The HIV protease inhibitor ritonavir increases lipoprotein production and has no effect on lipoprotein clearance in mice

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Abstract This study examined the effect of human immunodeficiency virus (HIV) protease inhibitor therapy on lipoprotein production and catabolism in vivo. The HIV protease inhibitor ritonavir was given to C57BL/6 mice fed either a basal low-fat diet or a Western type high-fat diet. Fasted mice were injected with Triton WR1339 followed by hourly blood collection to monitor lipoprotein production. Results showed that ritonavir increased VLDL triglyceride production by 30% over a 4 h period when mice were fed the low-fat basal diet. The ritonavir effect was more pronounced under high-fat feeding conditions, with a 2-fold increase in VLDL triglyceride production rate. Ritonavir did not alter hepatic expression levels of diacylglycerol acyltransferase or microsomal triglyceride transfer protein, but increased hepatic apolipoprotein B (apoB) secretion rates under both low- and high-fat dietary conditions. In contrast to its effect on lipoprotein production, ritonavir did not alter triglyceride-rich lipoprotein clearance from circulation under either dietary condition. Taken together, these results indicate that the hyperlipidemic effect of HIV protease inhibitors is a direct result of increased hepatic lipoprotein production. The mechanism appears to be related to their role in preventing proteasome-mediated degradation of apoB and activated sterol regulatory element binding proteins in the liver. —Riddle, T. M., N. M. Schildmeyer, C. Phan, C. J. Fichtenbaum, and D. Y. Hui. The HIV protease inhibitor ritonavir increases lipoprotein production and has no effect on lipoprotein clearance in mice. J. Lipid Res. 2002. 43: 1458–1463.

Supplementary key words anti-retroviral therapy • very low density lipoprotein synthesis • apolipoprotein B • high-fat diet

Highly active anti-retroviral therapy for human immunodeficiency virus (HIV)-infected patients has proven to be effective in reducing the morbidity and mortality of AIDS (1, 2). Typically, this regimen includes protease inhibitors that prevent viral protein processing and the maturation of the HIV virions (3). Unfortunately, despite the effectiveness of these drugs on reducing HIV titer, highly active anti-retroviral therapy is also associated with several undesirable adverse effects including hyperlipidemia, lipodystrophy, and insulin resistance (4, 5). Recent evidence points to a contributory role of protease inhibitors to these lipid abnormalities (4–9).

The mechanism by which HIV protease inhibitor therapy results in hyperlipidemia and insulin resistance remains incompletely understood. However, it appears that the various HIV protease inhibitors have dramatically different effects on hyperlipidemia and insulin resistance. For example, indinavir appears to induce insulin resistance without hyperlipidemia in HIV seronegative patients (9). In HIV subjects, indinavir treatment causes only mild hypercholesterolemia, and hypertriglyceridemia is less common (10). In contrast, both serum cholesterol and triglyceride levels are dramatically elevated in ritonavir-treated seronegative and HIV-positive patients (10, 11). These observations suggest that the various protease inhibitors may affect plasma lipid levels through different mechanisms. An understanding of the complete mechanism by which each of these protease inhibitors causes these metabolic abnormalities will improve our ability to predict and prevent adverse effects associated with the use of highly active anti-retroviral therapy for HIV-infected individuals.

Ritonavir is a prototype HIV protease inhibitor that causes the most severe hyperlipidemic effects in humans (5–10). In a previous study using the mouse as an animal model, we showed that ritonavir treatment increased serum triglyceride and cholesterol levels through increased fatty acid and cholesterol biosynthesis in the liver (12). Addi-
tionally, we showed that ritonavir-induced expression of genes relating to lipid metabolism can be attributed to the accumulation of activated sterol regulatory element binding proteins (SREBPs) in the liver (12). The mechanism is likely mediated by protease inhibitor suppression of proteasome degradation of the activated SREBPs (12, 13).

