Protease inhibitors used in the treatment of HIV infection have been causally associated with lipodystrophy and insulin resistance and were shown to alter adipocyte differentiation in cultured cells. We aimed to delineate the mechanism by which indinavir impaired adipocyte function. We report that indinavir altered neither the growth nor insulin sensitivity of 3T3-F442A preadipocytes, nor did it alter the initial step of their differentiation, i.e., clonal proliferation. However, adipose conversion was inhibited by indinavir (by 50–60%), as shown by 1) the decrease in the number of newly formed adipocytes; 2) the lower level of the adipogenic protein markers, sterol regulatory element-binding protein-1 (SREBP-1), peroxisome proliferator-activated receptor-γ (PPAR-γ), and the insulin receptor (IR); and 3) the lack of SREBP-1 and PPAR-γ immunoreactivity in the nucleus of most indinavir-treated cells. Partial adipose conversion also correlated with an accumulation of SREBP-1 at the nuclear periphery and an alteration in its electrophoretic mobility. Defective expression and nuclear localization of PPAR-γ probably resulted from the decreased level of nuclear SREBP-1. Indinavir also rendered 3T3-F442A adipocytes resistant to insulin for mitogen-activated protein kinase activation at a step distal to IR substrate-1 tyrosine phosphorylation. Hence, indinavir impairs differentiation at an early step of adipose conversion probably involving the process controlling SREBP-1 intranuclear localization. Indinavir Impairs Sterol Regulatory Element-Binding Protein-1 Intranuclear Localization, Inhibits Preadipocyte Differentiation, and Induces Insulin Resistance

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The use of retroviral protease inhibitors (PIs), in conjunction with other antiretroviral drugs, has dramatically improved the morbidity and mortality associated with HIV infection. However, patients on highly active antiretroviral therapy (HAART) frequently develop a lipodystrophic syndrome involving altered body fat distribution and alterations of glucose and lipid metabolism (1–4). At the clinical level, the patients develop peripheral lipoatrophy with subcutaneous adipose tissue loss in the face, buttocks, limbs, and arms that may or may not be associated with increased central adiposity (5,6). At the biological level, serum triglycerides and cholesterol are commonly increased in these patients. There is also a high prevalence of hyperinsulinemia and insulin resistance, in some cases resulting in diabetes (3,7). The pathophysiology of these clinical and biological alterations is currently unknown and is probably multifactorial. Lipodystrophic syndromes may appear in patients treated with reverse transcriptase inhibitors without PI. However, their biological features seem different and can be explained by the mitochondrial toxicity of these treatments (8–10). Increased levels of serum triglycerides and cholesterol have been observed in patients soon after the initiation of the PI treatment and long before the occurrence of lipodystrophy (1,11,12), and those higher levels are probably related to increased hepatic lipogenesis. Adverse effects of PIs on adipocyte differentiation and on the development of insulin resistance have also been observed in cultured cells (13–17), but the mechanisms have not yet been deciphered.

A defect in or a loss of white adipose tissue can occur in several unrelated genetic diseases, such as congenital lipoatrophic diabetes (18) and the Dunnigan-type familial partial lipodystrophy (19,20). Loss of adipose tissue can also be acquired as a result of diseases such as late-onset lipoatrophic diabetes (18). In all of these cases, lipodystrophy correlates with insulin resistance and altered lipid and glucose metabolism. Because the deleterious effects of PIs are difficult to investigate in patients receiving several classes of antiviral therapies, in vitro models should be used. The few studies carried out on cultured adipocytes show that the PIs can increase (13) or inhibit (15,16) fat storage of adipocytes. Carr et al. (2) proposed a
hypothesized explanation for the syndrome, implicating altered retinoic acid signaling. However, an alteration at the level of the peroxisome proliferator–activated receptor-γ (PPAR-γ)/retinoid X receptor (RXR) activation step can be excluded as a possible cause of the PI effect, as shown in three recently published articles in which mouse (16,17) and human (15) adipocyte differentiation was studied in vitro. On the other hand, it was reported in a recent study (14) that acute treatment with indinavir and other retroviral PIs selectively inhibited the function of the glucose transporter GLUT4, thus providing a possible explanation for the insulin resistance of HIV-infected patients treated with PIs.

Our study was undertaken with the aim of extending the above findings. Our results show that indinavir inhibited the adipocyte differentiation program of 3T3-F442A preadipocytes at an early step that might have involved the nuclear localization of sterol regulatory element-binding protein-1 (SREBP-1), and we showed that indinavir induced a state of insulin resistance.

