Hepatic steatosis : metabolic consequences

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Chapter 6

Ritonavir Impairs LPL-mediated Lipolysis And Decreases Uptake of Fatty Acids in Adipose Tissue

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Abstract

The use of the HIV protease inhibitor ritonavir (RTV) is frequently associated with hypertriglyceridemia and lipodystrophy. The aim of our study was to determine the mechanism underlying the observed hypertriglyceridemia.

Feeding female APOE*3-Leiden transgenic mice a western-type diet supplemented with RTV (35 mg/kg/day) for 2 weeks resulted in a 2-fold increase in fasting plasma triglyceride (TG) levels, which was specific for VLDL. RTV did not change the hepatic VLDL-TG production. Instead, RTV did increase the postprandial TG response to an oral fat load (AUC 25.5 ± 12.1 vs 13.8 ± 6.8 mM.h in controls; $P < 0.05$). Likewise, RTV hampered the plasma clearance of intravenously injected glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles ($t_{½}$ 19.3±10.5 vs 5.0±1.3 min in controls; $P < 0.05$), associated with a decrease of 44% in plasma LPL activity. Accordingly, RTV decreased the uptake of TG-derived fatty acids (FA) into adipose tissue, as well as the uptake of albumin-bound FA.

We conclude that RTV causes hypertriglyceridemia via decreased LPL-mediated clearance of VLDL-TG. In addition, RTV specifically impairs the uptake of FA in adipose tissue which may contribute to the lipodystrophy that is frequently observed in HIV-infected subjects on antiretroviral therapy.

Introduction

The introduction of highly active antiretroviral therapy (HAART) has considerably decreased morbidity and mortality associated with HIV-infection. This therapy, however, is associated with a lipodystrophy syndrome, which is characterized by changes in body fat distribution and metabolic abnormalities, such as hyperlipidemia and insulin resistance.[1,2] Studies in humans investigating the mechanism of HAART-induced hypertriglyceridemia reveal inconclusive results.[3-11] Some of these studies suggested that HAART increased VLDL-triglyceride (TG) production rates, whereas others suggested that antiretroviral treatment results in defective removal of VLDL-TG from plasma, either exclusively or in combination with increased VLDL-TG production rates. This discrepancy is difficult to resolve in humans, because the combination of drugs used in HAART does not permit a distinction between the effects of individual antiretroviral drugs. Since the HIV protease inhibitor ritonavir (RTV) is the antiretroviral drug that is associated with the most severe hypertriglyceridemic effects when used at therapeutic doses,[2,12] we aimed at conclusively elucidating the mechanism underlying hypertriglyceridemia induced by RTV. We used the APOE*3-Leiden transgenic mouse as an experimental model, because these mice have a humanized lipoprotein profile and are susceptible to diet- and drug-induced hyperlipidemia, obesity and atherosclerosis.[13-15] In contrast to wild-type mice, APOE*3-Leiden transgenic mice are highly sensitive to treatment with hypolipidemic drugs, such as statins, fibrates, and PPARα and PPARγ-agonists.[16] Similar to humans, APOE*3-Leiden transgenic mice have a much lower clearance rate of VLDL-TG than wild type mice. As a consequence, APOE*3-Leiden mice represent a suitable animal model for RTV-associated hyperlipidemia.

The first aim of the present study was to assess the effects of RTV on both VLDL-TG production and clearance rates. We used a low dosage of RTV that induced hypertriglyceridemia without causing toxicity, as measured by plasma alanine amino transferase (ALAT) levels. The second aim was to evaluate the effects of RTV on tissue-specific uptake of fatty acids (FA) derived from VLDL-TG and from the plasma free FA pool, by applying our recently described method using differentially labeled FA to quantify tissue-specific uptake of FA derived from VLDL-TG and from plasma free FA.[17] We found that RTV 1) decreased the clearance of VLDL-TG from plasma by decreasing lipoprotein lipase (LPL) activity, and 2) decreased the uptake of FA

derived from VLDL-TG and of albumin-bound FA in adipose tissue, but not in other organs.

Materials and methods

Animals

Female APOE*3-Leiden transgenic mice, housed under standard conditions with free access to water and food, were used for the experiments. Mice were fed a standard mouse chow diet (Hope Farms, Woerden, Netherlands) until 2 months of age. After this period they were fed a semi-synthetic western type diet (Hope Farms, Woerden, Netherlands) containing 15% saturated fat, 0.2% cholesterol and 40% sucrose for a 5 weeks run-in period. Mice were randomized and divided into 2 groups. One group was fed the western type diet with RTV (Norvir, Abbott, Kent, United Kingdom) added at a concentration of 35 mg/kg body weight/day for 2 weeks. The other group of APOE*3-Leiden transgenic mice was fed the western type diet without addition of RTV to serve as appropriate controls. On the basis of two papers investigating pharmacokinetic properties of HIV-protease inhibitors in mice[18,19] we designed a dose-finding study in which we showed that 35 mg/kg body weight/day did induce hypertriglyceridemia without causing liver damage as measured by plasma ALAT levels. Principles of laboratory animal care were followed and the animal ethics committee of our institute approved all animal experiments.

