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HIV protease inhibitors promote atherosclerotic lesion formation independent of dyslipidemia by increasing CD36-dependent cholesteryl ester accumulation in macrophages

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Protease inhibitors decrease the viral load in HIV patients, however the patients develop hypertriglyceridemia, hypercholesterolemia, and atherosclerosis. It has been assumed that protease inhibitor–dependent increases in atherosclerosis are secondary to the dyslipidemia. Incubation of THP-1 cells or human PBMCs with protease inhibitors caused upregulation of CD36 and the accumulation of cholesteryl esters. The use of CD36-blocking antibodies, a CD36 morpholino, and monocytes isolated from CD36 null mice demonstrated that protease inhibitor–induced increases in cholesteryl esters were dependent on CD36 upregulation. These data led to the hypothesis that protease inhibitors induce foam cell formation and consequently atherosclerosis by upregulating CD36 and cholesteryl ester accumulation independent of dyslipidemia. Studies with LDL receptor null mice demonstrated that low doses of protease inhibitors induce an increase in the level of CD36 and cholesteryl ester in peritoneal macrophages and the development of atherosclerosis without altering plasma lipids. Furthermore, the lack of CD36 protected the animals from protease inhibitor–induced atherosclerosis. Finally, ritonavir increased PPAR-γ and CD36 mRNA levels in a PKC- and PPAR-γ–dependent manner. We conclude that protease inhibitors contribute to the formation of atherosclerosis by promoting the upregulation of CD36 and the subsequent accumulation of sterol in macrophages.

Introduction

The Swiss HIV Cohort Study established a definite link between HIV protease inhibitors and dyslipidemia (1). HIV protease inhibitor–induced dyslipidemia is characterized primarily by elevations in plasma triglyceride levels and (to a lesser extent) plasma cholesterol levels (1–4). Stein et al. (5) documented that HIV protease inhibitors promote the formation of atherogenic lipoproteins and endothelial dysfunction. In addition, a recent report by Tabib et al. (6) documents accelerated coronary atherosclerosis and arteriosclerosis in young HIV-positive patients. This has led to the supposition that HIV protease inhibitor–induced dyslipidemia is a significant clinical concern and may contribute to accelerated atherosclerotic plaque formation (7–9). However, multiple mechanisms are involved in the initiation, progression, and rupture of atherosclerotic lesions (10–12), and although elevated plasma triglyceride and cholesterol levels are an important component of the disease process, these lipid factors fail to account for a significant number of clinically relevant atherosclerotic lesions, thereby indicating that other mechanisms must be involved (13).

The generation of lipid-laden macrophages is a key event in atherogenesis and is thought to be due in part to unregulated uptake of modified lipoproteins (14, 15). Aberrant sterol accumulation is influenced by scavenger receptors such as CD36 (16, 17). The exact role of CD36 in the development of atherosclerotic lesions is unclear; however, a natural polymorphism in the *CD36* gene in humans indicates that CD36 is likely to be an important receptor for the metabolism of lipoprotein in vivo (18). Monocytes/macrophages obtained from patients with a CD36 polymorphism bound less oxi-

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Nonstandard abbreviations used: scavenger receptor A (SRA); tris-buffered saline (TBS); LDL receptor (LDLR); disintegrations per minute (dpm).

dized LDL and accumulated less sterol than did control patients (19). Recent studies with CD36 null mice have confirmed the initial human findings and demonstrated that the presence of CD36 in macrophages promotes the accumulation of sterol and the formation of atherosclerotic lesions (16, 17).

To date the direct effects of HIV protease inhibitors on macrophage sterol accumulation have not been studied. The present studies use both in vitro and in vivo models to demonstrate that HIV protease inhibitors induce a specific increase in macrophage CD36 levels and that this promotes an increase in the accumulation of macrophage cholesteryl ester and atherosclerotic lesion formation. More importantly, the data demonstrate that HIV protease inhibitors can increase atherosclerotic lesion formation independent of frank dyslipidemia. The importance of these findings with regard to the therapeutic treatment of patients with HIV protease inhibitors is discussed.

Methods

Materials. RPMI 1640, DMEM high glucose medium, FCS, L-glutamine, trypsin-EDTA, penicillin-streptomycin, and OptiPrep were purchased from Life Technologies Inc. (Grand Island, New York, USA). Percoll, PVDF membrane, and Tween 20 were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Bradford reagent was purchased from BioRad Laboratories (Hercules, California, USA). The analytical silica gel TLC plates, heptane, petroleum ether, ethyl ether, acetic acid, and 2-propanol were from Fisher Scientific Co. (Pittsburgh, Pennsylvania, USA). [³H]acetate (specific activity 5.11 Ci/mmol) was from DuPont NEN Life Science Products (Boston, Massachusetts, USA). Anti–caveolin-1 IgG was obtained from BD Biosciences (San Diego, California, USA), anti-actin IgG was from Sigma-Aldrich, anti–mouse CD36 (IgM) was from BioDesign International Inc. (Kennebunkport, Maine, USA), and anti–scavenger receptor A (anti-SRA) was from Serotec Ltd. (Raleigh, North Carolina, USA) HRP-conjugated IgG's were supplied by Organon Teknika Corp. USA (West Chester, Pennsylvania, USA). Super Signal chemiluminescent substrate was purchased from Pierce Chemical Co. (Rockford, Illinois, USA). The analytical silica gel TLC plates, heptane, petroleum ether, ethyl ether, acetic acid, and 2-propanol were from Fisher Scientific Co. Glaxo Wellcome Inc. (Toronto, Ontario, Canada) provided the amprenavir, Merck and Co. Inc. (Whitehouse Station, New Jersey, USA) provided the indinavir, and Abbott Laboratories (Abbott Park, Illinois, USA) provided the ritonavir. The mouse feed was obtained from Harlan Teklad Laboratory (Madison, Wisconsin, USA). The triglyceride determination kit was from Wako Chemicals USA Inc. (Richmond, Virginia, USA). The CD36 morpholino (5′-ATGGGCTGTGACCGGAACTGTGGGC-3′) was from GeneTools LLC (Philomath, Oregon, USA). The LDLR and C57BL/6 mice came from The Jackson Laboratory (Bar Harbor, Maine, USA). The CD36 null mice, apoE null mice, and apoE/CD36 null mice were provided by Maria Febbraio (Weill Medical College of Cornell University, New York, New York, USA). All the animals were backcrossed into the C57BL/6 strain ten times.

