Human Immunodeficiency Virus Protease Inhibitors Accumulate into Cultured Human Adipocytes and Alter Expression of Adipocytokines*

Received for publication, July 30, 2004, and in revised form, September 27, 2004
Published, JBC Papers in Press, November 3, 2004, DOI 10.1074/jbc.M408687200

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Lipodystrophic syndrome is a major side effect of highly active antiretroviral therapy. Fat tissue redistribution is associated with changes in adipocyte gene expression and in circulating levels of adipocytokines involved in the development of insulin resistance. However, the evidence that HIV drugs accumulate into human adipocytes and have a direct effect on the expression of adipocyte-specific genes is still lacking. To address these questions, we used adipocytes derived from adult stem (hMADS) cells isolated from human adipose tissue. We showed by ELISA that two inhibitors of the HIV protease, lopinavir and ritonavir, accumulated at similar levels during the development of hMADS cells in adipocytes, whereas a non-nucleoside reverse transcriptase inhibitor, the nevirapine, accumulated at lower levels. Two fluorescent protease inhibitors then have been generated to investigate their subcellular localization. The data showed that HIV drugs accumulated into adipocytes and displayed various effects on hMADS cell-derived adipocytes. Indinavir, amprenavir, and nevirapine did not alter differentiation of precursor cells. In contrast, lopinavir, saquinavir, and ritonavir inhibited the development of preadipocytes into adipocytes. In adipocytes, amprenavir increased leptin expression and ritonavir was able to up-regulate tumor necrosis factor-α, interleukin 6, and leptin expression and down-regulate the expression of peroxisome proliferator-activated receptor γ and adiponectin. Intracellular accumulation and localization of HIV drugs into human adipocytes strongly suggest that adipose tissues store these drugs. Because ritonavir can alter the expression of insulin resistance-related cytokines in human adipocytes in a way parallel to the situation observed in vivo upon treatment of HIV-infected patients, we propose that protease inhibitors participate in insulin resistance through a direct effect on adipocytes.

Highly active antiretroviral therapy (HAART)† combines treatment with three classes of anti-HIV drugs: 1) protease inhibitors (PIs); 2) nucleoside reverse transcriptase inhibitors (NRTIs); and 3) non-nucleoside reverse transcriptase inhibitors (NNRTIs). These drugs have demonstrated their efficiency in improving the lifespan of HIV-infected patients. However, patients under HAART develop metabolic alterations including fat tissue redistribution with peripheral lipodystrophy and increased central adiposity. The lipodystrophic syndrome affects up to 60% treated HIV-infected patients and is emerging as a significant medical concern. The adipose tissue of lipodystrophic HIV-infected patients contains clusters of small adipocytes (2) that can result from a defect in differentiation of adipocyte precursors or from metabolic alterations of mature adipocytes. PIs and NNRTIs were detected in adipose tissue of patients, but evidence that HIV drugs accumulate and have a direct effect on the development of human adipocytes are still lacking. It has been recently shown that PIs can enter and accumulate in cultured mouse adipocytes (3, 4) and that PIs, but not all, inhibited differentiation of mouse adipocytes (3, 5, 6). These reports indicate a direct effect of PIs on adipogenesis. However, the effects of PIs were different according to the mouse preadipocyte clonal lines (3), indicating that studies with human adipocytes should be more appropriate to gain better insights into the molecular events that occur in human adipose tissue.

