Indinavir inhibits sterol-regulatory element-binding protein-1c-dependent lipoprotein lipase and fatty acid synthase gene activations

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Background: A syndrome characterized by hypertriglyceridaemia, hypercholesterolaemia, hyperinsulinaemia, and lipodystrophy has been found to be associated with highly active antiretroviral treatment (HAART) including protease inhibitors. A marker predicting this syndrome has been previously identified in the gene encoding the sterol-regulatory element-binding protein (SREBP)-1c, a regulator of triglycerides, cholesterol, insulin, and adipocytes.

Objective: A possible inhibition of SREBP-1c-dependent genes by the protease inhibitor indinavir and its possible reversal by the lipid-lowering drug simvastatin were studied.

Methods: The effects of indinavir and simvastatin on the inhibition/activation of SREBP-1c-dependent genes were compared with the effects of indinavir and simvastatin on the inhibition/activation of SREBP-1c-independent genes.

Results: Indinavir inhibited the SREBP-1c-dependent genes encoding the lipoprotein lipase (10^3 nmol/l resulted in an inhibition of 12.4%; $P = 0.0051$) and the fatty acid synthase (10^3 nmol/l resulted in an inhibition of 30.3%; $P = 0.036$) in a dose-dependent fashion but not the SREBP-1c-independent gene encoding the low-density lipoprotein receptor. Simvastatin antagonized the indinavir-induced SREBP-1c-inhibition.

Conclusions: Indinavir inhibits important effector genes of the SREBP-1c pathway, explaining major HAART-related adverse effects.

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Keywords: retrovirus, antiretroviral therapy, protease inhibitors, hyperlipidaemia, lipodystrophy, sterol-regulatory element-binding protein, SREBP, reporter gene assays

Introduction

Although highly active antiretroviral treatment (HAART) including protease inhibitors (PIs) has drastically lowered morbidity and immediate mortality in HIV-1-infected patients [1,2], it frequently induces hypertriglyceridaemia, hypercholesterolaemia, hyperinsulinaemia and lipodystrophy [3,4]; this, consequently, increases the risk of cardiovascular complications [5,6]. Recent studies revealed that HAART-related adverse effects are common and persist in patients remaining on treatment [3,6]. A single-nucleotide polymorphism in the gene encoding the sterol-regulatory element-binding protein (SREBP)-1c, also called adipocyte determination and differentiation factor (ADD)-1, has been recently found to be associated with this HAART-related syndrome [7]. Furthermore, PI drugs inhibit SREBP-1c/ADD-1 *in vitro* [8,9]. To determine the effect of the PI indinavir on SREBP-1c/ADD-1 and the consequences of a possible inhibition, effector genes specifically activated by SREBP-1c/ADD-1 were studied in cell culture in the presence of indinavir.

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Materials and methods

Indinavir sulfate and simvastatin were provided by Merck & Co. Inc., Rahway, New Jersey, USA. The inactive lactone prodrug simvastatin was converted into its active dihydroxy-open form (L-644128) by acidic hydrolysis according to the manufacturer's protocol. Aqueous solutions of indinavir sulfate and the active dihydroxy-open form of simvastatin were prepared.

Reporter gene constructs

Lipoprotein lipase gene

The pGL2–lipoprotein lipase (*LPL*) gene construct contained nucleotides −1910 to −9 (**a** of the **a**tg translation start site was assigned +1 in all the constructs) of the *LPL* gene promoter (GenBank X68111; nucleotides 1 to 1902 according to GenBank numbering), comprising three putative sterol-regulatory elements. The promoter was amplified by polymerase chain reaction (PCR) using the oligonucleotide sequences 5′-GG**GGTACC**TGCAGGAGTATTCTAT ATAAGATAG-3′ and 5′-CCC**AAGCTT**CGCGTC CCTCTGGAGGAGCTGCAAG-3′ (restriction sites are shown in bold), digested, purified, and ligated into the pGL2–Basic vector (Promega, Madison, Wisconsin, USA).

