Suppression of Preadipocyte Differentiation and Promotion of Adipocyte Death by HIV Protease Inhibitors*

Paul Dowell‡§, Charles Flexner¶, Peter O. Kwiterovich, and M. Daniel Lane‡

From the Departments of ‡Biological Chemistry, ¶Pharmacology and Molecular Sciences, and †Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Many human immunodeficiency virus (HIV)-infected patients taking combination antiretroviral therapy that includes HIV protease inhibitors experience atrophy of peripheral subcutaneous adipose tissue. We investigated the effects of HIV protease inhibitors on adipogenesis and adipocyte survival using the 3T3-L1 preadipocyte cell line. Several HIV protease inhibitors were found either to inhibit preadipocyte differentiation or to promote adipocyte cell death. One protease inhibitor, nelfinavir, elicited both of these effects strongly. When induced to differentiate in the presence of nelfinavir, 3T3-L1 preadipocytes failed to accumulate cytoplasmic triacylglycerol and failed to express normal levels of the adipogenic transcription factors CCAAT/enhancer-binding protein α and peroxisome proliferator-activated receptor γ. The level of the proteolytically processed, active 68-kDa form of sterol regulatory element-binding protein-1, a transcription factor known to promote lipogenic gene expression, also was reduced markedly in nelfinavir-treated cells, whereas the level of the 125-kDa precursor form of this protein was unaffected. The inhibitory effect of nelfinavir occurred subsequent to critical early events in preadipocyte differentiation, expression of CCAAT/enhancer-binding protein β and completion of the mitotic clonal expansion phase, because these events were unaffected by nelfinavir treatment. In addition, nelfinavir treatment of fully differentiated 3T3-L1 adipocytes resulted in DNA strand cleavage and severe loss of cell viability. In contrast, cell proliferation and viability of preadipocytes were unaffected by nelfinavir treatment. Thus, molecular or cellular changes that occur during acquisition of the adipocyte phenotype promote susceptibility to nelfinavir-induced cell death. When considered together, these results suggest that nelfinavir may promote adipose tissue atrophy by compromising adipocyte viability and preventing replacement of lost adipocytes by inhibiting preadipocyte differentiation.

Highly active antiretroviral therapy (HAART) has proven effective at reducing morbidity and mortality in HIV-infected individuals displaying symptoms of disease progression (1). Currently, the recommended therapy for such patients includes the use of one or two HIV protease inhibitors (PIs) combined with two nucleoside reverse transcriptase inhibitors (RTIs) or two nucleoside RTIs combined with one nonnucleoside reverse transcriptase inhibitor (2). Inhibition of the HIV protease prevents cleavage and maturation of the viral polypeptide precursor leading to production of noninfectious viral particles (reviewed in Ref. 3). The HIV reverse transcriptase is required to copy the viral RNA genome and inhibitors used to target this enzyme consist of nonnucleoside, noncompetitive inhibitors or chain-terminating nucleoside analogues (reviewed in Ref. 4).

Despite the clinical benefits of HIV suppression by HAART, a serious metabolic syndrome has arisen in treated patients. The syndrome often includes atrophy of subcutaneous adipose tissue, thus giving rise to the widely used term “lipodystrophy syndrome,” and increased visceral and dorsocervical adipose tissue (5–10). Other symptoms include dyslipidemia (6, 8, 9, 11), hyperglycemia (12, 13), and insulin resistance (6, 14). Emergence of the syndrome has been correlated temporally with the widespread use of PIs. However, similar symptoms have been reported in therapy naive HIV-infected patients (15) and in patients receiving non-PI containing antiviral regimens (16–19). The underlying cause of the syndrome may be a complex physiological response to multiple factors including one or more components of combination drug regimens, viral infection, or effective viral suppression. Currently, the cause of this syndrome, referred to hereafter as HIV/HAART-associated syndrome (HAS), is unknown. A commonly reported symptom of HAS appears to be alteration of adipose tissue depots.

