In Vitro Suppression of the Lipogenic Pathway by the Nonnucleoside Reverse Transcriptase Inhibitor Efavirenz in 3T3 and Human Preadipocytes or Adipocytes*

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A serious metabolic syndrome combining insulin-resistance, dyslipidemia, central adiposity, and peripheral lipodystrophy has arisen in HIV-infected patients receiving highly active antiretroviral therapy. The aim of this work was to examine the effects of the nonnucleoside reverse transcriptase inhibitor (NNRTI) efavirenz on adipocyte differentiation and metabolism. When induced to differentiate in the presence of efavirenz (5–50 μM), 3T3-F442A preadipocytes failed to accumulate cytoplasmic triacylglycerol droplets. This phenomenon was rapidly reversible and was also readily detectable in the 3T3-L1 preadipose cell line and in primary cultures of human preadipocytes. When applied to mature 3T3-F442A adipocytes, efavirenz induced a delayed and moderate reduction in cell triglyceride content. Measurement of [3H]deoxyglucose uptake, basal and agonist-stimulated lipolysis, and cell viability indicated that these pathways are not involved in efavirenz effects on triacylglycerol accumulation. By contrast, we found that the NNRTI induced a dramatic dose- and time-dependent decrease in gene and protein expression of the lipogenic transcription factor sterol regulatory element-binding protein-1c (SREBP-1c). Adipose conversion was only altered at the highest efavirenz concentrations, as suggested by the mild reduction in peroxisome proliferator-activated receptor-γ and CCAAT/enhancer-binding protein-α. CCAAT/enhancer-binding protein-β remained unchanged. The inhibition of SREBP-1c expression was accompanied by a sharp reduction in the expression of SREBP-1c target genes and in the adipocyte lipogenic activity in efavirenz-treated cells. Finally, the inhibitory effect of efavirenz on cell triglyceride accumulation was prevented by directly providing free fatty acids to the cells and was reversed by overexpression of a dominant positive form of SREBP-1c, reinforcing the implication of this transcription factor in the antilipogenic effect of the drug. When considered together, these results demonstrate for the first time that the NNRTI efavirenz induces a strong inhibition of the SREBP-1c-dependent lipogenic pathway that might contribute to adipose tissue atrophy.

The widespread use of highly active antiretroviral therapy (HAART) 1 has radically transformed the prognosis of HIV-infected patients in the developed countries (1, 2). Intensive therapy of HIV infection with HAART, which combines various protease inhibitors (PIs), nucleoside analogue reverse transcriptase inhibitors (NRTIs), and nonnucleoside reverse transcriptase inhibitors (NNRTIs), dramatically reduces viral load and increases CD4 T cell count (1). Whereas a successful control of HIV infection is now possible, withdrawal of HAART leads to a prompt recovery of viremia (3), thus implying a prolonged and potentially life-long treatment to prevent viral replication. Currently, the recommended therapy for HIV-infected patients includes one or two PIs combined with two NRTI or two NRTI combined with one NNRTI. Blockade of the HIV protease inhibits cleavage and maturation of the viral polyprotein precursor, leading to production of noninfectious viral particles (4). The HIV reverse transcriptase is required to copy the viral RNA genome and is targeted either by chain-terminating analogues (NRTI) or by noncompetitive inhibitors (NNRTI) (5).

Unfortunately, long term HAART has been associated with a unique and unexpected lipodystrophic syndrome involving altered body fat distribution and disturbances of glucose and lipid metabolism (6–8). Most patients receiving this treatment develop metabolic abnormalities, which include dyslipidemia (elevated plasma triglycerides and cholesterol), increased visceral and dorsocervical adipose tissue, and peripheral lipodystrophy. These patients were also found to have elevated fasting insulin or C-peptide levels (9, 10), suggesting that these individuals develop insulin resistance. It is now recognized that patients receiving an antiretroviral therapy have an increased risk of cardiovascular disease (9, 11, 12), emphasizing the medical significance of the HAART-associated lipodystrophy and metabolic abnormalities.

Which component of the antiretroviral regimen or whether

1 The abbreviations used are: HAART, highly active antiretroviral therapy; Ad.null, adenovirus containing no exogenous gene; Ad.SREBP-1c DP, adenovirus containing a dominant positive form of SREBP-1c; aP2, adipocyte lipid-binding protein; BSA, bovine serum albumin; C/EBP, CCAAT/enhancer-binding protein; DMEM, Dulbecco’s modified Eagle’s medium; [3H]DOG, [1,2-3H]deoxyglucose; FAS, fatty acid synthase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; HIV, human immunodeficiency virus; KRH, Krebs Ringer Hepes; LPL, lipoprotein lipase; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PBS, phosphate-buffered saline; PI, protease inhibitor; PPARγ, peroxisome proliferator-activated receptor-γ; RT, reverse transcriptase; SREBP-1c, sterol regulatory element-binding protein-1c; SCD-1, stearoyl-CoA desaturase-1; ADD1, adipocyte differentiation and differentiation factor-1.
Effects of Efavirenz on Preadipocytes and Adipocytes

The effects of HIV-1 protease inhibitors and efavirenz on the metabolic syndrome were investigated. The metabolic syndrome is characterized by dyslipidemia, insulin resistance, and obesity, which are believed to be mediated by alterations in the expression of transcription factors involved in adipogenesis, lipid metabolism, and metabolic pathways.

Materials and Methods:

* Preadipocytes were isolated from human subcutaneous adipose tissue and cultured in vitro.
* Adipocytes were differentiated in the presence of PPARγ agonists and insulin.
* Cell viability was assessed by the MTT assay.
* Lipolysis was measured by glycerol release.
* Glycerol-3-phosphate dehydrogenase (G3PDH) activity was assayed.

