Hepatic steatosis : metabolic consequences

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Chapter 7

Ritonavir Protects Against the Development of Atherosclerosis Despite an Atherogenic Lipoprotein Profile in APOE*3-Leiden Transgenic Mice

In preparation

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Abstract

The use of the HIV protease inhibitor ritonavir (RTV) is associated with the induction of cardiovascular risk factors such as dyslipidemia and insulin resistance. It is not clear whether this increase in cardiovascular risk factors may lead to an epidemic of premature cardiovascular disease in HIV-infected patients treated with antiretroviral drugs.

To investigate the potential effects of RTV administration on atherosclerosis development, we fed APOE*3-Leiden mice, which have a human-like lipoprotein profile, a Western-type diet with or without the addition of RTV (35 mg/kg/day). Every 4 weeks, plasma triglyceride (TG) and total cholesterol levels were measured. RTV administration increased plasma TG levels when compared to control mice ($P < 0.05$), but did not alter total cholesterol levels. Unexpectedly, after 19 weeks on the diet, the mean atherosclerotic lesion area in the aortic root was decreased by ~52 % in RTV-treated mice compared to control mice ($P < 0.05$), which was reflected by decreased lesion severity. In contrast, *in vitro* studies with peritoneal macrophages showed that RTV dose-dependently increased oxLDL and lipid association.

In conclusion, RTV decreased atherosclerotic lesion area and severity, even though RTV induced hypertriglyceridemia. We speculate that RTV may decrease atherosclerotic lesion formation via an alternative (*e.g.* cholesterol efflux-enhancing or anti-inflammatory) pathway on the cellular or molecular level.

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 increased cardiovascular risk factors, such as hyperlipidemia and insulin resistance.[1,2] At present, the relationship between HAART and the development of premature atherosclerosis in HIV-infected patients is unclear. Studies measuring intima-media thickness (IMT) as a surrogate marker for the development of atherosclerosis do not conclusively show a correlation between HAART and IMT.[3-6] Some recent studies observed a slightly increased risk for HIV-infected individuals treated with HAART for the development of atherosclerosis.[5,7,8] It should be noted that the characteristics of study cohorts bias results, since HIV-infected subjects have in general more cardiovascular risk factors such as opportunistic infections and smoking compared to the general population.[9] However, a large prospective observational study showed that HAART was independently associated with a 26% relative increase in the rate of myocardial infarction per year of exposure during the first 4-6 years of use.[10]

Since it is difficult to study the effect of specific antiretroviral drugs on the development of atherosclerosis in HIV-infected subjects, several mouse models have been used. A study in male apoE knockout (apoE$^{-/-}$) and low density lipoprotein receptor knockout (LDLr$^{-/-}$) mice showed promotion of atherosclerotic lesion formation by the HIV protease inhibitor ritonavir (RTV) accompanied by CD36-dependent cholesterylester (CE) accumulation in macrophages.[11] In female LDLr$^{-/-}$ mice this effect was significantly less pronounced[12] even though in general female LDLr$^{-/-}$ mice are more susceptible to development of atherosclerosis.[13] This observation in transgenic mice partly supports the hypothesis that the metabolic effects of RTV may ultimately translate into an increased incidence of cardiovascular disease in HAART-treated subjects.

In accordance with the studies in humans and mice, we observed in a previous study that RTV causes hypertriglyceridemia in APOE*3-Leiden mice.[14] This atherogenic lipoprotein profile was caused via inhibition of LPL-mediated lipolysis. APOE*3-Leiden transgenic mice have an attenuated clearance rate of VLDL-TG, which resembles the VLDL-TG metabolism of humans, rather than wild type mice. As a consequence, the APOE*3-Leiden mouse represents a suitable animal model to

study the effects of dyslipidemia on atherosclerosis development.[15] Therefore, the aim of the present study was to determine the effects of RTV on the development of atherosclerosis in this APOE*3-Leiden transgenic mouse model. In contrast to our expectations, we observed that RTV significantly decreased atherosclerotic lesion area and severity in APOE*3-Leiden mice, compared to control mice, independent of plasma cholesterol levels.

