HIV Protease Inhibitors Stimulate Hepatic Triglyceride Synthesis

James M. Lenhard, Dallas K. Croom, James E. Weiel, Deborah A. Winegar

Abstract—Hyperlipidemia may complicate the use of HIV protease inhibitors (PIs) in AIDS therapy. To determine the cause of hyperlipidemia, the effect of PIs on lipid metabolism was examined with HepG2 liver cells and AKR/J mice. In HepG2 cells, the PIs ABT-378, neflavinav, ritonavir, and saquinavir stimulated triglyceride synthesis; ritonavir increased cholesterol synthesis; and amphotrenavir and indinavir had no effect. Moreover, neflavinav increased mRNA expression of diacylglycerol acyltransferase and fatty acid synthase. The retinoid X receptor agonist LG100268, but not the antagonist LG100754, further increased PI-stimulated triglyceride synthesis and mRNA expression of fatty acid synthase in vitro. In fed mice, neflavinav or ritonavir did not affect serum glucose and cholesterol, whereas triglyceride and fatty acids increased 57% to 108%. In fasted mice, ritonavir increased serum glucose by 29%, cholesterol by 40%, and triglyceride by 99%, whereas neflavinav had no effect, suggesting these PIs have different effects on metabolism. Consistent with the in vitro results, neflavinav and ritonavir increased triglyceride 2- to 3-fold in fasted mice injected with Triton WR-1339, an inhibitor of triglyceride clearance. We propose that PI-associated hyperlipidemia is due to increased hepatic triglyceride synthesis and suggest that retinoids or meal restriction influences the effects of select PIs on lipid metabolism. (Arterioscler Thromb Vasc Biol. 2000;20:2625-2629.)

Key Words: HIV ■ hypertriglyceridemia ■ liver ■ protease inhibitor ■ retinoid

N nucleoside reverse transcriptase inhibitors (NRTIs),1 non-nucleoside reverse transcriptase inhibitors (NNRTIs), and HIV protease inhibitors (PIs) are 3 classes of antiretroviral drugs that are used in the treatment of patients with AIDS. NRTIs and NNRTIs inhibit HIV reverse transcriptase and suppress replication of the virus. PIs block proteolytic cleavage of the gag and gag-pol protein precursors required for maturation of the virus particle. Highly active antiretroviral therapy (HAART) includes combinations of a PI, an NRTI, and an NNRTI.1 HAART has been associated with numerous affects on lipid metabolism, including subcutaneous fat wasting, abdominal obesity, insulin resistance, and hyperlipidemia.2,3 Metabolic complications arising from HAART may be due to drug-drug interactions, exacerbation of preexisting conditions, reconstitution of immune system function, or a combination.3–5 Studies of hyperlipidemia in HIV-infected persons are complicated because dyslipidemia may occur in the absence of therapy (reviewed in Safrin and Grunfeld).6 Although HIV infection itself and treatment with reverse transcriptase inhibitors have been associated with altered metabolism,2–6 substantial evidence indicates a role for some PIs in causing metabolic complications.