Another lipid metabolic pathway that is regulated by proteasomes is the VLDL synthesis and secretion pathway in the liver. In this process, newly synthesized apolipoprotein B (apoB) can either be assembled with lipids in the endoplasmic reticulum to form lipoproteins or be degraded in the cytoplasm by proteasomes (14–17). Thus, protease inhibitor suppression of proteasome activities may also result in increased hepatic VLDL production. This hypothesis has been tested in a recent in vitro study with cultured human and rat hepatoma cells and with primary hepatocytes isolated from apoB transgenic mice (18). This study showed that HIV protease inhibitors were effective in inhibiting apoB degradation, causing intracellular accumulation of apoB in the liver (18). However, protease inhibitor-induced increase of VLDL secretion was observed only in cells treated with oleic acid and not in cells treated in the absence of fatty acids (18). Therefore, it remains uncertain if protease inhibitors can influence hepatic VLDL synthesis and secretion in vivo and if the effect can be modulated by dietary fat. This study was undertaken to examine the effect of ritonavir on hepatic lipoprotein production in vivo under both low-fat and high-fat dietary conditions. Additional studies were also undertaken to determine the effect of HIV protease inhibitors on lipoprotein clearance from circulation.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were housed according to institutional guidelines. The animals were fed either a standard mouse chow containing 4% fat and 0.04% cholesterol (Harlan Teklad Laboratories, Madison, Wisconsin) or a Western-type diet containing 21% milk fat and 0.15% cholesterol (TD8137 from Harlan Teklad). Six- to eight-week-old mice were used for the studies. Half of the mice from each dietary group were treated with 50 μl ritonavir (2 mg) every morning by stomach gavage. The other half of the animals in each group received 50 μl of a 22% ethanol solution, which served as vehicle control. The mice were treated similarly and then fasted for 4 h and then anesthetized with a mixture of ketamine (80 mg/kg, Fort Dodge Laboratories) and xylazine (16 mg/kg, The Butler Co.). A 100 microliter saline solution containing 12.5 mg of Triton WR1339 was then injected into the saphenous vein. The animals were allowed to recover, usually within a 20- to 30-min period. Blood samples were collected by retro-orbital puncture, both before Triton WR1339 infusion and at hourly intervals afterwards for 4 h for lipid and apoB determinations.

ApoB measurement

Two microliters of serum from mouse blood were electrophoresed in 4% to 15% SDS-polyacrylamide gradient gels, transferred to nitrocellulose paper, and then hybridized with a 1:5,000 dilution of rabbit anti-mouse apoB antiserum (BioDesign, Saco, Maine). The immunoreactive products were detected by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ) and then developed with enhanced chemiluminescence reagents purchased from Amersham Pharmacia Biotech. After exposure of the nitrocellulose paper to X-ray films, the image was scanned into the computer for quantitation with an ImageQuant program (Molecular Dynamics, Inc.).

Northern blot analysis of diacylglycerol acyltransferase mRNA in liver

Livers from control and ritonavir-treated mice were removed and frozen immediately on dry ice prior to processing. Total RNA from individual mouse liver was prepared using the cold guanidinium isothiocyanate method (20). Twenty micrograms of total RNA was electrophoresed in 1% formaldehyde-containing agarose gels, transferred to a Hybond-XL nylon membrane (Amersham Pharmacia Biotech), and hybridized with a 32P-labeled diacylglycerol acyltransferase (DGAT) cDNA probe (generously provided by Dr. Robert Farese, Jr., Gladstone Institute, San Francisco, CA). A 32P-labeled rat glycerolaldehyde-3-phosphate dehydrogenase cDNA probe was used as control for normalization of sample loading. Hybridization was carried out for 18 h at 42°C in a buffer containing 50% formamide, 5 × SSC (1 × SSC contains 15 mM sodium citrate, 150 mM NaCl), 1 × Denhardt’s solution, 1% SDS, and 2 mg/ml denatured salmon sperm DNA. Blots were washed once at 25°C for 15 min with 2 × SSC containing 0.1% SDS, and then two times at 42°C for 30 min in 0.2 × SSC buffer containing 0.1% SDS. The blots were exposed to Kodak phosphor imager screens, scanned with the Storm 84 Phosphorimager, and quantitated by computer image analysis (ImageQuant, Molecular Dynamics). Samples from chow-fed, vehicle-treated mice were taken as the baseline value for analysis.