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 experiment. 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 (n=14). One group of APOE*3-Leiden mice 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 19 weeks. The other group 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[16,17], we previously designed a dose-finding study in which we observed that RTV at a dose of 35 mg/kg body weight/day induced hypertriglyceridemia without causing liver damage as reflected by increased plasma levels of ALAT.[14] Principles of laboratory animal care were followed and the animal ethics committee of our institute approved all animal experiments.

Plasma lipid analysis

Every 4 weeks tail vein blood was collected into chilled paraoxon-coated capillary tubes to prevent *in vitro* lipolysis.[18] These tubes were placed on ice and immediately centrifuged at 4°C. Plasma levels of TG and total cholesterol were determined enzymatically using commercially available kits and standards (#310-A Sigma GPO-Trinder kit, St. Louis, MA, USA; CHOL MPR3, Boehringer, Mannheim, Germany).

Atherosclerosis analysis

After 19 weeks on the Western type diet, with or without the addition of RTV, mice were sacrificed. The hearts were perfused with ice-cold PBS, isolated, fixed in phosphate-buffered 4% formaldehyde, dehydrated and embedded in paraffin. The embedded hearts were cross-sectioned (5 µm) throughout the entire aortic root area. Sections were stained with hematoxylin-phloxine-saffron (HPS). Per mouse, 4 sections at 40 µm intervals within the valve area were used for quantification of atherosclerotic lesion area and characterization of lesion severity. Lesion area was determined using Image-Pro Plus version 3.0 analysis software (Media Cybernetics, U.S.). The atherosclerotic lesions were categorized for severity according to the American Heart System for humans[19], which has been adapted to categorize lesions in mice.[20] Three categories were discerned: no or very early lesions (type 0-1 lesions), moderate lesions that are fatty streaks containing only foam cells (type 2-3 lesions) or advanced lesions showing foam cells in the media and presence of fibrosis, cholesterol clefts, mineralization and/or necrosis (type 4-5 lesions). The number observed in each lesion category is expressed as a percentage of the total number of lesions present within one group of mice.[15]

In vitro lipid association studies with peritoneal macrophages

Four days after i.p. injection of Brewer's thioglycollate, peritoneal cells from APOE*3-Leiden transgenic mice were harvested into PBS. The cells were recovered after centrifugation, and resuspended in DMEM (Invitrogen) containing 10% fetal calf serum (Cambrex) and 1% penicillin and streptomycin. The cells were plated onto 24-wells plates (Costar, Corning Inc., Corning, NY, USA) at a density of $6.0*10^5$ cells/well. After incubation at 37°C under 5% CO_2 humidified air for 2 h, cells were washed to remove non-adhering cells. After o/n culturing, the macrophages were pre-incubated with RTV (0.1 or 1 µg/mL) or vehicle (0.5% ethanol) for 24 h. To determine the effect of RTV on the association of oxLDL with the macrophages, after 24 h of pre-incubation, cells were subsequently incubated with 50 µg/mL oxLDL[21] and 2 µCi/mL [1α,2α(n)-^{3}H]cholesterol (Amersham Biosciences, UK) as a tracer in presence of RTV or vehicle for another 24 h. Alternatively, to determine the effect of RTV on the association of 80 nm-sized TG-rich emulsion particles[22], after 24 h of pre-incubation with RTV or vehicle, cells were incubated with 380 µg TG/mL [^{3}H]TG and

[^{14}C]cholesteryl oleate (CO) labeled VLDL-like emulsion particles (380 µg TG/mL) for 3 h. After incubation with either oxLDL or TG-rich particles, cells were washed three times with ice-cold PBS, lyzed with 0.1 M NaOH and subsequently the amount of cell-associated radioactivity was determined.