Fatty acid synthase gene

The pGL2–fatty acid synthase (*FAS*) gene construct contained nucleotides −1485 to −1246 of the *FAS* gene promoter (GenBank X54671; nucleotides 1381 to 1620 according to GenBank numbering), comprising the key regulatory elements such as the sterol-regulatory element. The promoter was cloned into pGL2 following PCR (5′-GGA**GGTACC**GCGTTCCTT GTGCTCCAGCGCGC-3′; 5′-CAG**AAGCTT**CTG GACGGGACGCTGCTGCCGTCTCTC-3′).

Low-density lipoprotein receptor gene

The pGL2–low-density lipoprotein receptor (*LDLR*) gene construct contained nucleotides −328 to −61 of the *LDLR* gene promoter (GenBank L29401; nucleotides 380 to 627 according to GenBank numbering), comprising one sterol-regulatory element. The promoter was cloned into pGL2 following PCR (5′-AGCT**G GTACC**CGGAGACCCAAATACAACA-3′; 5′-TGT CC**AAGCTT**GAAACCCTGGCTTCCCGCGA-3′).

Confirmation of constructs

All constructs were confirmed by sequencing and contained sequences identical to those previously published and demonstrated to be functional [10–13]. In each experiment, the regulatory sequences of the inserts have been demonstrated to be inhibited by sterols as a negative and activated by simvastatin as a positive control.

Cell culture

Monolayers of human embryonic kidney (HEK)293 and hepatoma (Hep)G2 cells were set up (day 0, 5×10^6 cells/100 mm poly-D-lysine-coated Petri-dish) and cultured (37°C, 5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Paisley, UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma, St Louis, Missouri, USA), and 5% (v/v) fetal calf serum (FCS) (Gibco) for 15 h.

Transfection

HEK293 and HepG2 cells were independently cotransfected with 8.4 μg/dish of the pGL2–*LPL*, pGL2–*FAS* or the pGL2–*LDLR* luciferase reporter gene constructs, with 0.2 μg pRL–cytomegalovirus (CMV) (Promega), a plasmid encoding the *Renilla* luciferase, as an internal control for transfection efficiency, and with the vectors without inserts. The cells were incubated (7 h), treated with trypsin and transferred to medium A [DMEM supplemented with 5% (v/v) FCS, 100 U/ml penicillin, and 100 μg/m streptomycin] or medium B [DMEM supplemented with 5% (v/v) calf lipoprotein-deficient serum (LPDS) (Sigma), 50 μmol/l sodium mevalonate, 100 U/ml penicillin, and 100 μg/m streptomycin]. The cells were distributed over 96-well plates and incubated for 17 h. Each well contained 2×10^4 viable cells.

Indinavir effects

Twenty-four hours after transfection, indinavir was added at final concentrations of 0, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10^1, 5×10^1, 10^2, 2×10^2, 7.5×10^2, 10^3, 2×10^3, 5×10^3, 2×10^4, and 10^5 nmol/l. As a control of the inhibition of the SREBP-regulated reporter genes, the cells were incubated with 1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol (Sigma); as a control of the activation of the SREBP-regulated reporter genes, the cells were incubated with the dihydroxy-open form of simvastatin at final concentrations of 3×10^4 or 4×10^4 nmol/l.

Following incubation for 24 h, the media were discarded, the cells were washed with 1 × phosphate-buffered saline (PBS), and passive lysis buffer (25 μl/well; Promega) was added. The 96-well plates were shaken for 20 min. The luciferase and *Renilla* activities were determined by the Dual-Luciferase™ Reporter Assay System (Promega). Luciferase activities were normalized according to the *Renilla* activities.

Reversibility of indinavir effects

To determine whether indinavir-induced inhibition of the activation of the SREBP-dependent genes was reversible or not, two series of HEK293 cells were cotransfected with pGL2–*FAS* luciferase reporter gene constructs and with pRL–CMV. These experiments were identical to those described above except that two sets of cells were used instead of one. Both sets of cells

were first incubated with indinavir for 24 h at the concentrations specified above. Thereafter, indinavir was completely washed out with 1 × PBS. The first set of cells was incubated for another 24 h with fresh medium not containing indinavir. The second set of cells was incubated for another 24 h with fresh medium again containing indinavir at various concentrations. After incubation, the cells were harvested to determine the activation of the SREBP-dependent genes as described above.