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

Cell culture. THP-1 cells were grown in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine. The medium was changed every 48 hours. THP-1 cells were also grown in the presence of 100 nM PMA for 72 hours to induce differentiation to a macrophage phenotype (20). Human PBMCs were isolated as described (21). Aggregated LDL (50 μg/ml) was added to the culture medium as the source of exogenous modified LDL. To prepare aggregated LDL, 2 mg freshly isolated LDL was diluted in 1 ml PBS and vortexed for 60 seconds as described by Khoo et al. (22). Mouse peritoneal macrophages were isolated as described (23).

Cholesterol and cholesteryl ester mass quantification. Cholesteryl heptadecanoate was added to each vessel preparation to serve as an internal standard and the samples were then extracted with isopropanol-hexane (24). The extracted lipid was derivatized by suspending the dried lipid in N,O-bis(trimethylsilyl)trifluoroacetamide, trimethylchlorosilane, and acetonitrile (89:1:10). The material was heated at 80°C for 5 minutes, dried, suspended in iso-octane, and used for gas chromatography (Protocol T496125B, Supelco; Sigma-Aldrich). Authentic cholesterol (Sigma-Aldrich) was dissolved in iso-octane and used as a standard for the retention time of cholesterol. The samples were injected (splitless) into a 6890 gas chromatograph G2579A system (Agilent Technologies, Palo Alto, California, USA) equipped with an SGE HT5 aluminum-clad fused silica capillary column (12 m × 0.32 mm × 0.1 μm; Sigma-Aldrich). The gas chromatography temperature program was as follows: initial temperature was 220°C for 3 minutes. This was increased to 310°C (20°C increase/min) and then to 400°C (10°C/min) and held for 3.5 minutes. A mass-selective detector (model 5973; Agilent Technologies) was used in both scan and selected ion-monitoring modes to identify the samples.

SDS-PAGE and immunoblotting. Samples were concentrated by trichloroacetic acid precipitation and washed in acetone. Pellets were suspended in sample buffer containing 1.2% (vol/vol) β-mercaptoethanol and heated at 95°C for 3 minutes before being loaded onto gels. Proteins were separated in a 12.5% SDS-polyacrylamide gel using the method of Laemmli (25). The separated proteins were then transferred to PVDF membranes. Each membrane was blocked in TBS containing 5% dry milk for 1 hour at room temperature. Primary antibodies were diluted in TBS containing 1% dry milk and incubated with the PVDF membrane for 1 hour at room temperature. The PVDF membrane was washed four times, 10 minutes each, in TBS plus 1% dry milk.

The secondary antibodies (all conjugated to HRP) were diluted 1:20,000 in TBS plus 1% dry milk and incubated with the PVDF membrane for 1 hour at room temperature. The membrane was then washed and the bands were visualized by chemiluminescence.

