HIV Protease Inhibitor Induces Fatty Acid and Sterol Biosynthesis in Liver and Adipose Tissues Due to the Accumulation of Activated Sterol Regulatory Element-binding Proteins in the Nucleus*

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The mechanism by which human immunodeficiency virus (HIV) protease inhibitor therapy adversely induces lipodystrophy and hyperlipidemia has not been defined. This study explored the mechanism associated with the adverse effects of the prototype protease inhibitor ritonavir in mice. Ritonavir treatment increased plasma triglyceride and cholesterol levels through increased fatty acid and cholesterol biosynthesis in adipose and liver. Ritonavir treatment also resulted in hepatic steatosis and hepatomegaly. These abnormalities, which were especially pronounced after feeding a Western type high fat diet, were due to ritonavir-induced accumulation of the activated forms of sterol regulatory binding protein (SREBP)-1 and -2 in the nucleus of liver and adipose, resulting in elevated expression of lipid metabolism genes. Interestingly, protease inhibitor treatment did not alter SREBP mRNA levels in these tissues. Thus, the adverse lipid abnormalities associated with protease inhibitor therapy are caused by the constitutive induction of lipid biosynthesis in liver and adipose tissues due to the activation of SREBP in the nucleus.

The discovery and use of protease inhibitor therapy has significantly reduced the morbidity and mortality of AIDS as a consequence of HIV infection (1, 2). Unfortunately, the use of HIV protease inhibitors (PI) has also been associated with several undesirable side effects, including peripheral fat wasting and excessive central fat deposition (lipodystrophy), overt hyperlipidemia, and insulin resistance (3–7). These abnormalities in lipid metabolism may also lead to the increased incidence of accelerated atherosclerosis in HIV patients (8, 9). The mechanism by which PI therapy leads to lipid abnormalities remains unclear, thus limiting the options for effective treatment of HIV-infected individuals.

The urgency of designing agents that are less likely to induce lipid disturbances has prompted numerous studies aimed at understanding the relationship between PI therapy and abnormalities in lipid metabolism and preadipocyte differentiation. However, cell culture studies have yielded conflicting results with the PI either suppressing (10–12) or enhancing (13) preadipocyte differentiation in vitro. These studies also focused primarily on PI effects on transcription factors that are responsible for regulating lipid metabolism pathways and adipocyte differentiation genes, such as the peroxisome proliferator-activated receptor-γ (14), the CCAAT/enhancer-binding protein (15), and the sterol regulatory element-binding protein-1 (SREBP-1) (16, 17). Very little attention was paid to PI effects on lipogenesis per se. Accordingly, the lipodystrophic and hyperlipidemic effects of PI in vivo have not been defined. Additionally, the contribution of different dietary nutrients on PI-induced lipid abnormalities has not been addressed.

In this study, we examined the effect of ritonavir, a prototype PI that elicits potent lipid abnormalities in patients (18–20), on lipogenesis and plasma lipid concentrations in mice fed either a basal low fat/low cholesterol diet or a Western type high fat/high cholesterol diet. The results showed that ritonavir-induced lipodystrophy and hyperlipidemia are caused by PI-induced accumulation of activated SREBP-1 and SREBP-2 in the nucleus, thereby increasing the expression of genes responsible for lipid biosynthesis in adipocytes and in the liver.

EXPERIMENTAL PROCEDURES

Animal Maintenance—Six- to 8-week-old male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were housed according to our institutional guidelines. The animals were fed either a standard mouse chow containing 4% (w/w) fat and 0.04% (w/w) cholesterol (Harlan Teklad Laboratories, Madison, Wisconsin) or a Western type high fat diet containing 21% (w/w) anhydrous milk fat and 0.15% (w/w) cholesterol (TDX137 from Harlan Teklad). Half of the mice from each dietary group were gavaged every morning with 50 μl ritonavir (2 mg/day), while the other half of the animals received equal volume of a 22% ethanol solution as vehicle control. On the day of experiments, animals were gavaged with the test reagent and then fasted for 4 h before use.

Plasma Lipid Analysis—Blood samples were collected from mice under anesthesia after a 4-h fast. Plasma was obtained by centrifugation and then analyzed for cholesterol and triglyceride concentrations with kits purchased from Wako Chemicals (Richmond, Virginia). Lipoprotein distribution in plasma was analyzed by fast performance liquid chromatography with 2 tandem Supero 6 HR columns (Amersham Pharmacia Biotech). Two hundred μl of pooled plasma from four mice in each group were applied to the columns and 0.5 ml fractions were collected for cholesterol analysis. Lipoprotein elution profile was determined by comparison with standard VLDL, LDL, and HDL.