Plasma lipid analysis

In all experiments, tail vein blood was collected into chilled paraoxon-coated capillary tubes to prevent in vitro lipolysis.[20] These tubes were placed on ice and immediately centrifuged at 4°C. Plasma levels of TG, total cholesterol and free FA were determined enzymatically using commercially available kits and standards (#310-A Sigma GPO-Trinder kit, St. Louis, MA, USA; CHOL MPR3, Boehringer, Mannheim, Germany; #315 Sigma NEFA-C kit, St. Louis, MA, USA). FPLC analysis was performed on pooled plasma to determine the distribution of TG and cholesterol over the lipoprotein fractions using the AKTA purifier supplied with a Superose-6 column (Amersham Pharmacia Biotech).

Hepatic VLDL-TG production by Triton WR1339 injection

After the diet period mice were fasted overnight, anaesthetized (0.5 mL/kg Hypnorm; Janssen Pharmaceutica, Beerse, Belgium and 12.5 mg/kg midazolam; Roche, Mijdrecht, The Netherlands) and subsequently injected with Triton WR1339 (500 mg/kg body weight, 15% solution in 0.9% NaCl). Plasma VLDL clearance is completely inhibited under these circumstances.[21] Plasma TG were measured before injection of Triton and at 30, 60 and 90 min after injection and related to the body mass of the mice. Production of hepatic TG was calculated from the slope of the curve and expressed as µmol/h/kg body weight.

Postprandial TG response

After an overnight fast, mice were administered a 200 µL olive oil bolus through intra-gastric gavage. Blood samples were drawn just before and 1, 2, 4 and 8 h after olive oil bolus administration. TG concentrations were determined in plasma as described above and corrected for the plasma TG levels at t = 0.

In vivo clearance of VLDL-like TG-rich emulsion particles

The preparation and characterization of glycerol tri[^{3}H]oleate-labeled 80-nm-sized protein-free VLDL-like emulsion particles have previously been described.[22] This emulsion was stored at 4°C under argon and was used within 3 days. To study the in vivo serum clearance of the glycerol tri[^{3}H]oleate-labeled emulsions, fed mice were anaesthetized, the abdomen was opened and the emulsion (1 mg of TG) was injected intravenously via the vena cava inferior. Blood samples were taken via the vena cava inferior at 2, 5 and 10 min after bolus administration and the radioactivity in serum was determined by scintillation counting (Packard Instruments, Dowers Grove, IL). From these data the serum half-life of the glycerol tri[^{3}H]oleate was determined. The total plasma volumes of the mice were calculated from the equation: V (mL) = 0.04706 x body weight (g) as determined from ^{125}I-BSA clearance studies as previously described.[23]

Total plasma LPL activity

To determine the total LPL activity present in plasma, 4 h fasted RTV-treated mice and their controls were injected intravenously with heparin (0.1 U/g BW; Leo Pharmaceutical Products B.V., Weesp, Netherlands) and blood was collected after

10 min. The capillaries were kept on ice and were spun immediately at 4°C. The plasma was snap-frozen in liquid nitrogen and stored at -80°C until analysis of the LPL activity, as modified from Zechner.[24] A TG substrate mixture containing triolein (TO; 4.6 mg/mL), [^{3}H]TO (2.5 µCi/mL) essentially FA-free BSA (20 mg/mL; Sigma), Triton X-100 (0.1%; Sigma) and heat-inactivated (30 min at 56 °C) human serum (20%) in 0.1 M Tris-HCl, pH 8.6, was generated by 6 sonication periods of 1 min using a Soniprep 150 at 7 µm output, with 1 min intervals on ice. Ten µL of post-heparin plasma was added to 0.2 mL of substrate mixture and incubated for 30 min at 37 °C in the presence or absence of 1 M NaCl which completely inhibits LPL activity, to estimate both the LPL and HL levels. The reaction was stopped by the addition of 3.25 mL of heptane-methanol-chloroform (1:1.28:1.37, v/v/v), and 1 mL of 0.1 M K_2CO_3 in saturated H_3BO_3 (pH 10.5) was added. To quantify the [^{3}H]oleate generated, 0.5 mL of the aqueous phase obtained after vigorous mixing (20 s) and centrifugation (15 min at 3,600 rpm) was counted in 4.5 mL of Ultima Gold (Packard Bioscience, Meriden, CT). The LPL activity was calculated as the fraction of total lipolytic activity inhibited by 1 M NaCl and expressed as the amount of FA released per h per mL of plasma.

