Acipimox, an Inhibitor of Lipolysis, Attenuates Atherogenesis in LDLR-Null Mice Treated With HIV Protease Inhibitor Ritonavir

Wen Guo, Siu Wong, Jeffrey Pudney, Ravi Jasuja, Ning Hua, Lan Jiang, Andrew Miller, Paul W. Hruz, James A. Hamilton, Shalender Bhasin

Objectives—The advent of HIV protease inhibitors has greatly extended the life span of AIDS patients. With an aging HIV+ population, the cardiometabolic side effects of these drugs are becoming increasingly important clinical concerns. The purpose of this study was to test the hypothesis that inhibition of adipose lipolysis will retard atherogenic lesion development induced by the antiviral protease inhibitors.

Methods and Results—LDLR-null mice receiving ritonavir were compared with those receiving ritonavir plus lipolysis inhibitor acipimox or vehicle alone to determine how acipimox would affect ritonavir-induced atherogenesis. Intermittent high-fat high-cholesterol diet was used to facilitate optimal atheromatous lesion development. Drug effects were assessed as changes in aortic lesion score, plasma lipid and lipoprotein profile, body fat mass, and insulin-induced suppression of plasma fatty acid concentrations. Ritonavir increased aortic lesions, in association with decreased body fat mass, impaired antilipolysis action of insulin, and increased proatherogenic plasma lipoproteins. All these adverse effects were attenuated by cotreatment with acipimox.

Conclusions—Our results provide the first direct evidence that supports the hypothesis that dysregulation of adipose lipolysis is an important contributor to the proatherogenic role of selected HIV protease inhibitors. (Arterioscler Thromb Vasc Biol. 2009;29:2028-2032.)

Key Words: antiretroviral protease inhibitor ■ adipose tissue ■ insulin resistance ■ dyslipidemia ■ acipimox

The advent of HIV protease inhibitors (PIs) such as ritonavir brought a remarkable transition in AIDS therapy. Together with selected nucleoside reverse transcriptase inhibitors (NRTIs), PIs greatly improved viral suppression and changed HIV/AIDS from a rapidly fatal disease to a controllable chronic illness. Substantially increased life expectancy of HIV-infected patients has shifted focus to drug-associated metabolic disorders, such as lipodystrophy, insulin resistance, and coronary artery disease.1–5 Some of these metabolic abnormalities have been reproduced in HIV-negative humans and animal models after administration of PIs,6–9 suggesting that a proatherogenic phenotype is more associated with antiviral therapy than with viral infection alone.10

HAART patients with lipodystrophy generally display resistance to insulin regulation of lipolysis and glucose disposal.11–13 Direct binding of selected PIs to glucose transporter 4 (Glut4) acutely impairs insulin-stimulated glucose transport.14 Repeated administration of PIs can cause sustained impairment of insulin signaling, resulting in failure to suppress adipose lipolysis and to promote lipogenesis during postprandial periods.15,16 These changes are predicted to reduce body fat mass and further exacerbate systemic insulin resistance, contributing to proatherogenic dyslipidemia and premature atherosclerosis. Indeed, HIV-infected patients with lipodystrophy often have elevated plasma fatty acids (FFA) concentrations and a more proatherogenic lipid profile.17–19 This condition was found to be improved after inhibition of lipolysis by coadministration of acipimox,18,19 suggesting that lipolysis inhibitors may offer protection against HAART-induced premature atherosclerosis. For proof-of-concept, we tested how acipimox affects mouse aortic lesion development in LDLR-null mice treated with ritonavir, a PI known to induce atherogenesis in mouse models.20

Methods

To accelerate lesion development, mice were given an intermittent high-fat high-cholesterol diet (HFC) 2 days each week interspersed with standard chow. Ritonavir was provided orally twice daily (33 mg/kg/d), and acipimox was provided in drinking water (0.05% wt).
Acipimox retards ritonavir-induced arterial lesions (please see supplemental Figures I through IV). A, Cross-section of right subclavian and innominate arteries. B, Aortic en face lesion score: the percentage of lesion-covered surface area. C, Aortic-root lesion macrophage infiltration estimated by IHC staining for Mac3 (scaled 1 to 10).