Tissue Fatty Acid and Cholesterol Biosynthetic Rates—Mice were injected with 15 mCi of [3H]O and were exsanguinated after 1 h. The liver and brown and white adipose tissues were collected. The diglycerine-precipitable sterols were isolated from saponified samples (21, 22). The rates of sterol and fatty acid synthesis are expressed as nmol of [3H]O converted to sterol or fatty acids/μg of tissue.

Western Blot Analysis of Proteins—Liver and white adipose tissue

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† The abbreviations used are: HIV, human immunodeficiency virus; PI, protease inhibitor; SREBP, sterol regulatory element-binding protein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; ANOVA, analysis of variance; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CREBP, cAMP-response element-binding protein; CBP, CREBP binding protein.
were removed from control and ritonavir-treated animals. Tissues were immediately rinsed in ice-cold phosphate-buffered saline and then used for preparation of membrane and nuclear extracts using the procedure as described by Sheng et al. (23).

Aliquots of membrane proteins or nuclear extracts (200 μg of liver protein and 400 μg of white adipose protein) were electrophoresed in 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose paper, and hybridized with mouse monoclonal antibodies against SREBP-1 or SREBP-2, which have been shown previously to react with their respective mouse proteins (24, 25). Hybridoma cells producing these antibodies, which were originally prepared in the Brown and Goldstein Laboratory, were purchased from ATCC (Manassas, Virginia). Hepatic membrane proteins were also immunoblotted with LDL receptor antibodies (Santa Cruz Biotechnology, Inc.) to determine LDL receptor expression. The immunoreactive products were detected by incubation with either horseradish peroxidase-conjugated sheep anti-mouse IgG (for the SREBPs) or anti-goat IgG (for LDL receptor) and then developed with ECL reagent obtained from Amersham Pharmacien Biotech.

Preparation and Analysis of RNA—Total RNA was prepared as described previously (26). Ten μg of liver or adipose tissue RNA were electrophoresed in 1% formaldehyde-denatured agarose gels, transferred to nitrocellulose paper, and hybridized with 32P-labeled cDNA probes for mouse SREBP-1, SREBP-2, fatty acid synthase, and HMG-CoA reductase. These cDNA probes were obtained by polymerase chain reaction from first strand cDNA using mouse liver total RNA, using probes for mouse SREBP-1, SREBP-2, fatty acid synthase, and HMG-CoA reductase. These cDNA probes were obtained by polymerase chain reaction from first strand cDNA using mouse liver total RNA, using primers and conditions as described by Shimano et al. (27). A rat glyceraldehyde-3-phosphate dehydrogenase cDNA probe (28) was used as control for normalization of sample loading. Hybridization was performed with conditions as described previously (29). Blots were exposed to x-ray films at −70°C. The autoradiograph was scanned and then quantitated by computer image analysis (Scion, Frederick, MD). Hybridization signal for each experimental cDNA probe was normalized against that obtained with the glyceraldehyde-3-phosphate dehydrogenase cDNA probe. Samples from chow-fed, vehicle-treated mice were taken as the baseline value in each case.

Gross and Histology of Liver—Livers removed from mice were rinsed with cold phosphate buffered saline and immediately photographed. In other samples, sections of the liver were immediately embedded in freezing compound (HistoprepTM, Fisher Scientific), wrapped in foil, and submerged into liquid nitrogen for sectioning with a cryostat. The resulting tissue sections were stained with Oil Red O stain.

Statistical Analysis—Data are presented as mean ± S.E. For parametric data, means were compared by 1-way ANOVA followed by the Turkey test. For nonparametric data, the Mann-Whitney rank sum test was used.

RESULTS

Male C57BL/6 mice fed either a basal low fat/low cholesterol diet or a Western type diet containing 21% milk fat and 0.15% cholesterol were treated with 2 mg of ritonavir or an equal volume of an ethanolic solution without ritonavir for 10 days. At the end of the experimental period, blood samples were collected from fasting mice for plasma lipid analyses. We found that ritonavir treatment induced a 37% increase in both plasma triglyceride and cholesterol levels under basal dietary conditions (Table I). Whereas animals fed a Western type diet exhibited elevated plasma triglyceride and cholesterol levels; 67% higher plasma triglyceride and 78% higher cholesterol levels were observed in the ritonavir-treated mice compared with the vehicle-treated mice (Table I). Analysis of plasma lipoproteins from control and ritonavir-treated mice by fast performance liquid chromatography revealed that ritonavir treatment resulted in increase in all three major lipoprotein classes, including VLDL, IDL/LDL, and HDL under both control and Western type high fat dietary conditions (Fig. 1). The increase in VLDL was most pronounced when the animals were treated with ritonavir in combination with feeding a high fat diet (Fig. 1B). These results documented that the mouse is a good model to explore the mechanism for the lipid abnormalities associated with PI therapy.