Quantification of atherosclerotic lesions. Six-week-old male LDLR receptor (LDLR) null mice (The Jackson Laboratory) on a chow diet were given vehicle control (0.01% ethanol) or the following protease inhibitors in their drinking water: amprenavir (23 or 75 μg/mouse/day), indinavir (25 or 75 μg/mouse/day), or ritonavir (10 or 50 μg/mouse/day). After 0, 4, or 8 weeks of treatment, plasma was collected for quantification of cholesterol, cholesteryl ester, and triglyceride. Six-week-old male apoE null and apoE/CD36 double null mice were fed a chow diet and given vehicle (0.01% ethanol) or ritonavir (10 μg/mouse/day) in their drinking water for 6 weeks. The HDL cholesterol levels in all of the mice were 52 ± 9 mg/dl. The mice were then processed to quantify the surface area of atherosclerotic lesions. To quantify the lesions (26, 27), the aorta from the arch to the ileal bifurcation was collected, the extraneous tissue was dissected away, and the intimal surfaces were exposed by a longitudinal cut. The aortas were placed under a dissecting microscope equipped with a CCD camera attachment that captures the image in 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 atherosclerotic lesions were quantified with ImagePro software (Media Cybernetics, Silver Spring, Maryland, USA).
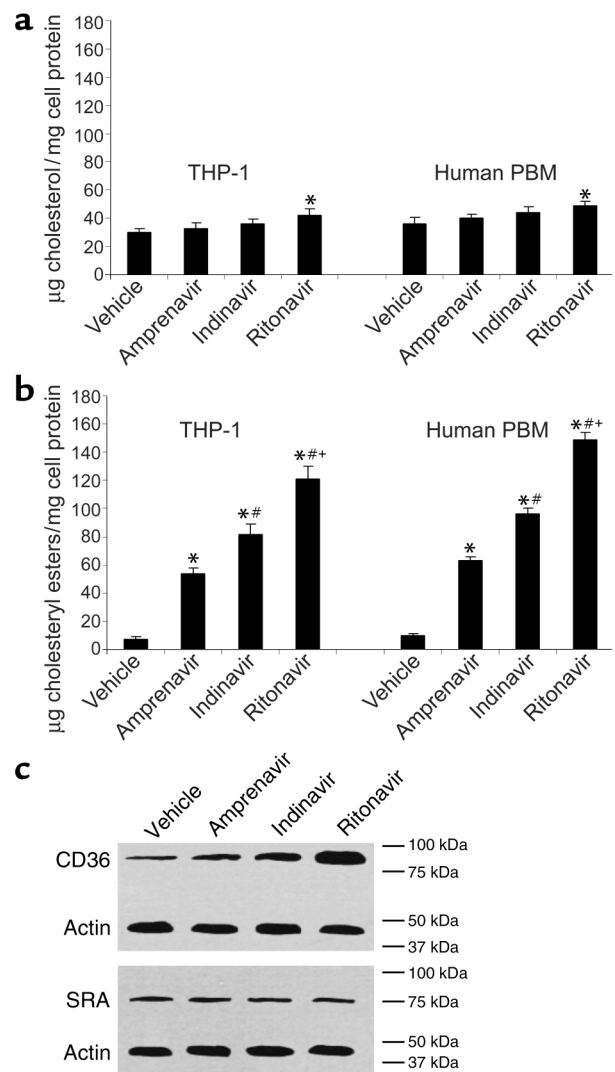
Statistics. Least squares ANOVA was used to evaluate the data with respect to sample, treatment, and time using the ANOVA procedure of Statistica (StatSoft, Tulsa, Oklahoma, USA). When appropriate, samples were compared within a given time using the Tukey HSD test. Means were considered different at $P < 0.01$.

Results

HIV protease inhibitors induce THP-1 cells and human PBMCs to accumulate cholesteryl esters. HIV protease inhibitors are thought to influence the development of cardiovascular disease primarily through increasing plasma triglycerides and plasma cholesterol levels (7). However, the direct effects of HIV protease inhibitors on macrophages have not been examined in detail. Because one of the prominent features of atherosclerotic lesions is the presence of lipid-laden macrophages (28), we determined whether HIV protease inhibitors could directly affect the amount of cellular cholesterol or cholesteryl esters associated with THP-1 cells, a model human monocyte/macrophage cell line (20), and with isolated human PBMCs. The cells were incubated in the presence of 30 ng/ml of amprenavir, indinavir, ritonavir, or vehicle for 24 hours along with 50 μg/ml of aggregated LDL (an exogenous cholesterol source), and then the total amounts of cholesterol and cholesteryl esters associated with the cells were determined by gas chromatography (29). Figure 1a demonstrates that only ritonavir treatment caused a signifi-
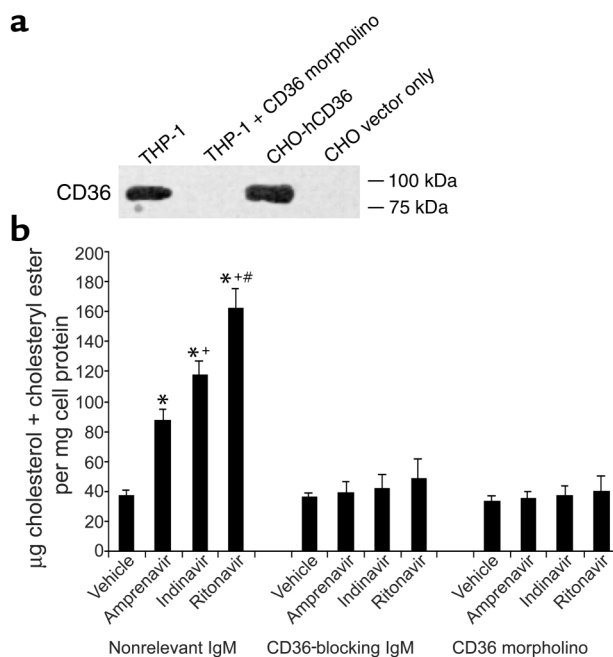