HIV-infected patients under HAART develop dyslipidemia and systemic insulin resistance. Adipose tissue is thought to play a critical role in insulin resistance, in particular, through the expression of adipocytokines such as leptin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL6), and adiponectin. Leptin is an important circulating satiety factor that regulates food intake via its action in the central nervous system (7), and insulin sensitivity correlates negatively with leptin in lipohypertrophic HIV-infected patients (8). It has also been reported that HIV-infected lipodystrophic patients exhibit 40% reduced plasma adiponectin levels compared with non-lipodystrophic patients leading to the proposal that adiponectin replacement therapy could improve the treatment of HAART-associated syndrome (9). The role of TNF-α in insulin sensitivity has been largely documented mainly using murine systems. In human, therapy; HIV, human immunodeficiency virus; hMADS, human multipotent adipose-derived stem; ELISA, enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor-α; IL6, interleukin 6; PPARγ, peroxisome proliferator-activated receptor γ; PIs, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; IDV, indinavir; KTV, ritonavir; SQV, saquinavir; APV, amprenavir; NVP, nevirapine; G3PDH, glycerol-3-phosphate dehydrogenase; MANT, N-methylanthraniloyl; HPLC, high pressure liquid chromatography; DAPI, 4’,6-diamidino-2-phenylindole; MDR, multi-drug resistance.
the increase of TNFα in obese subjects suggests a role of this cytokine in the development of insulin resistance (10). Recently, TNFα has been related to lipodystrophy and insulin resistance in HIV-infected and PI-treated patients (8). However, a direct effect of HIV drugs on the development of human adipocytes and on the expression of insulin-resistant-related genes has not been demonstrated. Importantly, there is no evidence that HIV drugs accumulate into human adipocytes that would indicate putative intracellular targets. Indeed, the data on the effects of HIV drugs on the differentiation of human adipocytes are still pending because of the lack of appropriate cellular models. Our laboratory has recently isolated from human adipose tissue adult stem cells able to differentiate at a high rate into adipocytes (11). These cells are termed multipotent adipose-derived stem (hMADS) cells. Therefore, we used hMADS cells to investigate the following: 1) the intracellular localization and accumulation of HIV drugs in undifferentiated and differentiated cells; 2) the effects of different PIs and nevirapine on adipocyte differentiation and on fully differentiated adipocytes; and 3) the ability of PIs to modify the expression of adipocytokines involved in the establishment of insulin resistance.

**EXPERIMENTAL PROCEDURES**

**Materials**—Nelfinavir, indinavir (IDV), ritonavir (RTV), and saquinavir (SQV) were obtained through the AIDS Research and Reference Reagent Program (National Institutes of Health). Amprenavir (APV) was kindly provided by GlaxoSmithKline Research and Development (Herfordshire, United Kingdom). Large amounts of RTV, LPV, IDV, SQV, and nevirapine (NVP) were obtained by extraction from commercially available tablets and capsules. The purity was assessed by 1H and 13C NMR and mass spectroscopy. PIs obtained from the National Institutes of Health were used as reference products for purification and to validate results obtained with drugs purified from tablets. Each PI was dissolved in Me2SO and diluted 1:1000 in culture medium.

**Cell Expansion and Differentiation**—hMADS cells were expanded in low glucose Dulbecco's modified Eagle's medium (Invitrogen) containing 10% heat-inactivated fetal calf serum (Dutscher S. A., Brumath, France). A detailed description of adipocyte differentiation of hMADS cells has been published recently (11). Confluent cells were maintained in serum-free medium (Dulbecco's modified Eagle/F-12 medium v/v) supplemented with 1 μM dexamethasone, 0.1 mM 3-isobutyl-1-methylxanthine, 10 μg/ml transferrin, 0.2 nM triiodothyronine (T3), 5 μg/ml human insulin, and 0.5 μM rosiglitazone. Three days later, Dex and 3-isobutyl-1-methylxanthine were omitted and media were changed every other day. Neutral lipid accumulation was assessed by Oil Red O staining as previously described (12). Glycerol-3-phosphate dehydrogenase (G3PDH) activity was determined as previously described (13).

**RNA Analysis**—Total RNA from hMADS cells was prepared using TriReagent (Euromedex) according to the recommendations of the supplier. 20 μg of total RNA were subjected to Northern blot analysis as previously described (14). Blots were successively hybridized with peroxisome proliferator-activated receptor γ (PPARγ) and β-actin probe to standardize signals. Quantification of the hybridization signal was performed using a PhosphorImager (Fuji-Bas1000) coupled to the MacBas, version 2.x bioimaging analyzer. The concentrations of the different adipocytokine mRNAs were measured by reverse transcription followed by real-time PCR using a Light-Cycler (Roche Diagnostics, Meylan, France) and the FastStart DNA Master SYBRGreen kit (Roche Diagnostics). Primers (Eurobio, Les Ulis, France) were selected to am-