The etiology of hyperlipidemia after PI therapy was explored by comparing rates of de novo lipid synthesis in control and ritonavir-treated mice fed either the low fat basal chow diet or a Western type high fat diet for 10 days. Under low fat dietary conditions, sterol synthesis rates in the liver were induced 2-fold after ritonavir treatment (Fig. 2, left panel). The feeding of a high fat/high cholesterol diet decreased sterol biosynthetic rates in the liver, which is consistent with previous reports (30). However, ritonavir treatment did elevatesterol synthesis rates in the liver under these conditions by ~2-fold (Fig. 2, right panel). In contrast, fatty acid biosynthetic rates in the liver, which were not suppressed by high fat feeding, were induced after ritonavir treatment under both dietary conditions (Fig. 3A). Although fatty acid synthesis rates under basal dietary conditions increased by only ~60% with ritonavir treatment (Fig. 3A), the difference was highly significant (p = 0.04). Interestingly, ritonavir treatment induced a 2.6-fold increase in fatty acid synthesis rates in mice fed the high fat diet (Fig. 3A).

Fatty acid synthesis rates in white adipose tissue were also induced by ritonavir treatment, albeit not to the same extent as those observed in the liver. When the animals were fed the basal low fat chow diet, fatty acid synthesis rates were only marginally increased (~30%) in white adipose tissue (Fig. 3B). Under high fat dietary conditions, ritonavir induced a ~70% increase in fatty acid synthesis rates in this tissue (Fig. 3B). Importantly, fatty acid synthesis rates were increased by 2.1-fold over basal levels when the animals received both the Western type diet and the protease inhibitor.

The most dramatic difference in fatty acid biosynthetic rates was observed in brown adipose tissue. When the animals were fed the basal low fat diet or the Western type high fat diet, ritonavir treatment increased fatty acid synthesis rates in this tissue by 2.8- and 2.0-fold, respectively (Fig. 3C).

Previous studies have clearly documented that fatty acid and cholesterol biosynthesis are regulated by SREBP-1c and SREBP-2 in vivo (21, 31). Thus, we compared SREBP-1 mRNA levels in liver and in white and brown adipose tissues of control and ritonavir-treated mice fed either the basal or the Western diet. However, no influence of PI on the mRNA levels of this SREBP was observed (Fig. 4). Because both SREBP-1 and SREBP-2 are synthesized as precursor forms that can be activated by proteases (32), immunoblot analysis was performed to determine the level of precursor and activated forms of SREBPs in the membrane and nucleus of liver and adipose

| Parameters          | Basal low fat diet | Western type high fat diet |
|---------------------|--------------------|----------------------------|
|                     | Vehicle-treated    | Ritonavir-treated          |
|                     | Vehicle-treated    | Ritonavir-treated          |
| Plasma triglycerides (mg/dL) | 62.07 ± 4.60 | 85.36 ± 10.31a | 57.64 ± 2.20 | 96.51 ± 16.23a |
| Plasma cholesterol (mg/dL) | 73.65 ± 7.27 | 100.63 ± 18.04a | 114.68 ± 7.70 | 204.63 ± 26.72a |
| Body weight (g)     | 21.10 ± 0.7       | 20.2 ± 0.3               | 23.1 ± 0.9 | 26.1 ± 0.4a |
| Liver weight (g)    | 0.85 ± 0.09       | 1.26 ± 0.05a             | 0.92 ± 0.06 | 1.51 ± 0.03a |
| Liver cholesterol (g/tissue) | 2.95 ± 0.02 | 3.17 ± 0.22             | 9.77 ± 1.30 | 20.54 ± 2.78 |
| Liver weight/body weight (%) | 4.01 ± 0.36 | 6.23 ± 0.21a | 3.99 ± 0.19 | 5.79 ± 0.16 |

* Statistically significant difference from the vehicle-treated control group fed similar diet at p < 0.05.
animals were fed the low fat diet (Fig. 5b). However, SREBP-1 levels in the nucleus of adipose tissue were too low after high fat feeding for the assessment of a ritonavir effect (Fig. 5c). Activated SREBP-2 in adipose tissue was undetectable regardless of the feeding conditions of the animals, which is consistent with the low level of cholesterol biosynthesis in this tissue.

