NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS PREVENT HIV PROTEASE INHIBITOR-INDUCED ATHEROSCLEROSIS BY UBIQUITINATION AND DEGRADATION OF PROTEIN KINASE C

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ABSTRACT

HIV protease inhibitors are important pharmacological agents used in the treatment of HIV infected patients. One of the major disadvantages of HIV protease inhibitors is that they increase several cardiovascular risk factors including the expression of CD36 in macrophages. The expression of CD36 in macrophages promotes the accumulation of cholesterol, the development of foam cells, and ultimately atherosclerosis. Recent studies have suggested that \( \alpha \)-tocopherol can prevent HIV protease inhibitor-induced increases in macrophage CD36 levels. Because of the potential clinical utility of using \( \alpha \)-tocopherol to limit some of the side-effects of HIV protease inhibitors, we tested the ability of \( \alpha \)-tocopherol to prevent ritonavir, a common HIV protease inhibitor, from inducing atherosclerosis in the LDLR mouse model. Surprisingly, \( \alpha \)-tocopherol did not prevent ritonavir-induced atherosclerosis. However, co-treatment with the nucleoside reverse transcriptase inhibitors (NRTIs), didanosine or D4T, did prevent ritonavir-induced atherosclerosis. Using macrophages isolated from LDLR mice we demonstrated that the NRTIs prevented the up-regulation of CD36 and cholesterol accumulation in macrophages. Treatment of LDLR mice with NRTIs promoted the ubiquitination and down-regulation of protein kinase \( \zeta \) (PKC). Previous studies demonstrated that HIV protease inhibitor activation of PKC was necessary for the up-regulation of CD36. Importantly, the in vivo inhibition of PKC with chelerythrine prevented ritonavir-induced up-regulation of CD36, accumulation of cholesterol, and the formation of atherosclerotic lesions. These novel mechanistic studies suggest that NRTIs may provide protection from one of the negative side effects associated with HIV protease inhibitors, namely the increase in CD36 levels and subsequent cholesterol accumulation and atherogenesis.

Keywords: CD36, macrophage, proteasome, LDLR
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INTRODUCTION

Highly active antiretroviral therapy (HAART) is essential to decreasing the morbidity and mortality of HIV-infected individuals (10, 22, 27). However, prolonged use of HAART is associated with hyperlipidemia, lipodystrophy, insulin resistance, and accelerated atherosclerosis (7, 10, 11, 17, 26). Of the various classes of compounds used in HAART, the HIV protease inhibitors appear to be associated with negative cardiovascular risk factors (7, 10, 11, 17, 26). Because of the importance of HIV protease inhibitors in HAART it is crucial to understand the mechanisms whereby HIV protease inhibitors induce cardiovascular side-effects.

HIV protease inhibitors affect multiple cellular mechanisms including inhibiting the degradation of the nuclear form of sterol regulatory element binding proteins (nSREBP) in the liver and adipose tissue (2, 15, 24). Accumulation of nSREBP in the liver increases fatty acid and cholesterol biosynthesis whereas in adipose tissue accumulation of nSREBP induces lipodystrophy, insulin resistance, and decreases leptin levels (2, 15, 24). HIV protease inhibitors also decrease the degradation of nascent apolipoprotein B by suppressing proteasome activity (16). Insulin resistance and diabetes are promoted by the ability of HIV protease inhibitors to attenuate glucose transporter GLUT4 activity in muscle and adipose (12).

In addition to the above effects, Dressman et al (5) demonstrated that HIV protease inhibitors contribute to the formation of atherosclerosis by promoting the up-regulation of CD36 and the subsequent accumulation of sterol in macrophages. CD36 is a class B scavenger receptor that facilitates the uptake of modified lipoproteins into macrophages which promotes foam cell formation and the development of atherosclerotic lesions. The use of CD36 blocking antibodies, CD36 morpholinos, and CD36 null mice clearly demonstrated that CD36 up-regulation was necessary for HIV protease inhibitor induced sterol accumulation in macrophages and atherosclerotic lesion formation (5). The fundamental finding that HIV protease inhibitors increase the amount of CD36 in macrophages has been confirmed by Munteanu et al (19).
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The reported mechanism for HIV protease inhibitor up-regulation of CD36 involved the up-regulation of peroxisome proliferator activated receptor gamma (PPARγ) (5). PPARγ has been previously reported to be up-regulated by molecules found in modified lipoproteins such as oxidized low-density lipoprotein (6, 20). Importantly, the up-regulation of PPARγ by HIV protease inhibitors and modified lipoproteins leads to increased CD36 expression which then promotes further uptake of modified lipoproteins, foam cell formation, and subsequently atherosclerotic lesions (6, 20). HIV protease inhibitors stimulated the expression of PPARγ via protein kinase C, but the entire molecular pathway has not been elucidated (5, 6). In contrast, Munteanu et al (19), using THP-1 monocytes, suggested that the primary mechanism for increasing the amount of CD36 in macrophages is due to the inhibition of the proteasome. This conclusion was based on the use of the proteasome inhibitor ALLN which significantly increased CD36 whereas only a modest increase in CD36 mRNA was observed in the presence of the HIV protease inhibitor, ritonavir (19). Presently it is unclear how many mechanisms contribute to HIV protease inhibitor-induced increases in the level of CD36 and the relative importance of each mechanism.