PIs are important therapeutically because they inhibit virus release from infected cells, reducing virus proliferation and subsequent infection.7 The currently available PIs include amphotrenavir (APV), indinavir (IDV), nelfavinav (NFV), ritonavir (RTV), saquinavir (SQV), and ABT-378 (lopinavir; delivered in combination with RTV to enhance systemic exposure to ABT-378). The antiviral activity among individual PIs is variable and correlates with the ability to inhibit the HIV protease (ie, IC50 0.10 to 20 nmol/L). Although these drugs contain a synthetic analog of the phenylalanine-proline amino acid sequence cleaved by the HIV protease, structural differences result in unique resistance and metabolic effects for each PI.7 Initial reports in 1997 indicated that a number of patients developed hyperlipidemia after PI therapy. Several studies specifically implicated RTV, RTV/SQV, or NFV therapy as having the greatest affects on lipid metabolism.2,3,8–14 For instance, in a recent clinical trial of 93 patients,8 PI-associated hyperlipidemia was most severe with RTV, followed by NFV and IDV therapy. In another clinical trial of 67 patients, median total cholesterol (CH) was greatest in NFV-treated patients, and the highest triglyceride (TG) level occurred in a patient treated with RTV.9 Likewise, Carr et al10 reported that patients who received RTV/SQV had higher serum lipid levels than patients who received IDV. Consistent with this observation, in a case report of a male patient treated with RTV, serum CH and TG levels were elevated by 4.5- and 14.5-fold, respectively, and this effect was reversible on switching to IDV.11 Serum TG levels have also been reported to be significantly greater in HIV-seronegative subjects treated with RTV than with APV12 and in AKR/J mice treated...
with NFV than with APV. A comparison of patients receiving NRTI, APV/NRTI, or IDV/NRTI revealed hypertriglyceridemia and liver abnormalities (grade 3 to 4) in 15%, 19%, and 29% of the subjects, respectively. In addition, recent studies in noninfected volunteers demonstrate that RTV increases serum TG and CH levels within 2 weeks of therapy. Thus, although patients with AIDS may have high levels of serum TG in the absence of therapy, these data indicate that PI therapy exacerbates dyslipidemia in the absence of infection. Careful analyses of the effects of infection and individual PI and NRTI on lipid metabolism are needed to improve the ability to predict, prevent, or treat side effects associated with HAART.

We report the effects of PI treatment on lipid synthesis using cultured hepatoma HepG2 cells and obesity-prone AKR/J mice. In vitro studies indicate that select PIs (ABT-378, NFV, RTV, SQV) stimulate de novo TG synthesis in liver cells. In vivo, RTV and NFV also increased serum TG levels in the presence of Triton WR-1339, an inhibitor of TG clearance. These studies suggest that PIs may increase hyperlipidemia, in part by stimulating hepatic TG synthesis.

**Methods**

**Materials**

Pls, LG100268, and LG100754 were obtained from the Medicinal Chemistry Department at GlaxoWellcome Inc. All drugs were determined to be ≥95% pure through HPLC or NMR analysis.

**In Vitro**

HepG2 cells were plated onto 24-well dishes and grown in normal growth medium (DMEM) containing 10% FBS to ~85% confluency. Medium was changed to growth medium containing 1% FBS, and the cells were incubated with PIs, LG100268, or LG100754 for 24 hours. Although PIs are often used in various combinations in the clinic, particularly with RTV, only the effects of individual PIs on lipid synthesis were studied. Lipid radiolabeling was initiated by adding [14C]acetate (5 μCi/well, 56 nmol) and incubating the cells for an additional 24 hours. Media and cells were harvested, and lipids extracted in chloroform-methanol (2:1). Radiolabeled lipids were analyzed with a normal phase HPLC system (Phenomenex Porasil silica column; gradient elution from 10% to 95% MTBE in hexane) equipped with a Radiomatic FLO-ONE βradiochromatographic detector. Peaks that correspond to TG, fatty acid (FA), and CH were identified through coelution with known standards and normalized to an internal standard.

Rat hepatocytes were prepared through perfusion of a liver lobe with collagenase as described previously. After isolation and washing, cells were suspended in DMEM containing 10 mmol/L HEPES, pH 7.4, 5% FBS, and 0.1 mg/mL gentamicin and plated onto 6-well dishes coated with collagen. Cells were incubated in a medium containing 5% lipoprotein-deficient serum. Drugs and standards and normalized to an internal standard.

**In Vivo**

Age- and weight-matched male AKR/J mice (Jackson Labs) were housed at 3 to 5 animals per cage at 72°F and 50% relative humidity with a 12-hour light/dark cycle and fed standard laboratory chow. Animals were dosed via oral gavage twice daily for 2 weeks with vehicle (0.5% methylcellulose with 0.1% Tween 80) or 30 mg/kg PI in vehicle. Clinically relevant doses for NFV and RTV are 750 mg TID and 600 mg BID, respectively. The average adult weight was 60 kg. At the end of the treatment period, the animals were fasted for 16 hours and injected intravenously with saline or 400 mg/kg Triton WR1339 (Ruger) as a 20-g/dL solution in saline. After 1 hour, the mice were anesthetized with isofluorane, blood was drawn via cardiac puncture, and fasting serum measurements were obtained with an automated chemistry analyzer (Technicon Axon). The mean (n=12 per treatment group), SEM, and P values were calculated by using a 2-tailed Student’s t test. A P value of <0.05 was considered to be significant. All research complied with the principles of laboratory animal care (NIH publication No. 85-23, revised 1985) and the GlaxoWellcome policy on animal use.