Figure 1
HIV protease inhibitors induce the accumulation of cholesteryl ester in THP-1 macrophages and human PBMCs. The human monocyte/macrophage cell line, THP-1, was cultured in 100 nM PMA for 72 hours to promote attachment and differentiation of the cells to a macrophage phenotype. In addition, we used freshly isolated and cultured human PBMCs (37) in these studies. The cells were incubated in the presence of 10% serum and 50 μg/ml of aggregated LDL along with 30 ng/ml of amprenavir, indinavir, ritonavir, or vehicle (ethanol) for 24 hours. The cells were lysed, lipids extracted, and processed to quantify total cellular cholesterol (**a**) or total cellular cholesteryl ester (**b**) by gas chromatography. Bars represent mean ± SE, $n = 4$ with triplicate measurements. *$P < 0.01$ compared with vehicle, #$P < 0.01$ compared with amprenavir, +$P < 0.01$ compared with indinavir. (**c**) THP-1 cells were lysed and 20 μg of protein was resolved by SDS-PAGE and immunoblotted with antibodies for CD36, SRA, and actin. Cross-reactive material was visualized by chemiluminescence. The exposure time was 2 minutes. The data are representative of five independent experiments. Essentially identical immunoblots were generated with human PBMCs (data not shown).
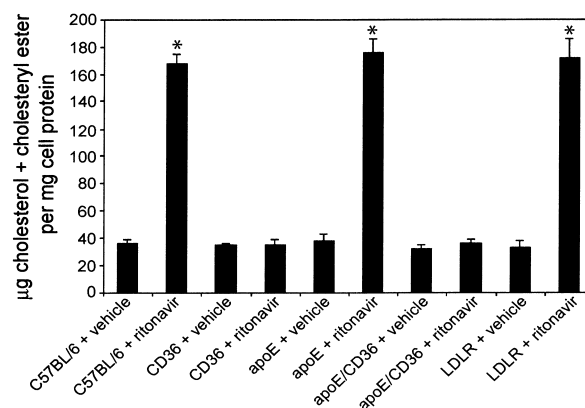
a

CD36 — 100 kDa — 75 kDa

b

cant increase in cellular free cholesterol. In contrast, Figure 1b illustrates that compared with the vehicle control (0.01% ethanol), each of the protease inhibitors significantly increased the amount of cell-associated cholesteryl ester. Interestingly, the increase in the amount of cell-associated cholesteryl ester correlated with the degree of dyslipidemia affiliated with the drugs, with amprenavir causing the least increase, followed by indinavir and then ritonavir (1, 30).

CD36 plays a role in HIV protease inhibitor–induced sterol accumulation. Multiple mechanisms can alter cellular cholesteryl ester levels, including new sterol synthesis and uptake of exogenous sterol from lipoproteins. To test whether HIV protease inhibitors were inducing new sterol synthesis, THP-1 cells were treated with vehicle or 30 ng/ml of amprenavir, indinavir, or ritonavir in the presence of [³H]acetate (31). The cells were then processed to isolate sterols and the amount of new sterol synthesized was determined by TLC (29). Each of the treatment groups synthesized 42,000 ± 2,401 disintegrations per minute (dpm) sterol per mg cell protein per 24 hours, indicating that global sterol synthesis was not altered. We next determined whether HIV protease inhibitors altered the levels of CD36 and SRA, two receptors involved in the uptake of exogenous lipoproteins and the formation of foam cells (15). THP-1 cells were treated as described above and analyzed by SDS-PAGE and immunoblot. We found that CD36 protein levels were increased in the presence of the HIV protease inhibitors, whereas the levels of SRA were not affected (Figure 1c). In addition, the relative increase in the level of CD36 (with amprenavir, 3.4-fold; indinavir, 6.2-fold; ritonavir, 13.1-fold) approximated the measured increase in cholesteryl ester accumulation (compare Figure 1b to Figure 1c). The actin immunoblots indicate that equivalent amounts of protein were applied to each lane.

To determine whether the increase in CD36 protein levels played a role in the increase in cholesteryl ester accumulation, CD36-blocking antibodies and a CD36 morpholino (which blocks transcription) were used. Figure 2a demonstrates that the CD36 antibody cross-reacts with a 90-kDa protein in CHO cells transfected with cDNA encoding human CD36 but not with CHO cells transfected with vector only. THP-1 cells contain the same 90-kDa cross-reacting band, and importantly, THP-1 cells treated with the CD36 morpholino did not produce the 90-kDa band. These data demonstrate

that the CD36-blocking antibody recognizes human CD36 and that treatment with a CD36 morpholino decreases CD36 protein below the level of detection. THP-1 cells were then incubated in the presence of 30 ng/ml of amprenavir, indinavir, or ritonavir for 24 hours along with CD36-blocking antibodies (20 μg/ml), nonrelevant isotype–matched antibodies (20 μg/ml), or a CD36 morpholino (25 nmol). At the end of the incubation period, the total amount of cholesterol and cholesteryl esters associated with the cells was determined by gas chromatography. Figure 2b demonstrates that the nonrelevant IgM antibody did not affect the ability of the protease inhibitors to induce sterol accumulation. However, both the CD36-blocking antibody and the CD36 morpholino completely inhibited the ability of the protease inhibitors to induce sterol accumulation.

The use of blocking CD36 antibodies and a CD36 morpholino are pharmacological approaches to demonstrate the involvement of CD36 in HIV protease–induced increases in macrophage cholesteryl ester. To be certain that an increase in CD36 protein levels is required for the increase in macrophage cholesteryl esters, we isolated peritoneal macrophages from various mouse models and then treated the isolated cells with HIV protease inhibitors as described above. After the treatment period the cells were processed and the amount of cholesterol and cholesteryl ester associated with the cells was quantified. Fig-

ure 3 demonstrates that treatment with vehicle (0.01% ethanol) did not alter the level of sterol in macrophages isolated from any of the mouse strains. Macrophages isolated from C57BL/6, apoE null, and LDLR null mice all accumulated sterol in response to treatment with ritonavir. In contrast, macrophages isolated from mice lacking CD36 (CD36 null and apoE/CD36 null mice) did not accumulate sterol in the presence of HIV protease inhibitors. These data strongly support the conclusion that CD36 was required for HIV protease inhibitor–induced increases in macrophage sterol levels.