Modulated lipolytic activity in plasma

To study the effect of RTV on LPL activity in plasma in situ, post-heparin mouse plasma (2.5% of the incubation volume) was incubated with a mix of [^{3}H]triolein-labeled 80 nm-sized VLDL-mimicking protein-free emulsion particles (0.25 µg TG/mL, prepared as described previously[22]) and excess FFA-free BSA (60 mg/mL) in 0.1 M Tris, pH 8.5. After 1 h of incubation 50 µL samples from the total 200 µL incubation volume were taken and added to 1.5 mL of extraction liquid (methanol-chloroform-heptane-oleic acid; 1404:1245:1001:1; v/v/v/v) and 0.5 mL of 0.2 N NaOH was added to terminate lipolysis. Generated [^{3}H]oleate was counted as described above and expressed as the amount of FA released per h per mL. In this assay, the lipolytic activity of plasma is determined towards a relatively low amount of emulsion particles instead of an excess of solubilized TG. Hereby, the modulated lipolytic activity of plasma is assessed, by allowing interference of the endogenous activators (e.g. apoCII) and inhibitors (e.g. apoCI and apoCIII) with the activity of LPL.

Tissue-specific FA uptake

To determine the effect of RTV on the uptake of FA from VLDL-TG by peripheral tissues in the fed state we used a steady-state approach, as described previously by Teusink et al.[17] In short, glycerol tri[^{3}H]oleate-labeled 80-nm-sized protein-free VLDL-like emulsion particles which are known to mimic endogenous VLDL-TG particles[22] and [^{14}C]oleate bound to albumin were continuously infused for 2 h. Blood samples were drawn at 1.5 h and at 2 h to determine steady-state specific activity in plasma. After 2 h infusion the mice were sacrificed and the liver, muscle, heart, and subcutaneous adipose tissue were taken out to determine the retention of [^{3}H]oleate and [^{14}C]oleate in these tissues as a measure for the uptake of FA from VLDL-TG and from albumin-bound FA, respectively. Values were corrected for specific activity of FA in the plasma and are expressed as retention of total plasma FA in nmol/mg tissue protein.

Statistical analysis

Results are presented as means ± SD for the number of animals indicated. Differences between experimental groups were determined by the Mann-Whitney U test. The level of statistical significance of the differences was set at $P < 0.05$. Analyses were performed using SPSS 12.0 for Windows software (SPSS, Chicago).

Results

*Ritonavir increases plasma TG specifically in the VLDL fraction in APOE*3-Leiden transgenic mice*

Plasma TG, cholesterol and free FA were measured in APOE*3-Leiden transgenic mice after a five-week run-in period on the western type diet (t=0) and, subsequently, again after 2 weeks of feeding the same diet with or without the addition of RTV (t=2 weeks). In RTV-treated mice plasma TG increased from 2.7 to 5.4 mM (Fig. 1A, $P < 0.05$) and plasma cholesterol from 12.7 to 15.3 mM (Fig. 1B, $P < 0.05$), whereas plasma lipid levels remained unchanged in the control group. The increase in plasma TG was mainly due to an increase in VLDL-TG (Fig. 1C), while cholesterol was mainly increased in the VLDL and IDL/LDL lipoprotein fractions (Fig. 1D). Plasma free FA increased significantly from 0.70 to 0.93 mM ($P < 0.05$) after 2 weeks on the western type diet with RTV added as is shown in Figure 2.