The accumulation of activated SREBPs in the nucleus suggests that genes responsive to these transcription factors may be constitutively induced after ritonavir treatment. To test this hypothesis, we used RNA isolated from the liver and adipose tissue of control and ritonavir-treated mice for Northern blot hybridization analysis with 32P-labeled cDNA probes for fatty acid synthase, HMG-CoA synthase, and HMG-CoA reductase. A 32P-labeled probe directed against glyceraldehyde-3-phosphate dehydrogenase was used as the control to normalize the amount of total RNA in each sample. Results showed a consistent increase in the mRNA levels for these lipid metabolism genes from 1.2- to 2.5-fold after ritonavir treatment (data not shown). The mRNA level of LDL receptor in the liver was too low for detection by Northern blot analysis under any of the treatment conditions. However, Western blot analysis of liver membranes with LDL receptor antibodies revealed ritonavir-induction of LDL expression in the liver under both basal and high fat dietary conditions (Fig. 6). Thus, the SREBP-1 and SREBP-2 accumulated in the nucleus of ritonavir-treated mice are functionally active, resulting in increased expression of genes involved in fatty acid and cholesterol metabolism.

Previous studies in transgenic mice documented that over-accumulation of either activated SREBP-1a, SREBP-1c, or SREBP-2 leads to liver enlargement and fatty liver (21, 27, 33–35). Although liver dysfunction associated with PI therapy has only been reported in patients treated with ritonavir together with saquinavir (36), or in patients treated with PI in combination with nucleoside analogue (37), we have used ritonavir-treated mice to determine whether this PI alone also induces changes in liver characteristics. We found that livers from ritonavir-treated mice were significantly enlarged in comparison to livers from vehicle-treated control mice (Fig. 7). Although the livers from mice fed the Western type diet without ritonavir treatment were also somewhat larger and more pale than normal mouse liver (compare panels 1 and 3 in Fig. 7), liver enlargement and discoloration were much more pronounced in livers of mice fed the Western type diet and receiving ritonavir treatment (Fig. 7, panel 4). Histologic analysis of the liver verified that the discoloration was due to ritonavir-induced lipid accumulation. Lipid deposition, as detected by Oil Red O staining, was minimal in control mice fed a low fat chow diet (Fig. 8, panel 1). Liver from ritonavir-treated mice displayed significant lipid deposition even in the absence of high fat diet (Fig. 8, panel 2). As expected, high fat feeding alone resulted in the appearance of Oil Red O-stained lipid droplets in the liver (Fig. 8, panel 3). However, massive lipid deposition was observed only in livers of ritonavir-treated mice that were fed a Western type diet (Fig. 8, panel 4). Increased hepatic lipid accumulation upon ritonavir treatment of Western type diet-fed mice was also observed by direct measurement of hepatic cholesterol levels in these animals (Table I). Thus, these results document a synergistic effect between a high fat diet and PI therapy in fat deposition in the liver. More importantly, the data suggest that hepatomegaly and hepatic steatosis are also abnormalities associated with PI therapy, especially in association with a Western type high fat/high cholesterol diet. Additional monitoring of liver functions in patients undergoing PI therapy is warranted.
The SREBP pathway for regulation of fat and cholesterol metabolism is well studied and shown to be initiated through proteolytic cleavage of precursor forms of the SREBPs in endoplasmic reticulum membranes. When cells are in need of sterol, the precursor SREBPs are hydrolyzed by a 2-step mechanism involving the membrane-bound serine protease S1P (17, 38–40) and a zinc metalloprotease S2P (41). The N-terminal fragment of SREBP liberated from S1P- and S2P-mediated hydrolysis is translocated to the nucleus where it binds to sterol regulatory elements and activates gene transcription (32, 42, 43). The nuclear SREBP can be rapidly degraded by an ALLN-sensitive proteasome-mediated mechanism, thus affording exquisite regulation of gene transcriptional activities (17).

Transgenic mice over-expressing the constitutively active nuclear forms of the SREBPs revealed that supra-physiological overexpression of SREBP-1 or SREBP-2 are capable of activating genes involved in the cholesterol and fatty acid biosynthesis cascades (16, 21, 31, 33). These studies also showed that SREBP-1 is primarily responsible for the induction of fatty acid biosynthesis (31), whereas SREBP-2 is more involved with cholesterol biosynthesis (21). Of particular importance to the current study is the observation that activated SREBP-1 transgenic mice displayed the classical features of generalized lipodystrophy, including disordered differentiation of adipose tissue with decreased white fat depots and brown fat resembling immature white fat, marked insulin resistance, and hyperlipidemia (34), similar to those observed in patients undergoing PI therapy.