**Results**

**Synthesis of Lipids After Treatment of Cultured Hepatoma Cells With PIs**

Initial experiments were performed in cultured human hepatoma HepG2 cells, which possess many of the normal biochemical functions of liver parenchymal cells, including the synthesis and secretion of plasma lipoproteins. To determine whether PIs influence hepatocyte lipogenesis, HepG2 cells were incubated with 2 and 10 μmol/L PIs in the presence of [14C]acetate. Intracellular lipids were isolated from cell lysates of PI-treated and untreated cells and separated through HPLC with standards to quantify TG, FA, and CH content. NFV and ABT-378 produced a concentration-dependent increase in intracellular TG synthesis with a maximal 136% and 146% increase (P<0.05), respectively, in TG derived from [14C]acetate at 10 μmol/L (Figure 1). RTV and SQV caused more moderate effects at 10 μmol/L, increasing intracellular TG by 82% and 96%, respectively (P<0.05). In contrast, APV and IDV did not stimulate TG synthesis (P>0.05) in these cells (Figure 1). Likewise, none of the PIs affected FA synthesis (data not shown). Although
NFV and LG100268 Stimulate Lipid Synthesis and Gene Expression in HepG2 Cells

Retinoids stimulate lipid production in HepG2 cells, in part by activating retinoid X receptors (RXRs). To investigate whether retinoids and PIs interact to increase hepatic lipid synthesis, HepG2 cells were treated with NFV in the absence or presence of the RXR agonist LG100268 or the RXR homodimer antagonist LG100754. As shown in Figure 2A, 100 nmol/L LG100754 did not significantly affect TG synthesis. However, relative to untreated cells, 100 nmol/L LG100268 or 10 μmol/L NFV increased TG synthesis (P<0.05) by 72% and 145%, respectively (Figure 2A). In cells exposed to both drugs simultaneously, cellular TG synthesis increased 335% compared with untreated cells (P<0.001). Similar but less pronounced effects were observed when other PIs were mixed with LG100268; in the presence of LG100268, 10 μmol/L RTV and SQV increased TG synthesis by 85±44% and 67±43%, respectively.

DGAT and FAS are key enzymes in the synthesis of TG and FA, respectively. NFV increased the expression of DGAT mRNA (47% at 1 μmol/L, P=0.024) to a greater extent than FAS mRNA (P=0.9, compared with untreated cells) in HepG2 cells (Figure 2B). In contrast, 100 nmol/L LG100268 significantly increased FAS mRNA (57%, P<0.001) but not DGAT mRNA (P=0.26, compared with untreated cells) expression in HepG2 cells (Figure 2B). Combining NFV and LG100268 did not significantly increase DGAT expression compared with either agent alone (P>0.05). However, FAS expression increased significantly (134%, P<0.001) in HepG2 cells treated with the 2 drugs simultaneously (Figure 2B). Taken together, these data suggest that PIs and LG100268 affect distinct molecular mechanisms.

Effects of Oral PIs on Metabolism in Postprandial AKR/J Mice

The subcutaneous administration of NFV (but not APV or SQV) to obese AKR/J mice increases postprandial serum TG and FA levels but not glucose or CH levels.13,14 Because the effects of oral dosing and RTV in fed mice are unknown, AKR/J mice were treated twice daily for 2 weeks with 30 mg/kg oral NFV or RTV, and serum was collected was nonfasted fats. Similar to the results obtained after the subcutaneous delivery of NFV to mice,13,14 oral NFV or RTV significantly increased (P<0.001) serum levels of TG and FA compared with control mice (Table 1). Furthermore, PI treatment had no significant effect (P>0.05) on serum concentrations of glucose or CH compared with control mice.

