Articletext
Abbreviations:

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; DMSO, dimethyl sulfoxide; \(^{3}\text{H-DOG}\), \([1,2-^{3}\text{H}]-\text{deoxyglucose}\); FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G3PDH, glycerol-3-phosphate dehydrogenase; HAART, highly-active antiretroviral therapy; HIV, human immunodeficiency virus; KRH, Krebs Ringer Hepes; LPL, lipoprotein lipase; MMLV-RT, Moloney murine leukemia virus-reverse transcriptase; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PBS, phosphate-buffered saline; PI, protease inhibitor; PPAR\(\gamma\), peroxisome proliferator-activated receptor-\(\gamma\); RT-PCR, reverse transcriptase-polymerase chain reaction; SREBP-1c, sterol regulatory element-binding protein-1c; SCD-1, stearoyl-CoA desaturase-1.
SUMMARY

Highly-active antiretroviral therapy (HAART) has largely reduced the morbidity and the mortality of HIV-infected patients, but a serious metabolic syndrome combining insulin-resistance, dyslipidemia, central adiposity, and peripheral lipoatrophy has arisen in treated individuals. HAART generally includes nucleoside reverse transcriptase inhibitors (NRTI) and protease inhibitors (PI). Since many studies have suggested that PI therapy is linked to the development of this metabolic syndrome, it is of importance to propose therapeutic strategies with less side effects, such as the use of the non-nucleoside reverse transcriptase inhibitors (NNRTI). The aim of this work was to examine the effects of the 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 anti-lipogenic 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.
INTRODUCTION

The widespread use of highly-active antiretroviral therapy (HAART) 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 (NRTI), and non-nucleoside reverse transcriptase inhibitors (NNRTI), dramatically reduces viral load and increases CD4 T cell count (1). While 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 include 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 non competitive 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 lipoatrophy. 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 the HIV infection itself is responsible for the HAART-associated metabolic syndrome is still a matter of controversy. Emergence of the syndrome has been correlated temporally with the widespread use of PIs, but similar symptoms have been observed in therapy naive HIV-infected patients (13), and in patients receiving treatments excluding PIs (14-16). The mechanisms underlying the onset of the syndrome may represent a complex response to independent factors, such as a specific drug or a combination of drugs, long-lasting viral infection, or effective viral suppression.
Studies on healthy subjects or on HIV-infected patients who have never received PIs have shown that dyslipidemia and insulin resistance can occur several months before the emergence of lipodystrophy (7,9). Conversely, glycemic and lipidic disturbances are also observed in genetic syndromes of generalized or partial lipodystrophy (17), and in transgenic models of lipoatrophy resulting from manipulation of major adipogenic transcription factors (18-20), suggesting that an initial lack of adipogenesis or a loss in adipose tissue may be involved in insulin resistance and metabolic complications.

A considerable sum of works has highlighted the molecular mechanisms of adipogenesis (21). Adipocyte differentiation involves a sequential and coordinated action of several transcription factors, that, in turn, regulate the expression of adipocyte-specific genes and proteins. At least two classes of transcription factors serve key roles in the regulation of adipogenesis, CCAAT/enhancer-binding proteins (C/EBPs), and peroxisome proliferator-activated receptor-γ (PPARγ). Following the initial and transient increase in C/EBPβ and δ, PPARγ and C/EBPα promote the expression of a number of adipose-specific markers, allowing acquisition of an enlarged rounded shape and the progressive accumulation of cytoplasmic triacylglycerol droplets. In addition, adipocyte differentiation is enhanced by the basic helix-loop-helix-leucine-zipper transcription factor SREBP-1c/ADD1 (sterol regulatory element-binding protein-1c/adipocyte determination and differentiation factor-1). SREBP-1c promotes lipogenic enzyme gene expression (22-24), transactivates the PPARγ promoter (25), and stimulates production of an unknown PPARγ ligand (26). Thus, PPARγ, C/EBPα, and SREBP-1c act in concert to induce and maintain the adipocyte phenotype.

Since the adverse effects of a single compound on a given tissue or cell type are difficult to determine in patients receiving a combination of several classes of antiretroviral drugs, in vitro models have been used to examine the exact influence of these treatments on adipocyte development or metabolism. In this regard, contradictory results have been published, few studies showing a positive (27), and more numerous ones showing a negative effect (28-33) of PIs on adipocyte differentiation. The preventing effects of PIs on adipogenesis have been related to a decreased expression in C/EBPα (31), or PPARγ (31,32), or to an altered expression or intranuclear localization of SREBP-1c (32,34,35). Interestingly, abnormal changes in SREBP-1c were also found in white adipose tissue of ritonavir-treated mice (36), and in lipoatrophic tissue from HIV-infected patients under HAART (37). Several authors have hypothesized that PI-
induced alterations in SREBP-1c may be a major phenomenon in the setting of the metabolic syndrome (37,38). In fat cells, in addition to their effects on adipogenesis \textit{per se}, PIs also impair insulin signaling events and glucose transport (39,40), increase lipolysis (30,40), and stimulate apoptosis (31,41).