**FIG. 1.** Accumulation and subcellular localization of HIV drugs in human adipocytes. A, hMADs cells were induced to undergo differentiation into adipocytes in the presence of indicated drugs, and ELISAs were performed at the indicated time points. Data are means ± S.D. from three independent experiments. B, time-lapse digital fluorescence microscopy was performed on preadipocytes and adipocytes derived from hMADS cells. APV-dansyl was added at time 0, and fluorescent images were taken at indicated time. C, intracellular localization of APV-dansyl and IDV-MANT in hMADS cell-derived adipocytes 30 min after exposure to the fluorescent PI. Three-dimensional deconvolved images (lower panels) and bright-light images (upper panels) are shown. MIA is the free form of MANT.
pify small fragments (80–200 bp) and to hybridize in different exons of the target sequences. The list of the primers and real-time PCR conditions for each mRNA assay is available upon request. Each assay was performed in duplicate, and the validation of the real-time PCR was assessed by evaluation of the melting temperature of the products and by the slope and error obtained with the standard curve. The analyses were performed using the Light-Cycler software.

**PIs Labeling and Time-lapse Fluorescence Microscopy—**Pyridine (0.178 mmol), N,N-dimethylaminopyridine (0.0158 mmol), and dansyl chloride (0.297 mmol) were added to amphenirin (0.198 mmol) in anhydrous dichloromethane under nitrogen atmosphere for 8 h at reflux temperature. The solvent was removed in vacuo, and the crude product was purified by flash chromatography on a silica gel (96/4, chloroform/methanol) to obtain the APV-dansyl, N,N-Dimethylaminopyridine (0.081 mmol) and N-methylsitoic anhydride (0.089 mmol) were added to indinavir (0.081 mmol) in anhydrous N,N-dimethylformamide under nitrogen atmosphere for 5 h at room temperature. The solvent then was removed in vacuo, and the crude product was purified by TCL (96/4, chloroform/methanol) to obtain the indinavir-N-methylanthraniloyl (IDV-MANT). All of the products were fully characterized by nuclear magnetic resonance and mass spectrometry. Details of the chemical procedure are available upon request. Cell images were recorded by using the ×40/0.65–1.35NA oil immersion objective lens. Image acquisition and analysis were performed using the Applied Precision Deltavision system (Applied Precision, Issaquah, WA) built on an Olympus IX-70 base. For APV-dansyl and IDV-MANT, excitation and emission filters were FITC-DAPI and DAPI-DAPI, respectively. The stability of the labeling has been investigated. For that purpose, intracellular APV-dansyl and IDV-MANT have been recovered from adipocytes 2 days after incubation and analyzed by HPLC-UV. No uncoupled PIs or metabolites were detected at the time of labeling. The stability of the labeling was stable and that the fluorescence reflected the PI localization.

**Figure 2. Effects of HIV drugs on differentiating hMADS cells.** A, undifferentiated cells were induced to differentiate into adipocytes in the presence of 10 μg/ml IDV, RTV, LPV, APV, and NVP or 4 μg/ml SQV. G3PDH activities were determined 10 days after treatment. Data are reported by taking as 100% the G3PDH activity of untreated cells MeSO (DMSO). The means ± S.D. are representative data from three independent experiments. Significant differences (p < 0.05, Mann-Whitney U test) between control (MeSO) and treated cells are denoted by asterisk. B, photo-micrographic records of hMADS cells induced to undergo differentiation for 10 days in the presence of 0.1% MeSO or 14 μg/ml RTV. Cells were stained with Oil Red O for lipid droplets and counterstained with eosin. The bar represents 50 μm.