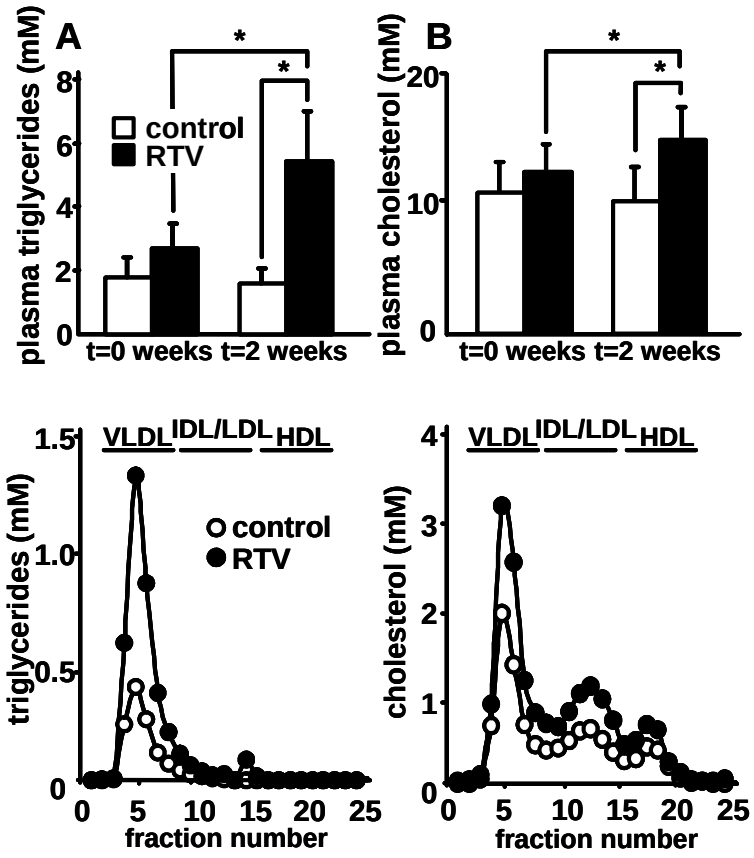

Figure 1. Ritonavir increases plasma TG and cholesterol. Plasma levels of TG (**A**) and cholesterol (**B**) were measured after a five-week run-in period and after 2 weeks of subsequent feeding with or without RTV administration through the diet. Values represent means ± SD of 8 mice per group. Lipoproteins in pooled plasma were fractionated by FPLC and eluted fractions were analyzed for TG (**C**) and cholesterol (**D**) distribution over the lipoproteins. * $P < 0.05$

Ritonavir does not change in vivo VLDL-TG production

To investigate whether the increase in plasma TG levels was due to increased hepatic VLDL-TG production, we injected fasted mice with Triton WR 1339, which completely inhibits lipolysis of VLDL-TG. However, as is shown in Figure 3A, after 2 weeks of dietary RTV administration no significant difference was observed in the rate of VLDL-TG production, when the RTV-treated mice were compared to the controls (139 ± 41 vs 177 ± 60 µmol TG/kg/h).

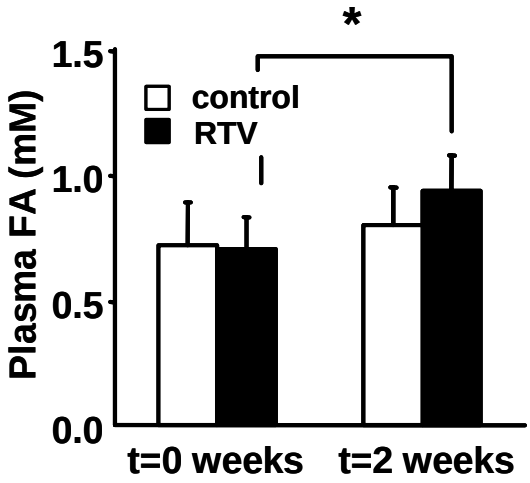

Figure 2. Ritonavir increases plasma free FA. Plasma levels of free FA were measured after a five-week run-in period and after 2 weeks of subsequent feeding with or without RTV administration through the diet. Values represent means ± SD of 8 mice per group. $P < 0.05$

Ritonavir increases postprandial TG response

Subsequently, we investigated whether the increase in postprandial plasma TG levels was caused by impaired postprandial clearance of TG. For this purpose, an intra-gastric bolus of olive oil was administered and subsequently plasma TG levels were determined. Figure 3B shows that RTV treatment caused a 2-fold increment in the postprandial TG response upon an intragastric olive oil administration (area under the curve 25.5 ± 12.1 vs 13.8 ± 6.8 mM.h; $P < 0.05$), which indeed suggests impaired TG clearance.

Ritonavir increases plasma half-life of TG-rich VLDL-like emulsion particles

To investigate whether the decreased clearance of TG indeed contributes to the hypertriglyceridemia observed in RTV-treated mice, mice were i.v. injected with glycerol tri[^{3}H]oleate-labeled protein-free VLDL-like emulsion particles. These particles mimic the metabolic behavior of TG-rich lipoproteins.[22,25] Because LPL is more abundantly expressed on the adipose tissue in the postprandial state compared to the fasted state[26], we used fed mice for this study. As is shown in Figure 4, the clearance of glycerol tri[^{3}H]oleate was markedly decreased in RTV treated mice when compared to the control group, which is evident from an approximately 4-fold

increase in serum half-life of glycerol tri[^{3}H]oleate ($t_{½}$ 19.3 ± 10.5 vs 5.0 ± 1.3 min; P < 0.05).