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>Triglycerides, mg/dL</th>
<th>Fatty Acid, mEq/dL</th>
<th>Glucose, mg/dL</th>
<th>Cholesterol, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo (12)</td>
<td>170±12</td>
<td>0.39±0.05</td>
<td>232±10</td>
<td>73±2</td>
</tr>
<tr>
<td>Nelfinavir (15)</td>
<td>267±16*</td>
<td>0.81±0.1*</td>
<td>241±9</td>
<td>73±1</td>
</tr>
<tr>
<td>Ritonavir (14)</td>
<td>278±21*</td>
<td>0.80±0.05*</td>
<td>226±10</td>
<td>76±2</td>
</tr>
</tbody>
</table>

Triglyceride, nonesterified fatty acid, glucose, and total cholesterol levels were measured in the plasma of postprandial AKR/J mice treated for 2 weeks with placebo or PI (30 mg/kg). The mice were 15 weeks old at necropsy.

*P<0.05 indicates significant differences between PI and placebo-treated mice with Student’s t test (values are mean±SEM, n=15).
PI Treatment Stimulates TG Synthesis in Fasted AKR/J Mice

To determine the effects of PIs on in vivo hepatic TG production, Triton WR1339, an inhibitor of TG clearance,17 was injected into mice treated for 2 weeks with placebo or 30 mg/kg oral NFV or RTV. To avoid confounding effects of food intake on hyperlipidemia, the mice were fasted for 16 hours before the injection with Triton WR1339. After Triton WR1339 injection (1 hour), TG levels were 2- to 3-fold higher in RTV- and NFV-treated mice compared with placebo-treated mice (Table 2), indicating these PIs stimulate TG synthesis in vivo.

It is worth noting that in contrast to fed mice (Table 1), serum glucose and CH levels were significantly (P < 0.001) greater in RTV-treated than in placebo-treated, fasted mice (Table 2). Also in contrast to fed mice (Table 1), NFV did not increase serum TG (Table 2) or FA (data not shown) levels relative to placebo-treated, fasted mice (in the absence of Triton WR1339).

Because APV and IDV did not stimulate lipid synthesis in HepG2 cells (Figure 1) and subcutaneous APV or SQV has little effect on TG in AKR/J mice,13,14 these PIs were not examined in the Triton WR1339 experiments. However, in a separate study of 16-hour-fasted AKR/J mice, the oral administration of APV (60 mg·kg⁻¹·d⁻¹) for 2 weeks had no significant effect on serum TG (P = 0.4), free FA (P = 0.1), or CH (P = 1.0) levels. These observations are consistent with recent reports that APV does not alter in vitro fat metabolism20,21 or serum lipid levels13,14 in fed mice.

Discussion

Although successful AIDS therapy includes the use of PIs, treatment with certain PIs is associated with fat redistribution, hyperlipidemia, or both.2,3,8–14 Investigations into the mechanisms that underlie these metabolic changes reveal varying effects depending on which cell type or PI is used. For example, recent studies show that NFV, RTV, and SQV, but not APV or IDV, reduce lipogenesis and stimulate lipolysis in adipocytes.20 In addition, unlike other PIs, IDV increases osteoblast-related alkaline phosphatase activity in mesenchymal stem cells.21 Considering the importance of liver in lipoprotein metabolism, there have been surprisingly few reports that examined the effects of PIs on hepatocytes. To test the possibility that increased hepatic TG synthesis contributes to the lipodystrophy syndrome, we examined the effects of PIs on lipid synthesis in cultured hepatocytes and AKR/J mice. The results showed that NFV and RTV increased serum TG levels in mice treated with Triton WR1339, indicating that these PIs stimulated TG synthesis in vivo. Consistent with these in vivo studies, ABT-378, NFV, RTV, and SQV, but not APV or IDV, increased TG synthesis and RTV increased CH synthesis in HepG2 cells. These and other observations1,7,12–14,20,21 indicate that select PIs affect multiple, distinct metabolic pathways, perhaps accounting for the different side effects observed for each PI in the clinic. This may be due to differences in affinity for cellular proteins (eg, proteases), cell permeability, tissue distribution, or metabolism of PI.

