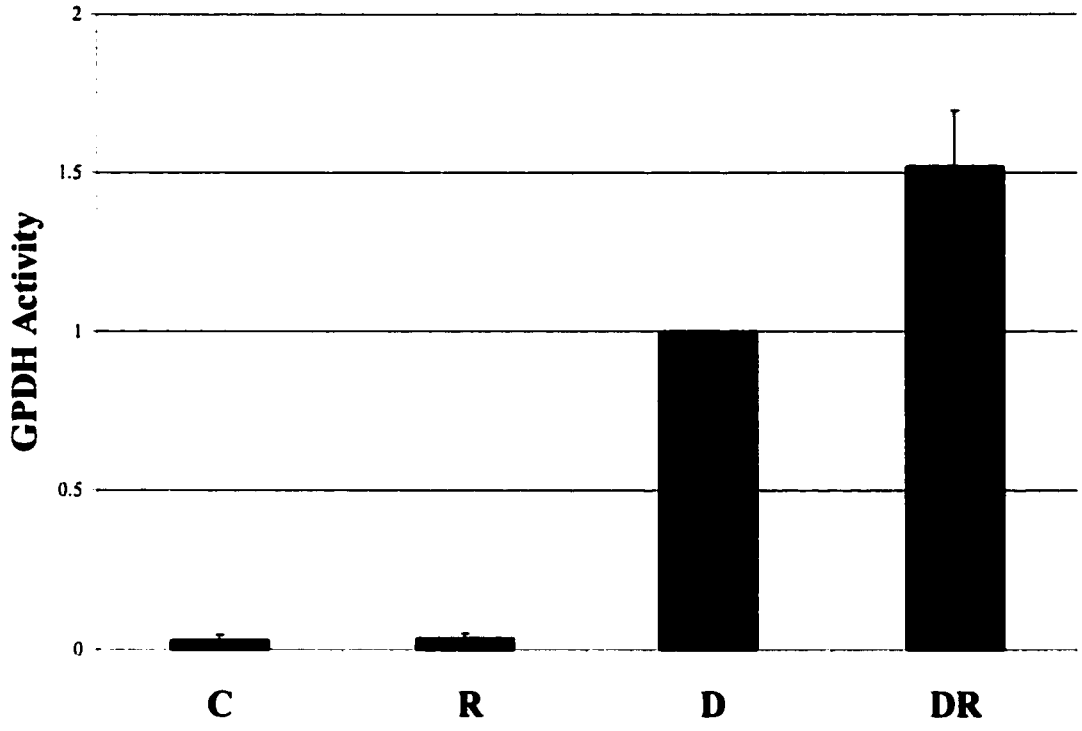

Triacylglycerol accumulation
(% above basal)
250
200
150
100
50
0
D
D + R
0
2
4
6
8
Day

Figure 4. Effect of ritonavir on GPDH specific activity in 3T3-L1 preadipocyte differentiation.

Confluent 3T3-L1 preadipocytes were induced to differentiate (D) or maintained as control preadipocytes (C), as described, in the absence or presence of 10 μg/ml ritonavir (R). Cells were lysed on day 8 and GPDH activity was measured as described. Data are expressed as means ± SEM (standard error of the mean) for 3 independent experiments, normalized to GPDH activity obtained under standard differentiation.


GPDH Activity
2
1.5
1
0.5
0
C
R
D
DR

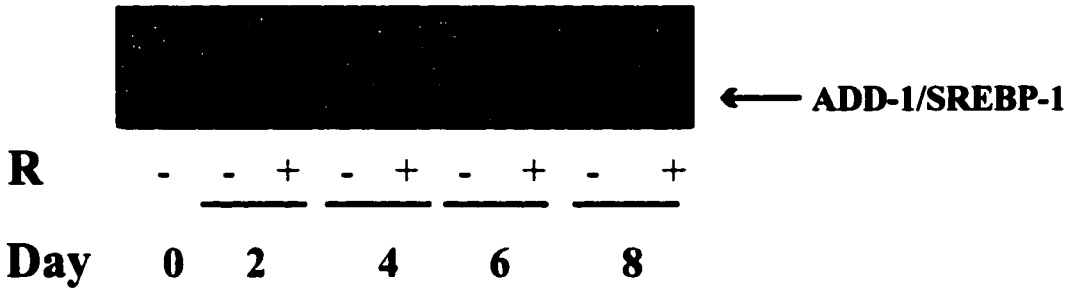
To investigate a possible mechanism by which ritonavir could augment TG accumulation and GPDH activity, protein expression of the 68-kDa active form of ADD-1/SREBP-1 was examined (Fig. 5). It has been shown that ADD-1/SREBP-1 positively regulates expression of lipogenic genes FAS, SCD-1/-2, and LPL (103,106). Recent evidence indicates that GPDH, the enzyme catalyzing the rate-limiting step in TG synthesis pathway, may also be a target gene of ADD-1/SREBP-1 (107).

