Modulation of the LDL receptor and LRP levels by HIV protease inhibitors

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Abstract Inhibitors of the human immunodeficiency virus (HIV)-1 protease have proven to be effective antiretroviral drugs. However, patients receiving these drugs develop serious metabolic abnormalities, including hypercholesterolemia. The objective of the present study was to identify mechanisms by which HIV protease inhibitors increase plasma cholesterol levels. We hypothesized that HIV protease inhibitors may affect gene regulation of certain LDL receptor (LDLR) family members, thereby altering the catabolism of cholesterol-containing lipoproteins. In this present study we investigated the effect of several HIV protease inhibitors (ABT-378, Amprenavir, Indinavir, Nelfinavir, Ritonavir, and Saquinavir) on mRNA, protein, and functional levels of LDLR family members. Our results demonstrate that one of these drugs, Nelfinavir, significantly decreases LDLR and LDLR-related protein (LRP) mRNA and protein levels, resulting in the reduced functional activity of these two receptors. Nelfinavir exerts its effect by reducing levels of active SREBP1 in the nucleus. The finding that Nelfinavir reduces the levels of two key receptors (LRP and LDLR) involved in lipoprotein catabolism and maintenance of vessel wall integrity identifies a mechanism that causes hypercholesterolemia complications in HIV patients treated with this drug and raises concerns about the atherogenic nature of Nelfinavir.—Tran, H., S. Robinson, I. Mikhailenko, and D. K. Strickland. **Modulation of the LDL receptor and LRP levels by HIV protease inhibitors.** ***J. Lipid Res.*** 44: **2003. 1859–1869.**

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Inhibitors of the human immunodeficiency virus (HIV)-1 protease have proven to be effective antiretroviral drugs to reduce morbidity and mortality in advanced HIV disease patients (1). However, patients receiving HIV protease inhibitors develop serious syndromes of metabolic abnormalities characterized by peripheral fat wasting, central adiposity, hypertriglyceridemia, hyperlipidemia, and insulin resistance (2–5). Reports have also indicated that patients undergoing therapy display elevated levels of total cholesterol, LDL, lipoprotein(a), and triglycerides in their plasma (6, 7). This is of concern (6, 8–11), insofar as high plasma LDL levels are known to increase the risk of developing atherosclerosis [reviewed in (12)].

Currently, there does not appear to be a single mechanism by which these drugs alter lipid and lipoprotein metabolism, and studies reveal that HIV protease inhibitors have multiple effects on cells. One effect that these inhibitors have is to increase the biosynthesis of fatty acids and lipoproteins. Liang et al. (13) found that high levels of Ritonavir and Saquinavir protect apolipoprotein B (apoB) from proteosomal-mediated degradation resulting in increased apoB secretion, while Lenhard et al. (14) noted that HIV protease inhibitors stimulated triglyceride synthesis (14). In support of these cell-based studies, Riddle et al. (15) found that mice treated with Ritonavir showed an increase in fatty acid and sterol biosynthesis. In addition to these effects, HIV protease inhibitors also increase expression of CD36 on macrophages, which results in increased cholesteryl ester accumulation within macrophages and increased lesion area in LDL receptor (LDLR)-deficient mice (16). Further, certain HIV protease inhibitors increase osteoclast activity (17), while others inhibit adipogenesis of human mesenchymal stem cells (17) and prevent the differentiation of 3T3-L1 preadipocytes (18).

A number of these pathways are regulated by a family of membrane-bound transcription factors, termed the sterol-regulatory element binding proteins (SREBPs) (19). This family of transcription factors modulates the transcription of genes involved in fatty acid, triglyceride, and cholesterol metabolism (20, 21), including genes involved in

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Abbreviations: LRP, LDLR-related protein; RAP, receptor-associated protein; SREBP, sterol-regulatory element binding protein.

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cholesterol biosynthesis and genes such as the LDLR, which are involved in cholesterol uptake. Cells acquire cholesterol either by de novo synthesis or by taking it up in the form of lipoproteins, such as LDL, and this internalization pathway involves endocytosis by the LDLR via clathrin-coated pits (22, 23). Internalized LDL particles are sorted to lysosomal compartments, where apoB is degraded into amino acids and its cholesteryl esters are hydrolyzed to form free cholesterol and fatty acids (24). The uptake of lipoproteins by the LDLR provides cholesterol required for maintaining cell function and regulates the concentration of cholesterol-rich lipoproteins in the circulation (23). Individuals with defects in the LDLR have excessively high plasma cholesterol levels that result in the development of atherosclerotic lesions and coronary artery disease (12, 25).