Microsomal triglyceride transfer protein determination

Livers from control and ritonavir-treated mice were removed and immediately rinsed in ice-cold phosphate buffered saline. The tissues were then used immediately to prepare membrane extracts. All procedures were performed on ice. The liver was placed in 10 vol of homogenization buffer consisting of 20 mM Tris-HCl (pH 7.4), 2 mM MgCl2, and 0.25 M sucrose. The buffer was supplemented just prior to use with a protease inhibitor cocktail consisting of 25 μg/ml N-acetyl-leucyl-leucyl-norleucinal, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 2 μg/ml aprotilin, and 0.5 mM PMSF (all from Sigma Chemical Co., St. Louis, MO). The liver was homogenized with a polytron and then centrifuged at 800 g for 15 min. The resulting supernatant was placed in a fresh tube and centrifuged again at 130,000 g for 90 min at 4°C. The pellet was resuspended by passing through a 26-gauge needle 10 times in a buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM MgCl2, 1% SDS, and 2% Triton X100. The resulting solution was then centrifuged at 100,000 g for 30 min. The protein content of the supernatant was determined by the Lowry method (21).

An aliquot of membrane protein (25 μg) was electrophoresed in a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and incubated with goat anti-microsomal triglyceride transfer protein (MTP) antiserum (kindly provided by Dr.
John Wetterau (Bristol-Myers Squibb, Princeton, NJ). Immuno-reactive products were detected by incubation with horseradish peroxidase-conjugated anti-goat IgG (BioRad, Hercules, CA), followed by enhanced chemiluminescence reaction with a kit obtained from Amersham Pharmacia Biotech. The nitrocellulose was exposed to Kodak films, and the image was scanned into a computer.

Lipoprotein clearance

Lipid emulsions containing triglyceride and cholesteryl esters were used to mimic triglyceride-rich lipoproteins for measurement of lipoprotein clearance from circulation (22, 23). The lipid emulsion was prepared by mixing 70 mg triolein, 3 mg cholesteryl olate, 2 mg cholesterol, 25 mg phosphatidylcholine, and 100 μCi [3H]triolein in organic solvent. After evaporation of the solvent to dryness under N₂, the lipid mixture was resuspended in 8.5 ml of buffer containing 10 mM Hepes (pH 7.4) and 150 mM NaCl and then sonicated in ice for 30 min. The density of the crude emulsion was adjusted to 1.10 gm/ml with solid KBr and then centrifuged at 71,150 g for 22 min at 20°C over a step gradient of 1.006, 1.02, and 1.065 gm/ml density solutions. The coarse material on the top of the gradient was removed and replaced with fresh 1.006 gm/ml density solution, and the sample was re-centrifuged at 86,100 g for 20 min at 20°C. The emulsion particles that floated to the top of the density gradient were isolated and used within 24 h. To determine plasma clearance of the lipid emulsion particles, control and ritonavir-treated male C57BL/6 mice were injected intravenously with 100 μl of the prepared emulsions through the tail vein. Blood samples were withdrawn by retro-orbital puncture at set time points over a 20 min period. Serum was obtained from each blood sample by centrifugation, and an aliquot of the serum sample was used for liquid scintillation counting.

Lipid analysis

Blood samples were collected from mice under anesthesia. Serum was obtained by centrifugation and then analyzed for triglyceride and cholesterol concentrations with kits obtained from Wako Chemicals (Richmond, Virginia).

Statistical analysis

Data are presented as mean ± SEM For parametric data, means were compared by one-way ANOVA followed by the Tukey test. For nonparametric data, the Mann-Whitney rank sum test was used.