Confluent 3T3-L1 preadipocytes were incubated in differentiation medium in the presence of 10 μg/ml ritonavir or vehicle (0.1% DMSO). At appropriate time points, cells were lysed and equal amounts of solubilized proteins were subjected to immunoblotting analysis. Under standard differentiation conditions, protein expression of the 68-kDa active form of ADD-1/SREBP-1 increased 3-fold on day 2 of differentiation ($p<0.05$) and returned to basal level on days 4, 6, and 8. Addition of 10 μg/ml ritonavir to the differentiation medium prolonged the expression of activated ADD-1/SREBP-1 on days 4 and 6 of the differentiation protocol. There was 2-fold more on day 4, and 3-fold more on day 6 of 68-kDa ADD-1/SREBP-1 ($p<0.05$), compared to that in the absence of ritonavir. The enhancing effect of ritonavir on ADD-1/SREBP-1 expression was no longer evident on day 8. On day 2 of differentiation, augmentation of ADD-1/SREBP-1 expression by ritonavir was not evident. In fact, expression of ADD-1/SREBP-1 in the presence of ritonavir was approximately 30% less than that of standard differentiation ($p<0.05$). There were no detectable changes in the level of the 125-kDa precursor form of ADD-1/SREBP-1 during differentiation in the absence or presence of ritonavir.

Figure 5. Effect of ritonavir on the protein expression of mature ADD-1/SREBP-1 during 3T3-L1 preadipocyte differentiation.

Confluent 3T3-L1 preadipocytes were induced to differentiate (D) as described in the absence or presence of 10 μg/ml ritonavir (R). Equal amount of solubilized protein (80 μg) was separated on SDS-PAGE, transferred, and immunoblotted with anti-ADD-1/SREBP-1 antibody (A). Densitometric analysis of ADD-1/SREBP-1 protein expression (B) is derived from data expressed as means ± SEM for 4 independent experiments.

A



B

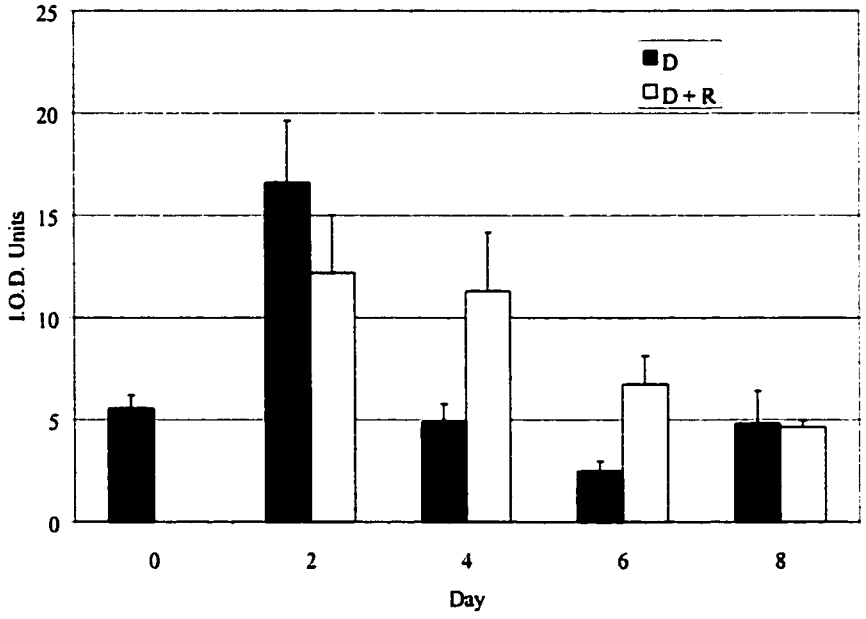


Figure 6. ADD-1/SREBP-1 mRNA expression in 3T3-L1 preadipocyte differentiation.

Confluent 3T3-L1 preadipocytes were induced to differentiate (D) or maintained as control preadipocytes (C), as described. Cells were lysed on day 8 and total RNA was collected as described. RNA was then electrophoresed, transferred, and probed with ADD-1/SREBP-1 fragment.

ADD-1/SREBP-1 mRNA

C D

Analysis of ADD-1/SREBP-1 mRNA expression (Fig. 6) in 3T3-L1 preadipocyte differentiation was also carried out. Preadipocytes (day 0) express very low levels of ADD-1/SREBP-1 mRNA. 3T3-L1 preadipocytes differentiated for 8 days in standard differentiation medium show a marked increase in message levels of ADD-1/SREBP-1.

To examine the effect of ritonavir on other markers of differentiation, protein expression pattern of the transcription factors PPARγ (Fig. 7) and C/EBPα (Fig. 8) and the intermediate marker aP2 (Fig. 9) was studied.

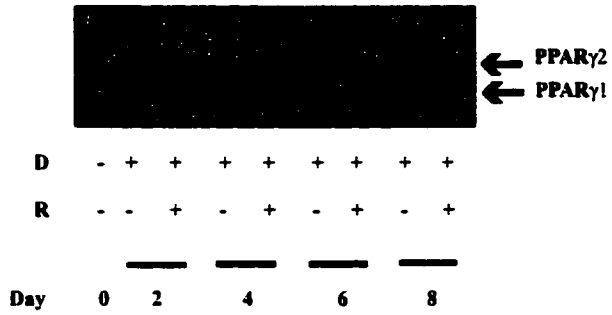
Upregulation of the PPARγ2 isoform (Fig. 7) was seen on day 2 of standard differentiation and the protein level gradually increased 9-fold to its highest level on day 8. The addition of 10 μg/ml ritonavir dampened protein expression of PPARγ2, keeping the protein level lower than that found on day 4 of standard differentiation. Inhibition of PPARγ2 by ritonavir was most significant on days 4 and 8 ($p<0.05$). For PPARγ1, protein expression was upregulated by day 2, increased approximately 2-fold to reach its peak on day 4, and remained at this level throughout the standard differentiation protocol. Ritonavir, when added to differentiation medium, attenuated PPARγ1 expression most significantly on days 4 and 6 ($p<0.05$), as seen by lower protein levels than that on day 2 of standard differentiation.