**RESULTS**

**HIV Drugs Enter and Accumulate into Human-cultured Adipocytes—**We first investigated by ELISA the ability of two PIs (RTV and LPV) and one NNRTI (NVP) to enter and accumulate in human adipocytes. The amounts of HIV drugs we used are in the range of those detected in the sera of HIV-infected patients under HAART (17–20). As shown in Fig. 1A, HIV drugs accumulated at different levels during adipocyte development. Whereas RTV and LPV accumulated at similar levels (4.1 ± 0.3 and 5.4 ± 0.8 nmol/10⁶ cells, respectively), the intracellular level of NVP was much lower (0.7 ± 0.03 nmol/10⁶ cells) despite a higher extracellular concentration. Two concentrations of RTV were used, and as shown in Fig. 1A, the intracellular accumulation was dose-dependent. The involvement of the multi-drug resistance (MDR) and MDR-associated protein transporters in preadipocyte accumulation of RTV has been investigated. The addition of 20 μM reserpine, a pharmacological inhibitor of the MDR1 and of MDR-associated protein transporter 1, for 3 h led to a higher accumulation of RTV (12 ± 0.8 nmol/10⁶ cell compared with 4.1 ± 0.3 nmol/10⁶ cell in the absence of reserpine, n = 3). This result strongly suggested that accumulation of the drugs into preadipose cells could be regulated via MDR transporters. We have previously shown that the antibodies used in the ELISAs are specific for the native form of the drugs and do not recognize metabolic forms (15, 16). In addition, HPLC-UV analysis indicated that the PIs stored in adipocytes were detected as the native forms. We studied the effects of PIs on fully differentiated adipocytes and the expression of both PPARγ and various adipocyto-
kines was analyzed. As shown in Fig. 3A, exposure to RTV but not to SQV, LPV, IDV, or APV inhibited PPARγ expression in mature adipocytes. TNFα was undetectable in untreated hMADS adipocytes. However, the expression of TNFα was reproducibly induced after treatment with RTV (Fig. 3B) and, at a less extent, after treatment with LPV and APV. The expression profile of leptin, IL6, and adiponectin has been investigated by quantitative RT-PCR. As shown in the Table I, a 5-day exposure of adipocytes to RTV decreased adiponectin expression and induced both leptin and IL6 gene expression. APV and LPV did not display any significant effect on adiponectin and IL6 expression. In contrast, leptin expression was dramatically induced upon the addition of APV. These data point out that PIs have a direct effect in human adipocytes on the expression of cytokines that are tightly associated with the development of insulin resistance. Fig. 4 summarizes the effects of the various HIV drugs on adipocyte differentiation of precursor cells and on the alteration of gene expression in adipocytes.

**DISCUSSION**

We have shown that APV and IDV, which belong to the same class of drugs, displayed different intracellular localization. It is well documented that, in peripheral blood mononuclear cells, the intracellular accumulation of HIV drugs depends of several parameters such as their chemical structures, their half-lives, or their affinity to drug transporters. Intracellular sequestration due to protein binding or ion trapping has also been suggested (21). APV was stored preferentially into lipid droplets, whereas IDV accumulated outside the lipid droplets. The lipophilic properties of APV and IDV could be responsible for their differential distribution, but active mechanisms cannot be ruled out. This observation indicates that adipose tissues can store PIs in treated patients. The capacity of adipocytes to mobilize and to release PIs remains to be investigated. It is interesting to note that the two labeled PIs were not detected into the nucleus. Even if we cannot exclude a role of the fluorphore on the localization of the drugs, these results indicate that nuclear proteins are not direct targets of PIs. This hypothesis does not exclude the effects of PIs in the cytoplasm on maturation of nuclear proteins. Altogether, these data strongly suggest that HIV drugs could have intracellular targets and direct effects on the development of human adipose cells.

Some HIV drugs, but not all, could inhibit differentiation of precursor cells isolated from human adipose tissue. Similar conclusions have been previously reported with precursor cells isolated from human bone marrow (22). In regard to the lipodystrophy associated with the use of PI, alternative therapies substituting NNRTIs (efavirenz or nevirapine) for protease inhibitors have been proposed (23). NNRTIs represent a promising approach to combine antiviral efficiency and limited adverse effects. However, recent papers have reported that efavirenz could accumulate in adipose tissue (24) and inhibit triacylglycerol accumulation in mouse preadipocyte clonal lines (25). We show in this paper that, despite a high extracellular concentration of nevirapine, the intracellular accumulation of this NNRTI was low compared with PIs. More interestingly, nevirapine did not interfere with lipid accumulation during adipocyte development of hMADS cells.