Studies have found that another hepatic member of the LDLR superfamily, the LDLR-related protein (LRP), participates in the removal of apoE-rich chylomicron remnants from the plasma (26–28). This class of lipoproteins serves to shuttle dietary cholesterol from the gut to the liver. LRP is a multifunctional receptor, whose functions include roles not only in lipid metabolism and in the homeostasis of proteinases and proteinase inhibitors (29), but also an important role in protecting vascular wall integrity by modulating platelet-derived growth factor (PDGF) receptor function (30–32).

Given the important role SREBPs play in regulating gene expression in cholesterol, fatty acid, and triglyceride metabolism, it is likely that some of the side effects reported for HIV protease inhibitors may be manifested either by altering SREBP levels or by modulating SREBP activation. Indeed, two studies (15, 18) have reported an effect of HIV protease inhibitors on SREBP activation; however, the results are contradictory. Dowell et al. (18) found that Nelfinavir prevented the differentiation of 3T3-L1 preadipocytes and decreased levels of cleaved forms of SREBP-1 in cell extracts when compared with untreated cells undergoing differentiation. In apparent contrast to these studies, Riddle et al. (15) found that Ritonavir treatment induced accumulation of the activated forms of SREBP-1 and SREBP-2 in the nucleus of livers and in adipose tissues of mice fed a Western-type high-fat diet.

The objective of the present study was to resolve this apparent discrepancy in order to generate insight into the mechanisms by which HIV protease inhibitors affect triglyceride metabolism and increase plasma cholesterol levels. Our studies demonstrate that Nelfinavir induces endoplasmic reticulum (ER) stress, resulting in activation of ER stress response genes and in a decrease in active SREBP-1 levels in the nucleus. This results in decreases in both LDLR and LRP mRNA and protein levels, reducing the activity of both of these receptors. In contrast to the effect of Nelfinavir on SREBP processing, Saquinavir and Ritonavir both increase active SREBP-1 levels in the nucleus. These results indicate that different HIV protease inhibitors have vastly different effects on gene expression by differential modulation of SREBP.

EXPERIMENTAL PROCEDURES

Antibodies and reagents

Mouse monoclonal antibodies [MAb 2A4 (33) and 1C6 (34)] against SREBP-1 and -2, respectively, obtained from American Type Culture Collection (ATCC, Rockville, MD) were kindly provided by Dr. Kelley Argraves (Medical University of South Carolina, Charlestown, SC). The LDLR-specific polyclonal antibody R711 was provided by Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX). Rabbit polyclonal anti-LRP (R2629) was prepared as described (35). A rabbit polyclonal anti-receptor-associated protein (RAP) (R438) was prepared by immunizing rabbits with purified human RAP, and the resultant antibody was affinity purified on RAP-Sepharose. VLDL receptor (VLDLR)-specific polyclonal antibody (R2623) has been described (36). Polyclonal anti-$\alpha_5\beta_1$ integrin antibody (R3847) was a gift from Dr. Kenneth Yamada [National Institute of Dental and Craniofacial Research, National Institutes of Health (NIH)] and has been described (37). Anti-KDEL monoclonal antibody was purchased from Stressgen. HRP-conjugated goat anti-mouse or anti-rabbit IgG antibodies were purchased from Amersham Pharmacia Biotech.

Human α_2M was isolated from plasma and activated with trypsin as described (38). Human RAP was expressed in bacteria as fusion proteins with glutathione *S*-transferase and purified as described previously (39). LDL was purchased from Intracel (Rockville, MD). HPLC-purified HIV protease inhibitor drugs ABT-378, Amprenavir, Indinavir, Nelfinavir, Ritonavir, and Saquinavir were provided by Deborah Winegar (Glaxo Wellcome, NC). Lipoprotein-deficient fetal bovine serum (FBS) was obtained from Cocalico Biologicals, Inc. (Reamstown, PA). Cholesterol, 25-hydroxycholesterol thapsigargin, and tunicamycin were purchased from Sigma Co. Calpain inhibitor I (ALLN) was obtained from Calbiochem.

Cell culture and HIV protease inhibitor treatments

Human HepG2 cells (ATCC no. HB-8065) were maintained in Eagle's minimum essential medium (EMEM) with 2 mM L-glutamine and Earle's balanced salt solution (EMEM/EBSS) (BioWhittaker) supplemented with 10% FBS (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2. Human lung fibroblast (WI-38) cell line was obtained from ATCC. These cells were kept in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. CHO-K1 cells were maintained in Ham/F12 medium supplemented with 5% Chinese hamster ovary (CHO) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HepG2 cells were plated on 100 mm dishes at a density of 0.75×10^6 cells/dish overnight at 37°C. Cells were washed with Dulbecco's phosphate buffer saline (DPBS) twice and incubated with fresh EMEM/EBSS medium containing 10% lipoprotein-free FBS (obtained from Cocalico Biologicals, Inc.) together with various HIV protease inhibitors that were dissolved in DMSO, or sterols (1 μg/ml of 25-hydroxycholesterol plus 10 μg/ml of cholesterol added in a final concentration of 0.2% ethanol) for 2–3 days.