In the current study, we found that PI therapy induces the accumulation of activated SREBP-1 and SREBP-2 in the nucleus of liver and adipose tissues. As a consequence, fatty acid synthesis rates in liver, white adipose tissue, and brown adipose tissue were found to be increased in PI-treated mice fed either chow or a Western type diet. This was evidenced by increased mRNA levels of SREBP-1 and SREBP-2 and increased protein levels of activated SREBP-1 and SREBP-2 in the nucleus of liver and adipose tissues. These findings suggest that PI therapy may be a potential target for the development of new therapies for the treatment of lipodystrophy and related disorders.
and cholesterol biosynthesis are increased in these tissues. Although hepatic LDL receptor expression was also found to be increased in ritonavir-treated mice due to the accumulation of activated SREBP-2 in the liver, the marginal increase in LDL receptor appeared to be unable to overcome the increase in lipid biosynthesis, thus resulting in the hyperlipidemia phenotype. It is possible that lipoprotein remodeling in circulation may also have been impaired after ritonavir treatment, resulting in the insufficient clearance of lipoproteins even in the presence of elevated hepatic LDL receptor. The latter possibility needs to be tested by direct experimentation.

Previously, lipodystrophy observed in inhibitor-treated patients was also shown to exhibit insulin resistance (3). Lipodystrophy observed in these patients may arise due to the accumulation of activated SREBP-1 in adipose (34). Although insulin resistance and diabetes were not observed in the current study (data not shown), due to the short duration of the current PI treatment period, insulin resistance and diabetes are common characteristics of animals with prolonged accumulation of activated SREBP-1c in brown and white adipose tissues (34, 44). Thus, it is anticipated that prolonged treatment with ritonavir will also lead to insulin resistance and diabetes. In fact, hepatic steatosis and hepatomegaly were already apparent in mice treated with ritonavir for only 10 days. Fatty liver leading to hepatic fibrosis and cirrhosis is a common side effect associated with insulin resistance and non-insulin-dependent diabetes mellitus (45). Taken together, these data strongly indicate that the major adverse effects of PI therapy, including lipodystrophy, hyperlipidemia, and insulin resistance, are the consequence of activated SREBP-1 and SREBP-2 accumulation in the nucleus of liver and adipose tissues.

The mechanism by which PI therapy leads to activated SREBP accumulation in the nucleus is not due to activation of SREBP gene expression per se. A second possibility is that PI therapy may facilitate the processing of precursor SREBPs to their activated forms. However, this possibility is unlikely because high fat/high cholesterol diet, which inhibits precursor SREBP processing (23, 32, 46), actually exacerbated the ritonavir effect on SREBP accumulation in the nucleus. Results showing no difference in precursor SREBP levels in membrane fractions of control and ritonavir-treated mice are also inconsistent with this possibility. Therefore, the most likely mechanism for PI-induced activated SREBP accumulation is PI suppression of activated SREBP degradation in the nucleus. As discussed above, this process is mediated by an ALLN-sensitive proteasome-mediated mechanism. PI has already been shown to suppress ALLN-sensitive proteasome activities in antigen presenting T cells (47). Thus, PI inhibition of proteasome activities may be a generalized phenomenon leading to most of the adverse effects associated with this class of therapeutic agents.

The hypothesis that lipid disturbances associated with PI therapy are due to suppression of activated SREBP degradation suggests that inhibition of SREBP biosynthesis or their activities may be a viable option to alleviate the adverse side effects. A recent study demonstrated that SREBP mRNA level can be reduced by polyunsaturated fatty acids (48). Inhibition of SREBP activities is also achievable by interfering with their ability to interact with co-regulatory proteins. For example, nuclear factor Y (NF-Y) interacts with SREBP and enhances its binding to promoter sites on lipid metabolism genes (49, 50). The activity of SREBP also requires their interaction with the co-activator CREBP binding protein CBP (51, 52). Thus, designing therapeutic agents that can inhibit SREBP interaction with NF-Y and CBP, or the histone acetyltransferase activities
of CBP and its associated factor P/CAF, may be a fruitful alternative strategy to suppress cholesterol and fatty acid biosynthesis. The combination of these agents and PI, along with additional dietary manipulations, should provide an effective regimen to treat HIV infection without the adverse effects associated with the current PI therapy.

In summary, this study showed that ritonavir induces lipid abnormalities via stabilization of activated SREBP-1 and SREBP-2 in the nucleus of liver and adipose tissues. Whether other protease inhibitors have similar effects remains to be determined.

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