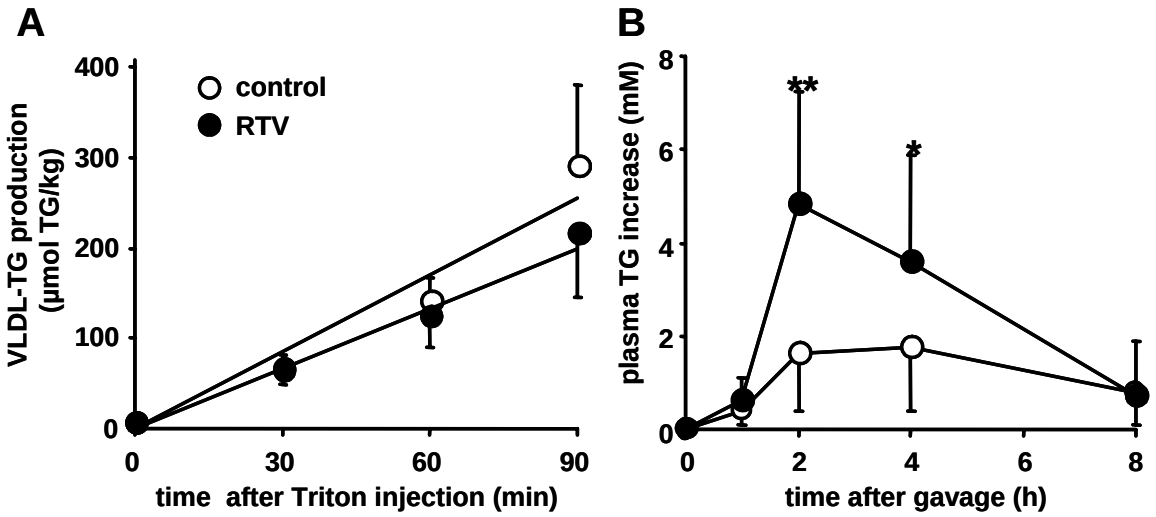

Figure 3. Ritonavir does not affect hepatic VLDL-TG production but increases the postprandial plasma TG response. **A**. After overnight fast, mice were anaesthetized and injected i.v. with Triton WR1339 (500 mg/kg BW) to completely block the peripheral lipolysis of VLDL-TG. Before and 30, 60 and 90 min after Triton injection blood samples were drawn. Plasma TG were determined and corrected for body weight and the values at T = 0. The slopes of the curves were calculated by linear regression to determine the rate of hepatic VLDL-TG production. Values represent means ± SD of 7 mice per group**.** **B**. After an overnight fast, mice were administered a 200 µL olive oil bolus through intra-gastric gavage. Blood samples were drawn before and at 1, 2, 4 and 8 h after the olive oil bolus and the levels of plasma TG were determined and corrected for the values at T=0. Values represent means ± SD of 8 mice per group. * P < 0.05, ** P < 0.01

Ritonavir decreases total LPL activity in post-heparin plasma

Impaired LPL-mediated TG hydrolysis can be due to decreased expression of LPL and/or by a direct effect of RTV on LPL activity. Therefore, we determined the effect of RTV on the total lipolytic activity in post-heparin plasma by incubation with a glycerol tri[^{3}H]oleate-containing substrate mixture. As shown in Figure 5A, the post-heparin HL activity in RTV-treated mice did not differ significantly from that of control mice (15.1 ± 3.7 vs 12.5 ± 3.7 µmol FA/h/mL). The post-heparin LPL activity, however, was significantly decreased by 44% in RTV-treated mice versus control mice (11.2 ± 3.3 vs 19.9 ± 11.1 µmol FA/h/mL; P < 0.05). This observation shows that

RTV impairs LPL-mediated TG lipolysis by lowering the total LPL activity present in plasma.

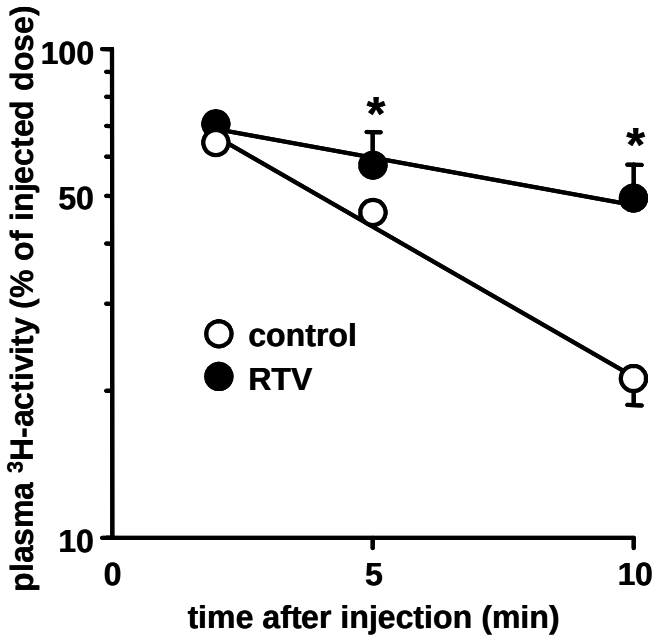

Figure 4. Ritonavir increases the plasma half-life of [3H]TG-labeled VLDL-like emulsion particles. Fed mice were injected via the vena cava inferior with glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles to investigate the plasma clearance. Blood samples were drawn at 2, 5 and 10 min after bolus administration and the amount of ^{3}H-activity in plasma was detemined. Values represent means ± SD of 3 mice per group. * P < 0.05

Ritonavir decreases the modulated lipolytic activity in post-heparin plasma

To study the modulated lipolytic activity in plasma, by allowing interference of the endogenous activators (e.g. apoCII) and inhibitors (e.g. apoCI and apoCIII) with the activity of LPL, we performed an additional assay in which the lipolytic activity of plasma is determined towards a relatively low amount of well-defined emulsion particles instead of an excess of solubilized TG. As is shown in Figure 5B the post-heparin modulated lipolytic activity is decreased significantly by 55% in plasma of RTV-treated mice as compared to control mice (19.0 ± 3.7 vs 42.8 ± 12.7 nmol FFA/h/mL; P < 0.05).