Retinoids cause hypertriglyceridemia in the clinic situation, possibly by activating RXR homodimers in liver.19 In support of this claim, we observed that the RXR agonist LG100268, but not the antagonist LG100754, stimulated TG synthesis in HepG2 cells. One proposal suggests that PIs alter RXR signaling by inhibiting the synthesis of 9-cis-retinoic acid.22 Three findings in the present report indicate that PIs may affect a molecular pathway other than RXR. First, the RXR antagonist LG100754 did not block NFV-stimulated TG synthesis in HepG2 cells. Second, LG100268 increased the expression of FAS and NFV the increased expression of DGAT in these cells. Third, LG100268 and NFV in combination increased TG synthesis in HepG2 cells more than the use of either agent alone. Thus, the response of HepG2 cells to both drugs may be due to effects on distinct signaling pathways. Nonetheless, the interaction between PIs and retinoid signaling pathways may be an important mechanism by which PIs affect lipid metabolism.21,23

If PIs inhibit TG clearance but not synthesis, then PIs should have no effect on TG levels in mice treated with Triton WR1339, an inhibitor of TG clearance. The observation that NFV and RTV increased serum TG levels in mice treated with Triton WR1339 indicates that these PIs stimulate TG synthesis in vivo. This is consistent with the in vitro studies that show PIs increase TG synthesis in HepG2 cells. Nonetheless, the data do not rule out the possibility that in addition to stimulating synthesis, PIs also block the clearance of TG. Indeed, Carr et al22 reported 63% homology between the ligand-binding domain of the HIV protease and LDL-related receptor. They proposed that PI binding to LDL-related receptor may inhibit chylomicron uptake and TG clearance.22 However, Purnell et al16 found that post–heparin lipase activity was unaffected by RTV in noninfected patients, indicating that an abnormality in lipoprotein metabolism plays a role in the lipid alterations observed in these patients.
lipase may be excluded as a cause of TG elevation. Moreover, the removal of remnant lipoproteins was unaffected by RTV in these patients, implicating increased VLDL secretion as a possible cause of hypertriglyceridemia in the clinic. Although our data indicate that PIs stimulate hepatic TG synthesis, further studies are needed to determine whether PIs affect lipid clearance.

The effects of various PIs on TG synthesis in HepG2 cells do not correlate with the effects in vivo, where RTV may be the most efficient in producing hypertriglyceridemia. One explanation for this is that different active metabolites (generated by cytochrome P4503A) or pharmacokinetic properties may be present in the clinic situation that are absent from cultured cells. Another possibility is PIs may affect fat metabolism in other tissues, such as adipocytes. Thus, in vitro analysis of a single cell type may explain only part of the effects that PIs have on the syndrome. Likewise, there are several differences between the in vivo studies reported here and those in the clinic situation. For example, the mice were treated for a shorter period of time (2 weeks) than is used in clinical studies and different effects may be observed over longer time periods. Other confounding factors may affect PI toxicity in the clinic, including drug interactions, viral load, and disease progression. Indeed, metabolic changes are observed in HIV-infected individuals treated with non–PI-containing regimens, including stavudine, indicating that factors other than PIs contribute to hyperlipidemia. Differences in diet and genetics may also influence PI toxicity.

Consistent with the hypothesis that the effects of PI on metabolism can be influenced by diet, PIs have different effects on lipid metabolism in mice fed a high- or low-fat diet. Similarly, the results of the present study indicate that NFV increased serum TG levels in fed, but not fasted, mice, whereas RTV increased serum glucose and CH levels in fasted, but not fed, mice. Thus, one hypothesis is that the variability of metabolic changes reported in the clinic situation may depend on food restrictions associated with select PIs. Because experiments analogous to those described in this report have not been carried out with human subjects, caution should be used when extrapolating the results from cell culture or rodent studies to humans. For example, HIV infection may cause dyslipidemia in the absence of therapy. Thus, how PIs interact with infection to alter lipid metabolism is unclear. Nonetheless, this study provides experimental evidence that serves to explain the elevation in serum TG levels observed in patients who receive PI therapy in the clinic; in particular, the results suggest that select PIs stimulate TG synthesis in hepatocytes. Further studies on the mechanisms of action for each PI and their abilities to cause metabolic changes in the clinic are needed. The results of these studies will allow safer and more effective therapy for AIDS to be used in the future.

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### References


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