Antagonization of indinavir effects by simvastatin

To determine whether indinavir-induced inhibition of the SREBP-dependent genes could be partially or entirely antagonized by statins, simvastatin in combination with indinavir was added to the cell culture medium. The net effects of the combination of simvastatin (constant final concentration of 3×10^4 nmol/l) and indinavir (final concentrations of 10, 5×10^1, 10^2, 2×10^2 nmol/l) were determined following 24 h of incubation with the respective combinations. The cells were harvested and the activation of the SREBP-dependent *FAS* gene was determined as described above.

Statistical methods

RepGene, a spreadsheet template for the management of reporter gene assays was used for planning and evaluation of the experiments [14]. The significance level of the effect of indinavir on *Renilla*-normalized luciferase activities was determined by analysis of variance (ANOVA) using StatView 4.5 (Abacus Concepts Inc., Berkeley, California, USA).

Results

Indinavir inhibited the *LPL* and *FAS* reporter gene activities, measured as normalized relative light units, in a dose-dependent fashion (Fig. 1a,b). ANOVA confirmed the significance of the interaction between the different indinavir concentrations and the inhibition of the *LPL* gene activity ($P = 0.0158$) as well as the interaction between the different indinavir concentrations and the inhibition of the *FAS* gene activity ($P = 0.0358$). Indinavir did not inhibit the *LDLR* reporter gene activity significantly.

In the *LPL* reporter gene experiments, inhibition of the gene activity was detectable starting from an indinavir concentration of 1 nmol/l. At a concentration of 10^3 nmol/l, indinavir inhibited the *LPL* gene activity from baseline by 12.4% but did not reach a plateau at this concentration. At the highest concentration tested (10^5 nmol/l) indinavir inhibited the *LPL* gene activity from baseline by 57.1% (difference baseline versus highest concentration: $P = 0.041$) (Fig. 1a).

In the *FAS* reporter gene experiments, inhibition of the gene activity was detectable starting from an indinavir concentration of 10^{-2} nmol/l. At a concentration of 10^3 nmol/l, indinavir inhibited the *FAS* gene activity from baseline by 30.3%. The effect reached a plateau at this concentration (Fig. 1b).

Two sets of HEK293 cells were identically prepared except that, following incubation with indinavir and washing of the cells, the first series was incubated with medium containing no indinavir, the second series with medium containing indinavir. After a second incubation period, no statistically significant differences regarding the indinavir-induced inhibition of the SREBP-dependent genes were detectable between the two series. It was, therefore, concluded that indinavir-induced effects were not reversible.

Toxicity of indinavir causing a decrease in the viability of the cells with increasing concentrations of indinavir was first excluded on the morphological level. Cell toxicity was not detected microscopically, even at high indinavir concentrations ($\geqslant 10^3$ nmol/l). Cell toxicity as an explanation for the decrease of the *LPL* and *FAS* gene activities attributed to indinavir was further excluded by incubating the cells with the active dihydroxy-open form of simvastatin, a strong activator of the SREBP pathways. When simvastatin was combined with various concentrations of indinavir, the cells were still able to upregulate the SREBP-1c/ADD-1-dependent genes, as shown for *FAS* (Fig. 2): increasing concentrations of indinavir (0, 10, 5×10^1, 10^2, and 2×10^2 nmol/l) decreased the rate of the gene activation induced by a constant simvastatin concentration (3×10^4 nmol/l) again in a dose-dependent fashion. At an indinavir concentration of 2×10^2 nmol/l combined with a simvastatin concentration of 3×10^4 nmol/l, the *FAS* gene activity decreased by 36.4% compared with the activation achieved with simvastatin alone. However, the combination of 3×10^4 nmol/l simvastatin with 2×10^2 nmol/l indinavir still resulted in a net activation of the gene, demonstrating that the cells maintained their ability to upregulate their SREBP-1c/ADD-1-dependent genes despite the presence of indinavir (Fig. 2).