As regards to the major side effects associated with the use of PIs, alternative therapies have been proposed to replace the PIs with abacavir (42,43), or by the NNRTI nevirapine (44-46) or efavirenz (47,48). These approaches appear successful to control HIV infection, particularly with the NNRTI (43). The most common side effects encountered with NNRTI are cutaneous rash and central nervous system disturbances such as dizziness, while these compounds are generally well tolerated at the metabolic level. However, recent works have reported that treatment with the NNRTI efavirenz can be associated with dyslipidemia (49-51), and that this compound can accumulate in adipose tissue (52). These observations prompted us to examine whether efavirenz alters adipocyte development and metabolism. Surprisingly, we demonstrate that efavirenz primarily reduces cell triglyceride accumulation in several \textit{in vitro} systems of adipogenesis, including a human model. This effect of efavirenz on triglyceride stores seems essentially related to a decrease in SREBP-1c expression, with a consecutive alteration in the lipogenic pathway.
EXPERIMENTAL PROCEDURES

Subjects

Adipose tissue samples were obtained from deep (mesenteric) or abdominal subcutaneous deposits from men (age 56 ± 6 years) or women (age 69 ± 3 years) undergoing surgical intervention. BMI ranged from 22.9 to 32.4 kg/m², and subjects had no metabolic or endocrine diseases. This study was approved by local ethics committee.

Cell culture and treatment

Murine 3T3-F442A preadipocytes were grown until confluence at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) with 4.5 g/L D-glucose, 10 % fetal calf serum (Life Technologies), and penicillin/streptomycin (100 U/ml penicillin/ 50µg/ml streptomycin). After confluence 3T3-F442A adipose conversion was obtained in the presence of the same culture medium supplemented with 170 nM insulin. 3T3-L1 adipocyte differentiation was initiated by addition of 100 µM methyl-isobutylxanthine, 170 nM insulin, and 250 nM dexamethasone for 48 h, then cells were refed by DMEM containing 10 % fetal calf serum and 170 nM insulin.

Human preadipocytes were obtained from adipose tissue samples by collagenase digestion as previously described (53). The floating adipocytes were discarded, and the infranatant containing the stromal vascular fraction was successively filtered through 150- and 25-µm nylon screens. The filtrate was centrifuged at 600xg for 10 min. After two washes, cells were plated into cell culture dishes at a density of 2-4 x 10⁴ cells/cm² with DMEM/Ham F-12 (1:1, v:v) supplemented with 10 % fetal calf serum and antibiotics, and cultured at 37°C under an atmosphere of air/CO₂ (95:5, v:v). After plating, cells were extensively washed and maintained under the same conditions until confluence (3-4 days following plating). To induce human preadipocyte differentiation, cells were then shifted in a chemically-defined medium consisting of DMEM/Ham F-12 supplemented with 80 nM insulin, 10 µg/ml transferrin, 0.2 nM L-T3, 100 nM hydrocortisone, and antibiotics, and for the first 3 days with 200 µM methyl-isobutylxanthine and 1 µg/ml troglitazone.

When mentioned, cells were cultured in the absence (dimethyl sulfoxide [DMSO] alone) or in the presence of efavirenz (dissolved in DMSO), at concentrations and periods of time
indicated in the “Results” section. The final concentration of DMSO never exceeded 0.025 \%. During the period of efavirenz exposure, the medium was changed daily for control and drug-treated cells. The powdered form of efavirenz was a generous gift of Bristol-Myers Squibb Laboratories.

**Cell extracts, biochemical determinations, and enzyme assays**

Cultured preadipose or adipose cells were washed twice with phosphate-buffered saline (PBS), harvested, and homogenized in Tris 25 mM, pH 7.5, EDTA 1 mM. A fraction of the homogenate was stored at −80°C. The remaining fraction was centrifuged at 10,000x\(g\) for 10 min at 4°C, and the supernatant was kept at −80°C until use. Aliquots of homogenates and supernatants were used to determine protein content by the method of Lowry (54), using bovine serum albumin (BSA) as a standard. Triglyceride content was determined on homogenates with PAP150 triglyceride kit (Biomérieux, Marcy L’Etoile, France).