RESULTS

Effect of ritonavir on triglyceride-rich lipoprotein secretion by liver

In agreement with results reported previously, treatment of Chow-fed mice with ritonavir resulted in an average 30% increase in serum cholesteryl and triglyceride levels compared with vehicle-treated controls, whereas there was an average 60% increase in serum cholesterol and triglyceride levels in ritonavir-treated mice when they were fed a high-fat and high-cholesterol diet. To determine if ritonavir treatment influences VLDL production by the liver, mice were fed either the low-fat basal chow or the high-fat/high-cholesterol diet for 7 weeks. Animals with similar triglyceride levels from each group were selected to measure VLDL production. A solution of Triton WR1339 was injected intravenously into fasting animals to prevent lipolysis and clearance of the triglyceride-rich lipoproteins. Serum was collected at hourly intervals for the measurement of triglyceride accumulation. The results of these studies showed that the amount of VLDL triglyceride produced within a 4 h period was 30% higher in the Chow-fed ritonavir-treated mice compared with Chow-fed animals receiving only the vehicle control without the protease inhibitor (Fig. 1A). Ritonavir-induced VLDL triglyceride production was more pronounced in animals fed the high-fat/high-cholesterol diet (Fig. 1B).

The increase in triglyceride-rich lipoprotein secretion suggests that apoB secretion may also be increased after ritonavir treatment. However, previous in vitro studies with HepG2 cells showed that protease inhibitors inhibited the secretion of apoB under basal conditions and that the addition of oleic acid was required to increase apoB secretion to above the control level (18). To determine the effect of ritonavir treatment on apoB in vivo, the accumulation of serum apoB after Triton WR1339 infusion was compared between control and ritonavir-treated mice fed either the basal diet or the Western type high-fat/high-cholesterol diet. Results showed that ritonavir increased apoB secretion by approximately 50% under basal low-fat dietary conditions (Fig. 2). Ritonavir treatment dramatically increased apoB secretion by >400% when the ani-
mals were fed the Western-type high-fat/high-cholesterol diet (Fig. 2).

The secretion of apoB synthesized in the liver is dependent on the availability of neutral lipids and on its lipida- 
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Fig. 2. Ritonavir effects on apolipoprotein B (apoB)-100 produc- 
tion and secretion in mice. Male mice maintained on a basal low-fat 
chow diet or a Western-type high-fat diet were treated daily with ve-

cle control or ritonavir for 6 weeks. Serum samples were obtained 
from these animals 4 h after injection of Triton WR1339. Pooled 
serum samples from six vehicle-treated control mice (− and solid 
bars) or six ritonavir-treated mice (+ and open bars) were electro-

phoresed in a 4% to 15% SDS-polyacrylamide gradient gel under 
reducing conditions, transferred to nitrocellulose, and hybridized 
to anti-apoB antisera. Immunoreactive bands were identified by 
incubation with horseradish peroxidase-conjugated anti-rabbit IgG 
and developed with enhanced chemiluminescence. The nitrocellu-

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lose paper was then exposed to X-ray films and the image was 
scanned into the computer for quantitative analysis. The relative 
level of apoB-100 accumulation in serum after ritonavir treatment 
was determined based on the amount of apoB-100 present from the 
time of Triton WR1339 injection (0 h) to 4 h afterwards, using sam-

ples from vehicle control as 100% apoB-100 accumulation during the same period.

Effect of ritonavir on triglyceride-rich lipoprotein clearance

In addition to the production and secretion of lipoproteins by the liver, lipoprotein clearance by peripheral 
tissues is also an important determinant of serum lipid homeo-

DISCUSSION

Results from this current study demonstrated that ritonavir-induced hyperlipidemia is due to increased produc-
tion of triglyceride-rich lipoproteins. The protease inhibitor 
has no effect on lipoprotein clearance from circulation. 
Moreover, the data revealed no difference between control 
and ritonavir-treated mice in the expression of enzymes 
responsible for triglyceride synthesis and its as-

Fig. 3. Expression of diacylglycerol acyltransferase (DGAT) 
mRNA in liver of control (− and solid bars) and ritonavir-treated 
(+ and open bars) mice fed either chow or Western-type diet. The 
top panel shows the results of Northern blot hybridization with 32P-
labeled cDNA for DGAT and glyceraldehyde-phosphate dehydroge-
nase (GAPDH). The bottom panel is a bar graph representing the 
quantitation of the hybridization signals. The relative level of 
DGAT mRNA was determined by normalizing sample loading dif-

ferences with GAPDH mRNA level. Values obtained from control 
mice fed chow diet were taken as 100%. Results represent data 
from three separate Northern hybridization experiments using dif-

ferent RNA samples.