RESEARCH DESIGN AND METHODS

Cell culture and methods. Powdered forms of indinavir (Merck Sharp & Dohme Laboratories), nelfinavir (Agouron Pharmaceuticals), and amprenavir (Vertex Pharmaceuticals) were provided by the sources indicated. Smith Kline Beecham Pharmaceuticals provided rosiglitazone (BRL 49653), 3T3-F442A preadipocytes (21) were provided by Professor H. Green (Boston, MA) and Dohme Laboratories), nelfinavir (Agouron Pharmaceuticals), and amprenavir (Vertex Pharmaceuticals) were provided by the sources indicated. Smith Kline Beecham Pharmaceuticals provided rosiglitazone (BRL 49653), 3T3-F442A preadipocytes (21) were provided by Professor H. Green (Boston, MA) and Dohme Laboratories, nelfinavir (Agouron Pharmaceuticals), and amprenavir (Vertex Pharmaceuticals) were provided by the sources indicated. Smith Kline Beecham Pharmaceuticals provided rosiglitazone (BRL 49653). 3T3-F442A preadipocytes were cultured as previously described (22). Passage numbers 6–12 were used in all studies. After plating (5 × 10^4 cells), cells were maintained for 2 days in Dulbecco’s modified Eagle’s medium with 10% FCS and 100 μg/ml streptomycin, and 14.5 mmol/l 4-biotin (medium A) containing 10% newborn calf serum (NCS) without or with indinavir (1–50 μg/ml). Subconfluent preadipocytes were cultured for 2 days in medium A supplemented with 5% NCS and 5% fetal calf serum (FCS). Adipocyte differentiation was then induced (day 0) using medium A containing 10% FCS and 100 mmol/l insulin and continued until day 8. In some experiments, the drug was added 2 days after the initiation of differentiation (day 2). Cell differentiation was initiated by rosiglitazone (1 μmol/l) in the absence of insulin. Because the stock solution of PIs and rosiglitazone was made in DMSO, we verified that at the highest concentration of the solvent tested (0.01%), it did not affect cellular function and preadipocyte differentiation.

Measurement of adipocyte differentiation. The differentiation of preadipocytes was evaluated by oil red O staining and by counting (based on their refractivity) the cells having accumulated lipid droplets after trypan blue coloring. Oil red O staining was performed by fixing cell monolayers in 10% formalin in isotonic buffer for 2 h, washing them in water, and staining them with a 0.0% (wt/vol) oil red O solution (80% isopropanol, 40% water) for 1 h at 22°C. Cells were then washed extensively to remove unbound dye. Cell differentiation was also estimated by the increased expression on Western blot of two early markers of adipogenesis, the transcription factors SREBP-1 and SREBP-2, and the differentiation was also estimated by the increased expression on Western blot of two early markers of adipogenesis, the transcription factors SREBP-1 and SREBP-2, and the increased expression of the insulin receptor (IR) protein. It was also taken as an index of adipocyte differentiation (26,27). It was noted that the experiments were based on cell number rather than on cell protein. Indeed, preadipocyte differentiation proceeds from a mitotic clonal expansion step (between day 0 and 2 of differentiation) followed by an arrest in cell division and cell hypertrophy (28). Between day 2 and 8 after initiation of differentiation, the total cell number increased 1.3-fold (Fig. 2F), whereas the cellular protein content was about 2-fold higher (301 ± 18 and 585 ± 24 μg/10^6 cells, respectively).