HIV protease inhibitors promote the generation of lipid-laden macrophages independent of dyslipidemia. The in vitro data established that HIV protease inhibitors increase the cellular level of macrophage cholesteryl esters in a CD36-dependent manner. These data implied that frank dyslipidemia is not necessary to generate lipid-laden macrophages and subsequently atherosclerotic lesions. To directly test this prediction, two doses of amprenavir, indinavir, or ritonavir were given via the drinking water to LDLR null mice for 0, 4, or 8 weeks. The LDLR null mice were maintained on a normal chow diet, thereby greatly alleviating diet-induced dyslipidemia and diet-induced atherosclerotic lesion formation. The lower doses of protease inhibitors did not alter plasma triglyceride (Figure 4a) or cholesterol/cholesteryl ester (Figure 4b) levels, whereas the higher doses of inhibitors induced a pronounced increase in
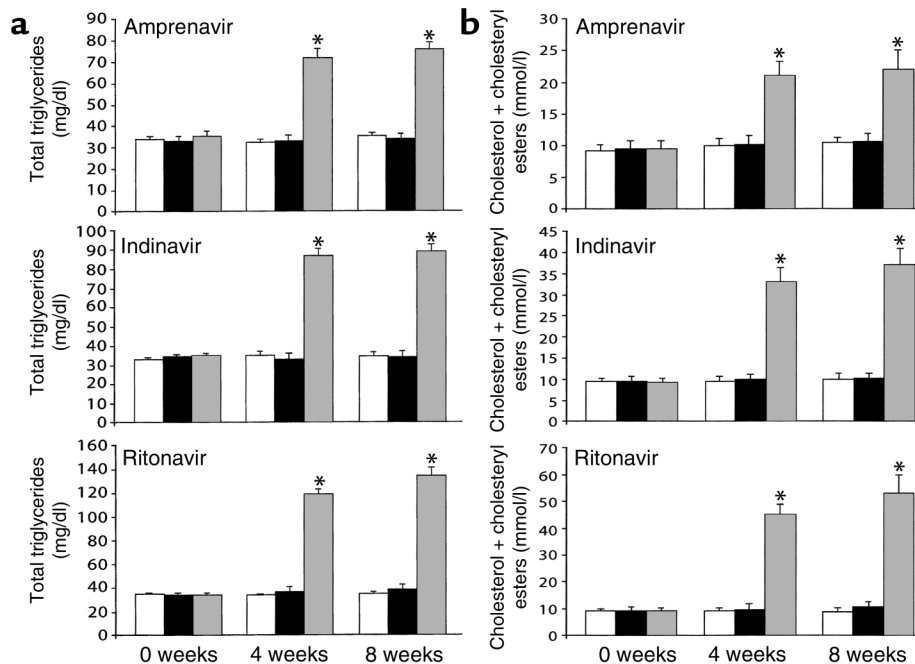
Figure 4
Effect of HIV protease inhibitors on plasma lipids. Six-week-old male LDLR null mice on a chow diet were given vehicle control (0.01% ethanol) or the following protease inhibitors in their drinking water: amprenavir (23 or 75 μg/mouse/day), indinavir (25 or 75 μg/mouse/day), or ritonavir (10 or 50 μg/mouse/day). The total plasma triglyceride (**a**) and cholesterol/cholesteryl ester (**b**) levels were determined after 0, 4, and 8 weeks of treatment with a commercial kit (Wako Chemicals USA Inc.) or gas chromatography. Bars represent mean ± SE, *n* = 8. *$P < 0.01$ compared with vehicle. White bars are vehicle controls, black bars are low-dose protease inhibitors, and gray bars are high-dose protease inhibitors.
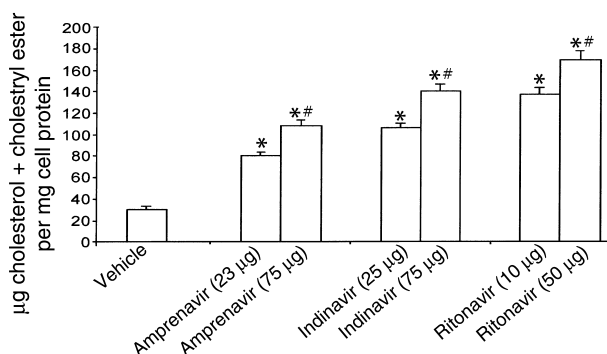
Figure 5

HIV protease inhibitors induce an increase in peritoneal macrophage cholesterol/cholesteryl ester levels. Six-week-old male LDLR null mice on a chow diet were given vehicle control (0.01% ethanol) or the following protease inhibitors in their drinking water: amprenavir (23 or 75 μg/mouse/day), indinavir (25 or 75 μg/mouse/day), or ritonavir (10 or 50 μg/mouse/day). After 8 weeks of treatment, peritoneal macrophages were isolated and cholesterol/cholesteryl ester mass was determined by gas chromatography. Bars represent mean ± SE, $n = 8$. *$P < 0.01$ compared with vehicle, #$P < 0.01$ compared with low dose of the same protease inhibitor.

plasma triglyceride and cholesterol/cholesteryl ester levels. These data demonstrate that we established doses of HIV protease inhibitors that promote dyslipidemia and doses that do not promote dyslipidemia, in the same animal model.