Fig. 4. Immunoblot analysis of microsomal triglyceride transfer 
protein (MTP) in membrane extracts from liver of control mice 
(lanes 1–3) and ritonavir-treated mice (lanes 4–6). Each lane con-
tained membrane extracts from an individual animal.
studies, experiments were designed to optimize the apoB experimental design of the two studies. In the in vitro and the in vitro studies may be explained by differences in fat diet. The apparent discrepancy between the in vivo and previously reported in vitro experiments (18). However, under high lipid throughput conditions, such as those observed in animals maintained chronically on a high-fat diet, protease inhibitor treatment resulted in additional increase of lipoprotein production above that observed with either high-fat diet or ritonavir treatment alone.

Another explanation for the differences observed between the current in vivo study and the previous in vitro study reported by Liang et al. (18) is the potential difference in regulation of hepatic lipid biosynthesis under in vivo and in vitro conditions. In the in vitro experiments, hepatocytes were obtained from apoB transgenic mice after an overnight fast (18). Under these conditions, the nucleus would be depleted of mature forms of both SREBP-1 and SREBP-2 (24), thereby resulting in the suppression of fat and cholesterol biosynthesis (18). Ritonavir treatment, which resulted in stabilization of the mature forms of SREBP-1 and SREBP-2 in the nucleus without affecting the proteolytic activation of the precursor SREBPs in membranes (12), would not be expected to induce lipid biosynthesis in liver cells cultured under these conditions. In contrast, under in vivo conditions where the mice were fed ad libitum, significant basal levels of activated SREBP-1 and SREBP-2 were present in the nucleus under normal conditions (12, 24). Ritonavir treatment prevented the proteasome-mediated hydrolysis of these transcription factors, thereby increasing their activities in promotion of endogenous lipid biosynthesis (12). The increased hepatic synthesis of fat and cholesterol, together with the stabilization of nascent apoB, may directly result in increased lipoprotein production after ritonavir treatment. In this regard, it is interesting to note that neither DGAT nor MTP expression level was altered by ritonavir treatment. Thus, these enzymes are apparently not rate-limiting in determining the amount of lipoproteins produced in the liver.

Results of the current study also revealed that ritonavir treatment has no effect on the clearance of triglyceride-rich lipoproteins from circulation in mice. Although physiology and several lipid metabolism pathways are different between mice and humans, the data are consistent with results demonstrating no abnormality in the clearance of remnant lipoproteins in ritonavir-treated normal human subjects (11). However, antiretroviral combination therapy was reported to reduce VLDL lipolysis in addition to increasing VLDL production in HIV-infected individuals (25). The discrepancy may be due to a difference between

![Fig. 5. Triglyceride-rich lipoprotein clearance from circulation of control and ritonavir-treated mice. A lipid emulsion containing \[^3H\]triolein was injected intravenously into control mice (open circles) or ritonavir-treated mice (solid circles) fed a basal low-fat chow diet (A) or a Western-type diet (B). Serum was collected for radioactivity measurements. Each data point represents mean ± SEM from eight mice.](image-url)
HIV-infected and non-infected subjects; Grunfeld et al. have demonstrated decreased VLDL triglyceride clearance and postheparin lipase activity in HIV-infected patients in comparison to normal individuals (26). Additionally, the combination therapy used routinely for treatment of HIV infection may also induce drug-drug interaction that adversely affects normal lipid metabolism in humans. These possibilities need to be assessed in more detail in future studies.

In summary, the current study, together with previous results reported by us and others (12, 18), indicate that ritonavir induced hyperlipidemia by increasing lipoprotein production by the liver via a mechanism related to its inhibition of proteasome-mediated hydrolysis of activated SREBP and apoB. Increased dietary fat content provided additional substrate for apoB-lipoprotein production, thereby exacerbating the adverse hyperlipidemia effects of the protease inhibitors. The latter observation suggests that reducing dietary fat intake may partially alleviate lipid abnormalities associated with HIV protease inhibitor therapy. However, as discussed above, the physiological difference between ritonavir-treated non-infected animals and HIV-infected individuals undergoing combination antiretroviral therapy requires additional testing in human subjects in a well-controlled clinical setting before any dietary recommendations can be made.

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