Expression of both p42 and p30 isoforms of C/EBPα was upregulated on day 2 of standard differentiation and gradually increased to a maximal level on day 8. Ritonavir, at a concentration of 10 μg/ml, downregulated C/EBPα protein expression to levels lower than that found on day 4 of standard differentiation (Fig. 8).

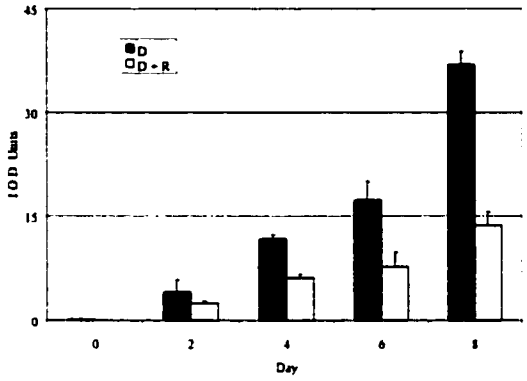
Figure 7. Effect of ritonavir on the protein expression of PPARγ during 3T3-L1 preadipocyte differentiation.

3T3-L1 preadipocytes were grown to confluence and induced to differentiate (D) as described, in the absence or presence of 10 μg/ml ritonavir (R) for up to 8 days. Cells were lysed and equal amount of solubilized protein (80 μg) was separated on SDS-PAGE, transferred, and immunoblotted with anti-PPARγ antibody (A). Densitometric analysis of protein expression of PPARγ2 isoform (B) and PPARγ1 isoform (C) is derived from data expressed as means ± SEM for 3 independent experiments.

A


PPARγ2
PPARγ1
D - + + + + + + + +
R - - + - + - + - +
Day 0 2 4 6 8

B


D
D + R
IOD Units
45
30
15
0
0
2
4
6
8
Day

C

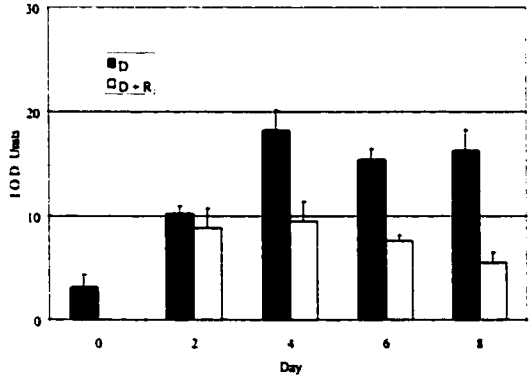
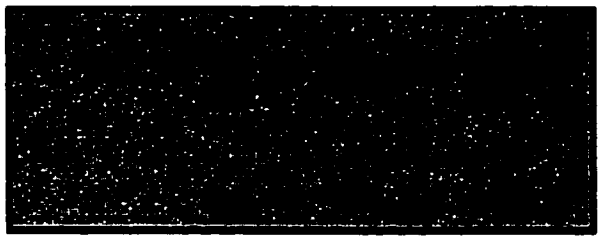

D
D + R
IOD Units
30
20
10
0
0
2
4
6
8
Day

Figure 8. Effect of ritonavir on the protein expression of C/EBPα during 3T3-L1 preadipocyte differentiation.

3T3-L1 preadipocytes were grown to confluence and induced to differentiate (D) as described, in the absence or presence of 10 μg/ml ritonavir (R) for up to 8 days. Cells were lysed and equal amount of solubilized protein (80 μg) was separated on SDS-PAGE, transferred, and immunoblotted with anti-C/EBPα antibody (A). Densitometric analysis of protein expression of the p42 isoform (B) and the p30 isoform (C) is derived from data expressed as means ± range for 2 independent experiments.