Immuno- and Western blotting. Subconfluent 3T3-F442A preadipocytes (0.4 × 10^6 cells) or confluent adipocytes (10^6 cells) were washed twice in phosphate-buffered saline (PBS), scraped into 100 μl Laemml buffer containing 100 mmol/l dithiothreitol, and boiled 5 min at 95°C. Equal amounts of cell lysates (corresponding to 10^6 cells) were subjected to SDS-PAGE and Western blotting with the appropriate antibody. Polyclonal antibodies against adipocyte determination and differentiation factor 1/SREBP-1 (K-10), PPAR-γ (H-100), and IRS β-subunit (C-19) were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody to phosphorylated extracellular regulated kinase (E-4; Santa Cruz Biotechnology) was used to detect activated mitogen-activated protein kinase (MAPK). Immune complexes were visualized by chemiluminescence (enhanced chemiluminescent kit; Amersham Pharmacia Biotech SA, Les Ulis, France). IR and the IR substrate (IRS-1)-tyrosine phosphorylation were checked after immunoprecipitation of the cell lysate with a monoclonal anti-phosphotyrosine (PY-20) antibody (Transduction Laboratories, Lexington, KY). 3T3-F442A preadipocytes (2 × 10^6 cells) were solubilized for 30 min at 4°C in lysis buffer (50 mmol/l HEPES, 50 mmol/l NaF, 100 mmol/l NaCl, 5 mmol/l EDTA, 5 mmol/l EGTA, 0.2 mmol/l NaVO_3, 1 μg/ml leupeptin, 0.2 mmol/l phenylmethylsulfonyl fluoride, 1% Triton X-100, pH 7.4), and cell proteins (corresponding to 10^6 cells) were incubated with the PY-20 antibody (5 μg/ml) for 2 h and with protein A/G Plus-agarose (25 μl) for 16 h at 4°C. Aliquots of the immunoprecipitates were processed for SDS-PAGE and Western blotting. Polyclonal antibodies to IR β-subunit and IRS-1 (C-20; Santa Cruz Biotechnology) were used to detect phosphorylated IR and IRS-1.

Confocal immunofluorescence microscopy. 3T3-F442A cells were grown and differentiated in the absence or presence of indinavir (10 μg/ml) on 13-mm glass cover slips. They were fixed and permeabilized with ethanol for 10 min at −20°C. The subcellular distribution of adipogenic markers was visualized by indirect immunofluorescence microscopy using polyclonal antibodies to SREBP-1 and PPAR-γ and secondary antibodies coupled to fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology). Lamin A/C was used as a marker of the inner nuclear envelope (30) and was identified using a monoclonal anti-lamin A/C primary antibody (Chemicon International, Temecula, CA) and Texas Red–coupled secondary anti-IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Primary and secondary antibodies were applied to coverslips in PBS with 1% bovine serum albumin for 15–30 min at room temperature. Confocal microscopy was performed using a Leica TCS-SP microscope (Laserotechnik, Germany) equipped with a 100× objective. A krypton-argon mixed-gas laser adjusted to 488 and 568 nm was used to excite either FITC or Texas Red, respectively. Double fluorescent images were acquired in a sequential mode. Serial optical sections of 1 μm were taken. Selected paired sections were then processed to produce single composite color-merged overlay images using Adobe Photoshop software (version 5.5).

Insulin signaling. Cell responsiveness to insulin was evaluated in preadipocytes and adipocytes by measuring both early and late events representative of the insulin-signaling pathways, i.e., increased tyrosine phosphorylation of both the IR β-subunit and IRS-1 and activation of MAPK, respectively (28,31). Cells were grown and differentiated (or not) in the absence or presence of indinavir or rosiglitazone (1 μmol/l), as described above. They were starved of FCS and insulin for 4 h (preadipocytes) or 6 h (adipocytes). Insulin (100 nmol/l) was then added (or not) for 10 min, cell lysates were prepared, and aliquots (corresponding to 10^6 cells) were immunoblotted with antibody. Cell number was determined in parallel.

Statistical analysis. Results are given as the mean ± SE of the indicated number of independently performed experiments.

RESULTS

Short- and long-term treatment with indinavir failed to alter the growth and insulin sensitivity of 3T3-F442A preadipocytes. In the first set of experiments, we examined the effect of indinavir on the proliferation and sensitivity to insulin of 3T3-F442A cells, which were maintained in a nondifferentiated state. As shown in Fig. 1, short-term treatment (30 min) of the cells with indinavir (10 and 50 μg/ml) did not modify the ability of insulin (100 nmol/l) to increase tyrosine phosphorylation of the IR β-subunit and IRS-1, nor did it modify insulin’s ability to activate MAPK (Fig. 1A–C, left panels). Also, preadipocytes cultured for 8 days in the presence of indinavir at 10 μg/ml were similarly responsive to insulin, with comparable results in terms of IR β-subunit and IRS-1 tyrosine phosphorylation and MAPK activation (Fig. 1A–C, right panels). At confluency, the cell number was similar in both untreated and treated cells (0.16 and 0.17 × 10^6 cells per 12-well dish, respectively), and both cell lines expressed...
FIG. 1. Short- and long-term treatment with indinavir failed to alter insulin sensitivity of 3T3-F442A preadipocytes. Left panels: Subconfluent 3T3-F442A preadipocytes were depleted from NCS for 4 h and incubated for 30 min in the absence or presence of indinavir at the indicated concentration (0–50 μg/ml). Some were stimulated with insulin (100 nmol/l) for 10 min. Right panels: 3T3-F442A cells were cultured for 8 days in the absence or presence of indinavir, depleted from NCS but not from indinavir for 4 h, and stimulated or not stimulated with insulin (100 nmol/l). Cell lysates were then prepared either for immunoprecipitation with an anti-PY-20 antibody at 5 μg/ml (A) or for SDS-PAGE analysis (B and C). Aliquots of the immunoprecipitate (IP) (corresponding to 10^6 cells) and of the cell lysate (corresponding to 10^7 cells) were immunoblotted with the indicated antibody at 0.1 μg/ml. Representative immunoblots from three (A) and four separate experiments (B and C) are shown.