Importantly, the study by Munteanu et al (19) detailed a finding of potentially great clinical significance, namely that α-tocopherol, in THP-1 monocytes, prevented ritonavir induced increases in CD36 levels by preventing the inhibition of proteasome activity. If α-tocopherol prevents HIV protease inhibitor-induced increases in CD36 levels in humans a simple and safe treatment would be available to counteract at least one of the negative side effects of HIV protease inhibitors. While the cell culture studies done by Munteanu et al (19) are intriguing, the in vivo effects of α-tocopherol was not determined. In the present study we tested the ability of α-tocopherol to prevent HIV protease inhibitors from increasing CD36 levels in an animal model. As a control, we used nucleoside analogue reverse transcriptase inhibitors (NRTIs), which are commonly used in combination therapy with HIV protease inhibitors. To our surprise, α-tocopherol did not prevent HIV protease inhibitor induced increases in CD36 or the development of atherosclerotic lesions. However, NRTIs completely prevented the up-regulation of CD36 and the development of atherosclerosis.
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**MATERIALS AND METHODS**

**Materials** RPMI medium 1640, DMEM high glucose medium, fetal calf serum, L-glutamine, trypsin-EDTA, and Penicillin-Streptomycin were purchased from Life Technologies Inc. (Grand Island, NY). Percoll, PVDF membrane, Tween 20, α-tocopherol, and chelerythrine chloride were purchased from Sigma (St. Louis, MO). Bradford reagent was purchased from BioRad (Hercules, CA). The anti-PKCα IgG was from BD Biosciences (San Diego, CA), the anti-actin IgG was from Sigma, the anti-mouse CD36 (IgM) was from BioDesign International (Kennebunk, ME), the anti-SRA (scavenger receptor, type A) was from Serotec, anti-ubiquitin was from Zymed Laboratories (San Francisco, CA), and Quality Control Biochemicals (St. Louis, MO) generated the SR-BI (scavenger receptor, type B, class I) antibody as a fee for service. Horseradish peroxidase conjugated IgGs were supplied by Cappel (West Chester, PA). Super Signal® chemiluminescent substrate was purchased from Pierce (Rockford, IL). Bristol-Myers Squibb provided the didanosine and Abbott Laboratories provided the ritonavir. The mouse feed was obtained from Harlan Tekland (Madison, WI). The protein kinase C assay kit was from Calbiochem (San Diego, CA). Adenosine 5’-triphosphate[$^{32}$P] (109 TBq/mmol) was from PerkinElmer (Boston, MA). The ubiquitin enrichment kit was from Pierce (Rockford, IL).

**Buffers** Sample buffer (5X) consisted of 0.31 M Tris, pH 6.8, 2.5% (w/v) SDS, 50% (v/v) glycerol and 0.125% (w/v) bromophenol blue. Tris-buffered saline (TBS) consisted of 20 mM Tris, pH 7.6, and 137 mM NaCl.

**Animals** All animals were housed in the University of Kentucky animal facilities. Animals were maintained in constant temperature conditions on a 14:10 light/dark cycle (lights on at 0400h) and were provided food and water ad libitum. The LDLR null mice were obtained from The Jackson Laboratory (Bar Harbor, ME). At six weeks of age mice were placed on a chow diet containing 0, 400 or 800 mg/kg of α-tocopherol and given either vehicle control (0.01% ethanol), ritonavir (50 µg/mouse/day), didanosine (75 µg/mouse/day), or D4T (75 µg/mouse/day) in the drinking water. Where indicated, chelerythrine chloride (5 mg/kg) was injected i.p. every 48 hours for the duration of the study (3). This regimen of ritonavir has previously been described to produce
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atherosclerotic lesions in male LDLR null mice without altering plasma cholesterol levels (5). Mouse peritoneal macrophages were obtained by lavage (29).

*Cholesterol and Cholesteryl Ester Mass Quantification*  Cholesterol and cholesteryl ester mass was quantified using a commercially available kit from Wako Chemicals (Richmond, VA) per manufacture’s instructions.