A



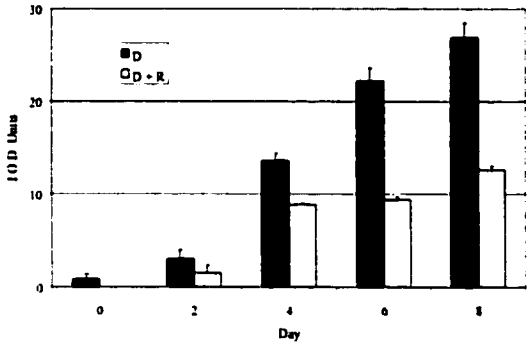
← p42

← p30

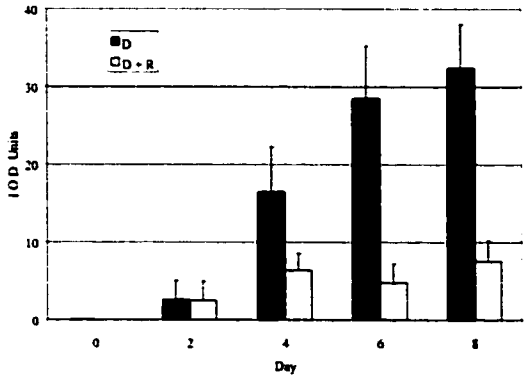
D	-	+	+	+	+	+	+	+	+
R	-	-	+	-	+	-	+	-	+

Day 0 2 4 6 8

B



C



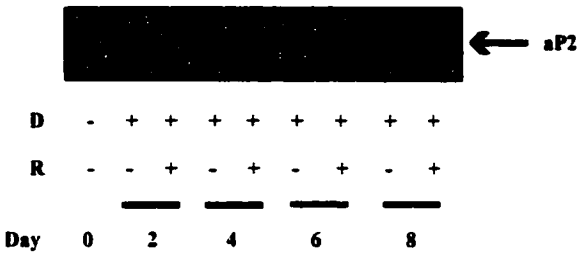
The aP2 gene has been shown to be negatively regulated by AEBP1 whose carboxypeptidase activity is required for this repression function (111). AEBP1 shares homology over a short stretch of amino acids present in the active site of HIV-1 protease to which ritonavir is targeted (29). Under standard differentiation conditions, protein expression of aP2 emerged on day 4 and gradually increased until reaching a peak on day 8. Consistent with data for PPARγ and C/EBPα, ritonavir partially inhibited aP2 expression on day 6 and 8 of the differentiation program (Fig. 9). The inhibitory effect of ritonavir on aP2 epxression was most significant on day 8 of differentiation ($p<0.05$).

To investigate the effects of ritonavir on human preadipocyte differentiation (Table 1), primary cultures of human intra-abdominal and subcutaneous preadipocytes were used. Preadipocytes isolated from patients undergoing intra-abdominal surgery were obtained and induced to differentiate as described. Preliminary results suggest that ritonavir enhances human intra-abdominal preadipocyte differentiation in primary culture, assessed by morphological appearance of cytoplasmic lipid droplets. Interestingly, human subcutaneous preadipocyte differentiation was inhibited in the presence of ritonavir.

Figure 9. Effect of ritonavir on the protein expression of aP2 during 3T3-L1 preadipocyte differentiation.

3T3-L1 preadipocytes were grown to confluence and induced to differentiate (D) as described, in the absence or presence of 10 μg/ml ritonavir (R) for up to 8 days. Cells were lysed and equal amount of solubilized protein (75 μg) was separated on SDS-PAGE, transferred, and immunoblotted with anti-aP2 antibody (A). Densitometric analysis of protein expression of aP2 (B) is derived from data expressed as means ± SEM for 6 independent experiments.

A



B

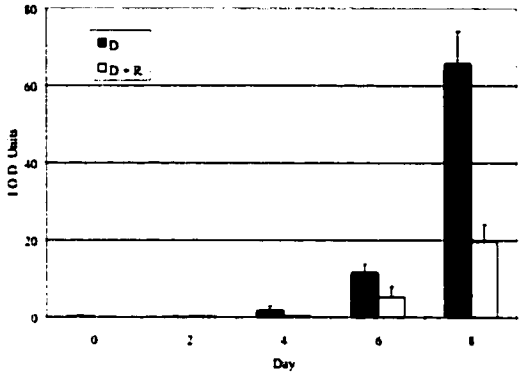


Table 1. Effect of ritonavir on human intra-abdominal and subcutaneous preadipocyte differentiation in primary culture.

Human preadipocytes were grown to confluence in primary culture and induced to differentiate as described (D), in the absence or presence of 10 μg/ml ritonavir (R). On day 16, degree of differentiation was determined by visual assessment of morphological changes under the light microscope.

	Subcutaneous Preadipocyte Differentiation		Intra-abdominal Preadipocyte Differentiation	
	Percent Differentiation (%)		Percent Differentiation (%)	
	D	**D + R**	**D**	**D + R**
Subject A	50	30	10	20
Subject B	50	30	N/A	N/A
Subject C	50	30	N/A	N/A
Subject D	50	30	N/A	N/A
Subject E	N/A	N/A	50	60

V. DISCUSSION

The data demonstrate that TG accumulation and GPDH activity are enhanced by ritonavir during 3T3-L1 preadipocyte differentiation. Ritonavir may act through a novel mechanism involving the 68-kDa active form of ADD-1/SREBP-1 to exert its positive effect on late markers of differentiation, as immunoblotting analysis showed that ADD-1/SREBP-1 expression was augmented in the presence of ritonavir. The enhancing effects of ritonavir on expression of ADD-1/SREBP-1 and late events of 3T3-L1 preadipocyte differentiation (TG accumulation and GPDH activity) are independent of PPARγ, C/EBPα, and aP2. In fact, ritonavir was found to attenuate expression of the transcription factors PPARγ and C/EBPα and the intermediate marker aP2.