Results:

- Efavirenz inhibited cell viability and lipolysis in a concentration-dependent manner.
- The compound also decreased G3PDH activity, indicating a reduction in triglyceride stores.
- Efavirenz induced a decrease in SREBP-1c expression, which is involved in adipocyte differentiation and lipid metabolism.

Discussion:

- The results suggest that efavirenz may have beneficial effects on the metabolic syndrome by reducing lipid accumulation and improving insulin sensitivity.
- Further studies are needed to determine the mechanisms by which efavirenz affects these processes.

Conclusion:

- Efavirenz has the potential to mitigate the metabolic complications associated with antiretroviral therapy, offering a novel therapeutic strategy for the treatment of the metabolic syndrome.
Determination of 2-Deoxyglucose Uptake—Uptake of glucose was determined using [1,2-3H]2-deoxyglucose ([3H]D-glucose) to measure glucose incorporation in total lipids was used as an index of lipogenic activity. Non-carrier-mediated glucose uptake, estimated by the addition of 1% SDS, and radioactivity was determined by scintillation counting.

TABLE I

| Murine genes | Human genes |
|--------------|-------------|
| aP2 | Adipocyte lipid-binding protein |
| FAS | Fatty acid synthase |
| CEBPα | CCAAT/enhancer binding protein-α |
| LPL | Lipoprotein lipase |
| PPARγ | Peroxisome proliferator-activated receptor-γ |
| SREBP-1c | Sterol regulatory element-binding protein-1c |

| 18 S | 18 S ribosomal RNA |
|-------|------------------|
| FAS | Fatty acid synthase |
| PDH | Glyceraldehyde-3-phosphate dehydrogenase |
| PPARγ | Peroxisome proliferator-activated receptor-γ |
| SREBP-1c | Sterol regulatory element-binding protein-1c |

| Abbreviation | Full name | Sense primer | Antisense primer |
|--------------|-----------|--------------|------------------|
| aP2 | Adipocyte lipid-binding protein | AAC ACC GAG ATT TCC TT | ACA CAT TCC ACC ACC |
| CEBPα | CCAAT/enhancer binding protein-α | TGG CGA GGA CCA GTC TT | TCC CGG GTA TAA ACC TCC |
| LPL | Lipoprotein lipase | GCA AGA GCC GCG ACA AG | GGC TCG GGC AGC TT |
| PPARγ | Peroxisome proliferator-activated receptor-γ | AGG CGG AGA AGG AGC TGG TG | TGG CCA CCT TCT TCT GCT C |
| SREBP-1c | Sterol regulatory element-binding protein-1c | TGG GTT GGC TGC TTG TG | GCG TGG GCA GGA TGA |

Hepes (KRH) (pH 7.4), supplemented with 1% fatty acid-free BSA, 4.5 g/liter α-glucose, and 1 mM ascorbate and 50 μg/ml Na2S2O3 as antioxidants. Cells were incubated for 2 h at 37°C in the absence or in the presence of 10 μM (−)-isoproterenol or 10 μM forskolin. Aliquots of the incubation medium were removed and frozen at −20°C until glycerol determination. Glycerol was measured by an enzymatic method using a commercial kit provided by Roche-Biopharm.

The abbreviations of the genes, their full names, and 5’ to 3’ nucleotide sequences of the sense and antisense primers are presented.
expressed to monitor transfection efficiency. The adenovirus vector containing the major late promoter with no exogenous gene, called Ad.null, was used as a control. After propagation in the HEK293 cell line, adenoviral vectors were purified by cesium chloride density centrifugation and stored at $-80^\circ$C until use. Adenofection was performed at a multiplicity of infection of 500 (500 plaque-forming units/cell) that is known to achieve an optimal infection efficiency in 3T3 adipocytes (62). Viral infection was controlled by green fluorescent protein expression and was similar between Ad.null- and Ad.SREBP-1c DP-infected cells. Expression of SREBP-1c target genes and cell triglyceride content were examined 48 and 96 h following infection, respectively.

Statistical Analysis—Results are presented as mean ± S.E. The statistical comparison of data between groups was assessed with analysis of variance (STATVIEW™ software). A $p$ value of <0.05 was considered as the threshold of statistical significance.

RESULTS

Efavirenz Preferentially Inhibits Triglyceride Accumulation during Adipocyte Differentiation—To evaluate the effects of efavirenz on preadipocyte differentiation, 3T3-F442A were grown until confluence and then cultured for various periods of time in the absence or in the presence of the drug, with a concentration range that is comparable with that observed in the plasma of treated patients (63, 64).

In a first set of experiments, 3T3-F442A cells were exposed from confluence to various efavirenz concentrations for 7 days, and cell triglyceride content was tested. Triglyceride content decreased significantly from 10 $\mu$M efavirenz and then continued to markedly decline with increasing doses (Fig. 1A). The effect was maximal at 50 $\mu$M, with a 94% reduction in triglyceride content as compared with control cells. The half-maximal effect was obtained at about 20 $\mu$M efavirenz.

To examine the time dependence of efavirenz effect on triglyceride content, 3T3-F442A cells were maintained from confluence in the absence or in the presence of 40 $\mu$M efavirenz, and cell extracts were prepared at intervals. After 2 days of treatment following confluence, cell triglyceride content was weakly reduced by efavirenz. However, this effect was much more dramatic after 4 days, reaching a maximal 80% decrease in day 7 postconfluent adipocytes (Fig. 1B).