Statistical analysis

Differences between experimental groups were determined by the Mann-Whitney U test for two independent samples. The differences in lesion severity were statistically tested using the Chi-Square 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

At the start of the experiment, and every 4 weeks thereafter, blood samples were taken to determine plasma levels of TG and total cholesterol. Throughout the whole study period, RTV administration significantly increased plasma TG levels approximately 2-fold compared to control mice and this effect was sustained until the end of the experiment ($P < 0.05$ for all time points; Figure 1A). In contrast, RTV did not affect plasma total cholesterol levels throughout the study period (Figure 1B).

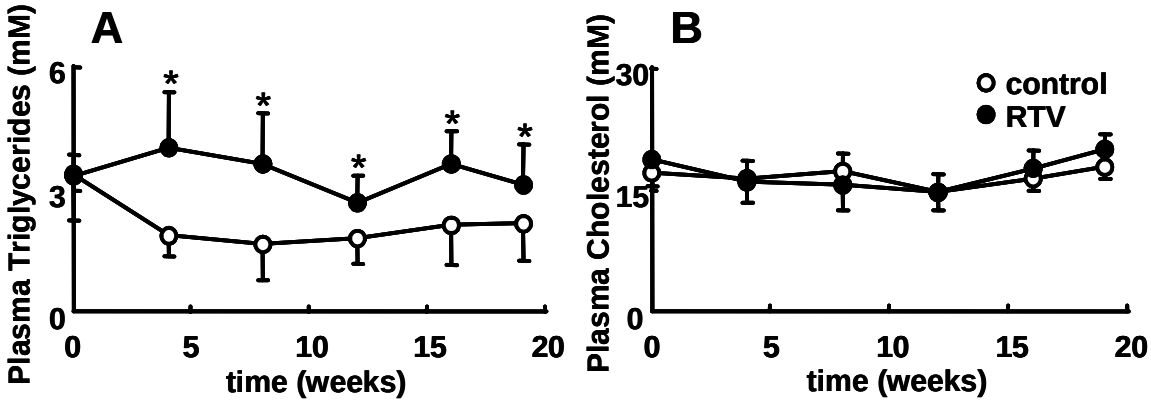

Figure 1. RTV increases plasma TG. Mice were fed a Western type diet without or with RTV added (35 mg/kg bodyweight/day). At baseline and every 4 weeks thereafter, plasma levels of TG (1A) and total cholesterol (1B) were measured after 4 h fasting. (n=14; * $P < 0.05$)

Ritonavir decreases the development of atherosclerotic lesions

After 19 weeks on the Western type diet, we sacrificed the mice to quantify the atherosclerotic lesion area and to determine atherosclerotic lesion severity in the aortic root (Figure 2A). RTV attenuated the development of atherosclerosis as indicated by a ~52% decrease in atherosclerotic lesion area compared to control mice (39.3 ± 41.4 x 10^3 μm^2 vs 82.6 ± 46.3 x 10^3 μm^2; $P < 0.05$; Figure 2B). This was reflected by a reduction in moderate (type 2-3) and advanced (type 4-5) lesions, concomitant with an increase in the percentage of segments with early (type 1) lesions, or no lesions at all (type 0) ($P < 0.05$; Figure 2C).