*SDS-PAGE and Immunoblotting*  Samples were concentrated by trichloroacetic acid precipitation and washed in acetone. Pellets were suspended in sample buffer that contained 1.2% (v/v) β-mercaptoethanol and heated at 95°C for 3 min before being loaded onto gels. Proteins were separated in a 12.5% SDS-polyacrylamide gel using the method of Laemmli (13). The separated proteins were then transferred to PVDF. The PVDF was blocked in TBS that contained 5% dry milk for 1 hr at room temperature. Primary antibodies were diluted in TBS that contained 1% dry milk and incubated with the PVDF for 1 hr at room temperature. The PVDF was washed four times, 10 min each in TBS+1% dry milk. The secondary antibodies (all conjugated to horseradish peroxidase) were diluted 1/20,000 in TBS+1% dry milk and incubated with the PVDF for 1 hr at room temperature. The PVDF was then washed and the bands visualized by chemiluminescence.

*Northern Blot Hybridization*  Total RNA was extracted from cells using TRIZOL (Invitrogen, Carlsbad, CA). RNA was quantified by spectrometry, and 20 μg of RNA was loaded onto a 5% denaturing agarose gel. Following electrophoretic separation, the RNA was transferred to nylon membranes and probed for peroxisome proliferator-activated receptor gamma (PPARγ), PKCα, and glyceraldehyde-3-phosphate dehydrogenase mRNA as previously described (5).

*Immunoprecipitations*  Protein A-Sepharose beads were first blocked by incubation for 4 hr at 4°C with a peritoneal macrophage cell lysate (200 μg/ml) plus 30 mg/ml of BSA in immunoprecipitation buffer (150 mM NaCl, 0.5 % Triton X-100, 50 mM Tris, pH 8.0). Blocked beads were then used to pre-clear the experimental
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fractions that had been adjusted to 0.5 % (v/v) Triton X-100. Pre-cleared fractions were incubated for 2 hr at 4°C with the appropriate antibody before adding blocked Protein A-Sepharose beads and incubating an additional 1 hr at 4°C. The beads were collected by centrifugation, washed four times in high salt (500 mM NaCl) immunoprecipitation buffer, and then placed in Laemmli sample buffer. Immunoprecipitated proteins were detected by Western blotting.

Quantification of atherosclerotic lesions  After 8 weeks of treatment, plasma was collected for cholesterol, cholesteryl ester, and triglyceride determinations. The mice were then processed to quantify the surface area of atherosclerotic lesions. Atherosclerotic lesions were quantified as we have done previously (4, 5). Briefly, the aorta from the arch to the ileal bifurcation was collected, the extraneous tissue dissected away and the intimal surfaces exposed by a longitudinal cut. The aortas were placed under a dissecting microscope equipped with a CCD camera attachment that captures the image directly to a computer file. Atherosclerotic lesions on the intimal aortic surface appear as bright white areas compared with the thin and translucent aorta. Areas of intima covered by atherosclerosis were quantified with ImagePro software 6.0 (Media Cybernetics Inc, Silver Spring, MD).

Statistics  Least squares analysis of variance was used to evaluate the data with respect to sample, treatment, time, and their interaction using the ANOVA procedure of Statistica. When appropriate, samples were compared using the Tukey’s HSD test. Means were considered different at p < 0.01.
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RESULTS

Effect of α-tocopherol on ritonavir-induced atherosclerosis. Munteanu et al (19) have reported that α-tocopherol can prevent HIV protease inhibitor-induced accumulation of cholesterol in THP-1 macrophages. These in vitro data suggested that α-tocopherol may be effective at preventing or reducing HIV protease inhibitor-induced atherosclerosis. To test if α-tocopherol can prevent the atherogenic effect of ritonavir, six week old male LDLR null mice feed a standard chow diet were treated with ritonavir (50 µg/mouse/day), vehicle control (0.01% ethanol), α-tocopherol (400 mg/kg), or ritonavir (50 µg/mouse/day) and α-tocopherol (400 mg/kg or 800 mg/kg) in the drinking water for 8 weeks as we described previously (5). LDLR null mice were used for these studies because this mouse model develops few atherosclerotic lesions in the absence of a high fat diet or HIV protease inhibitors as we previously demonstrated (5). Thus, the effects of ritonavir and α-tocopherol on atherosclerotic lesions can be studied independently of other factors that effect lesion formation (i.e, serum cholesterol). At the conclusion of the study, the ascending and descending aortas were removed and opened, and the area covered by atherosclerotic lesions quantified by image analysis (4, 5) (Figure 1). Animals treated with vehicle or α-tocopherol did not have significant lesions whereas animals treated with ritonavir developed significant lesions, as previously demonstrated by Dressman et al (5). Animals treated with ritonavir and α-tocopherol developed atherosclerotic lesions to the same extent as animals treated with ritonavir only. Importantly, two doses of α-tocopherol (400 and 800 mg/kg) did not protect animals from ritonavir-induced atherosclerotic lesion formation.