the same protein level of the IR β-subunit (not shown) and extracellular signal-regulated kinase 2 (ERK2) (Fig. 1C, lower right panel). The results indicated that short- or long-term treatment with indinavir at 10 μg/ml did not affect the cell growth and insulin sensitivity of 3T3-F442A preadipocytes.

**Treatment with indinavir altered the differentiation program of 3T3-F442A cells.** We then studied the effect of the drug on cell differentiation induced by the addition of FCS and insulin. We observed that long-term indinavir treatment with increasing concentrations of 1–50 μg/ml (i.e., 1.5–75 μmol/l) progressively decreased 3T3-F442A cells differentiation. This was shown by counting the cells containing lipid droplets (Fig. 2A, left panel) and by oil red O staining (right panel). Dose-response analysis showed that 10 μg/ml was the concentration of indinavir that maximally altered preadipocyte differentiation (50–60%) without altering cell viability; it was a concentration that correlated with that found in the patients’ serum (32). We next showed (Fig. 2B, left panel) that long-term treatment (8 days) of preadipocytes with indinavir did not affect the proliferation step of their differentiation program (day 0–2), which typically consisted in one to two rounds of cell cycles (29). At day 2, control cells began to exhibit an accumulation of cytoplasmic lipid droplets and the rounded morphology typical of differentiated adipocytes. In contrast, at day 2, indinavir-treated cells (10 μg/ml) revealed a decreased accumulation of lipids and a lower level of adipose conversion (45–55% less adipocytes formed) (Fig. 2B, right panel). This was observed at every stage of the differentiation program (days 2–8), was confirmed by oil red O staining of cytoplasmic lipids (not shown), and occurred regardless of the length of the treatment of preadipocytes with indinavir (up to 30 days).

To further study the antiadipogenic effect of indinavir, we used Western blot to measure the level of two fat cell–specific transcription factors, SREBP-1 and PPAR-γ, whose expression sequentially increases at early steps of adipocyte differentiation (33–35). As shown in Fig. 2C, SREBP-1 and PPAR-γ proteins were poorly expressed in nondifferentiated cells (at day 0), regardless of whether they were treated with indinavir. Their expression markedly increased in control cells at day 2 and was almost maximal at day 4. The expression of SREBP-1 and PPAR-γ was 30–60% lower in indinavir-treated cells compared with control cells, despite any delay after differentiation. Indinavir at 10 μg/ml also inhibited the increase in IR β-subunit expression (Fig. 2D, left panel) and IR binding activity (right panel) that occurs at final steps of differentiation (26,27). These data indicated that indinavir altered the adipocyte differentiation program at the morphologic and molecular levels. Of note is the observation that 3T3-F442A cells cultured for 8 days with indinavir (10 μg/ml) recovered a normal adipogenic potential if the drug was removed while preadipocytes were in the exponential growing phase, i.e., 4 days before reaching confluency and induction of differentiation (data not shown).

We then observed (Fig. 3) that once initiated, adipose conversion could be altered by indinavir. Indinavir (10 μg/ml) was added to the culture medium after the clonal expansion step (at day 2), when ~20% of the cells are differentiated. Cell differentiation then continued until day 8, and adipogenic markers were checked every 2 days. Although the total cell number similarly increased in control and indinavir-treated cells up to the 8th day of differentiation (not shown), the number of adipose cells (Fig. 3A) increased in control cells (up to fourfold) but not in indinavir-treated cells. Interestingly, indinavir did not decrease the protein expression level of SREBP-1, but it led to alterations in its electrophoretic mobility (Fig. 3B, upper panel). This could reflect an abnormal processing of SREBP-1 in indinavir-treated cells (23,36,37) or an altered phosphorylation state of SREBP-1 (38,39). By contrast, the change in protein expression of PPAR-γ (Fig. 3B, lower panel), which is known to be genetically controlled by SREBP-1 (34), was markedly inhibited in indinavir-treated cells.