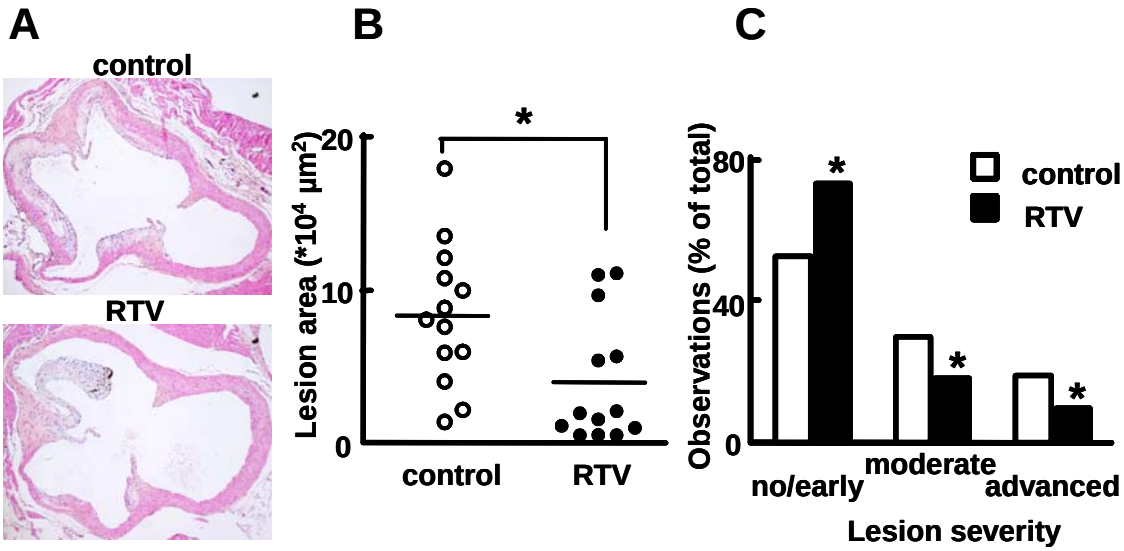

Figure 2. RTV decreases atherosclerotic lesion area and severity. After 19 weeks on the Western type diet with or without the addition of RTV (35 mg/kg body weight/day) mice were sacrificed and lesion area as well as lesion severity was determined. **A.** Representative overviews of the aortic root area of a control and a RTV-treated mouse. **B.** The lesion area was quantified in the aortic root area. **C.** The severity of lesions was determined in the aortic root area. (n=14; * $P < 0.05$)

Ritonavir increases the association of oxLDL with macrophages

To determine whether RTV induces decreased CD36 expression leading to decreased oxLDL uptake, and consequently, decreased atherosclerosis, we investigated oxLDL uptake by peritoneal macrophages. RTV dose-dependently enhanced the cell-association of oxLDL up to 28% at 1 µg/mL RTV ($P < 0.05$; Figure 3).

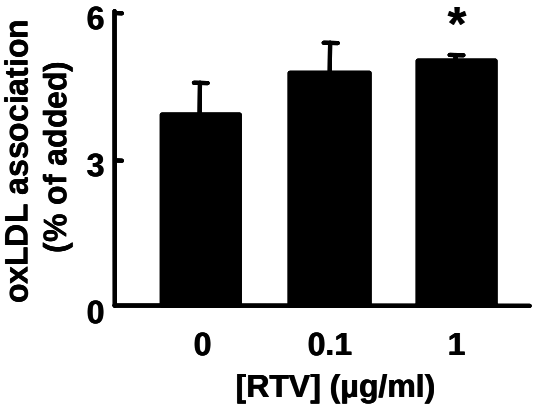

Figure 3. Ritonavir increases oxLDL association with peritoneal macrophages. After 24 h of pre-incubation with RTV or vehicle, cells were incubated with 50 μg/ml oxLDL and 2 μCi/ml [1α,2α(n)-^{3}H]cholesterol as a tracer in presence of RTV or vehicle for another 24 h. The amount of cell-associated [^{3}H]cholesterol was determined. (n=3; * *P* < 0.05)