Glycerol-3-phosphate dehydrogenase (G3PDH) activity was assayed by recording the initial rate of oxidation of NADH at 340 nm at 25°C (55).

Cell viability was assessed by testing lactate dehydrogenase activity in the culture medium. Media were assayed by measuring the rate of decrease in \(A_{340}\) in the presence of pyruvate, as described earlier (56).

**Lipolysis experiments**

Lipolysis was assessed as glycerol release from adherent 3T3-F442A adipocytes in 24-well plates. Adipocyte monolayers were washed with Krebs-Ringer buffer containing 12 mM Heps (KRH) (pH 7.4), supplemented with 1 % fatty acid-free BSA, 4.5 g/l D-glucose, and 1 mM ascorbate and 50 \(\mu\)g/ml \(Na_2S_2O_5\) as antioxidants. Cells were incubated for 2 h at 37°C in the absence or in the presence of 10 \(\mu\)M (-)-isoproterenol or 10 \(\mu\)M 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.

**\([^3H]\)-glucose incorporation in total lipids**

Measurement of \([^3H]\)-glucose incorporation in total lipids was used as an index of lipogenic activity. 3T3-F442A cell cultures in 12-well plates were rinsed three times with KRH buffer, then incubated for 1 h at 37°C in KRH containing 5 mM \(\text{[1-}^3\text{H}\text{]}\text{D-glucose (}^3\text{H-glucose)} (1 \(\mu\)Ci/well; 1 mCi/mmol; ICN Biochemicals, Orsay, France). Cells were washed with ice-cold PBS, harvested, and pelleted by centrifugation. After discarding the supernatant, cell total lipids
were extracted by the chloroform/methanol procedure of Folch et al. (57). The lipid-containing lower chloroform phase was finally evaporated and radioactivity was measured by scintillation counting.

**Determination of 2-deoxyglucose uptake**

Uptake of glucose was determined using [1,2-\(^3\)H]-deoxyglucose (\(^3\)H-DOG) (ICN Biomedicals, Orsay, France), a non metabolizable analogue of glucose. 3T3-F442A adipocytes cultured in 12-well plates were rinsed three times with KRH, then preincubated for 2 h at 37°C in 1 ml of the KRH buffer containing 0.1 % BSA. When mentioned, insulin was added during this preincubation period 1 h before measurement of hexose transport. Glucose uptake was initiated by addition of \(^3\)H-DOG (0.1 mM, 1 µCi/well). After 5 min at 37°C, the assay was stopped by aspiration, and cells were rinsed three times with ice-cold PBS. Cells were solubilized with 1 % SDS, and radioactivity was determined by scintillation counting. Non carrier-mediated glucose uptake, estimated by addition of 10 µM cytochalasin B in parallel wells, accounted for less than 3 % of total glucose transport.

**RNA analysis**

Total RNA was extracted from 3T3-F442A and 3T3-L1 cells by the method of Cathala (58), and from human cells by the procedure of Chomczynski & Sacchi (59).

For real time RT-PCR analysis, total RNA was first digested for 15 min at 37°C with 0.1 unit RNase-free DNase I (RQ1 DNase, Promega)/µg of nucleic acid in 40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM MgCl\(_2\), and 10 mM CaCl\(_2\). After phenol/chloroform extraction and ethanol precipitation, RNA (0.25-1 µg/sample) was reverse transcribed with Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) (200 units/µg) (Invitrogen, Inc.) in the presence of 10 µM random hexanucleotides (Amersham Biosciences), 400 µM of each dNTP in a final volume of 40 µl consisting of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl\(_2\), and 10 mM dithiothreitol. After a 1 h incubation at 42°C, MMLV-RT was heat-inactivated. To ensure that subsequent amplification did not derive from contaminant genomic DNA, a control without MMLV-RT was included in parallel for each RNA sample.