We next determined whether HIV protease inhibitors altered the level of cholesterol/cholesteryl ester associated with macrophages in vivo by isolating peritoneal macrophages (23) from the same group of mice described above and quantifying the level of cholesterol/cholesteryl ester by gas chromatography (29). All three protease inhibitors induced an increase in the level of peritoneal macrophage sterol compared with vehicle-treated animals (Figure 5). Importantly, the lower doses of protease inhibitors caused a dramatic increase in macrophage sterol levels without a concomitant increase in plasma lipids (Figure 4), whereas the higher doses increased plasma lipids (Figure 4) and further increased macrophage sterol levels.

The in vitro data demonstrated that HIV protease inhibitors increased the expression of CD36 in THP-1 cells. To determine whether HIV protease inhibitors stimulated an increase in the level of macrophage CD36 protein in vivo, we isolated peritoneal macrophages from LDLR null mice, generated cell lysates, and analyzed the proteins by SDS-PAGE and immunoblotting. Figure 6a demonstrates that HIV protease inhibitors did not alter the expression of SRA or actin. However, the inhibitors dramatically increased the amount of CD36 both at the low and high doses of inhibitor. In addition, the relative increase in the level of CD36 (with amprenavir, 3.1-fold; indinavir, 5.3-fold; ritonavir, 12.9-fold) approximated the measured increase in cholesteryl ester accumulation (see Figure 5). We next determined whether the HIV protease

inhibitors specifically affected macrophage CD36 protein levels or whether they also increased CD36 protein levels in other tissues. We isolated cardiac myocytes, adipocytes, and platelets and analyzed the levels of CD36 by immunoblot. HIV protease inhibitors did not alter the levels of CD36 in cardiac myocytes, adipocytes, or platelets, suggesting that these compounds specifically alter CD36 levels in macrophages (Figure 6b).

Because HIV protease inhibitors induced macrophages to accumulate sterol, we next determined whether HIV protease inhibitors promoted the formation of atherosclerotic lesions. To directly determine this, two doses of amprenavir, indinavir, or ritonavir were given in the drinking water to LDLR null mice for 8 weeks. At the conclusion of the study, the ascending and descending aortas were removed and opened, and the areas covered by lesions were
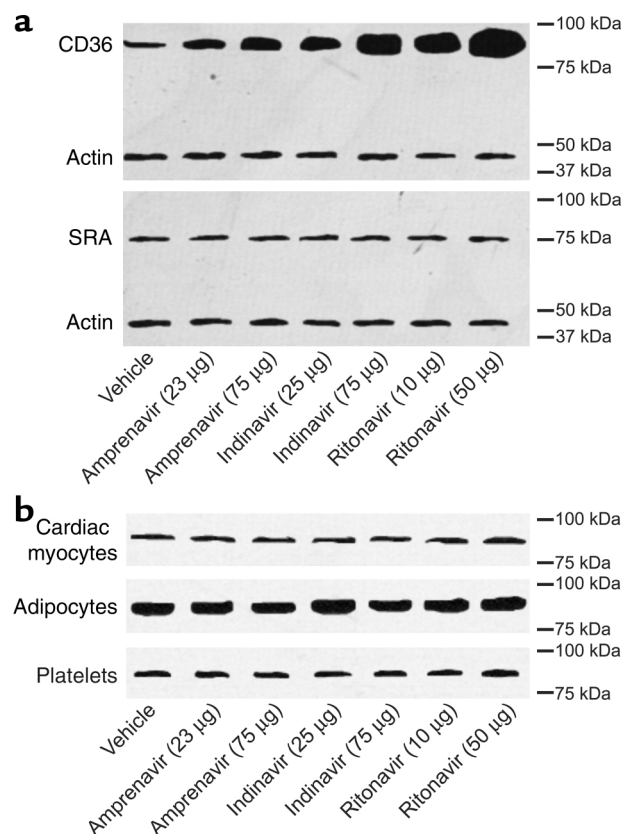
Figure 6

HIV protease inhibitors induce an increase of CD36 protein levels in peritoneal macrophages. Six-week-old male LDLR null mice on a chow diet were given vehicle control (0.01% ethanol) or the following protease inhibitors in their drinking water: amprenavir (23 or 75 μg/mouse/day), indinavir (25 or 75 μg/mouse/day), or ritonavir (10 or 50 μg/mouse/day). (**a**) After 8 weeks of treatment, peritoneal macrophages were isolated, the cells were lysed, and 20 μg of protein was resolved by SDS-PAGE and immunoblotted with antibodies for CD36, SRA, and actin. The data are representative of eight mice. (**b**) After 8 weeks of treatment, cardiac myocytes (38), adipocytes (39), and platelets (40) were isolated, the cells were lysed, and 20 μg of protein was resolved by SDS-PAGE and immunoblotted with antibodies against CD36. The data are representative of eight mice.
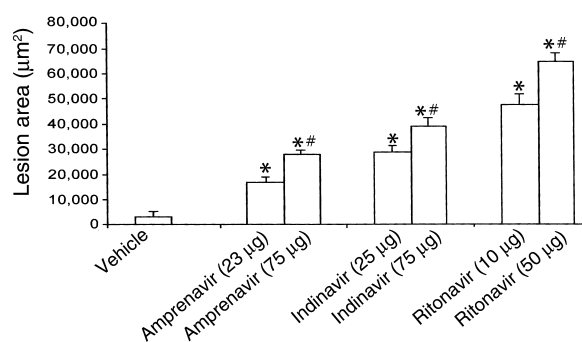
Figure 7