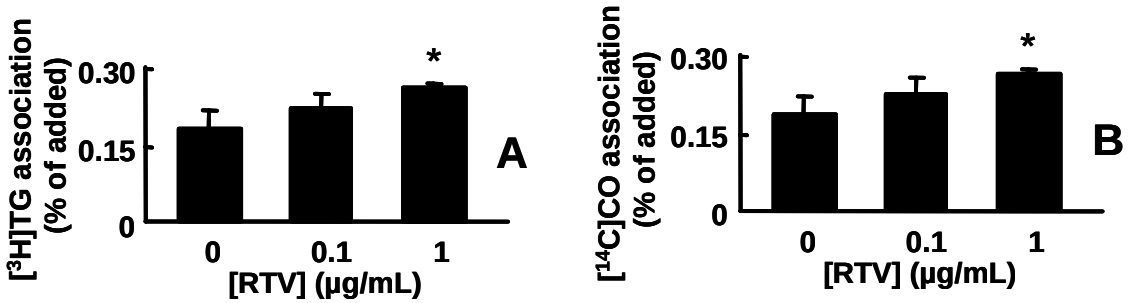

Figure 4. Ritonavir increases the association of TG-rich VLDL-like particles with peritoneal macrophages. After 24 h of pre-incubation with RTV or vehicle, cells were incubated with 380 μg TG/ml [^{3}H]TG/[^{14}C]cholesteryl oleate (CO) labeled VLDL-like particles for 3 h. The amount of cell-associated [^{3}H]TG and [^{14}C]CO was determined. (n=4; * *P* < 0.05)

Ritonavir increases the association of TG-rich VLDL-like particles with macrophages

We have previously observed that RTV-treatment of APOE*3-Leiden mice reduced the systemic expression of LPL, as reflected by reduced postheparin LPL levels.[14] To evaluate whether RTV would also reduce LPL expression specifically in macrophages, thereby reducing lipid uptake, we incubated peritoneal macrophages with TG-rich VLDL-like emulsion particles. However, RTV dose-dependently increased the association of both [^{3}H]TG and [^{14}C]CO (*P* < 0.05 ; Figure 4). Since the ratio between the uptake of [^{3}H]TG and [^{14}C]CO was similar for all conditions, and

was equal to their ratio in the emulsion itself, we conclude that RTV increases whole-particle association rather than selectively inducing the uptake of TG-derived fatty acids.

Discussion

The introduction of antiretroviral drug therapy has considerably increased the life span of HIV infected subjects. Consequently, long-term adverse drug effects become more clinically relevant in the considerations for the most optimal drug regimens. In this study we have conclusively shown that RTV decreases atherosclerosis in the aortic root in APOE*3-Leiden mice, despite the induction of dyslipidemia. Because there were no differences in plasma cholesterol levels between RTV-treated and control APOE*3-Leiden transgenic mice, this paradoxical effect of RTV was independent of plasma cholesterol levels.

We have previously shown that RTV induced hypertriglyceridemia, which was mainly confined to the VLDL fraction.[14] In that study we also found a small increase in plasma cholesterol after 2 weeks of RTV administration, also confined to VLDL. This initial increase in plasma cholesterol was probably secondary to the decreased clearance of VLDL, to which adaptation occurred during long-term administration of RTV (at the dose of 35 mg/kg body weight/day).

Previous studies in male apoE$^{-/-}$ and LDLr$^{-/-}$ mice showed, that HIV protease inhibitors such as RTV, promoted atherosclerotic lesion formation independent of dyslipidemia, which was explained by an increased CD36 expression in macrophages, thereby enhancing CD36-dependent cholesterylester accumulation.[11] On the other hand, a study in healthy volunteers, treatment-naive HIV-infected subjects and in human cell lines showed that antiretroviral therapy induced CD36 deficiency in monocytes.[23] Because we found that RTV decreased the formation of atherosclerotic lesions in our mouse model, we speculated that decreased CD36 expression on the macrophages could be the cause of decreased oxLDL uptake, and consequently of decreased development of atherosclerosis. Unexpectedly, we found that RTV dose-dependently increased oxLDL association with peritoneal macrophages, which is in accordance with the macrophage studies of Dressman *et al.*[11] In female LDLr$^{-/-}$ mice a much less pronounced effect of RTV administration on atherosclerosis development was observed.[12] In most animal models female mice are more susceptible to atherosclerosis than male mice.[13] Allred *et al*. suggested that the dissociation from

the usual gender difference in their RTV study is due to the pharmacological initiation of atherosclerosis.[12]

Another possible mechanism underlying the observed decrease in atherosclerosis could be decreased LPL expression. In our previous study, we observed that in postheparin plasma total LPL activity was considerably decreased by RTV administration (*i.e.* 50%).[14] Therefore, we speculated that decreased LPL activity on the macrophages in the vascular wall could lead to decreased lipid uptake and accumulation by macrophages. Interestingly, when we investigated this hypothesis *in vitro*, we found that RTV dose-dependently increased the association of both TG and cholesterylesters with macrophages. These findings indicate that RTV increases the whole-particle uptake of VLDL-like emulsion particles by macrophages. RTV apparently does not decrease LPL activity in all tissues, at least not in macrophages.