Reverse transcribed mRNAs were amplified on ICycler thermal cycler (Biorad Laboratories, Inc.), using the SYBR green fluorescence method, and were analyzed with the ICycler IQ\(^\text{TM}\) real time detection system software. PCR assay was performed under a final volume of 25 µl, starting from 5-100 ng of reverse transcribed total RNA, in the presence of 250
nM of each sense and antisense oligonucleotide, 125 µM of each dNTP, 2.5 mM MgCl₂, 7 nM fluorescein, 0.5 unit Taq DNA polymerase in its recommended buffer, including 1X SYBR green (Eurogentec). To verify that fluorescence generated by SYBR green incorporation into double strand DNA was not over-estimated by contaminations resulting from residual genomic DNA amplification and/or from primer dimer formation, controls without reverse transcriptase, and without DNA template nor reverse transcriptase were included in each experiment. Moreover, RT-PCR products were analyzed in a post-amplification fusion curve to ensure that a single amplicon was obtained. Ribosomal 18S RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used to normalize the initial amounts of cDNA in 3T3 and human cells, respectively. To measure PCR efficiency, serial dilutions of reverse transcribed RNA were amplified in the presence of the different sets of primers, then cycle threshold (Cₜ) was plotted against the initial amounts of reverse transcribed RNA, and the slope of the resulting curve allowed calculation of PCR efficiency. For all experiments, PCR efficiencies were close to one (PCR efficiency = 1.03 ± 0.03, n=84), indicating a doubling of DNA at each PCR cycle, as theoretically expected. Thus, taking into account standardization with 18S or GAPDH amplification products, it was possible to compare the relative levels of the tested transcripts between the different experimental conditions. Quantification of mRNA was carried out by comparison of the number of cycles required to reach reference and target threshold values (delta-delta Cₜ method, see http://www.bio.com/newsfeature, “introduction to real-time PCR” by M. Tevfik Dorak). Sequences of the sense and antisense oligonucleotides corresponding to the different tested genes are given in table 1.

**Western blot analysis**

Nuclear extracts were prepared from 3T3-F442A adipocytes as previously described (60). Proteins (30 µg/lane) were separated on a 7.5 % polyacrylamide SDS-gel, and electroblotted onto a 0.45 µm PVDF membrane (Immobilon-P, Millipore) in 0.1 % SDS, 192 mM glycine, and 25 mM Tris, pH 8.3. The membrane was depst in methanol, then blocked with 5 % BSA, 0.1 % Tween 20 in PBS for 1 h at room temperature. After washing in PBS containing 0.1 % Tween 20, the membrane was incubated overnight at 4°C with primary antibodies (rabbit polyclonal antibodies against SREBP-1c or C/EBPα; murine monoclonal antibody against PPARγ; all antibodies at a 1:1000 dilution; Santa Cruz Biotechnology Inc.) in 0.1 % Tween 20-PBS, 2 % BSA. After three washes with 0.1 % Tween 20-PBS, the membrane was incubated for 1 h at
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room temperature with horseradish peroxidase-conjugated anti-rabbit (1:20,000 dilution) or anti-mouse (1:2000 dilution) IgG (Sigma-Aldrich). The membrane was then washed in 0.1 % Tween 20-PBS. Detection of immune complex was performed using an enhanced chemiluminescence detection kit for Western blot (Amersham).

Adenofection experiments

The adenovirus vector containing the transcriptionally active dominant positive (DP) N-terminal part (amino acids 1-403) of rat SREBP-1c, called Ad.SREBP-1c DP, was generated as already described (61). Ad.SREBP-1c DP was under control of a cytomegalovirus promoter, and a green fluorescent protein was co-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°C until use. Adenofection was performed at a multiplicity of infection of 500 (500 plaque-forming units per 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 h 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 ANOVA (STATVIEW™ software). A P value <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, then cultured for various periods of time in the absence or in the presence of the drug, with a concentration range that is comparable to 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 µM efavirenz, then continued to markedly decline with increasing doses (Fig. 1A). The effect was maximal at 50 µM, with a 94% reduction in triglyceride content as compared to control cells. The half-maximal effect was obtained at about 20 µM 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 µM 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 post-confluent adipocytes (Fig. 1B).