Enhanced expression of the 68-kDa active form of ADD-1/SREBP-1 on days 4 and 6 of the 3T3-L1 preadipocyte differentiation program is consistent with the augmentation of TG accumulation and GPDH activity. ADD-1/SREBP-1 has been shown to be a direct positive regulator of the lipogenic enzymes FAS, LPL, SCD-1 and SCD-2 (103,106). In addition, recent data suggest that GPDH may be a target gene of ADD-1/SREBP-1 (107). The upregulation of TG accumulation and GPDH activity may result from the higher levels of mature ADD-1/SREBP-1 produced by ritonavir.

Protein expression of active ADD-1/SREBP-1 on day 2 of differentiation in the presence of ritonavir was downregulated when compared to standard differentiation. The enhanced levels on days 4 and 6 were no longer evident by day 8 and protein expression returned to basal level. The decreases observed may be due to limited effectiveness of ritonavir under the conditions of study.

It has been shown that PPARγ may be a direct target gene of ADD-1/SREBP-1 (105) and that ADD-1/SREBP-1 increases the transcriptional activity of PPARγ through the production of endogenous ligand (104). In this study, the data show that ritonavir enhances expression of mature ADD-1/SREBP-1 but partially inhibits the expected increase in protein expression of PPARγ during preadipocyte differentiation. One possible explanation may be the decreased protein levels of ADD-1/SREBP-1 on day 2 of differentiation in the presence of ritonavir. This drop in protein expression of ADD-1/SREBP-1 occurs at the crucial point in the differentiation program where upregulation of PPARγ is first noted. Enhanced expression of ADD-1/SREBP-1 by day 4 may be too late to affect PPARγ expression. In their studies, Fajas *et al.* showed that over-expression of ADD-1/SREBP-1 increased PPARγ mRNA in 3T3-L1 preadipocytes and adipocytes (105). An increase in message levels does not necessarily translate to an increase in protein expression. Furthermore, effects of a forced expression system may be more potent than that exerted by ritonavir.

Fajas *et al.* also showed that concomitant increases of PPARγ and ADD-1/SREBP-1 protein expression were observed in 3T3-L1 cells grown in medium depleted of cholesterol (105). Cholesterol depletion is a condition known to stimulate the production of active ADD-1/SREBP-1 (92). This work suggests that increased PPARγ expression was associated with activation of ADD-1/SREBP-1. Direct evidence of this association was not observed in 3T3-L1 preadipocytes, but only in a different cell type as seen by the six-fold increase of PPARγ expression in the hamster lung cell line CCL-39 transfected with a constitutively active form of ADD-1/SREBP-1 (105). Therefore, PPARγ expression mediated by ADD-1/SREBP-1 may be cell line specific and the effect

of overexpressing ADD-1/SREBP-1 may be more powerful than the transient effect of ritonavir.

At present, it is not known how ritonavir increases levels of active ADD-1/SREBP-1. It may do so by inhibiting the proteosomal activity responsible for the rapid degradation of the mature form in 3T3-L1 cells. In fact, ritonavir was found to inhibit a proteosomal chymotrypsin-like activity in $CD8^+$ lymphocytes as effectively as ALLN, an inhibitor of the proteasome (117). In HeLa cells, rapid degradation of ADD-1/SREBP-1 can be blocked by ALLN (96). The enhanced protein levels of ADD-1/SREBP-1 on days 4 and 6 of the differentiation program may possibly result from inhibition of the protein's degradation by ritonavir. Saquinavir, which has proteosomal inhibitory capacity (117), has been shown to enhance preadipocyte differentiation (29).

The protein expression profile of the active form of ADD-1/SREBP-1 during standard 3T3-L1 preadipocyte differentiation showed a 3-fold increase on day 2 and a restoration to basal level thereafter. This contrasts with mRNA expression of the gene. ADD-1/SREBP-1 mRNA levels were previously shown to markedly increase upon induction of differentiation and remain stable for the rest of the differentiation protocol (103). In our study, analysis of ADD-1/SREBP-1 gene expression showed that mRNA levels increased and the high message levels were maintained during standard differentiation of 3T3-L1 preadipocytes, consistent with previous findings. The results suggest that protein level of active ADD-1/SREBP-1 may be regulated post-translationally during standard differentiation of 3T3-L1 preadipocytes. Regulation may be via the degradation process mediated by proteosome activity and/or the production from the precursor form. The active form is generated in a 2-step proteolytic pathway

which is activated by low level of cellular cholesterol in hepatocytes (92). There are no obvious changes in cholesterol level during 3T3-L1 preadipocyte differentiation, and cholesterol alterations are not known to play a role in preadipocyte differentiation (103). Therefore, during differentiation, other novel pathways may exist to govern the formation and degradation of active ADD-1/SREBP-1.

The mature form of ADD-1/SREBP-1 is known to act as a transcription factor in the nucleus. In this study, protein expression of active ADD-1/SREBP-1 during standard 3T3-L1 preadipocyte differentiation was examined using total cell lysates. Thorough analysis of mature ADD-1/SREBP-1 protein expression using nuclear fractions which may contain a high concentration of the active form would perhaps reveal that the protein levels increase upon differentiation induction and remain at that level for the rest of the differentiation program. Another possibility is that the transient increase of protein levels on day 2 of differentiation, a crucial stage in the differentiation program at which important adipogenic transcription factors are induced, may be sufficient in the trans-activation of adipocyte-specific genes.