Effect of NRTIs on ritonavir-induced atherosclerosis. In HAART, patients are often treated with protease inhibitors in combination with other anti-retroviral drugs such as nucleoside reverse transcriptase inhibitors to increase the efficacy of anti-retroviral activity. Since we predicted that α-tocopherol would decrease protease inhibitor-induced atherosclerotic lesion size we also used two different nucleoside reverse transcriptase inhibitors, didanosine and D4T, presumably as negative controls that would not affect ritonavir-induced atherosclerosis. Six week old male LDLR null mice feed a standard chow diet were treated with didanosine (75 µg/mouse/day), D4T (75 µg/mouse/day), or ritonavir (50 µg/mouse/day) and didanosine or D4T
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(75 µg/mouse/day) in the drinking water for 8 weeks. Animals treated with didanosine or D4T alone did not develop significant atherosclerotic lesions (Figure 1). Surprisingly, animals treated with ritonavir and didanosine or D4T did not develop atherosclerotic lesions. To our knowledge these are the first data that indicate that nucleoside reverse transcriptase inhibitors can limit the atherogenic effects of ritonavir.

NRTIs prevent an increase in macrophage cholesterol and CD36 levels. Figure 1 clearly demonstrates that didanosine and D4T can protect mice from ritonavir-induced atherosclerosis, thus we next investigated the mechanism whereby protection is afforded by nucleoside reverse transcriptase inhibitors. We previously demonstrated that ritonavir induces the accumulation of cholesterol in macrophages and that cholesterol accumulation and atherosclerotic lesion formation was dependent on the up-regulation of CD36 (5). To determine if didanosine and D4T affected macrophage cholesterol accumulation and CD36 protein levels, peritoneal macrophages were isolated from mice treated as described in Figure 1. Consistent with previous studies (5), ritonavir increased the cholesterol associated with macrophages (Figure 2A) by 2-3 fold. Didanosine and D4T alone did not alter cholesterol levels compared to the vehicle control. However, didanosine and D4T both inhibited ritonavir-induced increases in macrophage cholesterol. Consistent with Figure 1 α-tocopherol did not affect macrophage cholesterol levels or prevent ritonavir-induced increases in macrophage cholesterol. Figure 2B illustrates that didanosine and D4T prevented ritonavir up-regulation of CD36 in macrophage. Again, α-tocopherol was ineffective at preventing ritonavir-induced up-regulation of CD36. Although it has been established that ritonavir induces the generation of atherosclerotic lesions in LDLR null mice by the up-regulation of CD36 (5) other proteins may effect lesion development. To determine if didanosine or D4T was altering other atherogenic proteins, we examined the levels of SR-BI and SRA. Importantly, none of the treatments altered the levels of SR-BI and SRA suggesting that these proteins were not involved in the protective effects of didanosine and D4T. The lower panels show that actin loads from the described samples and indicate equivalent protein loads in each well. These data suggested that the mechanism whereby didanosine and D4T prevented HIV protease inhibitor-induced atherosclerosis was by preventing the up-regulation of CD36 in macrophages.
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NRTIs promoted the loss of PKCα protein in macrophages. We previously used molecular and genetic models to demonstrate that ritonavir-induced CD36 up-regulation was mediated by a PKC-dependent increase in PPARγ which subsequently increased the expression of CD36 (5). To determine if didanosine and D4T prevented ritonavir-induced CD36 up-regulation and atherosclerotic lesion formation by a PPARγ mechanism we isolated mRNA from peritoneal macrophages obtained from LDLR null mice treated as described for Figure 1. Ritonavir increased PPARγ mRNA levels without altering PKCα mRNA levels (Figure 3A). Treatment with α-tocopherol did not inhibit ritonavir-induced up-regulation of PPARγ, in agreement with Figures 1 and 2. Importantly, didanosine and D4T prevented ritonavir-induced up-regulation of PPARγ mRNA. Since PKCα mRNA levels were not changed we next examined the levels of PKCα protein. Figure 3B demonstrates that didanosine and D4T in the absence or presence of ritonavir promoted the loss of PKCα protein while animals treated with α-tocopherol had normal levels of PKCα. These data demonstrated that didanosine and D4T caused the loss of PKCα which may be responsible for the inhibition of PPARγ up-regulation.