Induction of ER stress response

These experiments were carried out in CHO cell lines essentially as described (40). CHO-K1 cells were plated on 100 mm dishes at a density of 0.75×10^6 cells/dish and cultured overnight at 37°C. Cells were then washed with DPBS twice and incubated for two days with fresh Ham/F12 medium containing 5% CHO FBS together with Nelfinavir dissolved in DMSO. Following this preincubation, thapsigargin (300 nM in DMSO) or tunicamycin (2 μg/ml in DMSO) was added and incubated with the cells for 16 h before harvesting.

Quantitative PCR analysis of LDLR, LRP, and VLDLR

RNA was extracted from HepG2 cells that were treated with various HIV protease inhibitors for 3 days using Micro RNA isolation kit (Stratagene, San Diego, CA). Traces of DNA were removed by digestion with DNase-free RNase (Gene Hunter, Nashville, TN). Total RNA (1 μg) was reversibly transcribed to cDNA using random hexamers together with the superscript preamplification system for first-strand cDNA synthesis (Life Technologies, Inc.). Real-time quantitative PCR (TaqMan PCR), using a 7700 sequence detector (Perkin-Elmer Corp./PE Applied Biosystems, Foster City, CA), was used for quantification of LDLR, LRP, and VLDLR mRNAs as described previously (41). The amount of LDLR, LRP, or VLDLR mRNA was determined by amplification of the cDNA target using corresponding primers and TaqMan probes designed from the human LDLR, LRP, and VLDLR gene sequences by the Primer Express program from Perkin-Elmer Corp./PE Applied Biosystems, as shown in **Table 1**.

To normalize quantification of LDLR, LRP, or VLDLR mRNA for differences in the amount of total RNA added to each cDNA reaction, 18S rRNA served as a housekeeping gene, which was detected using the TaqMan rRNA Control Reagents (Perkin-Elmer Corp./PE Applied Biosystems). To minimize random errors, PCR amplification of each LDLR, LRP, and VLDLR gene and 18S rRNA were carried out in the same tube. Reaction mixtures contained 5 μl of cDNA product as template, 1× TaqMan Universal PCR Master Mix, 0.9 μmol/l for each LDLR, LRP, or VLDLR forward and reverse primers, 0.1 μmol/l, TaqMan probe, 0.05 μmol/l for each 18S forward and reverse primer, 0.1 μmol/l for the 18S TaqMan probe, and water to a final volume of 50 μl. The following temperature parameters were cycled 50 times: 15 s at 95°C, 1 min at 60°C. Input RNA amounts were calculated manually using the Comparative C_T method for both target genes and 18S. The amount of LDLR, LRP, or VLDLR mRNA was normalized by dividing the amount of 18S RNA for each sample.

Radioiodination of proteins

α_2M (250 μg) was radioiodinated using 20 μg IODO-GEN (Pierce Chemical Co.) and 0.5 mCi of $Na^{125}I$ for 5 min at room temperature. ^{125}I-α_2M was incubated with trypsin at 1:4 molar excess for 5 min at room temperature. After incubation, soybean trypsin inhibitor was added to the mixture and incubated for 5 more min. Radiolabeled α_2M-trypsin complexes were separated from the unincorporated iodine by gel filtration chromatography using a column prepacked with 10 ml of Sephacryl S200 (Amersham Pharmacia Biotech). The specific activity ranged from 2 to 10 μCi/μg of α_2M.

LDL was labeled with $Na^{125}I$ as described previously with some minor modifications (42). Briefly, 250 μg of LDL was diluted with 100 μl of a solution containing 150 mM NaCl and 1 mM EDTA at pH 8.0 and mixed with 56 μl of 2 M glycine, pH 10.0. A half millicurie of $Na^{125}I$ was added to the mixture and incubated for 5 min at room temperature. During the incubation, an aliquot of ICl solution at 33 mM was mixed with a small aliquot of 2 M NaCl solution at a ratio of 1:12.5. Nine microliters of this ICl-NaCl mixture was added into the LDL solution tube and incubated for 5 min on ice. Radiolabeled LDL was separated from the unincorporated iodine by gel filtration chromatography using a Sephadex G-25M column (Amersham Pharmacia Biotech). The specific activity is in the range of 2–20 μCi/μg of LDL.