The effects of indinavir on the *LPL* and *FAS* reporter genes were similarly detectable when liver cells (HepG2 cells) instead of HEK293 cells were transfected (data not shown). The effects of indinavir on the *LPL* and *FAS* reporter genes were also detectable when the cells were incubated in FCS or LPDS media. Indinavir, sterols or simvastatin did not influence the activity of the empty vectors (pGL, pRL) (data not shown).

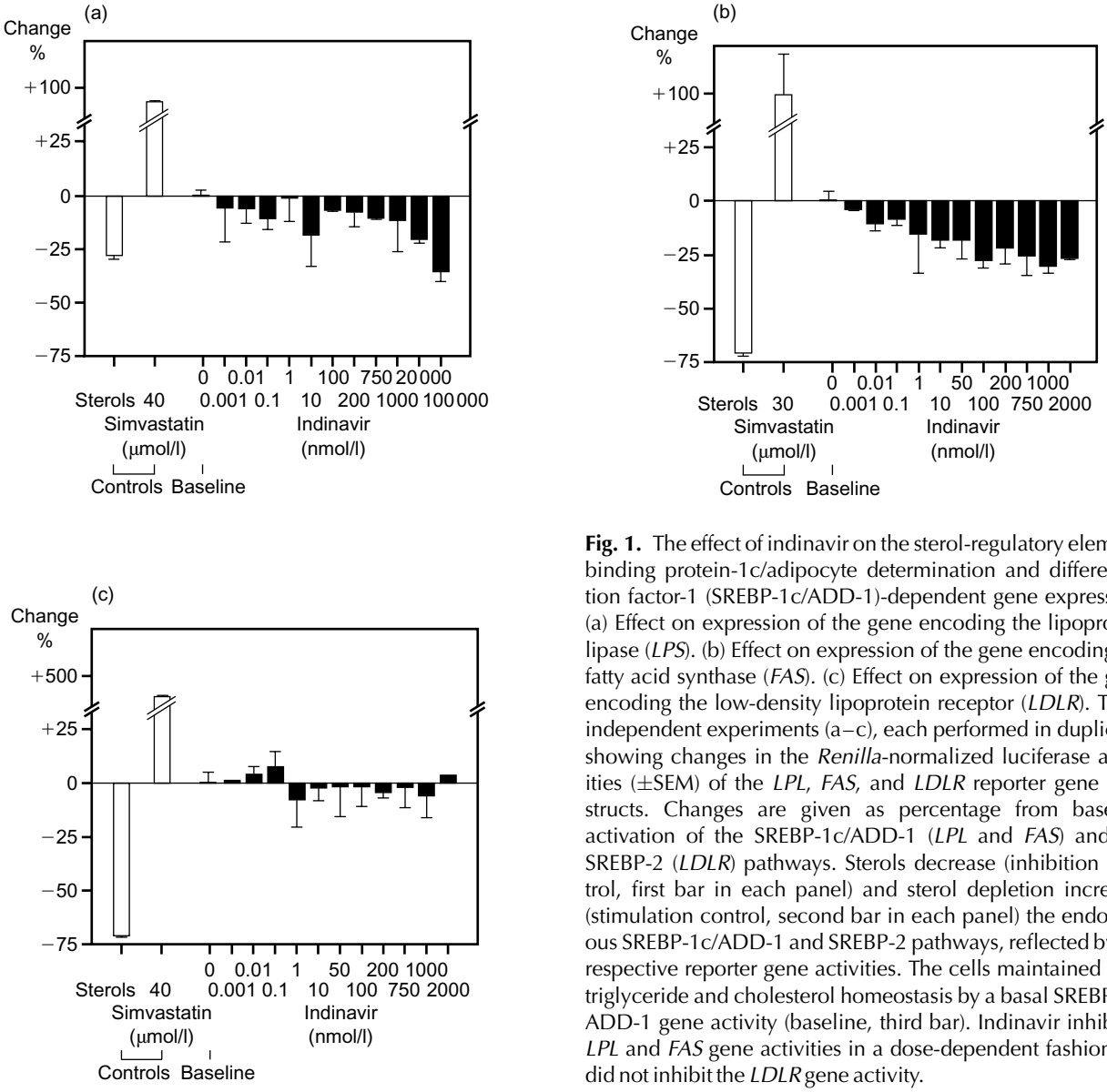

Fig. 1. The effect of indinavir on the sterol-regulatory element-binding protein-1c/adipocyte determination and differentiation factor-1 (SREBP-1c/ADD-1)-dependent gene expression. (a) Effect on expression of the gene encoding the lipoprotein lipase (*LPS*). (b) Effect on expression of the gene encoding the fatty acid synthase (*FAS*). (c) Effect on expression of the gene encoding the low-density lipoprotein receptor (*LDLR*). Three independent experiments (a–c), each performed in duplicate, showing changes in the *Renilla*-normalized luciferase activities (±SEM) of the *LPL*, *FAS*, and *LDLR* reporter gene constructs. Changes are given as percentage from baseline activation of the SREBP-1c/ADD-1 (*LPL* and *FAS*) and the SREBP-2 (*LDLR*) pathways. Sterols decrease (inhibition control, first bar in each panel) and sterol depletion increases (stimulation control, second bar in each panel) the endogenous SREBP-1c/ADD-1 and SREBP-2 pathways, reflected by the respective reporter gene activities. The cells maintained their triglyceride and cholesterol homeostasis by a basal SREBP-1c/ADD-1 gene activity (baseline, third bar). Indinavir inhibited *LPL* and *FAS* gene activities in a dose-dependent fashion but did not inhibit the *LDLR* gene activity.