These results drawn from biochemical analysis were also strengthened by cytochemical examination. Acquisition of an enlarged round shape, that usually precedes triacylglycerol accumulation, was slightly delayed in efavirenz-treated as compared to 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 to 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, ie the murine 3T3-L1 preadipose cell line, and primary cultures of stromal vascular fraction derived from human adipose tissue. 3T3-L1 preadipocytes were grown to confluence, then induced to differentiate in
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the absence or in the presence of various efavirenz concentrations. As shown in figure 1 C, triglyceride content was already decreased by 17% at 10 µM efavirenz, 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. 1 D). The influence of efavirenz on adipogenesis and lipid droplet accumulation was also examined during adipose conversion of human preadipocytes in primary cultures. Cells were grown to confluence in a serum-containing medium, 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. Figure 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. 2 C). 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 adipose 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 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 opposite, 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 post-confluent 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 3T3-442A adipocytes. In
this experiment, the drug was added at day 8 following confluence. As mentioned in table 2, efavirenz at 20 µM did not modify cell triglyceride accumulation. However, we observed an approximate 20% reduction in triglyceride content after a 4-day or 8-day exposure to 40 µM 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 from 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 20 or 40 µM of the NNRTI, then ³H-DOG uptake was determined. Neither basal nor maximal insulin-stimulated ³H-DOG were altered by a prior exposure to efavirenz (Table 3). Furthermore, we observed no effect of efavirenz (40 µM for 4 days) on EC₅₀ values of insulin for stimulating ³H-DOG uptake (EC₅₀ values of insulin were 1.10 ± 0.19 nM and 1.31 ± 0.40 nM in control and efavirenz-treated cells, respectively; n=4; P=0.66). Thus, efavirenz did not alter basal or insulin-stimulated glucose transport, precluding the possibility that this could represent a major mechanism for efavirenz-induced delipidation.
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 3). 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 non selective β-adrenoceptor agonist (-)-isoproterenol (10 µM) or of the adenylyl 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.

We then tested whether efavirenz impaired lipogenesis, ie de novo fatty acid synthesis from glucose. ³H-glucose incorporation into total lipids was used as an index of the lipogenic activity. Table 4 shows that following a 4- to 7-day treatment with efavirenz, there was a clear decrease in ³H-glucose incorporation into lipids. It was noteworthy that efavirenz effect tended to blur when the drug was applied to terminally differentiated cells. This anti-lipogenic effect of efavirenz was also dose-dependent, with a half-maximal concentration of about 20 µM (not shown).

Since efavirenz seemed to primarily alter the lipogenic pathway, we wondered whether the limiting effects of the NNRTI on cell triglyceride accumulation could be circumvented by directly providing exogenous fatty acids to the cells. From confluence, 3T3-F442A cells were maintained for 7 days without or with 40 µM efavirenz, and in the absence or in the presence of various concentrations of Intralipid as an exogenous source of free fatty acids. As expected, Intralipid addition provoked a moderate but significant increase in cell triglyceride accumulation, with a maximal effect at 100 µg/ml Intralipid (Fig. 4). Overall, the dramatic efavirenz-induced decrease in cell triglyceride content was prevented by Intralipid in a dose-dependent manner. As shown in figure 4, the effect of the NNRTI was completely abolished in the presence of 300 µg/ml Intralipid. This result demonstrates that the down-regulation of adipocyte lipid stores caused by the NNRTI can be prevented by directly providing fatty acids, and strongly suggests that the alteration in the lipogenic pathway is the primary site for efavirenz action.
Molecular mechanisms of efavirenz anti-lipogenic effects