Considerable progress has been made in understanding the molecular mechanisms of adipocyte biology using the 3T3-L1 preadipocyte cell line (20) as a model. 3T3-L1 preadipocytes, when growth-arrested at confluence, can be induced to differentiate into adipocytes in the presence of fetal bovine serum and a hormonal mixture that includes insulin, dexamethasone, and isobutylmethylxanthine (reviewed in Refs. 21–23). At least two classes of transcription factors serve important roles in regulating adipogenesis, CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) belonging, respectively, to the basic leucine zipper class of transcription factors and to the nuclear hormone receptor superfamily (reviewed in Refs. 22 and 24). After the
onset of differentiation, a cascade of gene expression begins with the rapid induction of C/EBP β and δ (25, 26). Concomitantly, synchronous re-entry into the cell cycle occurs, and cells proceed through a mitotic clonal expansion phase that consists of approximately two rounds of mitosis (27, 28). Near to or upon completion of mitotic clonal expansion, expression of C/EBPα (25) and PPARγ (29) is induced, and expression of C/EBPβ and δ begins to decline (25, 26). C/EBPα and PPARγ then promote sustained expression of numerous adipocyte genes, including that which encodes the fatty acid binding protein 422a/P2 (reviewed in Refs. 22, 24). The cells then become rounded and engorged with cytoplasmic triacylglycerol droplets. Both C/EBPα (30, 31) and PPARγ (29, 32), the latter in combination with the obligate heterodimeric partner and nuclear hormone receptor superfamily member, retinoid X receptor (RXR) α, have been shown to bind regulatory elements within the promoter of the 422a/P2 gene. Similar regulatory elements have been identified within the promoters of numerous other adipocyte genes through which transcriptional activation is achieved (reviewed in Refs. 22, 24). In addition, steroid regulatory element-binding protein-1 (SREBP-1, also referred to as ADD1) is expressed during 3T3-L1 differentiation (33, 34) and, like C/EBPα and PPARγ, is classified as a proadipogenic transcription factor. SREBP-1/ADD1, a member of the basic helix-loop-helix-leucine zipper transcription factor class, promotes lipogenic gene expression (34) and stimulates production of an unidentified PPARγ ligand (35). Thus, C/EBPα, PPARγ, and SREBP-1/ADD1 cooperatively promote adipogenesis and subsequent maintenance of the adipocyte phenotype.

Inhibition of preadipocyte differentiation by PIs has been reported recently. Several PIs, including amprenavir, indinavir, nelfinavir, and ritonavir, have been shown to inhibit triacylglycerol accumulation and expression of 422a/P2 mRNA in 3T3-L1 preadipocytes (36). Indinavir and saquinavir were demonstrated to inhibit glycerol-3-phosphate dehydrogenase activity, a late lipogenic marker of the adipogenic process, in primary human preadipocytes (37). Indinavir also has been shown to augment the inhibitory action of all-trans-retinoic acid on lipogenesis in the pluripotent mesenchymal stem cell line, C3H110T1/2 (38). A poorly understood mechanism whereby indinavir enhances retinoic acid receptor signaling has been proposed (38). Collectively, these findings demonstrate that PIs inhibit preadipocyte differentiation, although the precise molecular mechanisms involved remain unknown.

We conducted a detailed analysis of the effects of nelfinavir on both preadipocyte differentiation and adipocyte survival. Results from our investigation indicate that nelfinavir inhibits differentiation of 3T3-L1 preadipocytes at a point following two early, differentiation-associated events, C/EBPβ expression and mitotic clonal expansion, because these events were not affected by nelfinavir. Nelfinavir-dependent inhibition of adipogenesis was manifest by severe reductions in both triacylglycerol accumulation and expression of C/EBPα, PPARγ, SREBP-1/ADD1, and 422a/P2 protein. Furthermore, nelfinavir inhibited expression of the same adipogenic transcription factors and promoted cell death in fully differentiated 3T3-L1 adipocytes. Thus, nelfinavir and other PIs may promote adipose tissue atrophy by promoting adipocyte loss and/or preventing replacement of lost adipocytes by inhibiting preadipocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**3T3-L1 preadipocytes (20) were maintained in DMEM containing 10% calf serum (Intergen Co., Purchase, NY). Differentiation was induced as described (39) by incubating 2-day postconfluent cells (designated day 0) in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and a hormonal mixture composed of 520 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone and 167 μM insulin (termed MDI) for 48 h. Cells were then incubated in DMEM containing 10% fetal bovine serum and 167 μM insulin for another 48 h, after which they were maintained in DMEM containing 10% fetal bovine serum with a medium change every 48 h. All cell culture medium was supplemented throughout with 62.5 μg/ml penicillin, 100 μg/ml streptomycin, and 8 μg/ml amphotericin. All treatments were performed into culture medium, and in all experiments cells were exposed to an identical concentration of vehicle (0.1% v/v). Oil Red O staining was performed by fixing cell monolayers in 3.7% formaldehyde, washing in water and staining with a 0.6% (w/v) Oil Red O solution (60% isopropanol, 40% water) for 1 h at room temperature. Cell monolayers were then washed extensively with water to remove unbound dye. Trypan blue staining was performed by incubating cell monolayers in a 0.2% (w/v) trypan blue solution (0.15 mM NaCl) for 10 min at room temperature. Cell number data shown in Fig. 3C were determined by trypsinizing cell monolayers from 6-cm culture dishes followed by counting with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

**Cell Extract Preparation—**Whole cell extracts were prepared at the indicated times by washing cell monolayers from 6-cm plates once in phosphate-buffered saline (PBS, pH 7.5) followed by lysis in 1% SDS, 60 mM Tris-HCl (pH 8.0). Nuclear extracts were prepared according to the method of Dignam et al. (40). Protein concentrations of all samples were determined using the bicinchoninic acid assay (41).