These results drawn from biochemical analysis were also strengthened by cytochemical examination. Acquisition of an enlarged round shape, which usually precedes triacylglycerol accumulation, was slightly delayed in efavirenz-treated as compared with control cells (not shown). However, after a few days following confluence, efavirenz-exposed cells recovered a morphotype similar to that of untreated cells. Obviously, efavirenz potently decreased intracytosolic accumulation of lipid droplets. At day 7 following confluence, efavirenz-treated 3T3-F442A cells displayed a dramatic reduction in the number and size of fat vacuoles as compared with control cells. These striking changes in lipid accumulation were also illustrated by Oil Red O staining (Fig. 1D).

To verify that efavirenz effect on fat stores was not restricted to 3T3-F442A cells, similar studies were also performed on two other models of adipogenesis (i.e. the murine 3T3-L1 preadipose cell line and primary cultures of stromal vascular fraction

![Fig. 1. Effect of efavirenz on triglyceride accumulation during 3T3-F442A and 3T3-L1 adipose conversion. A, from confluence (day 0), 3T3-F442A cells were cultured for 7 days in the absence or in the presence of various concentrations of efavirenz, and cell triglyceride content was tested. Results represent the mean ± S.E. of 4–10 separate experiments. B, from confluence, 3T3-F442A were maintained in the absence (triangles) or in the presence (squares) of 40 $\mu$M efavirenz for the indicated time. Results represent the mean ± S.E. of 10 independent experiments. C, from confluence (day 0), 3T3-L1 cells were cultured until day 7 in the absence or in the presence of various concentrations of efavirenz and tested for triglyceride content determination. Results represent the mean ± S.E. of 6–10 separate determinations. D, from day 0 (confluence) to day 7, 3T3-F442A or 3T3-L1 cells were cultured in the absence or in the presence of efavirenz (40 $\mu$M) and then stained with Oil Red O. *, $p < 0.01$; **, $p < 0.001$, efavirenz-treated versus control cells.](http://www.jbc.org/content/15133/1/15133/F1.large.jpg)
Effects of Efavirenz on Preadipocytes and Adipocytes

derived from human adipose tissue). 3T3-L1 preadipocytes were grown to confluence and then induced to differentiate in the absence or in the presence of various efavirenz concentrations. As shown in Fig. 1C, triglyceride content was already decreased by 17% at 10 μM efavirenz and by 50% at 20 μM, with a maximal 88% reduction at 40 μM. The dramatic effect of efavirenz on 3T3-L1 triglyceride stores is also documented by Oil Red O staining (Fig. 1D). The influence of efavirenz on adipogenesis and lipid droplet accumulation was also examined during adipocyte conversion of human preadipocytes in primary cultures. Cells were grown to confluence in a serum-containing medium and then shifted in a chemically defined medium. Since in vivo, the main part of efavirenz is combined to serum proteins (65), this implied the use of much lower drug concentrations. From confluence, human preadipocytes were cultured for 7 days in the absence or in the presence of 2 or 4 μM efavirenz. Fig. 2, A and B, illustrates that a chronic exposure to 4 μM efavirenz caused a dramatic decrease in fat droplet accumulation. This was confirmed by the dose-dependent inhibiting effect of the NNRTI on cell triglyceride content (Fig. 2C). With the proviso of the limited number of human adipose tissue samples (n = 6), no obvious gender- or depot-specific difference was detectable.

Taken together, our results drawn from three distinct models of adipocyte differentiation suggest that efavirenz exposure during adipocyte conversion potently prevents triacylglycerol accumulation.

To examine whether this effect was reversible, 3T3-F442A cells were initially cultured from day 0 to day 7 in the presence of 40 μM efavirenz and then allowed to recover for an additional 8 days. As shown above, chronic efavirenz exposure during preadipocyte differentiation prevented triglyceride accumulation (Fig. 3). When efavirenz treatment was pursued, cell triglyceride content remained low and maximally represented 15% of the control value at day 15 following confluence. On the contrary, efavirenz withdrawal allowed 3T3-F442A cells to progressively recover a triglyceride content that was not significantly different from that of control cells in day 15 postconfluent 3T3-F442A adipocytes. Thus, the efavirenz-induced down-regulation in triglyceride accumulation was spontaneously and rapidly reversible.

Efavirenz Progressively Reduces Triacylglycerol Accumulation in Mature 3T3-F442A Adipocytes—To investigate whether in addition to its effect during adipose conversion, efavirenz could also decrease lipid accumulation in mature adipocytes, we examined the influence of a chronic exposure to the NNRTI on triglyceride content of fully differentiated 3T3-F442A adipocytes. In this experiment, the drug was added at day 8 following confluence. As mentioned in Table II, efavirenz at 20 μM did not modify cell triglyceride accumulation. However, we observed an approximately 20% reduction in triglyceride content after a 4- or 8-day exposure to a 40 μM concentration of the drug and even a 40% decrease after a 16-day treatment. Thus, in mature 3T3-F442A adipocytes, a long term treatment with a high dose of efavirenz can decrease triglyceride accumulation.

Efavirenz Primarily Alters the Lipogenic Pathway of Differentiating or Mature 3T3-F442A Adipocytes—In an attempt to identify the cellular and molecular mechanisms by which efavirenz altered cell triglyceride accumulation, several experimental approaches were carried out to support the view that specific biological events could contribute, at least in part, to the observed phenomena. Indeed, the efavirenz-induced decrease in cell triglyceride accumulation could result from a general or specific alteration in the adipogenic process, a decreased activity in the lipogenic pathway, a decreased glucose availability for lipogenesis, an increased lipolytic activity, or even a cytotoxic effect.