Our initial biochemical determinations (see Fig. 1) indicated that from a concentration of 10 µM, efavirenz significantly reduced triglyceride accumulation in differentiating adipocytes. This effect could be related to an alteration in specific events of the differentiation process. It was thus conceivable that efavirenz prevented cell lipid accumulation by a reduction in the expression and/or function of key adipogenic transcription factors. It is now well recognized that the marked and specific phenotypic changes occurring during adipocyte differentiation are related to variations in the expression level of many genes. Genetic reprogramming is under the control of key 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, that reflects the extent of cell differentiation (66), was also examined in parallel. As shown in figure 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, that 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 were 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. Figure 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, that 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
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PPARγ and C/EBPα mRNAs. Finally, C/EBPβ mRNA levels were not influenced by efavirenz treatment during the course of the culture. Thus, while 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, that 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, while 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 for 7 days (Fig. 6). In agreement with gene expression analysis, the mature nuclear form of SREBP-1c was sharply reduced by efavirenz, with a maximal decrease of 97 % at a concentration of 50 µM. A marked down-regulation of C/EBPα was only clearly detectable at the two highest concentrations of the antiretroviral compound, reaching 18 % and 3 % of control levels at 40 and 50 µM efavirenz, respectively. Likewise, PPARγ expression was affected only at high efavirenz concentrations, but with a maximal 50 % decrease at 50 µM 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 stearoyl-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 figure 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 µM, and reached a plateau at 50 µM, 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 mRNA 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 regulation 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. Interestingly, time-dependence experiments demonstrate 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, efavirenz induced a potent dose- and time-dependent down-regulation in SREBP-1c target gene expression, while 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 primary 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 to control cells, we observed a 82 % and 76 % decrease in SREBP-1c and FAS mRNA levels, respectively, while 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 (day 12 and day 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 Ad.null, or with Ad.SREBP-1c DP, under conditions that allowed an optimal expression of SREBP-1c in 3T3 adipocytes (62). Thereafter, efavirenz was pursued for 48 h before determination of mRNA levels, or for 96 h for triglyceride measurement.
Control dishes were infected in parallel with the Ad-null vector. As shown in figure 8, infection with Ad.SREBP-1c DP almost completely reversed the inhibitory effect of efavirenz on cell triglyceride content. FAS and SCD-1 mRNA levels were decreased by 10- and 12-fold by efavirenz exposure, respectively. Infection of 3T3-F442A cells with Ad.SREBP-1c DP was able to restore normal FAS mRNA levels (12.5-fold induction as compared to efavirenz-treated cells infected with Ad.null vector), and induced an 8-fold increase in SCD-1 mRNA abundance. Thus, the expression of a dominant positive form of SREBP-1c restored the adipocyte triglyceride stores of efavirenz-treated cells, likely through an induction of the lipogenic pathway.
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 HAART that exclude PIs (14-16), has underlined that other components of the antiretroviral regimen likely 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, that appears particularly associated with PI-containing regimens (69), highlights the concept that other antiretroviral compounds with less 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 likely 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 to accumulate 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 as regards 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, that 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 likely 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 antidiipogenic properties of the antiretroviral drug are probably located downstream this adipogenic transcription factor. Noticeably, the slightly delayed adipose 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 post-confluence. In keeping with this observation, the pattern of aP2 mRNA expression, that 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 explanations. 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, that are not fully expressed in differentiating cells (72). Thus, triglyceride synthesis is likely 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
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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 metabolised 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 anti-lipogenic 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, while 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. Though it is generally considered that PIs are likely 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 while 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 antilipogenic properties of efavirenz in adipocyte could be counteracted by sufficient availability of exogenous free fatty acids, that are likely present in vivo. However, the accumulation of efavirenz in adipose tissue (52) may favor an antiadipogenic effect in addition to its antilipogenic action. Thus, despite the sharp efavirenz-induced depletion in lipid stores detected on 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

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REFERENCES

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LEGENDS TO FIGURES

**Figure 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 ± 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 µM 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 µM), then stained with Oil Red O.

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

**Figure 2**: *Dose-dependent effect of efavirenz on triglyceride accumulation during human preadipocyte differentiation*

(A and B) Microscopic view (400X) of human preadipocytes cultured for 7 days from confluence in a chemically-defined medium in the absence (A) or in the presence (B) of efavirenz (4µM). (C) Triglyceride content of human preadipocytes cultured for 7 days in the absence or in the presence of 2 or 4 µM efavirenz. Results are expressed as the percentage of control triglyceride content and represent the mean ± S.E. of 4 independent determinations.

**Figure 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, while 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. Results are expressed as the percentage of control triglyceride value, and represent the mean ± S.E. of 4 independent experiments.
*
P<0.01; **, P<0.001; n.s., non significant, efavirenz-treated versus control cells, whatever the temporal sequence of efavirenz exposure. #, P<0.001, cells continuously exposed to efavirenz (D0 to D9, D0 to D12, or D0 to D15) versus cells with only an initial treatment with efavirenz (D0 to D7).

Figure 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 µM). Results represent the mean ± S.E. of 6 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.

Figure 5: Dose- and time-dependent effect of efavirenz on SREBP-1c, PPARγ C/EBPα and C/EBPβ mRNA levels and G3PDH activity 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, 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 SREBP-1c, PPARγ, and C/EBPα and β mRNA levels were determined by real time RT-PCR analysis. G3PDH activity was measured on cell supernatants. Control G3PDH activity were 27.6 ± 4.5, 254.1 ± 39.0, and 484.8 ± 31.8 nmol/min/mg in day 2, day 4, and day 7 post-confluent cells, respectively. Results are expressed as the percentage of control mRNA levels or G3PDH activity, and represent ± S.E. of 4-10 separate experiments.
*, P<0.05; **, P<0.01; ***, P<0.001, efavirenz-treated versus control cells.
Figure 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 in the “Experimental Procedures” section, 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.

Figure 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 4-5 separate experiments.

*, P<0.05; **, P<0.01; ***, P<0.001; n.s., non significant, efavirenz-treated versus control cells.