**Immunoblot Assays—**Data shown in Figs. 2 and 4 were obtained by staining 6-cm plates from each experiment with SDS-polyacrylamide gel electrophoresis. Two identical sets of protein extracts were run individually on 8% and 12.5% acrylamide gels to achieve maximum resolution for each series of immunoblots. After transferring to polyvinylidene fluoride membranes (0.45-micron pore size; Immobilon-P, Millipore), blots were probed with antibodies (see below) recognizing the proteins indicated. Blots were sequentially probed, stripped, re-blocked in 2% SDS, 0.1% 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.7) at 65 °C for 1 h, equilibrated in Tris-buffered saline (137 mM NaCl, 25 mM Tris-HCl, pH 7.6) + 0.1% Tween-20 (TTBS), and reprobed after blocking nonspecific protein binding by incubation for 30 min in 5% (w/v) nonfat dried milk dissolved in TTBS. In Fig. 2, extracts separated on the 12.5% gel were probed for C/EBPβ, C/EBPα, and PPARγ; those on the 8% gel were probed for PPARγ, RXRα, and SREBP-1. In Fig. 4, extracts separated on the 12.5% gel were probed for C/EBPα and 422a/P2, and those on the 8% gel were probed for PPARγ, RXRα, and SREBP-1. Commercially available primary antibodies recognizing the following proteins were used: PPARγ (Santa Cruz, catalogue number sc-7273), RXRα (Santa Cruz, catalogue number sc-553), and SREBP-1 (Santa Cruz, catalogue number sc-367); note this antibody recognizes both SREBP-1a and -1c (also known as ADD1)). Rabbit polyclonal antisera specific to C/EBPβ, C/EBPα, and 422a/P2 were generated in this laboratory. Protein detection was performed by ECL using commercially available reagents as per the manufacturer’s instructions (Amersham Pharmacia Biotech). Identical procedures were used to generate immunoblot data shown in Fig. 3B except 10 μg of nuclear extract was loaded per lane.

**Electrophoretic Mobility Shift Assays—**Assays were performed as described (28) with 10-μg nuclear extract except extracts were prepared according to the method of Dignam et al. (40). Recombinant C/EBPβ (38-kDa isoform) was generated using a TNT-coupled reticulocyte lysate system (Promega, Madison, WI).

**Fluorescence Microscopy and TUNEL Assays—**Cells grown on No. 1 coverslips (22 × 22 mm) were washed twice in ice-cold PBS and fixed in freshly prepared 1% paraformaldehyde on ice for 15 min. Coverslips were washed once with PBS and incubated in 70% methanol at −20 °C for 60 min to permeabilize cells. Coverslips were then washed three times in PBS, and 50 μl of TUNEL assay reaction mixture was pipetted gently onto coverslips. The mixture included 200 mM potassium cacodylate, 0.2 mM diethiothreitol, 0.25 mM cobalt chloride, 25 μM Tris-HCl (pH 6.1) supplemented with 0.5 nmol ChromaTide Alexa Fluor 488–5-dUTP (Molecular Probes, Eugene, OR), and 10 units terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals). Reactions were allowed to proceed for 90 min at 37 °C followed by one wash in 2× SSC to terminate reactions. Coverslips were washed twice in PBS, incubated in PBS + 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI), and washed again for 15 min at room temperature to stain nuclei and washed three times before mounting on microscope slides in Prolong Antifade solution (Molecular Probes). Samples were viewed at 630× magnification using a Zeiss Axioskop microscope, and images were obtained using IP Lab software (Scanalytics, Inc., Fairfax, VA). Percentage of cells exhibiting TUNEL reactivity (TUNEL index in Fig. 6) was determined by the number of TUNEL staining cells divided by the total number of cells (DAPI-stained cells). Five different fields were scored for each...
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Effects of Anti-HIV Drugs on Lipid Accumulation in Preadipocytes Induced to Differentiate. 3T3-L1 preadipocytes induced to differentiate with MDI (see “Experimental Procedures”) in the presence or absence of HIV protease inhibitors (20 μM) or stavudine (20 μM) were stained with Oil Red O after 6 days. A, preadipocytes were treated with protease inhibitors from D0–D2 (upper panel) or D0–D6 (lower panel) during the 6-day differentiation protocol. B, microscopic view (200×) of selected dishes shown in A. C, preadipocytes were treated with stavudine alone or stavudine in combination with the indicated protease inhibitors from D0–D6. NO MDI represents preadipocytes not induced to differentiate but cultured for an identical period of time in the presence of vehicle. MDI represents preadipocytes induced to differentiate in the presence of vehicle alone. IDV, indinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir.