For all other details, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Results

Ritonavir Accelerates Atherogenic Lesion Development, an Effect That Is Attenuated by Cotreatment With Acipimox

To determine the optimal treatment duration, we used high-resolution magnetic resonance angiography (MRA) to longitudinaly assess the progression of occlusive lesions in vivo in the innominate and right subclavian arteries (supplemental Figure 1A). As shown in Figure 1A, LDLR−/− mice treated with ritonavir developed lumen asymmetry in these arteries at an earlier phase (week 4) and had smaller luminal area for right subclavian (week 4) and innominate (week 8) than vehicle-treated mice. This effect was partially reversed by acipimox. In week 8, good concordance between occlusive vessels assessed by MRA and the presence of lipid-rich lesions was observed (supplemental Figure 1B). Because en face analysis of the aortic vessel revealed only sparse lesions at this time (not shown), the experiment was extended by 6 more weeks.

After 14 weeks of treatment, atheromatous lesions were found throughout the aorta, with a greater density in the arch around the origin of its major branches (supplemental Figures II and III). Mice receiving ritonavir had an approximately 2-fold increase in the percentage of lesion-covered surface area (Figure 1B). This effect of ritonavir was reversed by acipimox. We also estimated macrophage levels within the aorta root as an indicator of inflammatory responses. As shown supplemental Figure IV, lesions isolated from ritonavir-treated animals showed a remarkably greater staining intensity for Mac3, a macrophage marker commonly found in mouse atherosclerotic lesions.21 This effect was reversed by acipimox. The mean Mac3 staining intensity in the aortic root lesions (Figure 1C) closely correspond to the en face aortic artery lesion score (Figure 1B). This observation is in agreement with the in vitro findings that ritonavir stimulates macrophage activation.22 Further studies are required to determine whether increased macrophage infiltration is a direct effect of ritonavir on vascular wall inflammation or an indirect effect because of greater systemic lipid stress (see below).

Ritonavir-Associated Loss of Fat Mass Is Blocked by Cotreatment With Acipimox

Consistent with previous studies that reported lipoatrophy in wild-type mice treated with protease inhibitors,7,8 we observed a significant reduction in all 3 fat depots measured in mice receiving ritonavir (Figure 2A). Analysis of fat cell size distribution revealed a shift toward smaller fat cells in ritonavir treated mice (Figure 2B). These changes were reversed by acipimox. NMR measurements show that body fat mass initially increased in all groups after HFC was introduced. However, 6 weeks later, mice receiving ritonavir began to lose fat mass, whereas mice receiving vehicle or ritonavir plus acipimox did not change (Figure 2C). By the end of treatment, whole body fat mass was lower in the ritonavir group (4.29 g versus 5.8 g, P=0.031, compared to vehicle group) but not in the acipimox plus ritonavir group (5.8 g versus 6.1 g, P=0.791, compared to vehicle group).

Ritonavir also reduced adipose mRNA expression of adipocyte transcription factor (PPARγ), fatty acid synthase (FAS), and acetyl CoA carboxylase (ACC), and the changes were reversed by acipimox (supplemental Figure V). It is of
Ritonavir induces acute and chronic insulin resistance. The acute effect is caused by direct interaction with Glut4, whereas the chronic effect is related to impaired insulin signaling. To determine the effects of acipimox on ritonavir-induced insulin resistance, we performed insulin tolerance tests in mice after 8 weeks of treatment. As expected, when mice were tested in 2 hours after ritonavir dosing, there was a significant reduction in insulin-stimulated glucose disposal (Figure 3A, left panel), with AUC of 53.2 compared to 39.8 of the vehicle group ($P<0.05$, arbitrary unit). This effect was not reversed by acipimox (Figure 3A, AUC 61.8). We repeated the test in 16 hours after the last ritonavir dosing. In this case, there was no difference in acute plasma glucose lowering within the first 15 minutes (Figure 3A, right panel). After 30 minutes, the recovery of glucose concentration appears to be accelerated in ritonavir-treated mice with or without acipimox. These results suggest that the atheroprotective effect of acipimox is independent of immediate insulin-stimulated glucose disposal.