The adipocyte-specific gene aP2 was shown to be repressed by AEBP1 (111) which shares homology with the active site of the HIV-1 protease (29). AEBP1 has been implicated in binding to the AE-1 sequence in the promoter of aP2 and represses aP2 expression (111). Repression function of AEBP1 requires its carboxypeptidase activity. To explain the previous data indicating that ritonavir enhanced 3T3-L1 preadipocyte differentiation as assessed by TG staining positive for Oil Red O (29), we hypothesized that ritonavir would upregulate aP2 expression. Binding of ritonavir to AEBP1 may inhibit its carboxypeptidase activity. A loss in the carboxypeptidase activity of AEBP1

would lead to its inability to repress aP2 and this would translate into higher expression level of aP2. However, ritonavir showed no enhancing effect but on the contrary, partially inhibited aP2 expression. The AE-1 sequence in the promoter of aP2 also has binding site for C/EBPα. The inhibitory effect of ritonavir on aP2 expression is thus consistent with the attenuated expression of C/EBPα.

In another study, ritonavir, at a 50% higher dose, inhibit 3T3-L1 preadipocyte differentiation, as evident by the attenuated induction of TG accumulation and aP2 gene expression (118). The apparent discrepancy may be due to the differentiation protocols used. Zhang *et al.* differentiated 3T3-L1 preadipocytes in fetal bovine serum with a potent thiazolidinedione PPARγ agonist, whereas our group differentiated cells in calf serum without such an agonist. Their protocol also used dexamethasone at a 4-fold higher concentration and for a longer period of time (8 days). Other HIV-1 protease inhibitors, indinavir and saquinavir, were found to inhibit human breast preadipocyte differentiation, as assessed by their inhibitory effect on GPDH activity (119). This finding is in contrast to the expansion of breast fat in women with protease inhibitor-associated lipodystrophy.

The enhanced 3T3-L1 preadipocyte differentiation observed in this study is consistent with the clinical features of truncal adiposity in protease inhibitor-associated lipodystrophy. The results however do not explain peripheral fat atrophy of the syndrome. A uniform regional anatomic phenotype of the 3T3-L1 cell line is not known because of its embryonic origin. Preliminary data show that ritonavir exerts depot-

specific effects on primary human preadipocyte differentiation *in vitro*. Ritonavir, when added at a concentration of 10 μg/ml to standard differentiation medium, was found to inhibit human subcutaneous preadipocyte differentiation in primary culture, as assessed by morphological changes observed under the light microscope. In contrary, human intra-abdominal preadipocyte differentiation in primary culture was enhanced by the same concentration of ritonavir, as assessed by the same method.

Precedents exist for central versus peripheral preadipocytes having intrinsic differences and behaving differently in culture with respect to differentiation and apoptosis (120,121). Preadipocyte differentiation induced by insulin, cortisol, and IBMX, was significantly greater in primary culture derived from subcutaneous depot than that derived from intra-abdominal adipose tissue (122). Consistently, the addition of troglitazone or 15-deoxy-Δ-^{12,14}-prostaglandin J2 (a natural ligand of PPARγ), markedly increased differentiation of subcutaneous but not intra-abdominal preadipocytes, as assessed by TG accumulation and induction of GPDH activity and mRNA (14). This region-specific effect of troglitazone is most likely not due to PPARγ expression since protein levels were found to be comparable in both subcutaneous and intra-abdominal depots. Since PPARγ-dependent trans-activation of adipocyte-specific genes is mediated through the heterodimerization of PPARγ and RXRα (123,124), it is speculated that differential expression of RXRα may exist between the two anatomic sites. Another possibility which could explain the depot-specific effect of troglitazone may be the differential expression of co-repressor and co-activator of PPARγ and/or the differential interactions of these proteins with PPARγ in various regions (14). Preadipocytes isolated from different regions in the body also display site-specific susceptibility to apoptosis

(121). Studies show that intra-abdominal preadipocytes are more susceptible to apoptosis induced by serum deprivation or TNFα than their subcutaneous counterparts.