HIV protease inhibitors induced the formation of atherosclerotic lesions in LDLR null mice. Six-week-old male LDLR null mice on a chow diet were given vehicle control (0.01% ethanol) or the following protease inhibitors in their drinking water: amprenavir (23 or 75 μg/mouse/day), indinavir (25 or 75 μg/mouse/day), or ritonavir (10 or 50 μg/mouse/day). These mice were then used to quantify the surface area of atherosclerotic lesions. To quantify lesions, we first removed the aorta from the arch to the ileal bifurcation and dissected away extraneous tissue. The intimal surfaces were exposed by a longitudinal cut. The aortas were placed under a dissecting microscope equipped with a video camera attachment that captures the image in 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 atherosclerotic lesions were quantified with ImagePro software. This software analyzes differences in contrast to identify areas covered by lesions. Bars represent mean ± SE, $n = 8$. *$P < 0.01$ compared with vehicle, #$P < 0.01$ compared with low dose of the same protease inhibitor.

quantified by image analysis (26, 27). All of the animals treated with HIV protease inhibitors had significantly greater lesion area than did vehicle-treated animals (Figure 7). Animals treated with amprenavir had the smallest increase in lesion area and those treated with ritonavir had the largest increase in lesion area, consistent with the relative increase observed for CD36 and sterol levels in peritoneal macrophages. In addition, the higher doses of protease inhibitors caused an additional increase in lesion area compared with lower doses of the same protease inhibitors.

The data suggested that HIV protease inhibitors induced the upregulation of macrophage CD36, which then promoted the formation of atherosclerotic lesions independent of dyslipidemia. To directly test whether the increase in CD36 was responsible for the increase in atherosclerotic lesion formation, apoE null and apoE × CD36 double null mice were fed a chow diet and given vehicle or ritonavir (10 μg/mouse/day) in their drinking water for 6 weeks. The dose of ritonavir used did not induce dyslipidemia greater than that seen in vehicle-treated mice (data not shown). At the conclusion of the study, atherosclerotic lesion area was quantified in the ascending and descending aortas by image analysis (Figure 8). Vehicle-treated apoE null and apoE × CD36 double null mice had relatively small lesions, and importantly, the extent of the lesions was similar in both types of animals. Ritonavir-treated apoE null mice had a substantial increase in

lesion area compared with vehicle-treated control mice. In contrast, ritonavir did not induce atherosclerotic lesions in apoE × CD36 double null mice.

HIV protease inhibitors increase CD36 and PPAR-γ mRNA in a PKC-dependent manner. Previous studies have demonstrated that CD36 expression can be upregulated through activation of the transcription factor PPAR-γ (32). In addition, earlier studies have demonstrated that PKC is involved in the pathway responsible for the upregulation of CD36 expression (33). To determine whether ritonavir induced an increase in CD36 and PPAR-γ expression in a manner dependent on PKC, we treated human PBMCs as indicated and analyzed the amount of CD36 and PPAR-γ mRNA by Northern blot. Figure 9 demonstrates that ritonavir or aggregated LDL by themselves did not alter CD36 or PPAR-γ mRNA levels; however, the combination of ritonavir and aggregated LDL increased CD36 mRNA levels by 22-fold and PPAR-γ levels by 18-fold. Diacylglycerol (DAG) (20 μM) and PMA (100 nM), activators of PKC, stimulated an increase in CD36 and PPAR-γ mRNA independent of ritonavir. Importantly, two different PKC inhibitors, Go6976 (500 nM) and calphostin C (200 nM), inhibited the ability of ritonavir to increase CD36 and PPAR-γ mRNA. Finally, two different PPAR-γ activators, 15d-PGJ2 (1 μM) and ciglitazone (13 μM), stimulated the expression of CD36 and PPAR-γ mRNA, and the increase in expression was blocked by PKC inhibitors.

Discussion

The clinical data suggest that HIV protease inhibitors may promote accelerated atherosclerosis (6, 34, 35), and the current data provide one possible mechanism for this acceleration. Our studies demonstrate that three different HIV protease inhibitors, ritonavir, indinavir, and amprenavir, can increase the level of CD36 protein and the level of cholesteryl ester in THP-1 macrophages and human PBMCs. Importantly, the increase in cholesteryl ester accumulation was eliminated in the presence of CD36-blocking antibodies and a CD36 mor-
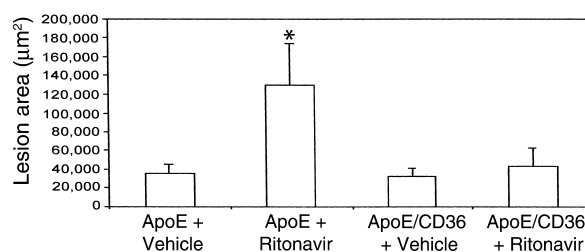
Figure 8

Ritonavir did not induce formation of atherosclerotic lesions in apoE × CD36 null mice. Six-week-old male apoE null and apoE × CD36 double null mice were fed a chow diet and given vehicle (0.01% ethanol) or ritonavir (10 μg/mouse/day) in their drinking water for 6 weeks. At the conclusion of the study the extent of atherosclerotic lesions was quantified as above. Data are presented as mean ± SE. $n = 12$. *$P < 0.01$ compared with vehicle.
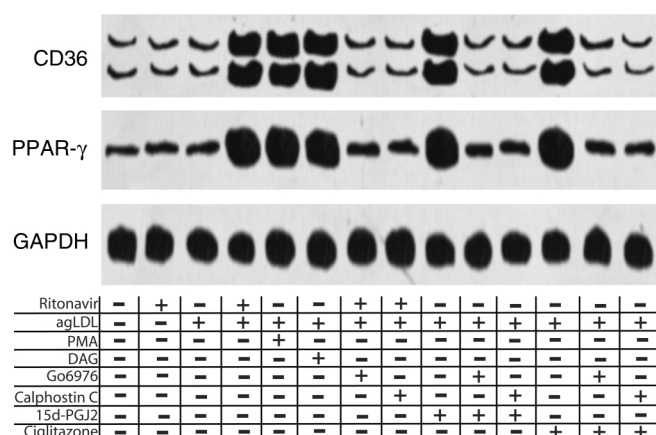
Figure 9