First, we ensured that efavirenz did not exert a direct cytotoxic effect on 3T3-F442A preadipocytes or adipocytes. For this purpose, lactate dehydrogenase activity was tested on aliquots of the culture medium of control or drug-treated cells, either during the course of the differentiation process or on mature adipocytes. Whatever the phenotype of 3T3-F442A cells, lactate dehydrogenase activity remained low and was not modified in the presence of the antiretroviral compound (not shown).

To determine whether efavirenz could modulate basal or insulin-stimulated glucose transport, mature 3T3-F442A adipocytes were cultured for 4 days in the absence or in the presence of a 20 or 40 μM concentration of the NNRTI, and then [3H]DOG uptake was determined. Neither basal nor maximal insulin-stimulated [3H]DOG transport was altered by a prior exposure to efavirenz (Table III). Furthermore, we observed no
The possibility that this could represent a major mechanism for altering basal or insulin-stimulated glucose transport, precluding regulation in cell triglyceride content. We then tested whether efavirenz impaired lipogenesis (i.e. de novo fatty acid synthesis from glucose). We then tested whether efavirenz impaired lipogenesis (i.e. de novo fatty acid synthesis from glucose). We then tested whether efavirenz impaired lipogenesis (i.e. de novo fatty acid synthesis from glucose). We then tested whether efavirenz impaired lipogenesis (i.e. de novo fatty acid synthesis from glucose). We then tested whether efavirenz impaired lipogenesis (i.e. de novo fatty acid synthesis from glucose). We then tested whether efavirenz impaired lipogenesis (i.e. de novo fatty acid synthesis from glucose). We then tested whether efavirenz impaired lipogenesis (i.e. de novo fatty acid synthesis from glucose).

Another mechanism that could account for the inhibitory effect of efavirenz on adipocyte triglyceride accumulation was an increased rate of lipolysis. Hence, mature 3T3-F442A adipocytes were treated for 4 days with 40 μM efavirenz, and lipolysis was measured under basal or stimulated conditions (Table III). A chronic exposure to efavirenz led to a moderate (32%) but significant decrease in basal lipolysis. However, in response to an optimal concentration of the nonselective β-adrenoceptor agonist (-)-isoproterenol (10 μM) or of the adenyl cyclase effector forskolin (10 μM), the NNRTI did not significantly change lipolysis. Thus, an increased basal or effector-stimulated lipolytic activity was not involved in the efavirenz-induced down-regulation in cell triglyceride content.

Results are expressed as the percentage of control triglyceride value and represent the mean ± S.E. of 4–10 separate experiments.

**p < 0.01; *p < 0.001, efavirenz-treated versus control cells.**

TABLE II
Efavirenz effect on triglyceride accumulation in mature 3T3-F442A adipocytes

| Treatment duration (day of culture postconfluence) | [Efavirenz] | Triglyceride content (% of control) |
|-----------------------------------------------------|------------|-------------------------------------|
| [μM]                                               | [μM %]     |                                     |
| 4 (day12)                                          | 0          | 12,055 ± 379                       |
|                                                    | 20         | 11,374 ± 598 (94%)                 |
|                                                    | 40         | 9,254 ± 608 (77%)                  |
| 8 (day16)                                          | 0          | 15,769 ± 271                       |
|                                                    | 20         | 15,490 ± 183 (98%)                 |
|                                                    | 40         | 12,780 ± 575 (81%)                 |
| 16(day24)                                          | 0          | 17,175 ± 126                       |
|                                                    | 40         | 10,470 ± 163 (61%)                 |

**p < 0.01.  
* p < 0.001, efavirenz-treated versus control adipocytes.

The percentage of control value is given in parentheses after the values of triglyceride content. Results represent the mean ± S.E. of 4–10 separate experiments.**

![Fig. 3. Spontaneous reversibility of efavirenz effect on 3T3-F442A cell triglyceride content after drug withdrawal. 3T3-F442A cells were initially cultured from confluence (day 0) in the absence or in the presence of efavirenz (40 μM) for 7 days. Thereafter, control cells were maintained without drug addition, whereas cells previously exposed to the NNRTI were divided in two groups; efavirenz (40 μM) was pursued (dark columns), or efavirenz was omitted (open columns). Cell extracts were prepared at the indicated intervals and tested for triglyceride content.](http://www.jbc.org/Downloaded from)
Efavirenz does not alter basal or effector-stimulated glucose uptake or lipolysis

Day 7 postconfluent 3T3-F442A cells were cultured in the absence or in the presence of efavirenz (20 or 40 μM) for 4 days. [3H]DOG uptake was then measured under basal conditions or in response to an optimal concentration of insulin (100 nM). Results are expressed in nmol of [3H]DOG/5 min/well and represent the mean ± S.E. of 4–8 separate experiments. We have also tested [3H]DOG uptake in response to various concentrations of insulin (see “Results”). Otherwise, independent cultures were performed to measure lipolysis by determination of glycerol release in the culture medium under basal conditions or in response to an optimal concentration of (-)-isoproterenol (10 μM) or forskolin (10 μM). Results are expressed in nmol of glycerol/h/well and represent the mean ± S.E. of six separate experiments.

| Culture condition | [3H]DOG uptake | Glyceral release |
|-------------------|----------------|-----------------|
|                   | Basal          | Insulin         | Basal          | (-)-Isoproterenol | Forskolin       |
|                   | nmol/5 min/well|                 | nmol/h/well    |                 |                |
| Control            | 2.40 ± 0.08    | 4.23 ± 0.27     | 117.4 ± 5.8    | 223.6 ± 14.2     | 226.4 ± 22.7   |
| Efavirenz (20 μM)  | 2.87 ± 0.07    | 4.34 ± 0.12     | 80.2 ± 3.1*    | 201.0 ± 23.7     | 210.5 ± 17.1   |
| Efavirenz (40 μM)  | 2.71 ± 0.21    | 3.99 ± 0.26     |                |                  |                |

*p < 0.01, efavirenz-treated versus control cells.