NRTIs promoted the ubiquitination of PKCα. The data shown in Figure 3 demonstrated that didanosine and D4T promoted the loss of PKCα protein without affecting the level of PKCα mRNA which suggested a post-translational mechanism is responsible for the loss of PKCα protein. A common mechanism to promote cytosolic protein degradation is the ubiquitination of proteins so that they are targeted to the proteasome. To determine if ubiquitination was involved in the loss of PKCα we immunoprecipitated PKCα from macrophages isolated from animals treated as described in Figure 1. Each immunoprecipitation was done with 500 µg of cell protein whereas only 20 µg of protein was analyzed in Figure 3 which likely accounts for the apparent lack of PKCα in Figure 3 and the presence of a small amount of PKCα in Figure 4. The precipitated material was then resolved by SDS-PAGE and immunoblotted using an anti-ubiquitin antibody. The amount of cross-reactive material was quantified by image analysis, normalized to the amount of actin in the original immunoprecipitation sample, and plotted in Figure 4A. The data demonstrate that didanosine and D4T...
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dramatically increased the amount of PKCα that has undergone ubiquitination. Importantly, the vehicle, ritonavir, and α-tocopherol samples did not affect PKCα ubiquitination. The non-NRTI treated samples contained approximately 90-fold more PKCα than the NRTI treated samples but had 5-8-fold less ubiquitinated PKCα (Figure 4B). These data suggested that didanosine and D4T prevented CD36 up-regulation by promoting the degradation of PKCα.

**In vivo inhibition of PKC prevents HIV protease inhibitor-induced increases in macrophage CD36.** Collectively, the data presented thus far suggest that didanosine and D4T protected against ritonavir-induced atherosclerosis by down-regulating PKCα and consequently preventing the up-regulation of CD36. To determine if the lack of functional PKCα can prevent ritonavir-induced atherosclerosis we treated six-week old LDLR null mice with 2.5 mg/kg chelerythrine, a PKC inhibitor along with ritonavir (50 µg/mouse/day) for 8 weeks. Peritoneal macrophages were isolated from the mice at the conclusion of the study and the extent of PKC activity determined with a commercially available enzymatic assay system. Figure 5A demonstrates that ritonavir increased PKC activity 6-fold above the vehicle control. Importantly, chelerythrine decreased basal PKC activity and ritonavir-induced PKC activity thereby demonstrating the effectiveness of chelerythrine at inhibiting macrophage PKC. Consistent with a decrease in PKC activity chelerythrine prevented ritonavir-induced up-regulation of PPARγ (Figure 5B) and an increase in the amount of CD36 protein (Figure 5C). These data support the concept that PKC is necessary for ritonavir-induced increases in CD36 protein levels.

**In vivo inhibition of PKCα prevents HIV protease inhibitor-induced atherosclerotic lesions.** The data suggested that didanosine and D4T inhibit ritonavir-induced atherosclerosis by causing the ubiquitination and down-regulation of PKCα. The PKC inhibitor, chelerythrine, prevented the up-regulation of PPARγ and CD36 in macrophage. To determine if the inhibition of PKC activity protected against ritonavir-induced atherosclerosis, LDLR null mice were treated with chelerythrine as described in Figure 5. Figure 6a demonstrates that chelerythrine prevented the accumulation of cholesterol in macrophages from mice treated with both ritonavir and chelerythrine. Dramatically, chelerythrine, like didanosine and D4T completely
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inhibited ritonavir-induced atherosclerosis. These data demonstrate that NRTI-induced depletion of PKC can account for the ability of NRTIs to prevent HIV protease inhibitor-induced atherosclerosis.
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DISCUSSION

The development of aspartyl endopeptidase protease inhibitors which catalyze the cleavage of the HIV gag and gag-pol polyproteins has been one of the most significant advances in HIV therapy (10, 22, 27). The usefulness of HIV protease inhibitors is tempered by significant side-effects, including lipodystrophy, hyperlipidemia, visceral adiposity, and insulin resistance (7, 10, 11, 17, 26). The ability of HIV protease inhibitors to interfere with the function of the proteasome results in an increase in sterol regulatory element binding proteins (SREBPs) and apoB both of which may contribute to lipodystrophy, hyperlipidemia, and cardiovascular diseases such as atherosclerosis (7, 10, 11, 17, 26). However, a study by Dressman et al (5) demonstrated that in mouse models, HIV protease inhibitors can promote atherosclerosis independent of changes in plasma lipid levels. The increase in atherosclerosis was shown to be dependent on the increase in macrophage CD36 protein levels (5). Studies by Munteanu et al (18, 19) confirmed that HIV protease inhibitors increase macrophage CD36 protein levels. This observation is potentially of clinical significance because reducing the dose of protease inhibitor to lessen the increase in plasma lipids may not correspond to a decrease in risk for cardiovascular disease.