HAART patients with lipodystrophy are reported to be less responsive to the antilipolytic action of insulin. Because lipolysis directly promotes hepatic VLDL secretion, we hypothesized that impairment of the antilipolytic action of insulin may be a relevant mechanism for ritonavir-induced atherogenesis. Accordingly, we compared glycerol release from adipocytes isolated from ritonavir and vehicle-treated mice, in response to a βagonist (isoproterenol, iso) and insulin. As shown in Figure 3B, insulin significantly suppressed iso-stimulated glycerol release in fat cells isolated from the vehicle group, but was not effective in fat cells isolated from the ritonavir group. Cotreatment with acipimox restored the cellular sensitivity to insulin (Figure 3B, right panel).

Acipimox Ameliorates Ritonavir-Induced Proatherogenic Plasma Lipid Profile

Increased plasma triglycerides (TAG) is typically found in HAART patients and PI treated rodents. This was also found in the mice receiving ritonavir in our study, and the effect was largely reversed by acipimox (Figure 4A). However, no detectable differences in FFA was found among the 3 groups (Figure 4B). This was not too surprising because steady-state plasma FFA is regulated by multiple endogenous mechanisms minute-to-minute and the antilipolysis effect of acipimox can diminish after the drug (dissolved in drinking water) intake diminishes during the day. Even so, sustained lowering of plasma TAG implies that long-term acipimox intake still results in an overall better regulated lipid homeostasis. To determine whether acipimox improves insulin suppression of lipolysis in vivo, we measured plasma FFA after a moderate dose of insulin (0.6 U/kg, i.v.). As shown in Figure 4C, ritonavir dampened the insulin suppression of plasma FFA but the effect was reversed by acipimox.

Dysregulation of plasma FFA and TAG predicts impaired VLDL metabolism. Indeed, as shown in Figure 4 (middle and lower panels), ritonavir increased VLDL-cholesterol. This effect was completely blocked by acipimox under nonfasting and partially blocked under fasting conditions. Note that antilipolysis effect of acipimox may decrease in the fasting period because the animals naturally drank less in the light cycle (8:00 AM to 4:00 PM). In addition, ritonavir moderately increase nonfasting LDL-cholesterol. This effect was again reversed by acipimox. There was a slight but significant increase in fasting HDL-cholesterol in animals treated with acipimox plus ritonavir. This is likely secondary to reduced VLDL secretion, as acipimox alone does not affect HDL metabolism.
This study provides the first in vivo evidence to support the hypothesis that the lipolysis inhibitor acipimox antagonizes the proatherogenic effect of HIV protease inhibitor ritonavir. The mechanistic framework of this hypothesis is shown in supplemental Figure VI. Briefly, this model envisions that HIV protease inhibitors impair insulin signaling for lipolysis regulation, causing increased FFA flux into the liver, which in turn increases VLDL output and promotes atherogenesis. This model predicts that atherogenesis will be attenuated if adipose lipolysis is suppressed by inhibitors that bypass the insulin signaling defects. Although partial support for this hypothesis has come from previous studies, our work provides the first demonstration that ritonavir-induced atherogenesis is attenuated by acipimox, a lipolysis inhibitor that acts independent of insulin signaling.

Plasma lipoprotein concentrations largely depend on the coordinated metabolic regulation in liver, adipose tissue, and muscle. Although many of the previous rodent studies documented strong drug effects on hepatic lipogenesis that can lead to VLDL hypersecretion, these findings were often confounded with high drug dosage and high-fat diet. In this work, we show that, with a moderate drug dose and dietary fat content, ritonavir induced dyslipidemia and atherosclerosis in LDLR−/− mice without significantly damaging the liver itself (supplemental Figure VII). Based on the findings that acipimox did not correct the ritonavir effect on insulin tolerance test, we suggest that muscle is not a direct contributor either. Instead, our results suggest that the proatherogenic effect of ritonavir can be primarily explained by the drug effects on adipose tissue.

First, we showed that long-term ritonavir dosing caused fat tissue loss and resulted in smaller fat cell size; implying increased fat mobilization. We also presented the first in vivo evidence that ritonavir induced expression of a stress responsive gene HO-1 in adipose tissue. Because FFA stimulates stress response in preadipocytes, we suggest that HO-1 may be induced as a result of increased adipocyte FFA release attributable to ritonavir-mediated insulin resistance. Second, we showed that ritonavir dampened in vivo plasma FFA response to insulin, which recapitulates the findings in HAART patients. However, we also provided the first evidence that long-term drug treatment dampened ex vivo fat cells sensitivity to the antilipolysis action of insulin. Most importantly, we provided compelling evidence that acipimox largely reversed these ritonavir-induced fat tissue malfunctions, in parallel to its suppression on ritonavir-induced atherogenesis.