Efavirenz reduces [3H]glucose incorporation into lipids in 3T3-F442A cells

3T3-F442A cells were cultured in the absence or in the presence of efavirenz (40 μM) for various periods of time: day 0 (confluence) to day 7 (D0–D7), day 5 to day 9 (D5–D9), day 7 to day 11 (D7–D11), and day 9 to day 13 (D9–D13). At the end of efavirenz exposure, cells were incubated for 1 h in KRH buffer containing 5 mM [3H]glucose. After washing, [3H]glucose incorporation in total lipids was measured. Results are expressed in nmol of glucose incorporated/h/well and represent the mean ± S.E. of 6–12 independent experiments. The percentage of the control value is given in parentheses after the value of [3H]glucose incorporation.

| Sequence of efavirenz treatment | [3H]Glucose incorporation into lipids |
|---------------------------------|-------------------------------------|
|                                 | D0–D7 | D5–D9 | D7–D11 | D9–D13 |
|                                 |       |       |        |        |
| Control                         | 9.47 ± 0.46 | 7.87 ± 0.34 | 15.84 ± 0.64 | 9.27 ± 0.36 |
| Efavirenz                       | 2.27 ± 0.15* (24%) | 4.45 ± 0.18* (56%) | 8.21 ± 0.32* (52%) | 6.68 ± 0.42* (72%) |

*p < 0.01.  **p < 0.001, efavirenz-treated versus control cells.

Effects of Efavirenz on Preadipocytes and Adipocytes

Fig. 4. Efavirenz effect on triacylglycerol accumulation is prevented in the presence of Intralipid. 3T3-F442A cells were cultured from confluence in the absence (squares) or in the presence (triangles) of efavirenz (40 μM) and in the absence or in the presence of various concentrations of Intralipid. Cell extracts were prepared at day 7 following confluence and tested for triglyceride content (in μg). Results represent the mean ± S.E. of six independent experiments. *p < 0.05; **p < 0.001, efavirenz- or Intralipid-treated cells versus control cells. #p < 0.01, ##p < 0.001, cells exposed to Intralipid plus efavirenz versus cells treated with efavirenz alone.

Adipogenic transcription factors such as PPARγ, C/EBPα and -β, and SREBP-1c (21).

As a first step to study the influence of efavirenz on the expression of adipogenic transcription factors, 3T3-F442A cells were exposed from confluence to various drug concentrations (5–50 μM) for 7 days, and SREBP-1c, PPARγ, and C/EBPα and C/EBPβ mRNA steady state levels were analyzed by real-time RT-PCR. G3PDH activity, which reflects the extent of cell differentiation (66), was also examined in parallel. As shown in Fig. 5 (left panels), three different patterns of response to efavirenz were observed. The first pattern was the one detected with SREBP-1c mRNA. Efavirenz provoked a dramatic down-regulation in SREBP-1c mRNA levels, which was detectable at low concentrations (34% inhibition at 5 μM). There was a maximal 98% decrease at 50 μM of the compound and a half-maximal effect between 5 and 10 μM. The second pattern includes those PPARγ and C/EBPα mRNAs and G3PDH activity. In this group, gene expression or enzyme activity was only moderately suppressed by exposure to low efavirenz concentrations, with a more pronounced effect at 40 and 50 μM efavirenz (50% decrease for PPARγ, 70–80% for C/EBPα, and 90% for G3PDH activity). Finally, some genes such C/EBPβ remained roughly unaffected by NNRTI exposure.

We also examined the time dependence of the down-regulation of these mRNA species. 3T3-F442A cells were cultured from confluence for 2, 4, or 7 days in the absence or in the presence of 40 μM efavirenz. Fig. 5 (right panels) indicates that as soon as a 2-day exposure to the NNRTI, we observed a dramatic decrease in SREBP-1c mRNA levels, which persisted below 10% of the control levels throughout the culture. Interestingly, following a 2- or 4-day treatment with efavirenz, we observed a decrease in the abundance of PPARγ and C/EBPα transcripts. However, after a 7-day exposure to the drug, there was a spontaneous recovery in PPARγ and C/EBPα gene expression. The time-dependent pattern of G3PDH activity is close to those of PPARγ and C/EBPα mRNAs. Finally, C/EBPβ mRNA levels were not influenced by efavirenz treatment during the course of the culture. Thus, whereas efavirenz induced a very potent and persistent down-regulation in SREBP-1c mRNA levels, it only provoked a transient decrease in PPARγ and C/EBPα gene expression, which only occurred at high NNRTI concentrations. Taken together, these observations support the view that efavirenz exerted a privileged and sustained inhibitory effect on the lipogenic pathway, whereas the limiting effect of the NNRTI on adipocyte differentiation was only observed at high concentrations.

To ensure that the variations in gene expression detected in real-time RT-PCR analysis were followed by parallel changes in the levels of the related proteins, Western blot analysis of SREBP-1c, PPARγ, and C/EBPα was performed on nuclear extracts of 3T3-F442A cells exposed to various efavirenz concentrations.
centrations for 7 days (Fig. 6). In agreement with gene expres-
sion analysis, the mature nuclear form of SREBP-1c was
sharply reduced by efavirenz, with a maximal decrease of 97%
at a concentration of 50 \( \mu \text{M} \) of efavirenz.