Discussion

In the present study, the influence of indinavir on the activation of effector genes involved in the triglyceride, cholesterol, and insulin metabolism was investigated.

The major findings were that indinavir (i) decreased the activities of the SREBP-1c/ADD-1-dependent *LPL* and *FAS* reporter genes in a dose-dependent manner and (ii) did not decrease the activity of the SREBP-1c/ADD-1-independent *LDLR* reporter gene.

Inhibition of the *LPL* and *FAS* reporter gene activities was detectable starting from an indinavir concentration of 10^{-1} nmol/l (*LPL*) or 10^{-2} nmol/l (*FAS*). For comparison: concentrations of 5×10^{1} to 10^{2} nmol/l indinavir inhibit viral spread by 95% in cell culture [15], concentrations of 10^{2}–10^{3} nmol/l indinavir correspond to physiological mean plasma concentrations in patients on treatment, and concentrations of 10^{3}–10^{5} nmol/l correspond to mean plasma concentrations usually not achieved in the steady state [16]. Since we observed clear decreases (12.4% for *LPL* and 30.3% for *FAS*) at an indinavir concentration of 10^{3} nmol/l, we expect that administration of indinavir in recommended doses (2.4 g daily) will result in changes in the expressions of the *LPL* and *FAS* genes *in vivo* as well. In contrast, a significant inhibition of the *LDLR* reporter gene activity was not detectable, even at an indinavir concentration of 2×10^{4} nmol/l. This result was not unexpected. We previously hypothesized that the pathophysiological mechanism to induce the hypertriglyceridaemia, hypercholesterolaemia, hyperinsulinaemia, and lipodystrophy syndrome observed in HIV-infected patients is mediated