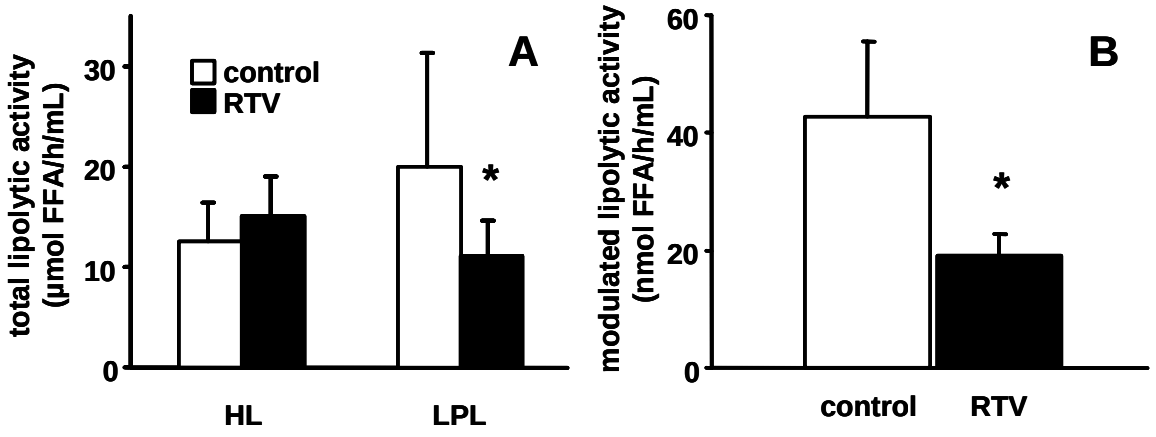

Figure 5. Ritonavir decreases total and modulated lipolytic activity in post-heparin plasma. Mice were fasted for 4 h and injected i.v. with heparin. After 10 min blood samples were drawn. A. The total lipolytic activity of post-heparin plasma was assessed by determination of [^{3}H]oleate production upon incubation of plasma with a substrate mix containing an excess of both [^{3}H]triolein and FA-free BSA as FA-acceptor. HL and LPL activities were distinguished in the presence of 1 M NaCl, which specifically blocks LPL. Values represent means ± SD of 9 mice in the RTV group and 10 mice in the control group. B. The modulated lipolytic activity of post-heparin plasma was assessed by incubation of plasma (2.5%) with [^{3}H]triolein-labeled VLDL-mimicking protein-free emulsion particles and excess FA-free BSA. After 1 h of incubation samples were taken and the modulated lipolytic activity was calculated as the amount of generated [^{3}H]oleate released per h per mL. Values represent means ± SD of 7 mice in the RTV group and 6 mice in the control group. * $P < 0.05$

Ritonavir decreases FA uptake in adipose tissue

The effect of RTV on the uptake of FA from VLDL-TG and albumin-bound FA by various tissues was studied during steady state infusion of glycerol tri[^{3}H]oleate TG-rich VLDL-like emulsion particles. RTV-treatment did not affect VLDL-TG derived FA uptake by the liver, skeletal muscle and the heart (Figure 6A). In adipose tissue, however, the uptake of VLDL-TG derived FA was significantly decreased (639 ± 220 vs 986 ± 80 nmol FA/mg tissue protein; $P < 0.05$). The uptake of FA bound to albumin was also decreased in adipose tissue of RTV-treated mice (514 ± 176 vs 1078 ± 194 nmol FA/mg tissue protein; $P < 0.05$), and not in the liver, skeletal muscle and the heart when compared to control mice (Figure 6B).