Cell binding and internalization assays

HepG2 cells were plated onto 12-well plates at 7.5×10^4 cells/well and incubated overnight. Cells were treated with different HIV protease inhibitors or sterol for 2–3 days in EMEM/EBSS containing lipoprotein-deficient FBS. The cells were then washed twice with DPBS to remove any trace of serum, incubated with an assay medium (EMEM/EBSS containing 20 mM Hepes, pH 7.4, 1.5% BSA, and various HIV protease inhibitors or sterol), and incubated for 1 h at 37°C. Then cells were incubated with 10 nM of ^{125}I-α_2M-trypsin or 5 μg/ml of ^{125}I-LDL in assay medium for 4 h at 37°C. After incubation, the cell monolayer was washed with DPBS three times and cells were detached from plates with 0.25 ml of trypsin-EDTA proteinase K solution (to dissociate ^{125}I-α_2M-trypsin/LRP or ^{125}I-LDL/LDLR complexes from the cell surface) and then pelleted by centrifugation (5,000 rpm for 5 min in an Eppendorf microfuge). The amount of internalized ^{125}I-α_2M-trypsin/LRP or ^{125}I-LDL/LDLR complexes was determined by measuring the radioactivity associated with the cell pellet in a Gamma counter. Nonspecific internalization was determined by measuring ^{125}I-α_2M-trypsin/LRP or ^{125}I-LDL/LDLR uptake in the presence of excess amounts of either RAP (1 μM) or LDL (0.5 mg/ml), respectively.

Cellular membrane and nuclear fractionations

Membrane and nuclear fractions were prepared as described previously (19, 43, 44). Briefly, HepG2 or CHO-K1 cells were treated with HIV protease inhibitors for 2–3 days. The treated cells were then incubated with 25 μg/ml ALLN for 3 h at 37°C. Cells were washed with cold DPBS three times on ice and extracted with 1 ml of buffer A [10 mM Hepes, pH 7.6, containing 10 mM KCl, 250 mM Sucrose, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, cocktail inhibitors (Roche)], 1 mM PMSF, and 25 μg/ml ALLN). Cells were scraped off the dish and placed on ice for 10 min. The cells were passed through a 22.5 gauge needle 20 times before being subjected to centrifugation at 1,000 *g* for 10 min at 4°C. The pellet was then resuspended in 0.1 ml of buffer B [20 mM Hepes, pH 7.6, containing 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 0.5 mM

TABLE 1. Oligonucleotide primers used in PCR

Primers	Oligonucleotide Sequence	Positions[a]
LDLR forward	5′-CTGGACCGGAGCGAGTACAC	1,333–1,352
LDLR reverse	5′-GACGCCGTGGGCTCTGT	1,463–1,479
LDLR TaqMan probe	5′-(FAM)-CCCAACCTGAGGAACGTGGTCGCT-(TAMRA)	1,363–1,387
LRP forward	5′-TGCCCTGGACCCTGACAA	13,942–13,959
LRP reverse	5′-GGCCCCCCATGTAGAGTGT	13,991–14,009
LRP TaqMan probe	5′-(FAM)-ACCAACTTCACCAACCCCGTGTATGC-(TAMRA)	13,964–13,989
VLDLR forward	5′-AACCAAGAGGAAGTTCCTGTTTAACT	1,717–1,742
VLDLR reverse	5′-TGACCAGTAAACAAAGCCAGACA	1,782–1,804
VLDLR TaqMan probe	5′-(FAM)-TGACTTGCGAGAGCCTGCCTCCAT-(TAMRA)	1,744–1,767

LDLR, LDL receptor; LRP, LDL receptor-related protein; VLDLR, VLDL receptor.

[a] The number indicated corresponds to residues within LDLR (GenBank accession number NM000527), LRP (NM002332), or VDLDR (NM003383) sequence.

EGTA, cocktail inhibitors (Roche)], 1 mM PMSF, and 25 μg/ml ALLN), and rotated at 4°C for 1 h. The dissolved pellets were centrifuged at 100,000 *g* in a TLA 100.3 rotor for 30 min at 4°C. The resulting supernatant was designated the nuclear fraction. The supernatant from the 1,000 *g* spin was used to prepare the membrane fraction by centrifugation at 100,000 *g* in a TLA 100.3 rotor for 30 min at 4°C. The resulting membrane pellets were resuspended in 0.1 ml SDS lysis buffer (10 mM Tris, pH 6.8, 100 mM NaCl, 1% SDS, 1 mM EDTA, 1 mM EGTA, 25 μg/ml ALLN, cocktail inhibitors, and 1 mM PMSF). Protein concentrations of membrane and nuclear fractions were determined by using the BCA kit (Pierce) and BSA as protein standard.