The mechanism(s) whereby HIV protease inhibitors increase CD36 is not completely clear. Dressman et al (5) reported that HIV protease inhibitors increased PPARγ via a PKC intermediate signaling step. Treatment of macrophages with oxidized LDL has been shown to result in an increase in PPARγ activity and subsequently the expression of CD36 thus the involvement of PPARγ is a plausible mechanism (5, 6). In vitro studies with human peripheral blood monocytes demonstrated that PKC inhibitors prevented ritonavir-mediated up-regulation of CD36 (5). In addition, the addition of a PPARγ agonist could overcome the PKC block suggesting that PKC was upstream of PPARγ (5). In contrast, Munteanu et al (18, 19) stated that the level of PPARγ mRNA in the monocyte cell line, THP-1, was not affected by therapeutic doses of ritonavir. Munteanu et al (18, 19) provided data that inhibition of the proteasome with ALLN increased CD36 levels and they suggested that ritonavir-induced increases in CD36 are caused by decreased proteasome degradation of CD36. Since different cells and different concentrations of ritonavir were used in the two studies it is difficult to directly compare the
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data. However, at the present time both mechanisms are possible and in fact it is conceivable that both mechanisms may be in part responsible for the increase in macrophage CD36.

Studies by Munteanu et al (19) also indicated that relatively high levels of α-tocopherol (50 µM) could prevent ritonavir-induced increases in CD36 in THP-1 cells. The authors provided data demonstrating that α-tocopherol could partially relieve the ritonavir-mediated inhibition of the proteasome. Although α-tocopherol has been shown to be ineffective in preventing cardiovascular disease (28) it was entirely possible that α-tocopherol could prevent HIV protease inhibitor induced atherosclerosis. Consequently, we tested this exciting possibility in our mouse model. To our surprise and disappointment α-tocopherol, even at a very high concentration, did not prevent ritonavir-mediated atherosclerosis or increases in macrophage CD36 levels. Despite convincing data with THP-1 cells (19), in vivo studies with a mouse model, could not recapitulate the effect of α-tocopherol on macrophage CD36 protein levels. However, we made the unexpected observation that the nucleoside reverse transcriptase inhibitors, didanosine and D4T, did prevent ritonavir-induced atherosclerosis.

NRTIs are incorporated into the viral genome during replication but are not efficiently removed because the viral reverse transcriptase does not have a proofreading exonuclease. NRTIs are a critical component of HAART, however toxicity has been observed related to inhibition of mitochondrial DNA replication (14) and fat redistribution syndrome (21). In the current study, the rationale for treating cells with ritonavir and didanosine or D4T was, presumably as a negative control for the putative effects of α-tocopherol. Stunningly, the NRTIs prevented ritonavir-induced increases in macrophage CD36 levels and ritonavir-induced atherosclerosis formation. Consistent with Dressman et al (5), ritonavir induced an increase in PPARγ and the NRTIs prevented this ritonavir-induced increase. The NRTIs did not affect the level of PKCα mRNA, however the NRTIs promoted the loss of PKCα protein in the presence or absence of ritonavir. By using anti-ubiquitin antibodies and immunoprecipitation we demonstrated that the NRTIs promoted the ubiquitination of PKCα which led to
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degradation of the protein by the proteasome. The mechanism of how NRTIs play a role in the ubiquitination of PKCa is not known.

Previous studies by Gogu et al (8, 9) demonstrated that zidovudine, the first drug in the 2',3'-dideoxynucleoside class approved by the FDA for treatment of AIDS prevented erythroid progenitor cell differentiation by inhibiting PKC. Although these investigators did not demonstrate the mechanism of PKC inhibition their findings are consistent with our didanosine data demonstrating the degradation of PKC. Interestingly, Gogu et al (9) also demonstrated that α-tocopherol could overcome the inhibitory effect of zidovudine on erythroid progenitor cell differentiation. Although it was not demonstrated it is possible that α-tocopherol prevented the degradation of PKC. If α-tocopherol prevented the degradation of PKC, based upon our current data one would predict that animals receiving ritonavir, didanosine, and α-tocopherol would develop atherosclerosis because didanosine no longer caused the degradation of PKC. The interactions between HIV protease inhibitors, nucleoside reverse transcriptase inhibitors, and α-tocopherol are complex and still incompletely understood.