RESULTS

Effects of Anti-HIV Drugs on Differentiation-dependent Triacylglycerol Accumulation—Under appropriate culture conditions, including incubation with a hormonal mixture (see the Introduction and “Experimental Procedures”), 3T3-L1 preadipocytes undergo differentiation and assume adipocyte characteristics that include a specific pattern of gene expression and accumulation of cytoplasmic, triacylglycerol-rich lipid droplets. Experiments were conducted to determine whether HIV PIs affect this process. Preadipocytes were induced to differentiate in the presence or absence of the PIs indinavir, ritonavir, nelfinavir, and saquinavir (20 μM). Six days after the onset of differentiation, when cytoplasmic lipid droplets are normally abundant, cells were stained with the lipophilic dye Oil Red O to determine the extent of triacylglycerol accumulation. Exposure to nelfinavir throughout the course of differentiation severely inhibited lipid accumulation, but this effect was not observed readily with 20 μM indinavir, ritonavir, or saquinavir under identical conditions (Fig. 1, A, lower panels, and B). The inhibitory effect of nelfinavir was not observed when preadipocytes were exposed to this drug only during the first 2 days of the differentiation program (Fig. 1A, upper panels). Similarly, exposure to nelfinavir from days 2–6 of the program did not inhibit lipid accumulation (data not shown). Thus, the inhibitory effect of nelfinavir was observed only when preadipocytes were exposed to this drug throughout the course of differentiation.

Adipose tissue abnormalities independent of PI-containing therapy regimens have been reported in some HIV-infected patients (15–19). The HIV reverse transcriptase inhibitor, stavudine, has been associated with such abnormalities in one group of patients (19). Therefore, the effect of stavudine on 3T3-L1 preadipocyte differentiation was examined. Stavudine at a concentration of 20 μM or stavudine in combination with indinavir, ritonavir, or saquinavir (20 μM) did not alter lipid accumulation noticeably (Fig. 1C). The inhibitory effect of nelfinavir did not appear to be altered when combined with stavudine (Fig. 1C). Under the conditions employed in the studies described herein, nelfinavir exhibited the most potent antiadipogenic effect of all the anti-HIV drugs tested. For this reason, further studies were conducted to more thoroughly examine the effects elicited by nelfinavir.

Nelfinavir Perturbs Adipogenic Protein Expression—The possibility that nelfinavir inhibits lipogenesis without affecting differentiation-associated protein expression was addressed. Preadipocytes were induced to differentiate in the absence or presence of nelfinavir, and whole cell extracts were prepared every 24 h during the 6-day differentiation protocol. Extracts containing equivalent amounts of total protein were then subjected to immunoblot analysis to assess expression levels of several proteins that are known to be induced during preadipocyte differentiation. Early induction of C/EBPβ was not affected by nelfinavir treatment because C/EBPβ protein levels at days 1 and 2 were similar in vehicle- and nelfinavir-treated cells (Fig. 2, C/EBPβ panel). C/EBPβ expression normally peaks by day 2 and then steadily declines as differentiation proceeds. Cells exposed to nelfinavir did exhibit a more rapid rate of decline of C/EBPβ than was clearly evident by day 5 (Fig. 2, C/EBPβ panel). Expression levels of the adipogenic transcription factors C/EBPα and PPARγ were reduced markedly in nelfinavir-treated cells when compared with those in vehicle-treated cells, with the latter exhibiting a pronounced induction of expression of these proteins beginning at day 2 (Fig. 2, C/EBPα and PPARγ panels). The protein level of RXRa, the heterodimeric partner of PPARγ, did not vary significantly in vehicle- and nelfinavir-treated cells except at days 5 and 6 where RXRa was reduced in nelfinavir-treated cells to a level similar to that observed in uninduced preadipocytes (Fig. 2, RXRa panel). Expression of the lipid binding protein, 422a/p2, was delayed by approximately 48 h in nelfinavir-treated cells, and the level of expression of this protein never achieved that of vehicle-treated cells (Fig. 2, 422a/p2 panel).