It is tempting to speculate about the mechanism(s) through which RTV decreases atherosclerotic lesion formation in the APOE*3-Leiden transgenic mouse model. It is possible, that RTV has anti-atherosclerotic effects at the cellular level in the arterial wall that overshadow the increased atherosclerotic risk induced by hypertriglyceridemia. For instance, an *in vitro* study with vascular smooth muscle cells showed that RTV inhibits platelet derived growth factor (PDGF)-induced DNA synthesis and chemotaxis.[24] PDGF is a major contributor to atherogenesis.[25] Furthermore, RTV inhibited PDGF-dependent downstream signaling such as Erk activation and these effects were not due to cytotoxicity of apoptosis.[24]

The upregulation of CD36 which was observed during RTV-treatment was shown to be accompanied by an increase in peroxisome proliferator-activated receptor-γ (PPARγ).[11] PPARγ is a ligand-activated nuclear transcription factor with pleiotropic effects on lipid metabolism and inflammation.[26] A study in apoE$^{-/-}$ mice showed that although the PPARγ agonist troglitazone upregulated the expression of CD36 in macrophage foam cells, this PPARγ agonist inhibited fatty streak lesion formation.[27] PPARγ has anti-atherogenic effects because it promotes cholesterol efflux via upregulation of ATP-binding cassette A1 (ABCA1) and ABCG1 and indirectly via upregulation of liver X receptor-α (LXRα) leading to decreased foam cell formation.[28-31] Furthermore, PPARγ agonists have anti-inflammatory effects on the macrophage[32-35], protecting against atherosclerosis. Taken together, the PPARγ-increasing activity of RTV could be involved in the paradoxical decrease in atherosclerotic lesion formation despite the presence of hypertriglyceridemia and upregulation of CD36 in

the APOE*3-Leiden mice.[27,36-38] We speculate that this anti-atherogenic effect of RTV is not observed in the apoE$^{-/-}$ mice because part of the anti-inflammatory and efflux-enhancing effects of PPARγ are caused by increased apoE expression via LXR activation.[39]

The Data Collection on Adverse Events of Anti-HIV Drugs (DAD) study showed that HIV-infected HAART-treated subjects are at a significantly greater risk of myocardial infarction.[10] In HAART-treated patients, however, the high prevalence of cardiovascular risk factors might overshadow the beneficial inhibitory effects of RTV on atherosclerosis.[8] The prevalence of the most significant risk factor, i.e. cigarette smoking, is high among HIV-infected patients with CHD (69 %).[9] Patients may already have some atherosclerotic lesion formation due to ageing.[9] In contrast, treatment of our mice with RTV started at young adulthood. More importantly, prior to initiation of HAART treatment, HIV-infected subjects may have been exposed to chronic systemic inflammation due to HIV-infection for many years. Several opportunistic infections may play a role in the pathophysiology of CHD. It has been suggested that Cytomegalovirus and *Chlamydiae pneumoniae* may promote atherosclerosis.[40] The APOE*3-Leiden mouse provides a good model to study the molecular effects of specific drugs such as RTV in a human-like lipoprotein metabolism setting, independent of the many complicating genetic and environmental factors that can influence the results in human studies.

In conclusion, RTV decreases the development of atherosclerosis in the aortic root of APOE*3-Leiden transgenic mice, despite the induction of hypertriglyceridemia. Because there were no differences in plasma cholesterol levels between RTV treated and control APOE*3-Leiden transgenic mice, this paradoxical effect of RTV was independent of plasma cholesterol levels. This observation indicates that plasma cardiovascular risk factors may not translate into the development of atherosclerosis under all conditions.

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