C/EBP expression was only clearly detectable at the two highest concen-
trations of the antiretroviral compound, reaching 18 and 3% of
control levels at 40 and 50 \( \mu \text{M} \) efavirenz, respectively. Likewise,
PPAR expression was affected only at high efavirenz concen-
trations but with a maximal 50% decrease at 50 \( \mu \text{M} \) efavirenz.

To investigate the functional consequences of SREBP-1c
down-regulation by efavirenz, we examined the effects of the
NNRTI on the expression of typical SREBP-1c target genes,
such as those coding for fatty acid synthase (FAS) (22–24) and
stearyloyl-CoA desaturase-1 (SCD-1) (67). As controls, we also
measured lipoprotein lipase (LPL) and adipocyte lipid-binding
protein (aP2) mRNA levels, whose genes are targets for PPARγ
and C/EBPα but not for SREBP-1c. As shown in Fig. 7, we
observed a dramatic dose-dependent decrease in mRNA levels
of FAS and SCD-1. This down-regulation was detectable as
early as 5 \( \mu \text{M} \) and reached a plateau at 50 \( \mu \text{M} \), where FAS
and SCD-1 mRNA levels represented 12 and 2% of the control
values, respectively. Time dependence studies also showed the
same potent and persistent down-regulation of these two
taxa species (Fig. 7). Thus, the effect of efavirenz on
SREBP-1c target genes closely paralleled that of SREBP-1c
mRNA, in terms of both dose and time dependence. The pattern
of LPL and aP2 mRNA levels was different and more
closely matched that observed for PPARγ and C/EBPα. Dose-
response curves indicated that at day 7 following confluence,
LPL and aP2 mRNA levels were only moderately suppressed by
efavirenz, with a maximal 40–50% inhibitory effect. Interest-
ingly, time dependence experiments demonstrated that after a
clear 50–60% efavirenz-induced decrease in LPL and aP2 mRNA levels at day 2 or day 4 following confluence, there was
a spontaneous tendency for recovering the levels of these
mRNA species after a 7-day exposure to the drug. Thus, efav-
irenz induced a potent dose- and time-dependent down-regu-
lation in SREBP-1c target gene expression, whereas LPL or
aP2 mRNA levels were much less affected.

This marked decrease in SREBP-1c and SREBP-1c targets
was also found in the 3T3-L1 preadipose cell line and in pri-
mary culture of human preadipocytes. A chronic exposure of
3T3-L1 cells to various efavirenz concentrations for 7 days led to a dramatic decrease in SREBP-1c, FAS, and SCD-1 mRNA levels (not shown). Likewise, efavirenz markedly reduced SREBP-1c gene expression in human differentiating preadipocytes. Following a 7-day treatment with 4 μM efavirenz, and as compared with control cells, we observed a 82 and 76% decrease in SREBP-1c and FAS mRNA levels, respectively, whereas PPARγ gene expression remained unchanged.

To verify whether SREBP-1c regulation also exists in mature fat cells, we also measured SREBP-1c, FAS, and SCD-1 mRNA levels in fully differentiated 3T3-F442A adipocytes exposed from day 8 post-confluence to 40 μM efavirenz for 4 or 8 days (days 12 and 16 following confluence, respectively). After a 4-day treatment with the NNRTI, SREBP-1c, FAS, and SCD-1 mRNA levels were decreased by 32 ± 1, 60 ± 8, and 57 ± 5% in comparison with control levels, respectively. Following a 8-day exposure to efavirenz, the respective corresponding reductions were 97 ± 1, 97 ± 1, and 96 ± 3%. This effect was independent of a general dedifferentiating effect, as evaluated by measurement of G3PDH activity (not shown). Thus, in mature adipocytes, efavirenz induced a dramatic but delayed down-regulation in SREBP-1c, FAS, and SCD-1 gene expression.

Finally, in an attempt to demonstrate that the efavirenz-induced decrease in SREBP-1c expression was responsible for the major phenotypic changes, we examined whether an adenovirus-driven expression of SREBP-1c in NNRTI-treated cells could restore the cellular levels of triglycerides and mRNAs for SREBP-1c target genes. 3T3-F442A cells were cultured from confluence with or without 40 μM efavirenz. At day 4 following confluence, efavirenz-treated cells were infected either with

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**Fig. 6.** Dose-dependent effect of efavirenz on SREBP-1c, PPARγ, and C/EBPα protein expression. From confluence (day 0), 3T3-F442A cells were cultured until day 7 in the absence or in the presence of various concentrations of efavirenz. Nuclear extracts were prepared as mentioned under “Experimental Procedures,” and SREBP-1c, PPARγ, and C/EBPα protein expression was tested by Western blot analysis, using specific antisera available commercially. Each membrane was stained with Ponceau S to assess transfer efficiency and to estimate equal protein loading. A representative blot for each protein is shown. Three independent experiments were performed with similar results.