Increased VLDL is a hallmark of HAART associated dyslipidemia. Our results also show that VLDL-c is increased by ritonavir under both fasting and nonfasting conditions. Previous studies demonstrated that PI increases VLDL secretion without affecting VLDL catabolism. If so, our results will suggest that insulin suppression of VLDL secretion is impaired by ritonavir under both fasting and nonfasting conditions. By increasing FFA flux into the liver, ritonavir may also increase secretion of TG-rich VLDL particles which may be more atherogenic than normal VLDL. Most importantly, we show that the effects of ritonavir on VLDL, fasting or nonfasting, were blocked by acipimox, in parallel with drug effects on aortic lesion development. This provides a clear link between adipose lipolysis and ritonavir-mediated dyslipidemia with drug-induced premature atherogenesis.

Finally, it is important to point out that although acipimox and niacin share the same receptor (GPR109A) pathway for lipolysis inhibition, niacin is, but acipimox is not, a precursor of NADH. For this reason, niacin is known to have profound atheroprotective effects beyond its function as a lipolysis inhibitor, most notably for its roles as a potent inhibitor for HDL catabolism and for hepatic lipid synthesis, as well as a general antioxidant. Such receptor-independent effects have not been reported for acipimox.

Overall, our observations have important clinical implications. We have recapitulated the major metabolic side effects of ritonavir in a mouse model and demonstrate that lipatrophy, proatherogenic dyslipidemia, and accelerated atherogenesis can be alleviated by concomitant administration of a lipolysis inhibitor. Further studies to explore the therapeutic applications of lipolysis inhibitors in treating the metabolic complications of PIs are warranted.

**Sources of Funding**

This research was supported by NIH grants RO1DK59261, RO1DK49296, RO1DK64572, and RO1DK078512-01.
Disclosures

None.

References

Acipimox, an Inhibitor of Lipolysis, Attenuates Atherogenesis in LDLR-Null Mice Treated With HIV Protease Inhibitor Ritonavir

Wen Guo, Siu Wong, Jeffrey Pudney, Ravi Jasuja, Ning Hua, Lan Jiang, Andrew Miller, Paul W. Hruz, James A. Hamilton and Shalender Bhasin

Arterioscler Thromb Vasc Biol. 2009;29:2028-2032; originally published online September 17, 2009;
doi: 10.1161/ATVBAHA.109.191304

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/29/12/2028

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/09/17/ATVBAHA.109.191304.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
1. Methods:

Animal model, drugs, and diets. Male LDLR null (LDLR\(^{-/-}\)) mice on a C57BL/6 background and wild-type C57/BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). At 14 weeks of age, the LDLR\(^{-/-}\) mice (n=48) were randomly assigned to one of three treatment groups (vehicle, ritonavir, or ritonavir plus acipimox). The protocol of animal use for this study has been approved by the IACUC of Boston University School of Medicine.

Ritonavir was administered by gavages twice daily at a dose of 33 mg/kg/day, similar to the dose used in published mouse studies. In preliminary experiments, the plasma ritonavir concentration, measured by a modified HPLC method, ranged from 3.46 – 7.93 \(\mu\text{M}\) (SD 0.86, n=8) in 2 h after drug ingestion, and from 0.23 – 1.83 \(\mu\text{M}\) (SD = 1.91, n = 6) in 5 h after drug ingestion. These values are well within the therapeutic range in humans taking ritonavir. Acipimox was supplied in drinking water (0.05% wt/wt) and changed twice a week. The average water consumption was about 4 ml per day, equivalent to 2 mg/day intake of acipimox. This dose has been shown to inhibit adipocyte lipolysis in rodents. In humans, acipimox is well tolerated beyond 2250 mg/day.