**FIG. 3.** RTV inhibits expression of PPARγ and induces expression of TNFα in mature adipocytes. Undifferentiated hMADS cells were maintained in adipocyte medium for 13 days with no addition of HIV drugs. At that stage, cells were differentiated and expressed PPARγ addition of HIV drugs. At that stage, cells were differentiated and expressed PPARγ. Subsequently, adipocytes were treated with 10 µg/ml IDV, RTV, LPV, and APV or 4 µg/ml SQV for 5 days (d18, day 18) and RNAs were prepared. A, the expression of PPARγ was analyzed by Northern blotting. Quantification of the signals was performed and standardized as described under "Experimental Procedures." Data are expressed by taking as 100% the signal obtained in 0.1% Me2SO (DMSO) treatment used as negative control. The means ± S.D. are representative data from three independent experiments. Significant differences (p < 0.05, Mann-Whitney U test) between control (Me2SO) and treated cells are denoted by asterisks. B, expression of TNFα was investigated by RT-PCR. Blotting was carried out in the same experiment and is representative of two independent experiments. Primers used for PCR amplification of TNFα were 5'-ATCTTCTCGAACCCTGAGTGA-3' and 5'-GGAGCTGCCCTCACCTT-3', and primer used for hybridization was 5'-CTGATGATGAAAATACT-3'. NT, not treated.

**TABLE I**

|        | Leptin | IL6  | Adiponectin |
|--------|--------|------|-------------|
| Me2SO  | 1      | 1    | 1           |
| LPV    | 1.1 ± 0.4 | 1.1 ± 0.2 | 1.1 ± 0.3 |
| APV    | 34 ± 15 | 0.9 ± 0.1 | 1.1 ± 0.3 |
| RTV    | 4.1 ± 0.7 | 3.3 ± 0.1 | 0.5 ± 0.1 |
hMADS cells accumulated less triacylglycerol droplets when exposed to RTV in agreement with its inhibitory effects previously reported on adipocyte differentiation of various mouse preadipocyte clonal lines (3, 5). We observed that lipid accumulation was inhibited, but the number of differentiated cells seemed to be similar in untreated and RTV-treated cultures. This is reminiscent of a previous report showing morphological changes in lipotrophic adipose tissue of HIV-infected patients where the clusters of adipocytes with few lipid droplets were observed. As suggested by the authors, these small adipocytes could be young regenerative cells replacing adipocytes lost by apoptosis (26). The inhibition of adipocyte formation induced by RTV could also be linked to a decreased expression of PPARγ known to be essential both for adipocyte differentiation and adipocyte survival (27).

RTV alters the expression of adipocytokines in mature hMADS adipocytes in a way parallel to the situation observed in lipodystrophic HIV patients developing insulin resistance. Since it has been shown that TNFα inhibits adiponectin gene expression on human adipose tissue fragments (28), it is tempting to postulate that the inhibition of adiponectin upon the addition of RTV on hMADS adipocytes was mediated by RTV-induced TNFα. It has also been reported that TNFα inhibits PPARγ expression in adipocytes (29) and that IL6 inhibits adiponectin (30). When subjected to an expression profile analysis, we have confirmed that adipocytes derived from hMADS cells express IL6 receptor, the gp130 signal transducer, and the two TNFα receptors, i.e. TNF receptors 1 and 2. Therefore, we could postulate that the up-regulation of IL6 and TNFα by RTV leads in adipose tissue to an autocrine/paracrine mechanism that could participate in the insulin resistance observed in lipodystrophic patients under HAART.

In conclusion, our data point out that HIV drugs display various effects on human adipocytes and that their storage alters the expression of adipocytokines involved in insulin resistance. Identification of molecular mechanisms implicated in adipocyte gene regulation by PIs might improve the treatment of HAART-associated syndrome.

ACKNOWLEDGMENT—We thank Cedric Matthews (UMR 6543 CNRS) for the time-lapse microscopy analysis.

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