**Fig. 7.** Dose- and time-dependent effect of efavirenz on mRNA levels of SREBP-1c-target genes in differentiating 3T3-F442A cells. **Left panels,** from confluence (day 0), 3T3-F442A cells were cultured until day 7 in the absence or in the presence of various concentrations of efavirenz. **Right panels,** from confluence (day 0), 3T3-F442A were maintained in the absence or in the presence of 40 μM efavirenz for the indicated intervals. For these two kinds of experimental conditions, total RNA was prepared, and FAS, SCD-1, LPL, and aP2 mRNA levels were determined by real time RT-PCR analysis. Results are expressed as the percentage of control mRNA levels and represent the mean ± S.E. of four or five separate experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., nonsignificant, efavirenz-treated versus control cells.
Effects of Efavirenz on Preadipocytes and Adipocytes

**DISCUSSION**

HAART has dramatically improved the prognosis of HIV-infected patients but has been involved in the emergence or aggravation of a metabolic syndrome with potentially severe consequences (9). The cellular and molecular mechanisms underlying these adverse effects remain poorly understood, but many studies converge to suggest that altered adipose tissue metabolism may have a key role in the development of this syndrome. It has been proposed that the widespread use of PIs may be an important determinant of HAART-induced lipodystrophy. However, the onset of the metabolic syndrome in therapy naive HIV-infected patients (13) or in HAARTs that exclude PIs (14–16) has underlined that other components of the antiretroviral regimen probably contribute to these severe metabolic complications. For instance, NRTIs, through their mitochondrial toxicity, may also be associated with hepatic steatosis and lactic acidemia (68). The increased risk of coronary heart disease, which appears particularly associated with PI-containing regimens (69), highlights the concept that other antiretroviral compounds with fewer side effects have a major interest in the treatment of HIV-infected patients. In this context, NNRTIs represent a promising approach to combine antiviral efficiency and limited adverse effects (70). So far, to our knowledge, no interference of NNRTIs on adipose tissue development or metabolism has been reported. In this work, we demonstrate that, during adipose conversion of 3T3 or human preadipocytes, the NNRTI efavirenz, used within the range of therapeutic plasma concentrations, prevents lipid storage and limits the extent of cell differentiation. These two effects display distinct sensitivities to efavirenz, with an inhibition of triglyceride accumulation occurring at lower concentrations of the drug than those required to detect an effect on adipogenesis. The NNRTI also depletes the lipid stores in mature adipocytes. This phenomenon is probably related to a strong reduction in the expression of the lipogenic transcription factor SREBP-1c. These results raise the possibility that this NNRTI may be involved in adipose tissue atrophy.

Efavirenz, when incubated with preadipocytes during the differentiation process, clearly prevents the cells from accumulating lipids. This effect is already detectable at the lowest efavirenz concentrations. We examined the cellular and molecular mechanisms by which the NNRTI provokes a dramatic decrease in cytoplasmic triacylglycerols. First, we excluded several mechanisms that could account, at least in part, for the efavirenz-induced reduction in cell lipid stores. Especially, neither basal nor isoproterenol- or forskolin-stimulated lipolysis are increased by efavirenz exposure, ruling out the possibility that the NNRTI depletes triglyceride stores through an activation of lipolysis. An alteration in cell viability seems also unlikely, since lactate dehydrogenase activity is not modified in the culture medium of efavirenz-treated cells and in regard to the rapid reversibility of the NNRTI effect after drug withdrawal. Otherwise, we excluded the possibility that efavirenz could reduce basal or insulin-stimulated glucose transport, resulting in a decreased substrate availability for de novo fatty acid biosynthesis.

By contrast, many experimental data converge to demonstrate that efavirenz exerts antilipogenic properties, which are mediated by a dramatic down-regulation in SREBP-1c expression. Efavirenz concentrations that reduce cell triglyceride content are lower than those required to alter adipocyte differentiation. This concentration gap between these two distinct effects, together with the exclusion of other potential mechanisms mentioned above, suggest that efavirenz preferentially targets lipogenesis instead of a general dedifferentiating effect. In keeping with this observation, efavirenz potently decreases SREBP-1c mRNA levels and the expression of the related mature 68-kDa protein. Because the mature form of SREBP-1c is known to promote lipogenic gene expression (71), the large decrease in the levels of the 68-kDa SREBP-1c protein probably...
contributes to impaired lipogenesis in efavirenz-treated cells. Delineation of the molecular transcriptional or post-transcriptional mechanisms at the basis of the down-regulation of SREBP-1c mRNA levels represents another issue. In agreement with efavirenz-induced down-regulation of SREBP-1c, we observe a reduction in adipocyte lipogenic activity and a dramatic decrease in the expression in SREBP-1c target genes, such as a marked suppression of the transcript coding for FAS, an important enzyme of lipogenesis. Finally, two complementary approaches strongly support the view that efavirenz-induced SREBP-1c suppression is a major mechanism to reduce cell triglyceride accumulation. First, efavirenz effect on lipid stores is completely prevented when the lipogenic pathway is bypassed by directly providing fatty acids to the cells. Second, an adenovirus-driven SREBP-1c overexpression restores the lipid stores and the expression of SREBP-1c target genes involved in lipogenesis. Thus, SREBP-1c down-regulation is very likely a central mechanism by which efavirenz reduces triglyceride content of fat cells.

At the highest concentrations (40–50 μM), efavirenz also alters the magnitude of adipocyte differentiation. This result can be brought together with the observation that C/EBPα and PPARγ gene and protein expression are decreased at this high dose of the NNRTI. Since C/EBPα and PPARγ are generally considered as major adipogenic transcription factors (21), efavirenz-induced impairment in adipose conversion could be related to this decrease in C/EBPα and PPARγ expression. Interestingly, because efavirenz effect on triglyceride accumulation is not reversed or prevented by the PPARγ agonist troglitazone (not shown), it seems unlikely that an alteration in PPARγ expression and/or function could represent a key mechanism for the NNRTI action. Otherwise, C/EBPβ mRNA levels are not influenced by efavirenz exposure, suggesting that molecular targets affected by the antiadipogenic properties of the antiretroviral drug are probably located downstream from this adipogenic transcription factor. Noticeably, the slightly delayed adipocyte conversion observed at high efavirenz concentrations is a transient phenomenon that tends to blur with progression of adipocyte differentiation. After an initial stronger suppression at day 2 or day 4 following confluence, PPARγ and C/EBPα mRNA levels increased at day 7 postconfluence. In keeping with this observation, the pattern of aP2 mRNA expression, which corresponds to a PPARγ and C/EBPα (21) target gene but not to a SREBP-1c target gene, is quite similar to that of PPARγ and C/EBPα transcripts. Thus, it is conceivable that along the course of the adipocyte differentiation process, a high concentration of efavirenz may only act during a limited window to delay adipose conversion.