The results indicated that indinavir inhibited both the initiation and completion of the differentiation program of 3T3-F442A preadipocytes at a step possibly involving SREBP-1 maturation.

**Indinavir altered the nuclear localization of SREBP-1 during adipogenesis.** SREBP-1 is synthesized as an inactive 125-kDa precursor protein anchored to the endoplasmic reticulum (ER) and the nuclear envelope, which is matured in two proteolytic steps producing a 68-kDa
FIG. 2. Indinavir inhibited the differentiation of 3T3-F442A preadipocytes. Cells were subcultured for 8 days in 12-well dishes in the absence (M) or presence (f) of indinavir. Cell differentiation was initiated at confluence (day 0) by adding 100 nmol/l insulin and 10% FCS. A: Dose-dependent effect of indinavir (1–50 μg/ml). Left panel: at day 6 of differentiation, cells were trypsinized and adipocytes were counted based on their refringency after trypan blue staining. Values are the mean ± SE of three independently performed experiments. Right panel: similarly treated cell monolayers were stained with oil red O as described in RESEARCH DESIGN AND METHODS.

B: Effect of indinavir (10 μg/ml) on the differentiation program. At the day indicated after differentiation (day 0–6), total cells (left panel) and adipocytes (right panel) were counted as described above. Values are the mean ± SE of four independently performed experiments. C and left panel of D: At the day indicated, whole cell lysates from control and 10 μg/ml indinavir-treated cells were prepared, and aliquots (corresponding to 10^4 cells) were immunoblotted with the antibodies against SREBP-1 (C, left panel), PPAR-γ (C, right panel), or IR β-subunit (D, left panel) at 1 μg/ml. A representative immunoblot from three separate experiments is shown. Right panel of 2D: at the day indicated, FCS- and insulin-depleted cells treated (f) or not treated (M) with indinavir (10 μg/ml) were incubated with ^125I-insulin (100 pg/ml) for 5 h in the binding buffer in the presence or absence of unlabeled insulin (1 μg/ml). Cells were washed and lysed in 0.5 mmol NaOH. Specifically bound ^125I-insulin was expressed as picograms per 10^6 cells. Indinavir did not increase the nonspecific binding of insulin. The results were the mean ± SE of three independently performed experiments.
fragment that enters the nucleus and directly activates gene transcription (23,36,37).

To evaluate whether indinavir acted at a step that controlled the nuclear localization of SREBP-1, we looked for the presence of the transcriptionally active SREBP-1 inside the nucleus using confocal microscopy (Fig. 4). The lamin A/C labeling was taken as a marker of the inner nuclear membrane (30). In preadipocytes treated and not treated with indinavir (Fig. 4, upper images), SREBP-1 expression was low and could be detected in the ER primarily at the poles of the nuclei. It was almost absent from the nucleus, in agreement with a low level of SREBP-1 processing in nondifferentiated cells. Cell differentiation (Fig. 4, left lower image) correlated in control cells with a strong labeling of SREBP-1 that was mostly intranuclear, consistent with the presence of the mature transcription factor (36). However, a faint cytoplasmic labeling of immature SREBP-1 persisted. By contrast, intranuclear labeling could hardly be detected in indinavir-treated cells examined 4 (not shown) or 6 days (Fig. 4, right lower image) after differentiation. Immunolabelling accumulated as a ring at the periphery of the nucleus, consistent with SREBP-1 being embedded in the nuclear membrane. Detailed analysis of the optical sections (Fig. 5) confirmed that SREBP-1 localization differed from that observed in nondifferentiated cells, and it further showed that SREBP-1 was homogeneously associated with the nuclear membrane. This atypical distribution of SREBP-1 concerned 50–60% of the indinavir-treated cells (125–175 nuclei of 250 observed), was not seen in untreated cells (~200 nuclei observed), and was observed in three independently performed experiments. This result may suggest that nonmature isoforms of SREBP-1 were sequestered at the nuclear membrane.