Immunoblot analyses

Proteins from membrane and nuclear fractions were solubilized in nonreducing SDS-PAGE sample buffer, boiled for 5 min, separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. Unoccupied protein binding sites were blocked by incubating the membranes with blocking buffer [5% nonfat dried milk in TBS-T (150 mM NaCl, 50 mM Tris-HCl, 0.1% Tween-20, pH 7.4)]. Antibodies were diluted in blocking buffer and incubated with membranes overnight in the cold room with shaking. The membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG (Biorad). Bound antibodies were visualized using a Supersignal kit (Pierce) and Hyperfilm (Kodak). The film was scanned as a TIFF file, and densitometry analyses were performed using NIH image 1.62 software. After film exposure, the blots were stripped by using an antibody stripping solution (Pierce) and reprobed with integrin antibody (Rab 3847) to show the protein loading in membrane fractions prepared from different HIV protease inhibitor-treated cells.

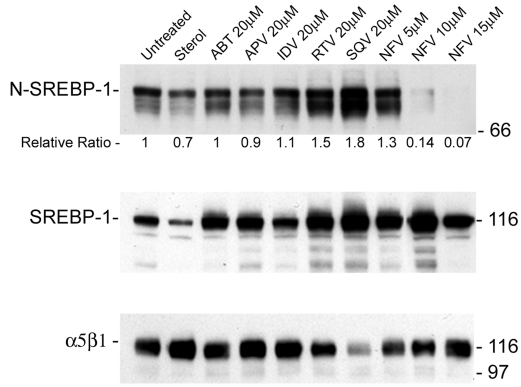

Fig. 1. Nelfinavir reduces the amount of active sterol-regulatory element binding protein (SREBP)-1 in the nuclear fraction, as revealed by immunoblot analysis. HepG2 cells were cultured for 3 days in lipoprotein-free media, lipoprotein-free media containing sterol (1 μg/ml of 25-hydroxycholesterol and 10 μg/ml of cholesterol in ethanol), ABT-378 (ABT), Amprenavir (APV), Indinavir (IDV), Ritonavir (RTV), Saquinavir (SQV), or Nelfinavir (NFV) at the concentrations shown. One hundred micrograms of total nuclear proteins (upper panel) or 50 μg membrane proteins (middle and lower panels) from treated cells were subjected to SDS-PAGE on an 8% polyacrylamide gel under nonreducing conditions and transferred to polyvinylidene difluoride (PVDF) membrane. The nuclear protein fractions were probed with SREBP antibody MAb 2A4 (upper panel), while the PVDF membrane containing membrane proteins was probed with SREBP monoclonal antibody 2A4 (middle panel) or $\alpha_5\beta_1$ integrin R3847 antibody (lower panel). Indicated at right are the molecular masses (in kDa) of marker proteins. The ratios of N-terminal SREBP-1 relative to control cells were determined by using quantitative densitometry. The results are representative of three independent experiments.

RESULTS

Levels of activated SREBP-1 in the nucleus are differentially altered by various HIV protease inhibitors

The processing of SREBP occurs by sequential proteolysis involving two distinct proteases, termed the site-1 and site-2 proteases, that ultimately releases the N-terminal domain of SREBP for export to the nucleus (45). To determine if HIV protease inhibitors alter levels of activated SREBP in the nucleus, HepG2 cells were cultured in lipoprotein-free media along with various HIV protease inhibitors. Following incubation, nuclear and membrane fractions were prepared and immunoblot analyses were performed using monoclonal antibody 2A4, which is specific for NH_2-SREBP-1 (46). Preliminary time course experiments revealed that the effects of the drugs were stabilized following 2 days of treatment, and thus for all experiments, 2–3 days of treatment with the drugs was selected. As a control for these experiments, cells were also cultured in lipoprotein-free media supplemented with sterol to prevent activation of SREBP. Representative results are shown in **Fig. 1**, where it is apparent that HepG2 cells cultured in the presence of Ritonavir and Saquinavir display increased levels of N-terminal SREBP-1 in the nucleus. In contrast, increasing concentrations of Nelfinavir significantly reduced the levels of N-terminal SREBP-1 present in the nuclear fraction. As a control for protein loading, integrin α5β1 levels are shown for each membrane fraction (Fig. 1). Taken together, our data show that higher levels of Nelfinavir specifically decrease NH_2-SREBP-1 levels in the nucleus, while in contrast, Ritonavir