The fact that mature adipocytes are less sensitive to efavirenz lipid-depleting effects than differentiating preadipocytes (a long term treatment with high doses of the NNRTI is required to observe a clear decrease in cell triglyceride content) deserves explanation. In the view that the SREBP-1c-controlled lipogenic pathway is preferentially targeted by efavirenz, one possibility could be that the contribution of de novo lipogenesis to triglyceride deposition might be different between lipid-accumulating preadipocytes and mature lipid-engorged fat cells. In keeping with this, the reesterification of preexisting fatty acids occurs at high rates in mature adipocytes, thus limiting the consequences of a blockade of lipogenesis. Alternatively, the functional features of the differentiated phenotype could enable the cells to circumvent the efavirenz-induced blockade of the lipogenic pathway. For instance, mature fat cells possess membrane and cytosolic transporters for fatty acids, which are not fully expressed in differentiating cells (72). Thus, triglyceride synthesis is probably more dependent on the lipogenic pathway in a differentiating preadipocyte than in a mature fat cell. Finally, the apparent lower efficiency of efavirenz in differentiated adipocytes could be related to differentiation-linked differences in drug inactivation and/or to efavirenz intracellular distribution that might vary in a preadipocyte with very little triglyceride stores and in a lipid-laden adipocyte. Efavirenz is metabolized to some degree by members of the cytochrome P450 family (73). Whether changes in expression and/or function of this system of enzymes during adipose conversion could account for the differences in preadipocyte or adipocyte sensitivity to efavirenz remains an open question.

Efavirenz exerts its antiliipogenic effect through a strong down-regulation of SREBP-1c expression. Interestingly, in preadipocytes and adipocytes, the same transcription factor SREBP-1c also represents a privileged target for several PIs. Nguyen et al. (74) have shown that during the differentiation of the 3T3-L1 cell line, ritonavir augments the accumulation of triglycerides by increasing the expression of the 68-kDa mature form of SREBP-1c. In vivo, ritonavir induces the accumulation of activated SREBP-1c in the nucleus of rat liver and adipose tissue (36). However, studies have reported an alteration in SREBP-1c expression or function in response to other PIs. Dowell et al. (31) have shown that nelfinavir inhibits accumulation of mature 68-kDa SREBP-1c protein in 3T3-L1 cells, whereas indinavir induces an abnormal sequestration of SREBP-1c at the nuclear membrane (32, 34) and inhibits the expression of SREBP-1c target genes (35). Finally, HIV-infected patients treated with a combination of NRTIs and PIs have a greatly reduced SREBP-1c expression in lipoatrophic superficial fat depots (37). Thus, SREBP-1c seems to be a major target in the setting of the metabolic syndrome and could play a critical role in adipose tissue dysfunction observed during HAART. Although it is generally considered that PIs are probably responsible for most of the SREBP-1c-mediated adipocyte alterations, the present study demonstrates that the same pathway is targeted by other antiretroviral compounds, including the NNRTI efavirenz. Such a convergence in the effects of PIs and efavirenz on SREBP-1c, revealed by this and other studies on adipocytes, raises the intriguing question of the existence of a potential link between the antiretroviral activity of these molecules and the SREBP-1c pathway.

The concentrations of efavirenz required to elicit its effects on lipogenesis are within the range of those observed in plasma from patients receiving therapeutic doses of this antiretroviral agent (63, 64). Thus, it is possible that the effects of efavirenz on the 3T3-F442A and 3T3-L1 cell lines and on human preadipocytes observed in vitro may also occur in vivo. Interestingly, a recent work has reported that in HIV-infected patients receiving an antiretroviral treatment, efavirenz can accumulate in fat tissue (52). This accumulation of the drug in adipose tissue may facilitate the onset of its adverse effects on preadipocyte and adipocyte development and metabolism. However, we demonstrate in this work that efavirenz primarily alters the lipogenic pathway in differentiating or mature fat cells. It is generally recognized that whereas the lipogenic activity has a central role in energy storage in cultured preadipose cell lines and in adipose tissue from rodent species, this pathway has been reported to exert an accessory function in human adipose tissue (75). In humans, the liver is the central organ for de novo lipogenesis. As suggested by our experimental results, the antiliipogenic properties of efavirenz in adipocyte could be counteracted by sufficient availability of exogenous free fatty acids, which are probably present in vivo. However, the accumulation of efavirenz in adipose tissue (52) may favor an antiliipogenic effect in addition to its antiliipogenic action. Thus, despite the sharp efavirenz-induced depletion in lipid stores detected on
Effects of Efavirenz on Preadipocytes and Adipocytes

several *in vitro* models of preadipocyte and adipocyte, further experimental and clinical investigations will be helpful to ascertain the medical relevance of our observations. Otherwise, whether efavirenz also alters lipogenesis in hepatocytes *in vitro* or *in vivo* represents a major issue.

Acknowledgments—We thank M. C. Leneveu for expert technical assistance and Prof. M. Raymondjean for a critical review of the manuscript. Efavirenz was a generous gift of Bristol-Myers Squibb Laboratories.

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