SREBP-1 mRNA levels are induced during differentiation of 3T3-L1 preadipocytes (33, 34). SREBP-1 message is translated to produce a 125-kDa membrane-spanning precursor protein that is proteolyzed, thereby releasing a 68-kDa protein fragment that translocates to the nucleus (reviewed in Ref. 42). The nuclear-localized, mature 68-kDa SREBP-1 protein functions as a transcription factor. Nelfinavir treatment had little effect on the level of 125-kDa SREBP-1 protein (Fig. 2, upper SREBP-1 panel). In vehicle-treated cells, expression of the mature 68-kDa form of SREBP-1 decreased by day 2, remained constant on days 3 and 4 and then increased markedly on days 5 and 6 (Fig. 2, lower SREBP-1 panel). The normal differentiation-dependent fluctuations in the expression level of the 68-kDa SREBP-1 were not mirrored in nelfinavir-treated cells. Rather, the onset of these fluctuations appeared to be delayed in nelfinavir-treated cells. Furthermore, the increased level of expression of the 68-kDa SREBP-1 observed by day 6 in vehicle-treated cells was not achieved in nelfinavir-treated cells (Fig. 2, lower SREBP-1 panel).
When considered together, immunoblot analyses indicate that nelfinavir treatment leads to disruption of the expression patterns of several proteins normally associated with preadipocyte differentiation including C/EBPα, PPARγ, and 422/aP2. Additionally, the maturation or accumulation of the mature 68-kDa form of SREBP-1 is altered severely in nelfinavir-treated cells. Nelfinavir did not appear to be generally toxic as the number of cells present under differentiating conditions and in the presence of nelfinavir appears to have been reduced to differentiate in the presence of vehicle or 20 μM nelfinavir, and whole cell extracts were prepared every 24 h for 6 days. Extracts from preadipocytes not induced to differentiate (0 days after MDI) were also prepared for comparative purposes. Portions of each sample containing 100 μg of total protein were electrophoresed on SDS-containing polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Immunoblot analysis was conducted using antisera specific to the protein indicated in each panel. Note that the immunoblots shown represent two complete sets of extracts run on individual 8% and 12.5% gels. The two resultant membranes were then sequentially stripped and reprobed multiple times with different antisera (see “Experimental Procedures” for details). Each membrane was stained with Coomassie Blue to assess transfer efficiency and to estimate equal protein loading. A portion of one representative blot is shown in the bottom panel (STAIN). Three independent experiments were conducted with similar results, and data from one experiment are shown. Note that two isoforms each of C/EBPα (38 and 18 kDa), C/EBPβ (42 and 30 kDa), and PPARγ (γ1, 55 kDa; γ2, 58 kDa) are expressed.

**Nelfinavir Disrupts Adipogenesis at a Point after Expression of C/EBPβ**—The finding that nelfinavir inhibits preadipocyte differentiation but does not interfere with the early induction of C/EBPβ raised the possibility that C/EBPβ function was perturbed. DNA binding assays were used to assess the functionality of nuclear C/EBPβ protein from preadipocytes induced to differentiate in the absence or presence of nelfinavir. An oligonucleotide corresponding to the C/EBP-binding site in the C/EBPα gene promoter (43) was utilized as a probe. Samples were prepared from cells at 33 and 46 h after the onset of differentiation as C/EBPβ binding activity is detected readily in differentiating preadipocytes at these time points (28). As expected, nuclear extract from preadipocytes not induced to differentiate contained little detectable C/EBP binding activity (Fig. 3A, lane 2). Extracts from cells at 33 and 46 h after the onset of differentiation exhibited strong binding activity that was not altered when the cellular source of the extracts was exposed to nelfinavir (Fig. 3A, lanes 4–7). It should be noted that C/EBPs α, β, and δ can bind the response element used in these experiments. Most of the C/EBP binding activity was shown to contain C/EBPβ (homodimers or C/EBPβ-containing heterodimers) by the ability of specific antisera to supershift these complexes (Fig. 3A, lanes 8–11). The diffuse character of bands results from a complex mixture of homo- and heterodimers composed of C/EBPβ (38- and 18-kDa forms), C/EBPα and, for the 46 h time point, C/EBPγ (42- and 30-kDa forms; Ref. 28). Indeed a much more compact band composed of recombinant 38-kDa C/EBPβ homodimers was evident in the same experiment (Fig. 3A, lane 1). Immunoblot analysis of the same extracts used in the DNA binding assays indicated that the nuclear protein levels of C/EBPβ were similar in vehicle- and nelfinavir-treated cells at both time points examined (Fig. 3B, lanes 4–7). These results are consistent with the finding that nelfinavir treatment does not alter C/EBPβ protein levels at early time points (Fig. 2, C/EBPβ panel). Thus, DNA binding activity and protein levels of nuclear C/EBPβ do not appear to be altered in preadipocytes treated with nelfinavir.