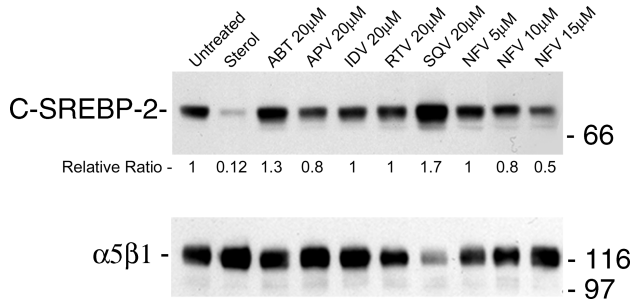

Fig. 2. Immunoblot analyses of COOH-SREBP-2 from cells treated with various human immunodeficiency virus (HIV) protease inhibitors and sterol. HepG2 cells were cultured for 3 days in lipoprotein-free media, lipoprotein-free media in the presence of sterol (1 μg/ml of 25-hydroxycholesterol and 10 μg/ml of cholesterol in ethanol), ABT-378, Amprenavir, Indinavir, Ritonavir, Saquinavir, or Nelfinavir at the concentrations shown. Proteins from membrane fractions (50 μg) were electrophoresed on an SDS-containing 8% polyacrylamide gel under nonreducing conditions and transferred to PVDF membrane. The PVDF membrane was probed with SREBP-2 monoclonal antibody 1C6 (upper panel), or $\alpha_5\beta_1$ integrin Rab3847 antibody (lower panel). Indicated at right are the molecular masses (in kDa) of marker proteins. The results are representative of two independent experiments.

and Saquinavir appear to increase levels of active SREBP in the nucleus. These results reveal that various HIV protease inhibitors differentially regulate SREBP activation, consistent with recent reports showing accumulation of activated SREBP-1 in the liver of mice given Ritonavir (15), and decreased levels of cleaved SREBP-1 in Nelfinavir-treated 3T3-L1 cells induced to differentiate, when compared with untreated cells (18). Virtually identical results were obtained when similar experiments were performed with WI-38 fibroblasts (data not shown), indicating that the effect is likely to be general, and not restricted to HepG2 cell lines.

The data presented in Fig. 1 suggest that Nelfinavir impairs the processing of SREBP-1, which could occur if site-1 or site-2 proteases were sensitive to this drug, although this seems unlikely, insofar as these proteases have been identified as serine proteinase and metalloproteinase, respectively (21). To determine if cleavage by site-1 protease is altered by HIV protease inhibitors, we examined the levels of C-terminal SREBP-2 in cells treated with various HIV protease inhibitors, because generation of this fragment requires site-1 protease activity. Membrane fractions from HepG2 cells treated with various HIV protease inhibitors were prepared and analyzed by immunoblot analyses using monoclonal antibody 1C6, which is specific for the COOH-SREBP-2 fragment. As shown in **Fig. 2**, Nelfinavir decreases COOH-SREBP-2 protein levels, whereas Saquinavir increases COOH-SREBP-2 levels. Because generation of the carboxyl-terminal fragment of SREBP-2 is catalyzed by site-1 protease, these results suggest that Nelfinavir and Saquinavir alter the cleavage of SREBP by site-1 protease activity either directly or indirectly. The results also highlight that activation of SREBP-1 and SREBP-2 is enhanced by Saquinavir and inhibited by Nelfinavir.

Nelfinavir induces increase in ER stress response genes

Another protein that is processed by site-1 and site-2 proteases is a membrane-bound transcription factor termed ATF6 (40) that is involved in the ER stress response mechanism. Once cleaved, ATF6 activates transcription of genes such as *GRP78*, *GRP94*, and *calnexin*, which encode chaperone molecules that restore the folding of proteins in the ER. To further test the effect of Nelfinavir on site-1- and site-2-mediated proteolysis, we examined the level of heat shock proteins GRP94 and GRP78 after treatment with thapsigargin or tunicamycin