**Indinavir inhibited the increase in nuclear PPAR-γ expression during adipogenesis.** We observed that quiescent preadipocytes treated or not treated with indinavir for 8 days did not express intranuclear PPAR-γ. This was shown by Western blotting of whole cell lysates (Figs. 2C and 3) and by immunofluorescence (Fig. 4, lower images). The induction of differentiation in control 3T3-F442A cells resulted in a marked increase in expression of PPAR-γ at the intranuclear level. As expected, the increase of PPAR-γ expression was lower in indinavir-treated cells: 50–60% of the nuclei (of 250 nuclei observed) remained unlabeled (Fig. 4), whereas the other nuclei were stained at lower levels than those seen in the control cells. This was also shown by Western blotting, regardless of whether the treatment was performed for 8 days before the induction of differentiation (Fig. 2C) or the drug was added at day 2 after initiation of differentiation (Fig. 3B). This suggested that almost all cells were vulnerable to the deleterious effect of indinavir.

**Rosiglitazone reversed the effect of indinavir on adipocyte differentiation.** To obtain further information on the mechanism by which indinavir altered adipogenesis, we verified whether indinavir-pretreated cells could succeed in differentiating when forced to do so by rosiglitazone, a potent activator of PPAR-γ (40,41). We observed that rosiglitazone (1 μmol/l) promoted adipose conversion of control cells whether insulin was present (not shown) or absent (Fig. 6A, left and right panels) in the differentiation medium. We also observed that pretreating the cells with indinavir (10 μg/ml) did not inhibit adipose conversion when promoted by thiazolidinedione. These results confirmed that indinavir altered the differentiation program at a step preceding PPAR-γ activation.

**In differentiated adipocytes, indinavir induced a resistance to insulin.** We next evaluated whether indinavir could alter the ability of the cells to respond to insulin at a maximally effective concentration. Thus, we checked whether proximal and distal steps of insulin signaling were affected by the drug. We showed (Fig. 7A, left panel) that despite decreased expression of the IR in indinavir-treated cells (Fig. 2D, left panel), the increase in IRS-1 tyrosine phosphorylation induced by insulin (100 nmol/l) was similar in control and indinavir-treated cells at 6 days after initiation of differentiation. However, in these cells, the activation of the MAPK by insulin was almost blunted (Fig. 7A, right panel). The effect of indinavir was dose-dependent (data not shown) and reversible because indinavir-treated preadipocytes almost completely recovered their ability to respond to insulin (for MAPK activation) when differentiated in the absence of the drug (Fig. 7B, left panel). As depicted in Fig. 6B, rosiglitazone almost totally reversed the adverse effect of indinavir on insulin signaling. The results thus indicated that in addition to altering the differentiation program of 3T3-F442A cells, indinavir rendered adipose cells selectively resistant to
FIG. 4. Indinavir altered the subcellular localization of adipogenic transcription factors. Confocal immunofluorescence microscopy was performed on cells treated with indinavir (10 µg/ml) for 8 days using primary antibodies against SREBP-1 (green labeling) and lamin A/C (red labeling). 3T3-F442A preadipocytes cultured for 8 days in the absence (left images) or presence (right images) of indinavir were grown and differentiated on glass coverslips without removing the drug. At day 0 and 6 of differentiation, the cells were fixed in methanol and incubated for 20 min at room temperature with anti-rabbit polyclonal antibodies directed against SREBP-1 (four upper images) or PPAR-γ (four lower images) at 20 µg/ml, together with the anti-mouse monoclonal antibody against lamin A/C (10 µg/ml). SREBP-1 was visualized with polyclonal antibodies directed toward the NH2-terminal DNA binding domain, which is common to both the precursor and mature forms. Secondary antibodies coupled to FITC or Texas Red (5 µg/ml) were then added for 20 min in the dark. Confocal analysis of double-labeling immunofluorescence was performed, and optical sections 3 (or 4) were selected for presentation. The bars represent 10 µm. Only background fluorescence was observed when primary antibodies were omitted. The images are representative of at least three experiments.
insulin for a distal cellular event by acting in the differentiation program at a step preceding PPAR-γ activation.