Ritonavir has been shown to induce aortic lesions in LDLR\(^{-/-}\) mice given a standard mouse chow. However, the lesions thus formed are microscopic and unlikely to cause clinical events. In contrast, an ad libitum atherogenic diet induces massive aortic lesions in LDLR\(^{-/-}\), as well as other mouse models of atherogenesis, and hence may mask the pro-atherogenic effects of the antiviral drugs (preliminary studies, data not shown). To overcome this problem, we used a modified dietary regimen in which a high fat, high cholesterol (HFC) diet (Harlan Teklad TD.94509) was fed on either two (28% of the time, n = 8) or three (43% of the time, n = 8) days each week interspersed with standard chow (PurinaOne 5051) on the remaining days. Additional pilot tests showed that the pro-atherogenic effect of ritonavir was properly detected under these dietary conditions (data not shown). The HFC diet contains 36.9% kcal from fat, 20.4% kcal from protein, and 41.2% kcal from carbohydrate, with 1.25% wt/wt cholesterol. These diet regimens better mimic the usual dietary fluctuations in free-living animals and humans and allow efficient detection of atherogenic effects of ritonavir (see below).

Body composition measurement and fat cell size determination. Longitudinal changes in fat mass were measured in mice using EchoMRI-700/100 whole body composition analyzer (Echo Medical Systems, Houston, Texas). After euthanasia, fat pads were dissected from the subcutaneous, epididymal and perirenal depots. Fat pad weight was expressed as % total body mass. A small section of each fat pad was fixed in buffered 10% formalin and cut into 10 \(\mu\text{m}\) sections after parafilm embedding. Fat cell distribution was imaged and analyzed using Metamorph software.

Magnetic resonance angiography (MRA). Mice anesthetized with Isoflurane (0.5%~2% in oxygen) were immobilized inside an MR probe designed for mouse imaging on an 11.7 T Avance spectrometer (Bruker, Billerica, MA). MRA was performed using the 3D FLASH (Fast Low Angle SHot) angiographic sequences. Innominate and right subclavian branch arteries were selected as the comparison sites because mouse atherogenesis typically begins near this branch as a result of the relatively fast and more turbulent blood flow in these areas.
Aortic lesion detection. The aorta was dissected from the arch to the ileal bifurcation, the extraneous tissue was removed, and the intimal surfaces were exposed longitudinally. The aorta was incubated for 5 minutes with Sudan IV (0.5% wt in acetone) and washed three times with 75% ethanol. Sudan IV imparts a red color to the lipid-rich lesions. The aortas were digitized with a CCD camera and lesions on the intimal aortic surface were quantified using Image J software. Representative results of en face staining are shown in Figure II-III. The calculated lesion score shown in Figure 1B are means of N = 8 for each group that were fed HFC 2 days per week. Except for Figure IIIIB, all other results shown in this work are from animals given a HFC for 2 days per week.

Histology and Immunohistochemistry. Separate experimental mice were prepared for this experiment with 2 d/wk HFC with daily ritonavir dosing and acipimox the same as described above. Experiment was ended after 12 week of treatment. Immediately after mice were euthanized, a catheter was inserted into the left ventricle of the heart and vascular system was flushed 20 ml with PBS followed with 20 ml PBS-buffered 10% formalin. The perfusion was controlled through syringe pump at rate of was 5 ml/min. The heart and the whole aorta was then taken out and dissected clean. The aorta was cut off midway between the root and the Innominate artery. The aorta root was dissected out as ~ 2 mm ring near the valve and embed in paraffin after dehydration. Serial 5 μm sections covering the 300 μm of the proximal aorta, starting from the sinus, were collected. Tissue sections were rehydrated and subjected to antigen retrieval by incubation with 0.1 M sodium citrate (pH 4.5 – 6.2) at 120°C using a pressure cooker. After antigen retrieval, tissue slices were process using Zymed HistoMouse-SP Kit (AEC, Broad Spectrum, Invitrogen Cat#959544), following the manufacturer’s instructions. Antibody for Mac3 was purchased from BD Biosciences (#550292; San Jose, CA). The first antibody was diluted in 1% BSA at experimentally optimized mixing ratio (1:20 dilute) and incubated with lesion sections at 4°C over-night. Positive controls were performed using mouse lymph nodes. Negative controls were performed the same but with mouse IgG. The tissue sections were mounted and photographed using Olympus camera (magnification specified by an in-photo mark, Figure IV). Results were estimated by multiple individuals blinded to the test conditions. Each data point shown in Figure 1C is an average of 3 estimates on the same section. Tissue sections from six different animals were analyzed for each group. These mice were given HFD two days per week for 14 weeks.