The current study along with the previous study by Dressman et al (9) strongly points to PKC as being a major regulatory point in the effects of protease inhibitors and NRTIs on the expression of macrophage CD36. To further explore the role of PKC we treated mice with chelerythrine, a PKC inhibitor, in the presence or absence of ritonavir. Since chelerythrine was only given to intact mice and the peritoneal macrophage isolated and immediately assayed for PKC activity this indicates that chelerythrine was effective at inhibiting PKC activity in vivo. Chelerythrine was effective at inhibiting PKC phosphorylation activity, PPARγ up-regulation, and an increase in CD36 protein levels. Importantly, chelerythrine prevented ritonavir-induced macrophage cholesterol accumulation and ritonavir-induced atherosclerotic lesion formation. It is important to point out that the ability of chelerythrine to prevent atherosclerosis is most likely limited to ritonavir-induced atherosclerosis and not atherosclerosis caused by the other multitude of risk factors.
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Although we were unable to confirm the protective effect of α-tocopherol with regards to ritonavir-induced increases in macrophage CD36 with our in vivo studies, we did discover that NRTIs can prevent an increase in macrophage CD36 levels. Interestingly, NRTIs increased the ubiquitination of PKC which resulted in the degradation of PKC and consequently prevented the up-regulation of PPARγ and the increase in macrophage CD36. This mechanism was surprising because it has been suggested that HIV protease inhibitor increases in CD36 are due to inhibition of the proteasome (18, 19). However, HIV protease inhibitor effects on the proteasome are complicated. For instance, Schmidtke et al (25) has demonstrated that ritonavir inhibited the chymotrypsin-like activity of the proteasome but increased the trypic activity of the proteasome. This finding offers one explanation as to why NRTIs could induce the degradation of PKC in the presence of HIV protease inhibitors. Alternatively, Piccinini et al (23) demonstrated that the use of a single protease inhibitor (as we did in the current studies) only had minimal impact on proteasome activity. In fact, Piccinini et al (23) showed that the use of three HIV protease inhibitors in combination only caused approximately 43% inhibition of the proteasome. Therefore another possible explanation for our results is that the concentration of ritonavir used in our studies did not sufficiently inhibit the proteasome to affect the degradation of PKC. Finally, Andre et al (1) has demonstrated that concentrations of ritonavir that do not affect proteasome function can inhibit the presentation of antigen to cytotoxic T lymphocytes, thus it is possible that HIV protease inhibitors and NRTIs impact PKC independent of the proteasome.

The mechanism whereby NRTIs promote the ubiquitination of PKC is unknown. Our current working hypothesis is that HIV protease inhibitors and NRTIs both impact on PKC and since PKC is upstream of PPARγ the ability of PPARγ to cause an increase in macrophage CD36 protein levels is increased by HIV protease inhibitors and decreased by NRTIs. This hypothesis does not exclude the contribution of other possible mechanisms nor should it be extended to other non-HIV drug mechanisms of effecting macrophage CD36 protein levels and atherosclerosis.

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FIGURE LEGENDS

Figure 1: Nucleoside reverse transcriptase inhibitors but not α-tocopherol prevented ritonavir-induced atherosclerosis. Six week old male LDLR null mice were fed a chow diet containing 0, 400, or 800 mg/kg of α-tocopherol and were treated with ritonavir (50 µg/mouse/day), vehicle control (0.01% ethanol), didanosine (75 µg/mouse/day), or D4T (75 µg/mouse/day) for 8 weeks as described in the text. At the conclusion of the study, the ascending and descending aortas were removed and opened, and the areas covered by atherosclerotic lesions quantified by image analysis (4, 5). Bars represent mean ± SE, n=15. *P<0.01 compared to vehicle, #P < 0.01 compared to ritonavir alone. Similar data were obtained with indinavir and amprenavir (data not shown) with a gradation of response seen as ritonavir > indinavir > amprenavir.

Figure 2: Didanosine and D4T prevent ritonavir-induced increases in macrophage cholesterol and CD36. Peritoneal macrophages were isolated from mice treated as described in Figure 1. A) Total cholesterol was quantified with a commercially available kit and data presented as total cholesterol per mg of cell protein. Bars represent mean ± SE, n=15. *P<0.01 compared to vehicle, #P < 0.01 compared to ritonavir alone. B) Cells were lysed and 20 µg of protein were resolved by SDS-PAGE, transferred to nylon, and Western blotted with antibodies for the indicated proteins. The proteins were detected by chemiluminescence. The exposure times for CD36 and SR-BI was 1 minute, SRA was 3 minutes, and actin was 0.5 minutes. Representative data from 8 mice are shown. Similar data were obtained with indinavir and amprenavir (data not shown) with a gradation of response seen as ritonavir > indinavir > amprenavir.