**Miticic Clonal Expansion Is Unaffected by Nelfinavir**—Adipogenesis is thought to be a culmination of at least two key events. These events include an initial cascade of gene induction and a critical mitotic clonal expansion phase, the latter of which is completed within the first three days of the differentiation process (see the Introduction). Thereafter, differentiating preadipocytes exit the cell cycle, and regulated adipogenic gene expression continues as the cells acquire adipocyte characteristics. Therefore, it was of interest to determine whether nelfinavir treatment prevents mitotic clonal expansion. Preadipocytes were induced to differentiate in the absence or presence of nelfinavir, and 3 days later cell number was determined. Cell number was also determined from additional dishes of preadipocytes not induced to differentiate but grown in the absence or presence of nelfinavir for an identical period of time. Preadipocytes not induced to differentiate, in the presence of vehicle or nelfinavir, were similar in cell number (Fig. 3C). Cell number increased almost 4-fold in vehicle-treated differentiating preadipocytes, consistent with approximately two rounds of mitosis (Fig. 3C). A similar, albeit slightly diminished, increase in cell number also was observed in preadipocytes induced to differentiate in the presence of nelfinavir (Fig. 3C). Microscopic images presented in Fig. 1 support this finding. Note that the number of cells present under differentiating conditions and in the presence of nelfinavir appears to have
increased upon comparison with cells that were not induced to differentiate (Fig. 1B, compare NO MDI and MDI/NFV/D0-D6). Therefore, nelfinavir does not significantly affect the mitotic clonal expansion phase of preadipocyte differentiation despite inhibitory effects on both triacylglycerol accumulation and post-C/EBPβ adipogenic gene expression.

**Nelfinavir Perturbs the Fully Differentiated Adipocyte Phenotype**—Adipose tissue mass is determined by the rate of preadipocyte differentiation, the rate of adipocyte loss, and adipocyte size. Changes in adipose tissue mass could arise from accelerated preadipocyte differentiation, the rate of adipocyte loss, and adipocyte size. Therefore, increases in adipose tissue mass could arise from alterations in any of these processes. Therefore, the effects of anti-HIV drugs on fully differentiated adipocytes were examined. Preadipocytes were induced to differentiate using the standard 6-day protocol as described above (see “Experimental Procedures”). At day 6, adipocytes were exposed to the following anti-HIV drugs at concentrations of 20 μM: stavudine, indinavir, nelfinavir, ritonavir, and saquinavir. Adipocytes were treated with vehicle or the indicated drug, and cells were cultured for an additional 6 days. At day 12 of the experimental protocol, cells were stained with Oil Red O. Adipocytes exposed to nelfinavir, ritonavir, or saquinavir retained less Oil Red O stain when compared with vehicle-treated cells, indicating a decrease in total cytoplasmic triacylglycerol per culture dish (Fig. 5). This effect was most pronounced in adipocytes treated with nelfinavir. Cells exposed to stavudine or indinavir (20 μM) did not appear to differ noticeably from vehicle-treated cells (Fig. 5). Undifferentiated preadipocytes cultured for a period of 12 days and stained with Oil Red O are shown for comparison (Fig. 4A and B). Microscopic examination of adipocytes treated with nelfinavir revealed a decrease in the number of triacylglycerol droplets and many patches devoid of cells (Fig. 4B). A noticeable increase in the number of floating, detached cells was observed 48 h after exposing adipocytes to nelfinavir (data not shown). In contrast, adipocytes exposed to vehicle remained attached to the culture dish, contained numerous lipid droplets and were present in a continuous monolayer (Fig. 4B). Although ritonavir and saquinavir appeared to have a similar effect on mature adipocytes (Fig. 4A), particularly at higher drug concentrations (data not shown), the effects of nelfinavir were most pronounced.

A detailed analysis of the effects of nelfinavir on adipogenic protein expression in 3T3-L1 adipocytes was undertaken. As described above, adipocytes at day 6 of the differentiation protocol were treated with vehicle or nelfinavir, and whole cell extracts were prepared every 24 h thereafter for 6 days. Immunoblot analysis was performed on equal amounts of total protein to examine the effects on adipocyte protein expression. After 3 days, C/EBPα protein levels were almost completely suppressed in nelfinavir-treated adipocytes, although partial suppression was observed after only 24 h (Fig. 5, C/EBPα panel). A similar effect of nelfinavir treatment on PPARγ protein was observed, but protein levels became undetectable only after 5 days of treatment (Fig. 5, PPARγ panel). RXRα protein levels were relatively constant until 4 days after nelfinavir treatment when increasing diminished levels were present (Fig. 5, RXRα panel). 422/aP2 protein expression persisted in nelfinavir-treated cells until day 5 when levels began to decline (Fig. 5, 422/aP2 panel). Levels of the 68-kDa form of SREBP-1 were lower in nelfinavir-treated cells by 24 h and were unde-
nelfinavir promotes loss of adipocyte viability—As indicated above, a noticeable increase in the number of floating, detached cells was observed 48 h after exposing adipocytes to nelfinavir. Subcutaneous adipocyte apoptosis in tissues from HIV-infected patients experiencing symptoms of HAS has been reported (44). Therefore, experiments were carried out to determine whether nelfinavir induces signs of apoptosis in 3T3-L1 adipocytes. The terminal deoxynucleotidyl TUNEL assay was used to detect DNA strand cleavage, a generally accepted marker for apoptosis (reviewed in Refs. 45 and 46). TUNEL is used frequently as a means to detect apoptosis in a variety of cells and has been reported to detect this cellular process in the 3T3-L1 cell line (47). Adipocytes at day 6 of the differentiation protocol were treated with vehicle, nelfinavir, or stavudine. Two days after the onset of treatment, cells were assayed for TUNEL reactivity and stained with DAPI to visualize cell nuclei. Positive staining for TUNEL was detected in less than 2% of adipocytes exposed to either stavudine or vehicle alone (Fig. 6A). In contrast, approximately 15% of nelfinavir-treated adipocytes stained positive for TUNEL reactivity (Fig. 6, A, panel K, and B). No TUNEL reactivity was observed in preadipocytes treated with vehicle, stavudine, or nelfinavir (Fig. 6A, panels D–F), indicating that nelfinavir induces DNA strand cleavage only after preadipocytes have differentiated into adipocytes. Trypan blue dye exclusion experiments were conducted to determine whether adipocytes remain viable after nelfinavir treatment. Adipocytes exposed to nelfinavir for 6 days exhibited widespread trypan blue staining, indicating that a majority of the cells were either dead or dying (Fig. 6C). In contrast, adipocytes treated with vehicle exhibited little or no trypan blue staining (Fig. 6C). Therefore, nelfinavir promotes DNA strand cleavage in adipocytes within 48 h and induces extensive loss of cell viability over a 6-day treatment period.