DISCUSSION

The aim of the present study was to analyze the effect of indinavir on adipocyte differentiation and insulin resistance. To this end, we chose to analyze the differentiation program of 3T3-F442A cells, a murine preadipocyte cell line that differentiates fully at confluency in the sole presence of insulin and FCS and that is highly responsive to insulin (21). This cellular model is believed to accurately reflect the in vivo biological features of adipocyte differentiation (21,33). We first reported that the long-term treatment (up to 30 days) with indinavir did not affect the function of preadipocytes; it modified neither cell growth nor sensitivity to insulin for early and distal metabolic effects, such as IR and IRS-1 tyrosine phosphorylation and MAPK activation. This indicates the drug’s lack of toxicity.
in preadipocytes when used in long-term experiments at concentrations similar to circulating levels of PIs in HIV-infected patients under HAART (32). This was confirmed by the inability of the drug to alter the proliferation step of the differentiation program (day 0–2 of differentiation). However, adipose conversion is clearly inhibited by indinavir, as demonstrated by the decreased accumulation of cytoplasmic lipid droplets and the expression of the adipogenic markers SREBP-1, PPAR-γ, and IR. This was also shown by the observation that adding indinavir to naive cells that have passed the initial phase of cell differentiation (i.e., clonal proliferation) inhibited their ability to complete adipose conversion. The sensitivity of the cells to the drug does not depend on the length of the treatment because similar inhibition was observed in cells treated from 4 to 40 days (up to 10 passages). Furthermore, inhibition was seen when indinavir was added in the course of the differentiation program. The effect of indinavir was dose-dependent and reversible if the drug was removed before preadipocytes reached confluency. This is consistent with the drug acting at an early step in the adipocyte differentiation program, which controls differentiation but not the proliferation event.

The in vitro effect of indinavir on adipogenesis has been evaluated in a few studies, and discordant results have been reported. When studying the differentiation of 3T3-L1
INHIBITORY EFFECTS OF INSTRUMENTAL MANIPULATION ON ADIPOCYTE DIFFERENTIATION

A

insulin

- + + +

blo t : PY-20

none

ind

IRS-1

B

insulin

- + + +

blo t : activated MAPK

none

ind

pERK-1

pERK-2

FIG. 7. Indinavir reversibly altered the effect of insulin on MAPK activation but not IRS-1 tyrosine phosphorylation. A: 3T3-F442A preadipocytes were grown and differentiated in the absence or presence of indinavir (10 μg/ml). At day 6 of differentiation, cells were depleted of FCS and insulin (but not of indinavir) for 16 h and stimulated or not stimulated with insulin (100 nmol/l) for 10 min at 37°C. Whole cell lysates were prepared, and aliquots (corresponding to 10^6 cells) were immunoblotted with the PY-20 and the activated-MAPK monoclonal antibodies and the polyclonal antibodies against ERK2 (0.1 μg/ml), as indicated. Representative immunoblots from three separate experiments are shown. B: After a treatment of 6 days with indinavir, cells were trypsinized and grown in the absence of the drug for 4 days up to confluency. Differentiation was then initiated and followed until day 6. The activation of MAPK was then tested at day 6, as described in the legend of Fig. 1. Immunoreactivity of ERK2 was taken as an index of MAPK expression. Representative immunoblots from three separate experiments are shown.

Preadipocytes, Gagnon et al. (13) and Zhang et al. (16) observed that indinavir either increased or decreased lipid droplet accumulation in adipocytes. Using cells activated and not activated with thiazolidinediones, which act as potent inducers of the terminal steps of the differentiation program (40,41), Zhang et al. (16) and Wentworth et al. (15) reported that indinavir moderately (~30%) decreased the differentiation of 3T3-L1 or human preadipocytes. Moreover, they reported that indinavir did not directly affect the PPAR/RXR activation process. In fact, studying the effect of indinavir at various steps in the adipocyte conversion program in a cell line whose differentiation is easily obtained (21) allows us to detect more precisely the deleterious effect of indinavir. In 3T3-F442A cells, we showed, as have others, that indinavir does not directly induce PPARγ inactivation. More interestingly, we observed that the drug acted early in the differentiation program, at the level of SREBP-1 maturation. Using confocal microscopy immunofluorescence, we showed a mislocalization of SREBP-1, which accumulated at the nuclear periphery, suggesting a defect in proteolytic maturation. A possible hypothesis to explain these results is that indinavir inhibits the S2P protease, which cleaves the partially proteolyzed transcription factor, allowing its nuclear penetration and the activation of gene transcription (23,42). Whether the antiprotease activity of indinavir directly inactivates S2P or affects another protease required for its activation (43) remains to be determined. Otherwise, it cannot be excluded that the loss of SREBP-1 intranuclear localization induced by indinavir resulted from alterations in the mechanisms involved in the nuclear import of SREBP (44). The effects observed with indinavir on the alteration of cell differentiation and on SREBP nuclear localization are also seen in cells treated with two other PIs (nelfinavir and amprenavir, 15 μmol/l), though to a lesser extent (data not shown). This suggests that the alteration resulted from a class-specific rather than an indinavir-specific phenomenon. Two studies recently reported that nelfinavir (45) and rotonavir (46) modulated the expression level of the 68-kDa mature form of SREBP-1 during the differentiation process. Nelfinavir was also shown to inhibit adipocyte conversion of 3T3-L1 preadipocytes and to induce cell apoptosis (45).