Liver histology was performed using formalin-fixed tissue samples, paraffin-embedded, followed with standard sectioning and H&E staining.

Insulin tolerance test. Mice were tested between 6 – 8 weeks after drug intervention. Acute and chronic drug effects were tested after administration of the last ritonavir dose in 2 h or 16 h, respectively. Insulin tolerance tests were performed as we described before except that insulin dose was slightly reduced (0.6 U/kg). Acute insulin response is measured as sequential decrease in plasma glucose concentrations. Accumulative insulin response is estimated by the area-under-curve (AUC) of the glucose – time relationship, using the GraphPad Prism software. The two insulin tolerance tests were performed 2 weeks apart to allow the mice a full recovery between tests.

Lipolysis: Wild-type C57BL/6 mice (male, 2 month old) were treated with ritonavir (50 mg/kg/day) for 4 weeks. Fat cell isolation and lipolysis assay were performed as previously described. Lipolysis was initiated by adding 50,000 fat cells into 0.5 ml KRH-buffer (pH 7.4, glucose 5 mM) containing vehicle (0.1% ethanol), Isoproterenol (0.5
μM), insulin (1.7 nM), ritonavir (10 μM), and acipimox (0.05%), as noted. After incubation at 37°C for 90 min with gentle shaking (60 Hz), 50 μl of solution was removed for measurement of glycerol. Results were expressed as the amount of glycerol released per min per 10^6 cells.

**Plasma lipid measurement.** Because food intake affects plasma lipid concentrations, blood sampling was performed on Friday, the last day of chow diet in the week. Mice were fasted from 8:00 AM to 3:00 PM, which approximates an overnight fast in humans. Insulin sensitivity was assessed as plasma FFA responses before and 30 min after insulin injection (0.6 U/kg, tail-vein). Preliminary studies indicated a linear insulin effect on plasma FFA from 15 min to 45 min post injection. Blood was withdrawn from retroorbital vein under light isoflurane anesthesia. Plasma FFA was measured using commercial reagents (Wako NEFA Kit). Plasma samples used for FPLC analysis were withdrawn on the 4th day of chow diet either at 10:00 – 11:00 am (non-fasting) or at 3:00 - 4:00 pm (fasting from 8:00 am to 4:00 pm). The FPLC analysis was performed in the Emory Lipid Research Laboratory of Cardiovascular Specialty Labs, Inc (Atlanta, Georgia). Results are presented as mean +/- SE, with N = 8 for each treatment group. All blood work was done in week 8th – 13th of drug treatment.

**Liver and adipose tissue mRNA analysis.** Total RNA isolation for liver was done using RNeasy Kit and that for fat tissue was done using RNeasy kit for lipid rich tissue, following manufacturer’s instruction. Both kits were from Qiagen Inc. First strand cDNA synthesis and real-time PCR were done as described in our previous work 13.

**Statistics:** Data are presented as means ± SE. Comparison of means between two groups was performed by using Students' t-test. One-way ANOVA was used for comparison of multiple groups. If ANOVA revealed a significant overall effect at the alpha level of 0.05 or less, individual groups were compared by Tukey's HSD test. All statistics were conducted using the SPSS program.