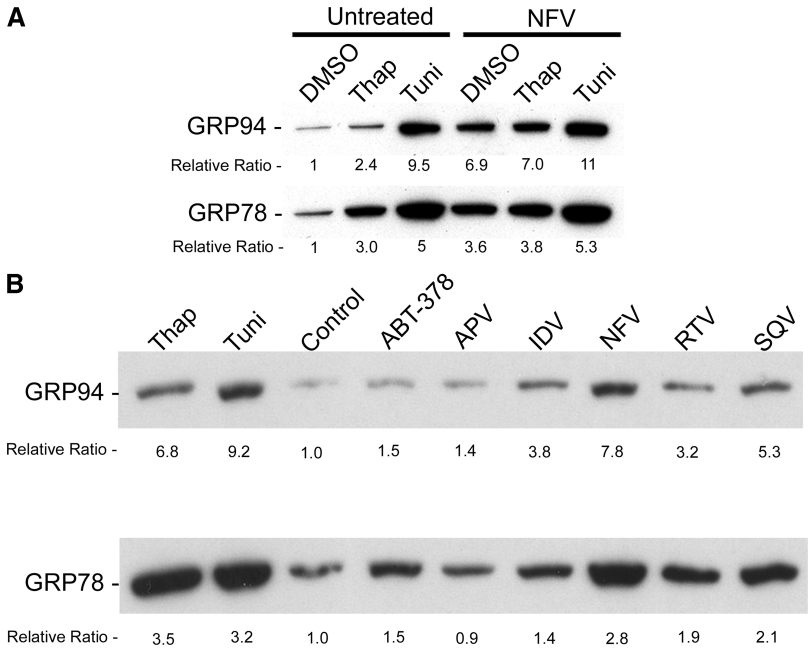

Fig. 3. Immunoblot analyses of GRP94 and GRP78 from CHO-K1 cells treated with Nelfinavir. A: CHO-K1 cells were treated in the presence (NFV) or absence (Untreated) of 15 μM Nelfinavir for 2 days and then incubated with thapsigargin (300 nM) or tunicamycin (2 μg/ml) for an additional 18 h in the presence or absence of 15 μM Nelfinavir. At this time, membrane fractions were prepared, and 50 μg of protein from the membrane fractions was electrophoresed on an SDS-containing 8% polyacrylamide gel under nonreducing conditions and transferred to PVDF membrane. The PVDF membrane was then probed with anti-KDEL antibody (0.55 μg/ml). The results are representative of three independent experiments. Numbers are indicated as fold increase relative to the untreated cells, determined by quantitative densitometry. B: CHO-K1 cells were treated for 18 h with thapsigargin (300 nM) or tunicamycin (2 μg/ml) as described above, or for 2 days with ABT-378 (20 μM), Amprenavir (20 μM), Indinavir (20 μM), Nelfinavir (15 μM), Ritonavir (20 μM), or Saquinavir (20 μM). Following treatments, membrane fractions were prepared, and 50 μg of protein from the membrane fractions was electrophoresed on an SDS-containing 8% polyacrylamide gel under nonreducing conditions and transferred to PVDF membrane. The PVDF membrane was then probed with anti-KDEL antibody (0.55 μg/ml). The results are representative of two independent experiments. Numbers are indicated as fold increase relative to the untreated cells and determined by quantitative densitometry.

to induce ER stress. For these experiments, we used CHO-KI cell lines, as these have been well-characterized for the involvement of site-1 and site-2 protease in the ER stress response (40). Following treatment, membrane fractions were prepared and immunoblot analyses were performed using an anti-KDEL antibody that is specific for GRP94 and GRP78. As shown in **Fig. 3A**, levels of both GRP78 and GRP94 are up-regulated by thapsigargin and tunicamycin, in agreement with previous studies (40). Nelfinavir did not inhibit the up-regulation of GRP78 and GRP94 induced by thapsigargin and tunicamycin (Fig. 3A), as would be expected if Nelfinavir directly inhibits site-1 or site-2 proteases. Interestingly, Nelfinavir induced up-regulation of GRP78 and GRP94 in cells (Fig. 3A), revealing that this HIV protease inhibitor induces ER stress. On the basis of these results, we also examined the effect of other HIV protease inhibitors on the induction of the ER stress response, and the results of this experiment are shown in Fig. 3B. In addition to Nelfinavir, both ABT-378 and Saquinavir also induced the ER stress response, as evidenced by increased production of GRP94 and GRP78. Thus, one mechanism by which certain HIV protease inhibitors may alter gene expression is via induction of the ER stress response.