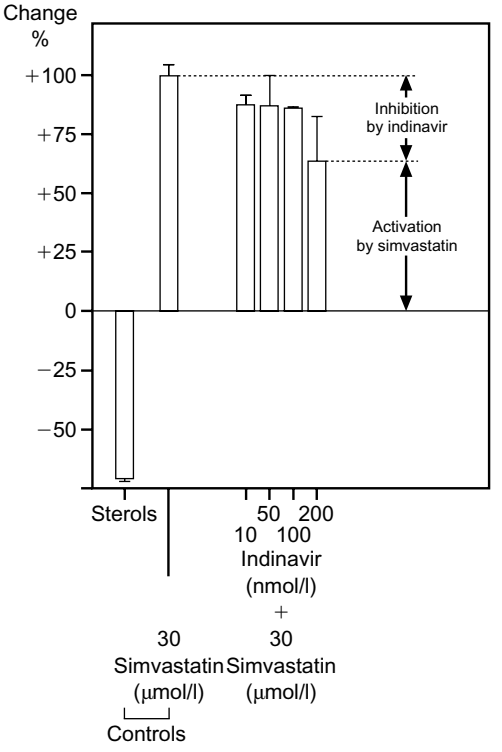

Fig. 2. Antagonization of the indinavir-induced inhibition of sterol-regulatory element-binding protein (SREBP)-dependent genes by simvastatin. Experiments performed in duplicates showing changes in *Renilla*-normalized luciferase activities (±SEM) of the fatty acid synthase (*FAS*) reporter gene constructs. Changes are given as percentage from the respective baselines. If the activation by simvastatin is defined as baseline, indinavir inhibits the simvastatin-induced activation of the *FAS* gene activity in a dose-dependent fashion by 12.1% (indinavir at a final concentration of 10 nmol/l), 12.7% (5×10^1 nmol/l), 13.5% (10^2 nmol/l), and 36.4% (2×10^2 nmol/l). If the *FAS* gene activity without simvastatin is defined as baseline, simvastatin entirely antagonizes the indinavir-induced inhibition of the *FAS* gene activity resulting in a net activation of the *FAS* gene of 87.4% (indinavir at a final concentration of 10 nmol/l), 86.9% (5×10^1 nmol/l), 86.1% (10^2 nmol/l), and 63.2% (2×10^2 nmol/l).

by the SREBP-1c/ADD-1 pathway rather than by the SREBP-2 pathway [7]. Since the gene encoding the *LDLR* is activated by SREBP-2 but much less by SREBP-1c/ADD-1 [12], the lack of a significant inhibition of the *LDLR* reporter gene activity by indinavir is in line with our hypothesis.

Using other experimental approaches, PI have been shown to inhibit SREBP-1c/ADD-1 [9], insulin-stimulated glucose uptake [17], insulin signalling [9,18] and adipocyte determination and differentiation [9,19,20], and to induce adipocyte apoptosis [21] and modulate proteosome activity [22]. In particular, the results of Caron *et al.* demonstrate a clear indinavir-induced impairment of SREBP-1 [9]. Caron *et al.* focused on the indinavir-induced inhibition of insulin effects and adipocyte determination and differentiation, which are both mediated by SREBP-1c/ADD-1. Our results demonstrate the indinavir-induced inhibition of the lipoprotein and fatty acid metabolism, both also mediated by SREBP-1c/ADD-1, and are, therefore, in agreement with the observations of these authors. Thus, the indinavir-induced inhibition of effector genes regulated by SREBP-1c/ADD-1 leads to hypertriglyceridaemia, hypercholesterolaemia, hyperinsulinaemia and lipodystrophy: the syndrome observed in HIV-infected patients on HAART.

SREBP-1c/ADD-1 plays a central role in the regulation of triglycerides, cholesterol, insulin, and adipose tissue formation. Administration of PIs, associated with hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia and peripheral lipodystrophy, affects SREBP-1c/ADD-1 and, therefore SREBP-1c/ADD-1-dependent effector genes. SREBP-1c/ADD-1 specifically activates the genes encoding the LPL and FAS [10,11,23–25] as well as the adipocyte determination and differentiation [26].