**References**


2. Supplemental Data

A: 3D abdominal thoracic artery tree

B: Lipid staining of branch of right subclavian and innominate arteries

Figure IA: Mouse thoracic aorta with its major branches was viewed by MRA (left). The innominate (2) and right subclavian (1) artery branch (middle) are shown in the middle panel, and the right panel shows the cross-section of innominate (2) and right subclavian arteries near their origin. B: Lipid staining of innominate and right subclavian branch in mice treated for 8 weeks for vehicle (a), ritonvir (b) and ritonvir co-treated with acipimox (c). The arrow marks indicate the lipid-rich lesions stained red after incubation with Sudan IV (bar = 500 mm).
Figure II. Upper panel: isolated mouse aorta arch with the branches intact. The whitish areas (marked with arrows) reflect athermanous lesions (bar = 1 mm). Lower panel: the en face of aorta arch with lipid lesions stained by Sudan IV (bar = 2 mm). The images are representative of results from N ≥ 8 independent animals. These mice were fed with HFC for 2 days per week. Ritonavir was given twice a day at a dosage of 33 mg/kg/day. Acipimox was given in drinking water at 0.05%. Experiment was completed in 14 weeks.
Figure III: en face detection of lesion distribution throughout the aorta arch and the descending aorta, stained red with Sudan IV (bar = 2 mm). A: isolated aorta from mice treated with HFC for 2 days per week. B: isolated aorta from mice treated with HFC for 3 days per week. All were treated for 14 weeks with drug conditions as described for Figure S2. These results indicate that increased dietary fat intake resulted greater lesion deposits. Since the pro-atherogenic effect of ritonavir and atheroprotective effect of acipimox against ritonavir was clearly demonstrated under both dietary conditions, the following studies were conducted only on animals fed HFC on the 2 days per week schedule.
Figure IV. positive and negative control of IHC staining for Mac3. A&B: lymph node from normal mice was embedded in wax and sectioned into 5 μm. After antigen retrieve, tissue slice was incubated with anti-Mac3 (1:20, A) or mouse IgG (1:20, B) overnight. Subsequent 2nd antibody reaction and substrate color development were performed using Zymed Kit following manufacturer's instruction. The intense dark rusty red spots (arrow) indicates positive staining for Mac3. C: a representative aorta root section after treated with IgG the same as the negative control B. It is noticed that IgG alone stained bright red color in areas of mechanical defects, such as the large necrotic core in (arrow). However, IgG staining is negligible at the foam cell rich areas near the lesion surface, indicating low probability of nonspecific false positive staining in this area.
Figure IV-D (Vehicle group): showing mild to moderate presence of Mac+ macrophages in the area overlaying the lesion core.
Figure IV-E (ritonavir group): showing intense presence of Mac+ macrophages in the shoulder area as well as the area overlaying the lesion core.
Figure IV-F (ritonavir plus acipimox group): showing intense Mac3+ staining at the shoulder area but very mild in the area overlaying the lesion core.
Figure IV-G (Vehicle group): showing moderate to intense Mac+ staining at the shoulder but mild in the area overlaying the lesion core.
Figure IV-H (ritonavir group): enlarged lesion core overlaid with think layers of Mac+ staining.
Figure IV-I (ritonavir plus acipimox group): enlarged lesion with large necrotic core. Moderately intense Mac+ staining near the shoulder. The color within the necrotic core is artificial, possibly due to the rugged texture in the sample area (compared to IgG stained negative control, not shown).
Figure IV-J (Vehicle group): mild to moderate Mac+ staining overlying the lesion core.
Figure IV-K (ritonavir group): intense Mac+ staining in the thickened intima area overlying a moderate size lesion.
Figure IV-L (ritonavir plus acipimox group): moderate Mac+ staining in the area overlaying the lesion core.
Figure V: Effects of ritonavir and acipimox on adipose tissue mRNA expression for heme oxygenase-1 (HO-1), HO-2, PEPCK, fatty acid synthase (FAS), PPARgamma, and acetyl CoA carboxylase (ACC). Among which, HO-2 is a constitutive heme oxygenase not subjected to exogenous regulations. It is not surprising that HO-2 is not affected by ritonavir nor acipimox. PEPCK has recently been implicated in lipolysis regulation. However, our results do not suggest an involvement of this enzyme in ritonavir-mediated metabolic effects, at least not at the mRNA level. The observation of suppressed PPARgamma, FAS and ACC by ritonavir suggest that decreased lipogenesis may also contribute to fat mass loss. However, the fact that all these changes were blocked by acipimox suggest that ritonavir modulates fat tissue function through mechanisms that involve lipolysis regulations.
Figure VI. Illustration of the hypothetical mechanism of ritonavir-mediated proatherogensis and how it is reversed by acipimox.
Figure VII: Liver is not a primary target of ritonavir under the current experimental conditions. H&E staining of liver slice show similar and mild signs of steatosis in all, which was not aggravated by ritonavir or acipimox (A). Neither ritonavir nor acipimox affected liver size and lipid storage (B). Neither ritonavir nor acipimox had significant impacts on insulin receptor substrates IRS1&2 or metabolic genes PEPCK, FAS, and ACC.