SREBP-1 is a known regulator of multiple genes involved in the metabolism of cholesterol, triglycerides, and fatty acids, particularly in the liver (33,34,47–50). It also controls the expression of PPARγ, which is an obligatory step in adipocyte differentiation (34,41,47). The defective maturation of SREBP could thus fully explain the defective expression of PPARγ in indinavir-treated cells. This hypothesis is further supported by the observation that rosiglitazone, a potent PPARγ agonist (41), almost reverses the inhibitory effect of indinavir on adipocyte differentiation.

In addition to its effect on SREBP-1 maturation and adipocyte differentiation, indinavir induces insulin resistance regardless of whether the cells have succeeded in adipocyte conversion. These cells have been found to be resistant to insulin for MAPK activation. Our observation that the early steps in the insulin-signaling pathway (i.e., the tyrosine phosphorylation of IRS-1 together with phosphatidylinositol 3-kinase activation) (not shown) are preserved in indinavir-treated cells is in accordance with a recently published study describing altered transport activity of GLUT4 with preservation of IR and IRS-1 tyrosine phosphorylation and Akt activation (14). The mechanism of the resistance observed in the present study is not clearly understood. However, the actions of insulin and SREBP-1 are known to be strongly related (34,48,49,51). Indeed, insulin regulates SREBP-1 by increasing gene expression, protein processing, and phosphorylation through the MAPK pathway (38,39,52), and conversely, SREBP-1 mediates the effect of insulin by increasing the expression of insulin target genes, such as PPARγ or FAS (35,49,51), resulting in enhanced insulin action and sensitivity. In light of these data, correlations may exist among the ability of indinavir to inhibit insulin signal transduction at the level of MAPK activation and the altered expression and processing of SREBP.

We report here that in cultured cells, indinavir has the ability, in the absence of any other drugs used in HIV therapy, to inhibit lipid accumulation in fat cells without impeding their growth. Such an effect would explain the subcutaneous lipatrophy observed in patients with HAART that includes PIs (1–7,53). In many cases, the subcutaneous lipatrophy also associates with visceral adipose tissue accumulation (1,5,6,12,53,54). Therefore, it must be postulated that the same agent can exert opposite effects on adipose tissue from different locations. Several explanations can be given for this discrepancy. The sensitivity to insulin is greater in subcutaneous tissue than it is in visceral adipose tissue (55). If PIs induce insulin resistance, thus impeding the maintenance of the adipocyte
differentiated state, the subcutaneous tissue will be more sensitive to the drug, resulting in a decreased content in cellular lipids. Otherwise, human subcutaneous adipocytes have been shown to be more sensitive to PPAR-γ agonists with regard to differentiation than have omental adipocytes, probably because of their different levels of PPAR-γ expression (56,57). Therefore, if PIs impede PPAR-γ expression, as reported in this study, subcutaneous adipocytes will be selectively affected in their differentiation process. Otherwise, we found that thiazolidinediones can reverse the inhibitory effect of indinavir on adipocyte differentiation, these drugs could be proposed as therapeutic agents to reverse the lipodystrophy of treated HIV patients.

In conclusion, we report that indinavir exerted a deleterious effect on adipocyte differentiation, probably acting at the step of SREBP-1 maturation and nuclear localization, which is critical for adipocyte conversion and precedes PPAR-γ expression. The precise mechanism of this alteration remains to be determined. Our findings, which localized more precisely the action of indinavir in the differentiation process of adipose tissue, provide a possible explanation for the subcutaneous cellular fat loss observed in PI-associated lipodystrophy and should help in designing therapeutic tools, such as PPAR-γ agonists, that can bypass this step in adipogenesis.

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