SREBP-1c/ADD-1 controls the peripheral clearance of triglyceride- and cholesterol-rich lipoproteins via regulation of *LPL* [25,27–29]. Inhibition of *LPL* results in hypertriglyceridaemia and hypercholesterolaemia (Fig. 3), as observed in inherited LPL deficiency syndromes (Fig. 3) [30–33]. SREBP-1c/ADD-1 controls fatty acid synthesis via regulation of *FAS* [11,24] and the determination and differentiation of adipocytes [26,27]. SREBP-1c/ADD-1 controls the insulin effect via regulation of a series of genes such as those encoding LPL [10], FAS [11,24], and glucokinase [34](Fig. 3). Inhibition of these genes results in a decreased insulin effect and, thus, insulin resistance [34,35]. Plasma insulin then increases in compensation [36]. Consequently, inhibition of *LPL*, *FAS* and the gene encoding the glucokinase results in hyperinsulinaemia, as observed in inherited defects affecting, for example, the genes encoding LPL [30–32] and glucokinase [37] (Fig. 3). In line with these observations are our previous findings of a significant, parallel increase in plasma cholesterol and plasma insulin levels in HIV-1-infected subjects treated with PIs [7].

Inhibition of fatty acid synthesis and adipocyte determination and differentiation results in decreased fatty acid synthesis and adipose tissue formation [9,19,20] and might, therefore, explain particular aspects of peripheral lipodystrophy in humans (Fig. 3).

Proteolytic cleavage of SREBP-2 activates the gene encoding the LDLR strongly whereas proteolytic cleavage of SREBP-1c/ADD-1 activates *LDLR* much less [12]. Similarly, simvastatin activates the SREBP-2-dependent *LDLR* strongly (approximately 500%, Fig. 1c) but activates the SREBP-1c/ADD-1-dependent