Figure 8: SREBP-1c transgene expression reverses efavirenz effect on triacylglycerol accumulation and gene expression

3T3-F442A cells were cultured from confluence in the absence or in the presence of efavirenz (40 µM). At day 4 following confluence, efavirenz-treated cells were infected either with Ad.null, or with Ad.SREBP-1c DP, and efavirenz was pursued. Control cells were infected with Ad.null. Since cells were cultured in the presence of insulin, and because SREBP-1c expression is directly stimulated by insulin at the transcriptional level (76), the effect of SREBP-1c DP on cells untreated by efavirenz could not be assessed in this experiment. However, the positive effect
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of SREBP-1c DP on FAS or SCD-1 gene expression has been shown previously in 3T3 insulin-deprived adipocytes (62).

After 48 h (day 6 after confluence), total RNA was prepared to evaluate FAS and SCD-1 mRNA levels by real time RT-PCR analysis. After 96 h (day 8 after confluence), cell homogenates were obtained to determine triglyceride content. Results are expressed as the percentage of control values, and represent the mean ± S.E. of two independent experiments performed in duplicate.

*, P<0.05; **, P<0.01, efavirenz-treated versus control cells.

#, P<0.05; ##, P<0.001, efavirenz-treated cells infected with Ad.null versus efavirenz-treated cells infected with Ad.SREBP-1c DP.
Table 1: **Primer sequences of genes tested in real time RT-PCR**

The abbreviations of the genes, their full names, and 5’ to 3’ nucleotide sequences of the sense and antisense primers are presented.

<table>
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<tr>
<th>Murine genes</th>
<th>Sense primer</th>
<th>Antisense primer</th>
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<tr>
<td>aP2 adipocyte lipid-binding protein</td>
<td>AAC ACC GAG ATT TTC TTT ACC ACC ACC AG</td>
<td>ACA ACC GAG ATT TCC TT</td>
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<tr>
<td>C/EBPα CCAAT/enhancer binding protein-α</td>
<td>CTG CGA GCA CGA GAC GTC TAT AG</td>
<td>TCC CGG GTA TGC AAA GTC ACC</td>
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<tr>
<td>C/EBPβ CCAAT/enhancer binding protein-β</td>
<td>GCA AGA GCC GCG ACA AG</td>
<td>GCC TCG GGC AGC TGC TT</td>
</tr>
<tr>
<td>FAS fatty acid synthase</td>
<td>TGC TCC CAG CTG CAG GC</td>
<td>GCC CGG TAG CTC TGG GTG TA</td>
</tr>
<tr>
<td>LPL lipoprotein lipase</td>
<td>AGG ACC CCT GAA GAC AC</td>
<td>GCC ACC CAA CTC TCA TA</td>
</tr>
<tr>
<td>PPARγ peroxisome proliferator-activated receptor-γ</td>
<td>AGG CCG AGA AGG AGA AGC TGT TG</td>
<td>TGG CCA CCT TTT TTG TGC GTC C</td>
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<tr>
<td>SCD-1 stearoyl-CoA desaturase-1</td>
<td>TGG GGT GGC TGC TGG TG</td>
<td>GCC TGG GCA GGA TGA AG</td>
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<tr>
<td>SREBP-1c sterol regulatory element-binding protein-1c</td>
<td>GGA GCC ATG GAT TGC ACA TT</td>
<td>GCT TCC AGA GAG GAG GGC AG</td>
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<tr>
<td>18S 18S ribosomal RNA</td>
<td>GGG AGC CTG AGA AAC GGC</td>
<td>GGG TCG GAG TGG GTA ATT T</td>
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<tr>
<th>Human genes</th>
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<th>Antisense primer</th>
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<tr>
<td>FAS fatty acid synthase</td>
<td>ACC TCC AAG GAC ACA GTC ACC AT</td>
<td>CAG CTG CTC CAC GAA CTC AA</td>
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<td>GAPDH glyceraldehyde-3-phosphate dehydrogenase</td>
<td>ACC CAC TCC TCC ACC TTT G</td>
<td>CTC TCG TGC TCT TGC TGG G</td>
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<td>PPARγ peroxisome proliferator-activated receptor-γ</td>
<td>CAG TGG GGA TGT CTC ATA A</td>
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<tr>
<td>SREBP-1c sterol regulatory element-binding protein-1c</td>
<td>GGA GCC ATG GAT TGC ACA TT</td>
<td>AGG AAG GCT TCC AGA GAG GA</td>
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</table>
Table 2: Efavirenz effect on triglyceride accumulation in mature 3T3-F442A adipocytes

3T3-F442A were differentiated with fetal calf serum and insulin. Once differentiation was achieved, day 8 post-confluent adipocytes were cultured in the absence or in the presence of efavirenz (20 or 40 µM) for 4, 8, and 16 days. At these intervals, cells extracts were prepared to determine triglyceride content. The percentage of the control value is given in parentheses after the values of triglyceride content. Results represent the mean ± S.E. of 4-10 separate experiments.