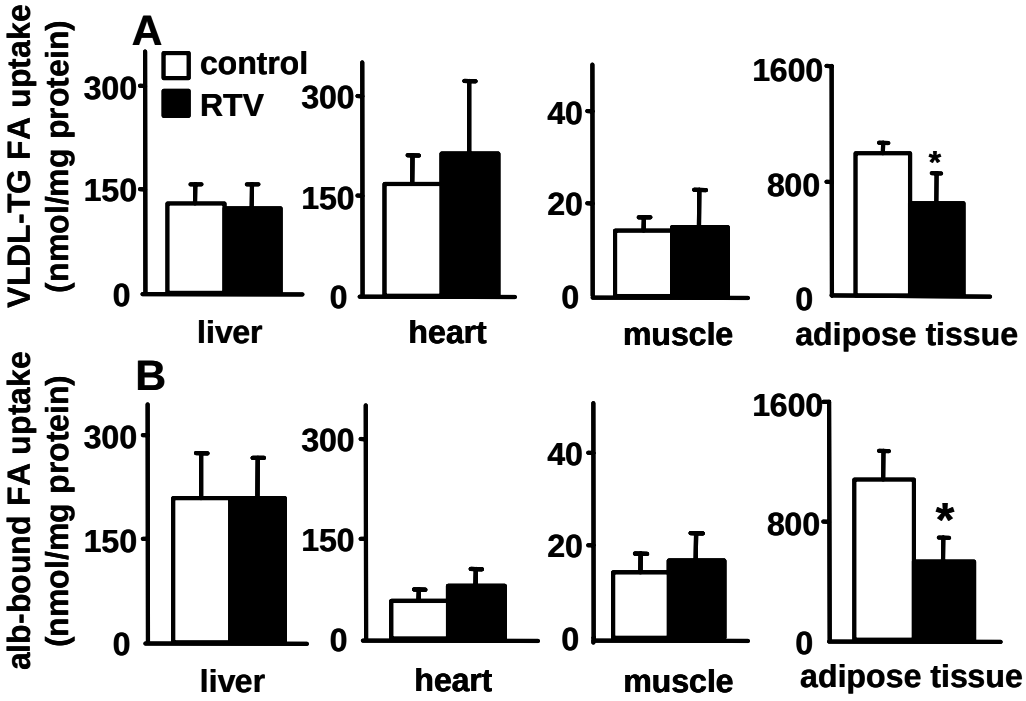

Figure 6. Ritonavir specifically decreases the uptake of FA by adipose tissue. Fed mice were anaesthetized and infused with a mixture of glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles and [^{14}C]oleate bound to albumin for 2 h to reach steady state specific activity in the plasma. After 2 h of infusion mice were bled and the organs were dissected to determine the uptake of VLDL-TG derived and albumin-bound FA. Values represent means ± SD of 7 mice per group. * $P < 0.05$

Discussion

In this study, we investigated the mechanism underlying the hypertriglyceridemia caused by RTV administration in APOE*3-Leiden transgenic mice with a human-like lipoprotein profile. Our data demonstrate that RTV clearly inhibits LPL-mediated TG clearance, which is supported by multiple lines of evidence. First, RTV increased postprandial hypertriglyceridemia indicating defective clearance of TG-rich lipoproteins. Second, RTV decreased the plasma clearance of i.v. injected TG-rich VLDL-like emulsion particles. Third, RTV decreased post-heparin plasma total LPL activity. In addition, the uptake of FA derived from VLDL-TG, as well as albumin-bound FA, was decreased selectively in adipose tissue where LPL is highly expressed in the postprandial state.

Human studies remain inconclusive with respect to the underlying mechanism of RTV-induced hypertriglyceridemia.[3-11] Purnell *et al.* showed that RTV decreased hepatic lipase activity, although there was no difference in post-heparin LPL levels

between RTV- and placebo-treated healthy subjects.[27] In contrast, a study by Baril *et al*.[3] showed that RTV caused decreased LPL activity while no differences in the amount of apolipoprotein CII (cofactor for LPL) or apolipoprotein CIII (inhibitor of LPL) were found, indicating a direct effect of RTV on the LPL enzyme as we now conclusively show in our study. Shahmanesh *et al*.[10] showed a significant decrease in the fractional catabolic rate of VLDL-TG in individuals treated with RTV either alone or in combination with other antiretroviral drugs, due to a decreased activity of LPL even in the postabsorptive state. Another study in HIV-negative subjects treated with RTV showed a trend towards decreased fat clearance as measured by an intravenous fat tolerance test after a 10 h fast.[5] A recent study by Sekhar *et al*.[9] revealed marked abnormalities in the ability of HIV lipodystrophy patients to metabolize dietary TG suggesting an impairment of the function of LPL. In humans it is impossible to conclusively show the direct effects of the individual drugs on the lipid metabolism, because HAART-treated patients are usually on a therapy regimen of at least three drugs. Moreover, in humans there is considerable heterogeneity in both environmental and genetic background.

To conclusively determine the mechanism underlying RTV-induced hypertriglyceridemia we used the APOE*3-Leiden transgenic mouse as our model. Studies in AKR/J mice[28] and in C57BL/6 wild type[29] mice showed an effect of RTV only on hepatic VLDL-TG production rate. In contrast to AKR/J and wild type mice, the APOE*3-Leiden transgenic mouse has a lipoprotein profile with close resemblance to the human profile.[13-15] In these mice plasma cholesterol levels can be titrated to any desired level by varying the amount of cholesterol in the diet. In contrast to wild-type mice, APOE*3-Leiden transgenic mice are highly sensitive to treatment with hypolipidemic drugs, such as statins, fibrates, and PPAR-α and γ-agonists.[16] These observations imply that the APOE*3-Leiden transgenic mice on a western type diet represent a suitable animal model for hyperlipidemia.