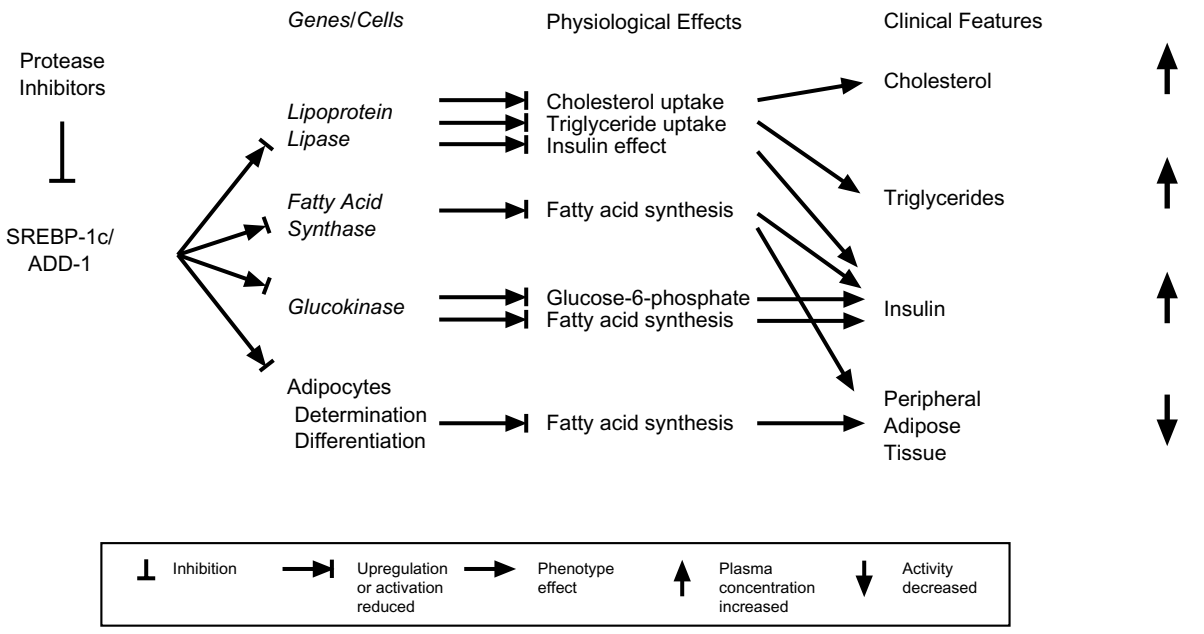

Fig. 3. Protease inhibitor-induced changes in the sterol-regulatory element-binding protein-1c/adipocyte determination and differentiation factor-1c (SREBP-1c/ADD-1)-regulated pathway. SREBP-1c/ADD-1 controls the genes encoding the lipoprotein lipase (LPS), fatty acid synthase (FAS) and the glucokinase as well as the adipocyte determination and differentiation. In subjects treated with highly active antiretroviral therapy (HAART), inhibition of the SREBP-1c/ADD-1-mediated *LPL* activity is expected to result in a combined hyperlipoproteinaemia (increases in triglycerides and cholesterol) similar to that observed in LPL deficiency syndromes [30–32,43]. Inhibition of SREBP-1c/ADD-1-mediated *FAS* activity is expected to result in a decrease in fatty acids and in an increase in insulin [19,20]. SREBP-1c/ADD-1 is regulated by insulin [36,44,45] and has an insulin-mimicking effect [34]. Inhibition of SREBP-1c/ADD-1, therefore, results in decreased activity of various insulin-dependent genes (including the gene for glucose 6-phosphate; [34,36]) and, hence, in reactive hyperinsulinaemia. In HAART-treated subjects, inhibition of SREBP-1c/ADD-1-mediated adipocyte determination and differentiation alters peripheral adipose tissue directly and may, therefore, contribute to HAART-related peripheral lipodystrophy [9,19,20].

LPL and *FAS* genes much less (approximately 100%, Fig. 1a,b). Inhibition of the SREBP-2-dependent *LDLR* gene (as in familial hypercholesterolaemia caused by *LDLR* gene defects) results in hypercholesterolaemia but usually not in hypertriglyceridaemia [38]. In HAART-associated hyperlipidaemia, however, hypertriglyceridaemia is the predominant phenotype. In line with this latter observation, in our cell culture experiments, indinavir inhibited *LDLR* much less, indicating that the SREBP-1c/ADD-1-mediated mechanisms play the predominant role in the development of HAART-associated adverse effects.

We previously cloned and characterized the promoters of the differentially spliced genes of *SREBP-1*, *SREBP-1a* and *SREBP-1c/ADD-1* and characterized the *SREBP-2* gene [39]. Very recently, we identified in the gene encoding SREBP-1c/ADD-1 a marker predictive of the hypertriglyceridaemia, hypercholesterolaemia, hyperinsulinaemia and lipodystrophy syndrome [7]. Although, in our experience, other cell lines such as HepG2 cells are more difficult to transfect than HEK293 cells, analogous experiments carried out using HepG2 cells instead of HEK293 cells resulted in similar inhibitory effects on *FAS* activation. Hence, there is strong evidence from others [8,9] as well as from our own previous [7] and present results that inhibition of SREBP-1c/ADD-1 is involved in the development of the HAART-related hyperlipidaemia, hyperinsulinaemia, and lipodystrophy syndrome. Nevertheless, it remains to be clarified how PIs mediate the inhibition of SREBP-1c/ADD-1. The HIV-1 protease (GenBank 230883) and the site-1 protease (GenBank 4506775), a sterol-regulated protease that cleaves the SREBP molecules [40], share a sequence homology at their catalytic sites. Despite this sequence homology, the HIV-1 protease and the site-1 protease belong to different classes of proteases. Therefore, because of considerable differences in the three-dimensional structures of the HIV-1 protease and the site-1 protease and based on steric models, it is unlikely that PI influence the SREBP pathway by direct inhibition of the site-1 protease. Similarly, these considerations apply to the hypothesis that PIs interact with the adipocyte-enhancer binding protein [41]. Furthermore, if the site-1 protease was inhibited, we would expect quantitatively similar effects regarding the impairment of the activation of SREBP-1c/ADD-1- and SREBP-2-dependent genes. However, this was clearly not the case in our experiments.

A possible explanation for the differential inhibition of SREBP-1c/ADD-1- and SREBP-2-dependent genes is based on recent experiments suggesting differences in the regulation of the amount of mature SREBP-1c/ADD-1 and SREBP-2. While SREBP-1c/ADD-1 is controlled by two independent mechanisms, by cleavage activation and by the rate of mRNA degradation [42], there is no evidence that SREBP-2 is regulated by the latter [29].

In summary, our demonstration of the inhibition of SREBP-1c/ADD-1-dependent genes explains the major metabolic effects described in association with HAART.

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