<table>
<thead>
<tr>
<th>Treatment duration (days) [day of culture post-confluence]</th>
<th>[Efavirenz] (µM)</th>
<th>Triglyceride content (µM) (% of control)</th>
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<tbody>
<tr>
<td>4 [day 12]</td>
<td>0</td>
<td>12,055 ± 379</td>
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<tr>
<td></td>
<td>20</td>
<td>11,374 ± 598 (94 %)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>9254 ± 608* (77 %)</td>
</tr>
<tr>
<td>8 [day 16]</td>
<td>0</td>
<td>15,769 ± 271</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>15,490 ± 183 (98 %)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>12,780 ± 575** (81 %)</td>
</tr>
<tr>
<td>16 [day 24]</td>
<td>0</td>
<td>17,175 ± 126</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>10,470 ± 163** (61 %)</td>
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</table>

*, P<0.01; **, P<0.001, efavirenz-treated versus control adipocytes.
Table 3: Efavirenz does not alter basal or effector-stimulated glucose uptake or lipolysis

Day 7 post-confluent 3T3-F442A cells were cultured in the absence or in the presence of efavirenz (20 or 40 µM) for 4 days. $^3$H-DOG uptake was then measured under basal conditions, or in response to an optimal concentration of insulin (100 nM). Results are expressed in nmol $^3$H-DOG/5 min/well and represent the mean ± S.E. of 4-8 separate experiments. We have also tested $^3$H-DOG uptake in response to various concentrations of insulin (see text). 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 glycerol/h/well and represent the mean ± S.E. of 6 separate experiments.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>$^3$H-DOG uptake (nmol/5 min/well)</th>
<th>Glycerol release (nmol/h/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Insulin</td>
</tr>
<tr>
<td>Control</td>
<td>2.40 ± 0.08</td>
<td>4.23 ± 0.27</td>
</tr>
<tr>
<td>Efavirenz (20 µM)</td>
<td>2.87 ± 0.07</td>
<td>4.34 ± 0.12</td>
</tr>
<tr>
<td>Efavirenz (40 µM)</td>
<td>2.71 ± 0.21</td>
<td>3.99 ± 0.26</td>
</tr>
</tbody>
</table>

*, $P<0.01$, efavirenz-treated versus control cells.
**Table 4: Efavirenz reduces $[^3]$H-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, day 5 to day 9, day 7 to day 11, and day 9 to day 13. At the end of efavirenz exposure, cells were incubated for 1 h in KRH buffer containing 5 mM $[^3]$H-glucose. After washing, $[^3]$H-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 $[^3]$H-glucose incorporation.

<table>
<thead>
<tr>
<th>Sequence of efavirenz treatment</th>
<th>D0-D7</th>
<th>D5-D9</th>
<th>D7-D11</th>
<th>D9-D13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.47 ± 0.46</td>
<td>7.87 ± 0.34</td>
<td>15.84 ± 0.64</td>
<td>9.27 ± 0.36</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>2.27 ± 0.15* (24 %)</td>
<td>4.45 ± 0.18** (56 %)</td>
<td>8.21 ± 0.32** (52 %)</td>
<td>6.68 ± 0.42* (72 %)</td>
</tr>
</tbody>
</table>

*, $P<0.01$; **$P<0.001$, efavirenz-treated versus control cells.
Figure 2

A

B

C

Triglyceride content (% of control)

0 20 40 60 80 100

0 2 4

Efavirenz concentration (μM)

*
Figure 3

Triglyceride content (% of control)

Efavirenz

Days of culture  D7  D9  D12  D15

+  -  +  -  +  -  +

**  **  #  **  **  #  **

n.s.
Figure 4

![Graph showing the relationship between Intralipid concentration (μg/ml) and Triglyceride content (μM). The graph includes data points and error bars, with statistical significance indicated by symbols (# and ##).]
Figure 6

SREBP-1c

C/EBPα

PPARγ

Efavirenz concentration (μM)
Figure 7

mRNA levels (% of control)

FAS

SCD-1

LPL

aP2

Efavirenz concentration (μM)

Day of culture D2 D4 D7
In vitro suppression of the lipogenic pathway by the non-nucleoside reverse transcriptase inhibitor efavirenz in 3T3 and human preadipocytes or adipocytes
Khadija El Hadri, Martine Glorian, Christelle Monsempes, Marie-Noëlle Dieudonné, René Pecquery, Yves Giudicelli, Marise Andreani, Isabelle Dugail and Bruno M. Fève

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