An *in vitro* study in human and rat hepatoma cells and primary hepatocytes from mice showed that protease inhibitor treatment inhibits proteasomal degradation of nascent apoB.[30] However, protease inhibitors also inhibited secretion of apoB. The concentrations of drugs used in these *in vitro* studies are much higher than the maximal plasma concentrations in subjects taking these drugs.[31] RTV may affect different components of the lipid metabolism depending on the dosage used. The dosage we used in our mice was 2 times higher than what an average adult would

receive per kg/day. Taking into account the much faster metabolic rate in mice it is clear that we used a low physiological dosage in our mice. Unfortunately, we did not have the opportunity to assess plasma RTV concentrations. It may be that at superphysiological concentrations RTV affects VLDL-TG production rate as well.

In the present study, RTV impaired FA uptake in adipose tissue under steady state conditions while infusing glycerol tri[^{3}H]oleate-labeled VLDL-like particles together with albumin-bound ^{14}C-labeled FA. Before tissues can take up FA derived from VLDL-TG, these TG have to be lipolyzed by LPL. In the current study we show that RTV decreased plasma LPL activity by 44%. As expected, due to decreased LPL activity the adipose tissue of RTV-treated mice took up significantly less FA derived from VLDL-TG compared to control mice under fed conditions. In the fed state LPL is more abundant in adipose tissue than in muscle[17,26] explaining why no change is seen in the uptake of VLDL-TG derived FA in muscle. In addition to decreased uptake of FA derived from VLDL-TG, the adipose tissue of RTV-treated mice also took up less albumin-bound FA, a process independent of LPL. The active transport of FA into tissues occurs mainly via CD36. CD36 functions as a high affinity transporter of long-chain FA in adipose tissue and the muscle.[32,33] Serghides *et al*.[34] have shown that CD36 deficiency was induced by antiretroviral therapy both in healthy humans and in HIV-infected subjects. They also showed that RTV significantly decreased CD36 levels in THP1 and C32 cells. The observed decrease in the uptake of albumin-bound FA in adipose tissue as we observed is in accordance with a decrease in CD36 levels. Another study showed that in murine peritoneal macrophages CD36 can be upregulated by protease inhibitor therapy leading to increased uptake of cholesterol and cholesteryl esters.[35] The difference in outcome of these studies may be a matter of different concentrations that are used in the *in vitro* studies. Many protease inhibitors, especially RTV, are very poorly soluble and difficult to handle in an *in vitro* assay.[36] Alternatively, it may be that the same drug exerts different effects in different types of cells.

In accordance with decreased FA uptake by peripheral tissues we found an increase of ~16% in plasma FA levels in RTV-treated mice. As we have shown recently[37], increased plasma FA levels can directly impair LPL activity most probably via product inhibition, because free FA can bind to the active site of LPL. In the present study plasma free FA levels are slightly but significantly increased, therefore, in addition to

direct impairment of LPL activity RTV may also be contributing indirectly to decreased LPL-mediated lipolysis via increased plasma FA.

Lipodystrophic HAART-treated HIV-infected patients showed an increased postprandial TG and FA response compared to non-lipodystrophic HIV-infected patients and healthy controls most likely caused by inadequate trapping of FA into adipose tissue.[38] Decreased postprandial adipose tissue FA uptake was already observed in our study after 2 weeks of drug administration, even though no obvious lipodystrophy as measured by weighing fat pads was observed yet. The flux of FA to adipose tissue mediated by LPL is an important determinant of adipogenesis. Deletion of LPL in adipose tissue in leptin-deficient *ob/ob* mice has been shown to prevent excessive storage of TG in the adipose tissue.[39] In contrast, the absence of apoCIII, the natural LPL inhibitor, enhances fatty acid uptake from plasma triglycerides in adipose tissue, which leads to higher susceptibility to diet-induced obesity.[40] In mice that were administered RTV for a much longer period generalized lipoatrophy was shown in male mice, while this lipodystrophy was restricted to the gonadal depot in female mice.[41] The investigators proposed that the lipodystrophy in these mice is caused, at least in part, by reduced PPARγ function. PPARγ transcriptionally activates a number of genes that are essential for adipogenesis, lipid storage and metabolism, including CD36.

The cause of the HAART-associated hypertriglyceridemia as observed in humans may be multifactorial in nature due to the use of different protease inhibitors simultaneously in combination with antiretroviral drugs of other classes. We propose that the main mechanism by which RTV increases plasma TG is by decreasing the LPL-mediated clearance of TG-rich lipoproteins. In the present study we directly show that RTV decreases the uptake of VLDL-TG derived FA and albumin-bound FA specifically in adipose tissue, an effect that may well contribute to HAART-associated lipodystrophy.

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