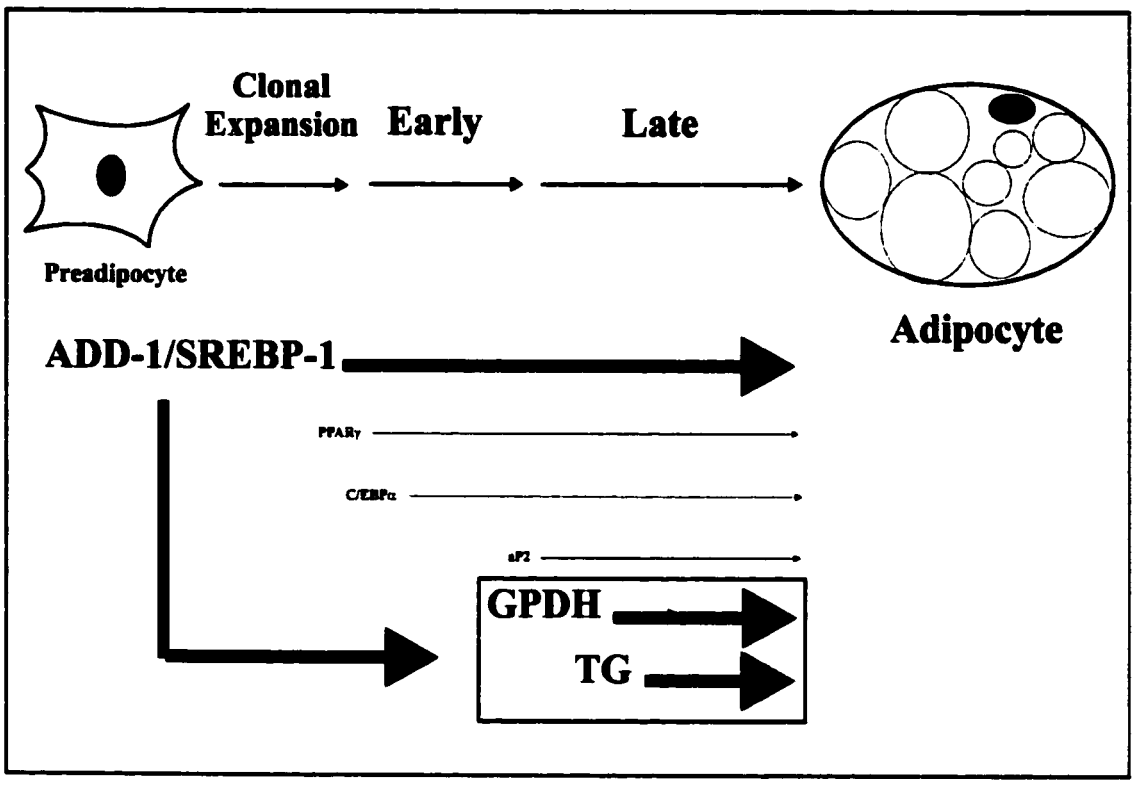
Results from clinical studies further indicate that preadipocytes display depot-specific response to external stimulation. Troglitazone, a thiazolinedione that acts as an insulin-sensitizing agent, when given to patients with type 2 diabetes, decreased intra-abdominal fat mass but did not induce changes in total body fat (125). In another study, the same drug was found to increase fat accumulation in subcutaneous adipose tissue rather than in intra-abdominal depot in type 2 diabetes patients (126). These data suggest that troglitazone preferentially promotes *in vivo* subcutaneous preadipocyte differentiation.

Data on the effects of ritonavir on human preadipocyte differentiation in this study are still in the preliminary stage. Percentage of differentiation was determined by visual assessment of morphological changes as seen under the light microscope. Quantitative analysis of ritonavir-mediated effects on differentiation markers are needed to solidify the effects observed visually. However, our results and findings in the studies of depot-specific differences of adipose tissue indicate that ritonavir display differential interference with the preadipocyte differentiation program in different regions of the body. The depot-specific effects of a pharmacologic agent have been observed previously, as seen in the clinical studies of the drug troglitazone. The apparent inhibitory effect of ritonavir on subcutaneous preadipocyte differentiation may partially explain the peripheral fat atrophy feature of the lipodystrophy syndrome. The possibility of ritonavir interfering with lipolysis, lipogenesis, or apoptosis cannot be ruled out and thus more studies are needed to elucidate how this protease inhibitor may be responsible

for peripheral fat atrophy. The enhancing effect of ritonavir on intra-abdominal preadipocyte differentiation in primary culture suggests that ritonavir may act to augment preadipocyte differentiation in the truncal areas. This enhancing effect may be mediated through a pathway that increases levels of active ADD-1/SREBP-1. Effects of ritonavir on lipolysis, lipogenesis, and apoptosis in intra-abdominal adipose tissue should also be examined to investigate the drug's action on the central obesity of the syndrome.

This study shows that ritonavir acts to augment TG accumulation and GPDH activity. The enhancing effects of ritonavir on the late events of 3T3-L1 preadipocyte differentiation may be mediated through ADD-1/SREBP-1, as the protein level of the active form increases in the presence of ritonavir. The positive effect of ritonavir on differentiation is not dependent on the transcription factors PPARγ and C/EBPα and the intermediate marker aP2 as seen by the partial inhibition of these proteins by ritonavir. Ritonavir is proposed to enhance 3T3-L1 preadipocyte differentiation by augmenting the protein level of the 68-kD active form of ADD-1/SREBP-1 which acts to augment TG storage and GPDH activity through a mechanism that does not depend on upregulation of the aP2 gene and the adipogenic transcription factors PPARγ and C/EBPα. Preliminary results suggest that ritonavir preferentially enhances intra-abdominal preadipocyte differentiation in primary culture over subcutaneous preadipocyte differentiation, as assessed by morphological changes observed under the light microscope. Ritonavir's effects in primary culture on human preadipocyte differentiation markers such as the

Figure 10. Proposed pathway through which ritonavir acts to enhance 3T3-L1 preadipocyte differentiation.