Figure 3: Didanosine and D4T promoted the loss of PKCα in macrophage. A) mRNA was isolated from peritoneal macrophages obtained from LDLR null mice treated as described for Figure 1. Northern blot analysis was then performed to detect mRNA for PPARγ, PKCα, and GAPDH by autoradiography. Representative data from 8 mice are shown. B) Peritoneal macrophages obtained from LDLR null mice treated as described for Figure 1 were lysed and 20 µg of protein were resolved by SDS-PAGE, transferred to nylon, and Western
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blotted with antibodies for the indicated proteins. The proteins were detected by chemiluminescence. The exposure time for PKC was 5 minutes and actin was 0.5 minutes. Representative data from 8 mice are shown. Similar data were obtained with indinavir and amprenavir (data not shown) with a gradation of response seen as ritonavir > indinavir > amprenavir.

Figure 4: Didanosine and D4T promoted the loss of PKC in macrophage. Peritoneal macrophages obtained from LDLR null mice treated as described for Figure 1 were lysed and 500 µg of total protein was immunoprecipitated with 3 µg of PKC IgG. A) The precipitated material was then resolved by SDS-PAGE and immunoblotted using an anti-ubiquitin antibody. The amount of cross-reactive material was quantified by densitometry and normalized to the amount of actin in the original immunoprecipitation sample. The data are presented as the relative intensity of the ratio ubiquitinated PKC (ubi-PKC) to actin. Bars represent mean ± SE, n=15. *P<0.01 compared to vehicle or ritonavir alone, #P < 0.01 compared to NRTI alone. B) Equal amounts of the immunoprecipitated material were Western blotted for PKC and ubiquitinated PKC. In addition, 20 µg of the starting lysate material was Western blotted for actin. Similar data were obtained with indinavir and amprenavir (data not shown) with a gradation of response seen as ritonavir > indinavir > amprenavir.

Figure 5: In vivo inhibition of PKC prevents HIV protease inhibitor-induced increases in macrophage. Six-week old LDLR null mice were treated with 2.5 mg/kg chelerythrine, a PKC inhibitor, along with ritonavir (50 µg/mouse/day) for 8 weeks. Peritoneal macrophages were isolated from the mice at the conclusion of the study. A) The extent of PKC activity was determined using a commercially available enzymatic assay system (see Methods). Bars represent mean ± SE, n=6. *P<0.01 compared to vehicle, #P < 0.01 compared to ritonavir alone. Note both chelerythrine and ritonavir + chelerythrine were significantly different than vehicle only (P<0.01). B) mRNA was isolated from peritoneal macrophages obtained from LDLR null mice treated as described above. Northern blot analysis was then performed to detect mRNA for PPARγ, PKC, and GAPDH by autoradiography. Representative data from 6 mice are shown. C) Peritoneal macrophages obtained from
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LDLR null mice treated as described above were lysed and 20 µg of protein were resolved by SDS-PAGE, transferred to nylon, and Western blotted with antibody for the indicated protein. The proteins were detected by chemiluminescence. The exposure times for CD36 and SR-BI was 1 minute, SRA was 3 minutes, and actin was 0.5 minutes. Representative data from 6 mice are shown. Similar data were obtained with indinavir and amprenavir (data not shown) with a gradation of response seen as ritonavir > indinavir > amprenavir.

Figure 6: In vivo inhibition of PKCα prevents HIV protease inhibitor-induced atherosclerotic lesions.

Six-week old LDLR null mice were treated with 2.5 mg/kg chelerythrine, a PKC inhibitor along with ritonavir (50 µg/mouse/day) for 8 weeks. Peritoneal macrophages were isolated from the mice at the conclusion of the study. A) Total cholesterol was quantified with a commercially available kit and data presented as total cholesterol per mg of cell protein. Bars represent mean ± SE, n=15. *P<0.01 compared to vehicle, †P < 0.01 compared to ritonavir alone. B) At the conclusion of the study, the ascending and descending aortas were removed and opened, and the areas covered by atherosclerotic lesions quantified by image analysis (4, 5). Bars represent mean ± SE, n=15. *P<0.01 compared to vehicle, †P < 0.01 compared to ritonavir alone. Similar data were obtained with indinavir and amprenavir (data not shown) with a gradation of response seen as ritonavir > indinavir > amprenavir.
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Figure 4

A) Relative Intensity of Ubi-PKCα/Actin (arbitrary units)

B) Western Blot Analysis:
- PKCα
- Ubi-PKCα
- Actin

Vehicle, Ritonavir, α-Tocopherol, Ritonavir + α-Tocopherol, Didanosine, Ritonavir + Didanosine, DHT, Ritonavir + DHT