Evidence is presented to suggest that the HIV PI, nelfinavir, may contribute to adipose tissue atrophy by promoting adipocyte cell death and preventing replacement of lost adipocytes by inhibiting preadipocyte differentiation. The concentration of nelfinavir required to elicit these effects in vitro is within the range of that observed in plasma from patients administered therapeutic doses of nelfinavir (reviewed in Ref. 3). Thus, it is possible that the effects of nelfinavir on the 3T3-L1 cell line observed in vitro may also occur in vivo.

The simplest explanation for PI-associated adipose tissue abnormalities would entail a common mechanism. Our initial studies with other PIs did reveal some effects similar to those elicited by nelfinavir. For example, ritonavir and saquinavir (20 μM) were capable of reducing the amount of cytoplasmic triacylglycerol in 3T3-L1 adipocytes as measured by Oil Red O staining (Fig. 4A), and this effect was more pronounced at higher drug concentrations (data not shown). 3T3-L1 adipocytes treated with ritonavir or saquinavir (20 μM) also exhibited signs of cell death and TUNEL reactivity, although to a lesser degree than that observed with nelfinavir (data not shown). In addition, we were able to reproducibly observe a robust inhibitory effect of indinavir (20 μM) on triacylglycerol accumulation in differentiating 3T3-L1 preadipocytes when isobutylmethylxanthine, an agent used to elevate cAMP levels, was not included in the hormonal induction mixture (data not shown). It is not clear why reduced cAMP levels in combination with indinavir are required to elicit an inhibitory effect by this PI on adipogenesis. Regardless, each PI examined in our investigation appeared either to inhibit lipid accumulation during preadipocyte differentiation or to promote adipocyte cell death.

A detailed analysis of the antiadipogenic influence of nelfinavir was conducted in an attempt to identify potential mechanisms. Nelfinavir appears to inhibit preadipocyte differentiation at a point following C/EBPβ protein expression and mitotic clonal expansion because these events were not inhibited by nelfinavir. In contrast, the levels of C/EBPα and PPARγ protein, which normally are expressed after C/EBPβ, were markedly reduced in nelfinavir-treated cells. C/EBP regulatory elements have been identified in the promoter regions of both the C/EBPα (43) and PPARγ (48, 49) genes, and C/EBPβ is thought to activate expression of both genes during preadipocyte differentiation. Therefore, nelfinavir may prevent the normal differ-
Effects of Nelfinavir on 3T3-L1 Preadipocytes and Adipocytes

Nelfinavir induces TUNEL reactivity and loss of viability in adipocytes. A, preadipocytes not induced to differentiate (NO MDI, panels A–F) and day 6 adipocytes (ADIPOCYES, panels G–L) were treated with vehicle (+VEH), nelfinavir (+NFV, 20 μM), or stavudine (+STVD, 20 μM) for 48 h. Cells were then fixed, permeabilized, assayed for TUNEL reactivity, and stained with DAPI. After mounting coverslips on microscope slides, cells were viewed by fluorescence microscopy. B, graphical representation of TUNEL assay data. Data shown are the means ± S.E. of three experiments. *, p values of 0.007 and 0.006 compared with vehicle- and stavudine-treated adipocytes, respectively. C, adipocytes were stained with trypan blue after 6 days of treatment with vehicle (VEH) or 20 μM nelfinavir (NFV). Images of culture dishes are shown above microscopic images.