Clonal
Expansion
Early
Late
Preadipocyte
Adipocyte
ADD-1/SREBP-1
PPARγ
C/EBPα
aP2
GPDH
TG

adipogenic transcription factors PPARγ and C/EBPα and the enzyme GPDH should be examined. Dose-dependent experiments are necessary to establish that increased levels of active ADD-1/SREBP-1 are due to specific effects of ritonavir. Examination of molecular target(s) of ritonavir is needed to elucidate its mechanism to enhance preadipocyte differentiation. Investigation of ritonavir's effects in primary culture would be one step closer to the *in vivo* situation. However, limitations exist in any cell culture model system. In the body, protease inhibitors may be metabolized to intermediate compounds which may act as the active agents. It is also possible that protease inhibitors may interfere with other tissues which may indirectly affect adipose tissue. In addition, these drugs may exert their effects *in vivo* in conjunction with other compounds or hormones. Future strategies may include *in vivo* examination of ritonavir's depot-specific effects on preadipocyte differentiation and total body fat distribution to obtain direct evidence of ritonavir's role in lipodystrophy syndrome.

Taken together, the data in this study suggest that ritonavir enhances 3T3-L1 preadipocyte differentiation by prolonging activation of ADD-1/SREBP-1. Preliminary results indicate that ritonavir preferentially augments human intra-abdominal preadipocyte differentiation in primary culture over subcutaneous preadipocyte differentiation, consistent with truncal fat accumulation and peripheral fat atrophy, the clinical features of HIV-1 protease inhibitor-associated lipodystrophy. Understanding the mechanism of these drugs in the lipodystrophy syndrome would help in the design of potent protease inhibitors without the deleterious side effects.

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VII. APPENDIX A

List of Abbreviations

15-d-PGJ2	15-deoxy-Δ-^{12,14}-prostaglandin J2
AE-1	Adipocyte enhancer 1
AEBP	Adipocyte enhancer-binding protein
ADD-1	Adipocyte determination and differentiation-dependent factor-1
ALBP	Adipocyte lipid binding protein
ALLN	N-acetyl-leucyl-leucyl-norleucinal
B	Biotin
BSA	Bovine serum albumin
bHLH	Basic helix-loop-helix
cAMP	Cyclic adenosine 5'-monophosphate
CCAAT	DNA sequence to which C/EBPs bind to
cDNA	Complementary dioxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
c-myc	Cellular homolog of the oncogene v-myc of avian myelocytomatosis virus strain 29
CRABP-1	Cytoplasmic retinoic-acid binding protein type 1
CTP	Cytidine 5'-triphosphate
DEPC	Diethyl pyrocarbonate
DHAP	Dihydroxyacetone phosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
F12	Ham's F12
FAS	Fatty acid synthase
FBS	Fetal bovine serum
gas	Growth arrest-specific
PDGF	Platelet-derived growth factor
FGF	Fibroblast growth factor
FKBP51	FK506 binding protein 51
GLUT	Glucose transporter
GPDH	Glycerol 3-phosphate dehydrogenase
H_2O	Water
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
IBMX	Isobutylmethylxanthine
LPL	Lipoprotein lipase
LRP	Lipoprotein-receptor-related protein
MOPS	3-(N-morpholino)propanesulfonic acid

mRNA	Messenger ribonucleic acid
NIH	National Institutes of Health
NaCl	Sodium Chloride
NAD	Oxidized form of nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
P450	P450 cytochrome
P	Pantothenate
PBS	Phosphate buffer saline
PPAR	Peroxisome proliferator activated receptor
Pref-1	Preadipocyte factor-1
PSN	Penicillin Streptomycin Nystatin
Rb	Retinoblastoma
RNA	Ribonucleic acid
RXR	Retinoid receptor
S1P	Site-1 protease
S2P	Site-1 protease
SCAP	SREBP cleavage-activating protein
SCD	Stearoyl-CoA desaturase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SREBP-1	Sterol regulatory element binding protein 1
TEMED	N-tetramethyl-1,2-diaminoethane
TG	Triacyglycerol
TZD	Thiazolidinedione
WD	Tryptophan – Aspartate
Wnt	Secreted glycoproteins whose name is derived from *wingless* and *int-1* genes

VIII. APPENDIX B

Curriculum Vitae

Name:	Anh T. Nguyen
Date of birth:	November 5, 1975
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Education:	**Bachelor of Science (Honours Biochemistry)** University of Ottawa Ottawa, Ontario 1994-1998
Awards:	**Undergraduate Biochemistry Research Excellence Award** University of Ottawa 1998 **Entrance Scholarship for Academic Excellence** University of Ottawa 1994
Publication:	Nguyen AT, A Gagnon, JB Angel, A Sorisky. 2000. Ritonavir increases the level of active ADD-1/SREBP-1 protein during adipogenesis. AIDS. 14:2467-2473.
Abstracts:	Nguyen AT, A Gagnon, JB Angel, A Sorisky. The HIV-1 protease inhibitor ritonavir enhances 3T3-L1 adipocyte differentiation without altering PPARγ protein expression. In: Program & Abstracts of the Endocrine Society's 81st Annual Meeting, San Diego, CA, June 12-15, 1999:P2-209. Nguyen AT, A Gagnon, JB Angel, A Sorisky. Enhanced 3T3-L1 adipocyte differentiation by the HIV-1 protease inhibitor ritonavir. In: Abstracts of the CDA/CSEM Professional Conference and Annual Meetings, Ottawa, ON, Oct. 13-16, 1999:183.

IX. APPENDIX C

Contributions of Collaborators

Thanks to Dr. AnneMarie Gagnon for her contribution in the triacylglycerol experiments.