Ritonavir increases CD36 and PPAR-γ mRNA in a PKC-dependent manner. Human PBMCs were incubated in the presence of 10% serum and 50 μg/ml aggregated LDL (agLDL) and/or 30 ng/ml ritonavir as indicated for 24 hours. In addition, some sets of cells were also treated with PMA (100 nM), (diacylglycerol [DAG]; 20 μM), Go6976 (500 nM), calphostin C (200 nM), 15d-PGJ2 (1 μM), or ciglitazone (13 μM). The cells were then lysed, mRNA was isolated, and the relative amounts of CD36, PPAR-γ, and GAPDH mRNA were determined by Northern analysis. Shown are representative data from three independent experiments.

pholino. Furthermore, peritoneal macrophages isolated from CD36 null mice did not accumulate sterol, whereas peritoneal macrophages isolated from CD36 wild-type mice did accumulate sterol, thereby demonstrating that the increase in CD36 was responsible for the increase in cellular cholesterol. In addition, the relative increase in CD36 protein levels correlated with the relative increase in cholesteryl ester levels, further implicating CD36 as a target for HIV protease inhibitor–induced changes. These in vitro data demonstrate that HIV protease inhibitors have direct effects on macrophage sterol regulation.

To determine whether the in vitro findings were relevant in vivo, we treated LDLR null mice with two different doses of HIV protease inhibitors. The doses for ritonavir, indinavir, and amprenavir were titrated to produce models that develop dyslipidemia as judged by an increase in plasma triglycerides and sterol levels. In addition, we titrated the doses for each protease inhibitor so they did not cause dyslipidemia. These models allowed us to elucidate the effects of three different HIV protease inhibitors in the absence of frank dyslipidemia. The use of the LDLR null mouse model was critical for these studies because LDLR null mice do not develop extensive atherosclerotic lesions when fed a chow diet, thereby allowing us to determine the effects of HIV protease inhibitors on the development of atherosclerotic lesions.

A major novel finding of these studies is that doses of HIV protease inhibitors that do not cause dyslipidemia cause an increase in the amount of CD36 and cholesterol/cholesteryl ester associated with peritoneal macrophages. Importantly, these same doses of HIV protease inhibitors cause a significant increase in atherosclerotic lesion area without an increase in plasma lipid levels. Interestingly, the higher doses of HIV protease inhibitors caused more of an increase in atherosclerotic lesion area, probably as a result of lipidemia that developed after treatment, although this has not been directly demonstrated. Finally, we used another animal model of atherosclerosis, the apoE null and the apoE × CD36 double null mice, to determine whether

the increase in CD36 levels was responsible for the increase in atherosclerotic lesion formation. At doses of protease inhibitors that did not induce dyslipidemia, apoE mice developed significant lesions, whereas apoE × CD36 double null mice did not develop lesions. Taken together, the data support the hypothesis that HIV protease inhibitor–induced increases in CD36 promote atherosclerotic lesion formation independent of dyslipidemia.

In an initial attempt to understand how HIV protease inhibitors stimulate the upregulation of CD36 protein levels, we examined the effects of ritonavir on PPARs, transcription factors that have been shown to increase CD36 expression (32). Our data demonstrate that ritonavir in the presence of aggregated LDL can induce the expression of PPAR-γ and CD36 mRNA in a PKC-dependent manner. It is not clear how HIV protease inhibitors induce an alteration in the level of macrophage CD36 without inducing a change in CD36 levels in platelets, cardiac myocytes, or adipocytes. Although our data suggest that the upregulation of PPAR-γ is responsible for the increase in CD36 protein levels, contributions by other regulatory mechanisms have not been ruled out. For instance, ritonavir has been demonstrated to affect the levels of a different transcription factor, SREBP-1, in the liver and adipocytes, resulting in an increase in fatty acid and sterol biosynthesis (2). Alternatively, the protease inhibitors may be preventing the degradation of CD36 and thereby increasing the total cellular mass of the protein. Liang et al. (36) recently demonstrated that ritonavir protects apoB from degradation by proteasomes in the liver. Although many of these other studies examined only ritonavir, the experiments we report here used three different protease inhibitors, which suggests that the results reflect an effect of HIV protease inhibitors as opposed to the nonspecific effect of a single compound.

A major novel aspect of these studies is the establishment of a mechanistic link between a clinically relevant treatment for HIV and a significant detrimental side effect of using these protease inhibitors in patients.

Although it is difficult to extend animal studies to humans, these studies strongly suggest that caution should be used when monitoring the effects of HIV protease inhibitors in patients because atherosclerosis may be promoted without a corresponding increase in marker plasma lipids. A more useful test may be to screen PBMCs from patients for an increase in CD36. Future studies will focus on the complex issue of how HIV protease inhibitors specifically increase CD36 levels in macrophages.

Acknowledgments

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