Fully differentiated 3T3-L1 adipocytes exhibited a 6% loss in cell number (data not shown) and signs of DNA strand cleavage within 48 h after treatment with nelfinavir. Surprisingly, 3T3-L1 preadipocytes exposed to nelfinavir proliferated normally and showed no signs of cell death or DNA strand cleavage even after extended drug exposures of up to 6 days (data not shown). These results suggest that some cellular or molecular change occurs during differentiation that sensitizes adipocytes to nelfinavir-induced cell death. A rapid reduction in adipogenic protein expression, initially observed with C/EBPα and followed by PPARγ and mature SREBP-1, was also observed when adipocytes were exposed to nelfinavir. The observed down-regulation of adipogenic protein expression could be an indication of drug-induced dedifferentiation. However, dedifferentiation without cell death does not offer a complete explanation because the majority of adipocytes are dead or dying after six days of drug treatment (Fig. 6C). Although some degree of dedifferentiation may occur, the primary response of adipocytes to nelfinavir appears to be loss of cell viability. Further experiments will be required to determine whether adipocyte death is a consequence of nelfinavir-induced loss of adipogenic protein expression or vice versa.

Although positive TUNEL reactivity was observed in response to nelfinavir, it should be noted that this assay has been shown to detect necrotic as well as apoptotic cells (51). Furthermore, in additional studies using 3T3-L1 adipocytes, we have been unable to clearly demonstrate nelfinavir-induced procaspase 9 cleavage or DNA laddering (data not shown), two independent indicators of apoptosis. Thus, we cannot definitively determine which of the two cellular death processes is occurring. Regardless, a clear loss of adipocyte viability in response to nelfinavir was observed. When considered together, the effects of nelfinavir on 3T3-L1 preadipocytes and adipocytes are distinct but commonly antiadipogenic.

The molecular mechanism responsible for the antiadipogenic effects of nelfinavir and other PIs is not known. Recently, amprenavir, indinavir, and ritonavir were shown to inhibit insulin-stimulated glucose uptake in 3T3-L1 adipocytes by interfering with GLUT4 transporter function (52). Inhibition of glucose transport has been reported to promote apoptosis in some cultured cell lines (53). Studies conducted in our laboratory demonstrated that indirect inhibition of glucose uptake by antibody-mediated insulin depletion in obese (ob/ob) mice leads to adipose-specific cell death (54). Thus, restriction of a glucose energy source in some cell lines and tissues may promote cell death. Nelfinavir-dependent inhibition of GLUT4 activity in 3T3-L1 adipocytes may provide a mechanism for the cell death observed in our experiments. However, it is difficult to explain the observation that indinavir had little or no effect on 3T3-L1 adipocyte viability under our experimental conditions. Furthermore, 3T3-L1 preadipocytes induced to differentiate in the presence of nelfinavir would not be expected to express GLUT4, because expression of this glucose transporter

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does not occur until after C/EBPα is expressed (55, 56). Therefore, attenuation of GLUT4 activity is not likely to play a role in PI-induced inhibition of 3T3-L1 preadipocyte differentiation. Future studies will be required to address these possibilities and to examine the intriguing hypothesis that some symptoms of HAS are due to PI-induced antagonism of GLUT4 activity.

We conclude from our studies that nelfinavir and other PIs exert antiadipogenic influences on the model 3T3-L1 cell line. Although nelfinavir elicited the most potent antiadipogenic effects on the 3T3-L1 cell line in our investigation, all PIs have been associated with HAS in treated patients (reviewed in Ref. 57). Differential drug concentrations and drug penetration at the site of action would be important determinants of drug effect in vivo. Clinical manifestations of HAS could be multifactorial, reflecting the contributions of drug treatment to multiple biochemical pathways. The relevance of our findings to in vivo adipose tissue homeostasis remains to be determined.

Data from a recently completed prospective study of HIV-infected patients suggest that PIs, but not nucleoside RTIs (lamivudine), promote metabolic abnormalities (hyperglycemia, hyperinsulinemia, and hyperlipidemia) before detectable changes in body composition (fat and lean body mass, truncal and appendicular, measured by dual energy x-ray absorptiometry; Ref. 58). These findings do not exclude the possibility that PIs may have direct, atrophic effects on subcutaneous adipose tissue subsequent to or detectable only after the development of metabolic abnormalities. Additional studies designed to test hypothetical mechanisms and longitudinal data from ongoing and future clinical studies will be required to firmly establish this key point. The need to acquire a thorough understanding of the factors leading to HAS is paramount because the detrimental effects of this syndrome threaten to erode strident gains in effective viral suppression and in lifespan extension of HIV-infected patients.

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