LDLR and LRP protein levels are regulated by HIV protease inhibitors

The elevation in plasma LDL levels that often accompanies HIV protease inhibitor therapy could result from alterations in LDLR levels, as this is one of the genes regulated by SREBP. Therefore, we performed experiments to examine the effect of HIV protease inhibitors on the levels of the LDLR. Membrane fractions prepared from HepG2 cells cultured in lipoprotein-free media (untreated) or in lipoprotein-free media supplemented with indicated concentrations of HIV protease inhibitors for 2–3 days were subjected to immunoblot analysis. As shown in **Fig. 4**, Nelfinavir treatment causes a significant decrease in LDLR protein levels in a dose-dependent manner. Interestingly, immunoblotting revealed that Ritonavir, Saquinavir, and Nelfinavir all decreased levels of LRP as well. Nelfinavir was especially potent, having a noticeable effect even at 5 μM. As a control for these experiments, cells were also incubated with media containing sterol, as this treatment is known to decrease expression of the LDLR. Curiously, we noted a reproducible effect of sterol on LRP levels: cells cultured in the presence of sterol expressed less LRP than sterol-deprived cells. This is well known and characterized for the LDLR, but is unexpected for LRP, because this receptor is not thought to be regulated by sterols, as it does not appear to contain a sterol-regulatory element within its promoter region (47). None of the drugs appeared to alter the protein levels of the VLDLR, $\alpha_5\beta_1$ integrin, or RAP in a reproducible manner, as assessed by immunoblot analysis. Similar effects of HIV protease inhibitors on LDLR and LRP protein levels were also observed in WI-38 fibroblasts (data not shown). In summary, the results indicate that LRP protein levels are sensitive to several HIV protease inhibitors, including Nelfinavir, and that LDLR protein levels are also decreased in a dose-dependent manner by Nelfinavir.

HIV protease inhibitors regulate LDLR and LRP mRNA levels

To determine whether HIV protease inhibitors regulate LDLR and LRP at the transcriptional level, we utilized quantitative PCR to measure the mRNA level of these two receptors in control and treated cells. As in the above experiments, cells were cultured in lipoprotein-free media, and as a control, cells were also cultured in the presence of sterol. The results (**Fig. 5A**) show that LDLR mRNA level is decreased when cells are cultured in the presence of Amprenavir and Nelfinavir. The effect of Amprenavir is curious, as it does not appear to alter the LDLR protein levels. As a control for these experiments, cells were also

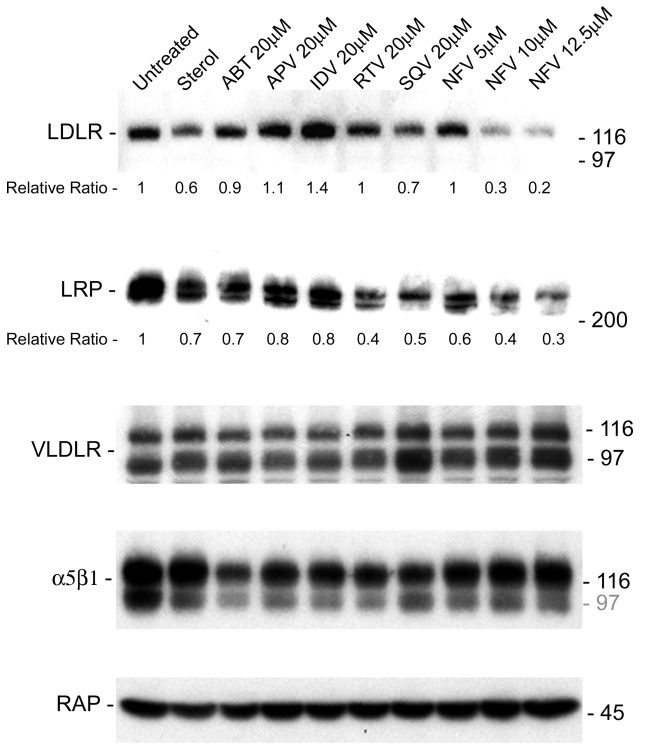

Fig. 4. Immunoblot analyses reveal effects of Nelfinavir on LDL receptor (LDLR) and LDL receptor-related protein (LRP) levels. HepG2 cells were cultured in lipoprotein-free media (untreated), lipoprotein-free media supplemented with sterol (1 μg/ml of 25-hydroxycholesterol and 10 μg/ml of cholesterol in ethanol), ABT-378 (20 μM), Amprenavir (20 μM), Indinavir (20 μM), Ritonavir (20 μM), Saquinavir (20 μM), or Nelfinavir (5, 10, 12.5 μM) for 3 days.Thirty micrograms of protein prepared from extracts of cellular membrane fractions were electrophoresed on an SDS-containing 4–12% polyacrylamide gel under nonreducing conditions and transferred to PVDF membrane. The membrane was probed sequentially with antibodies against LDLR (R711), LRP (R2629), VLDL receptor (VLDLR) (R2623), $\alpha_5\beta_1$ integrin (R3847), or receptor-associated protein (R438). Indicated at right are the molecular masses (in kDa) of marker proteins. Numbers below the protein bands are fold change relative to the untreated cells determined by quantitative densitometry. The figure shown is representative of five independent experiments.

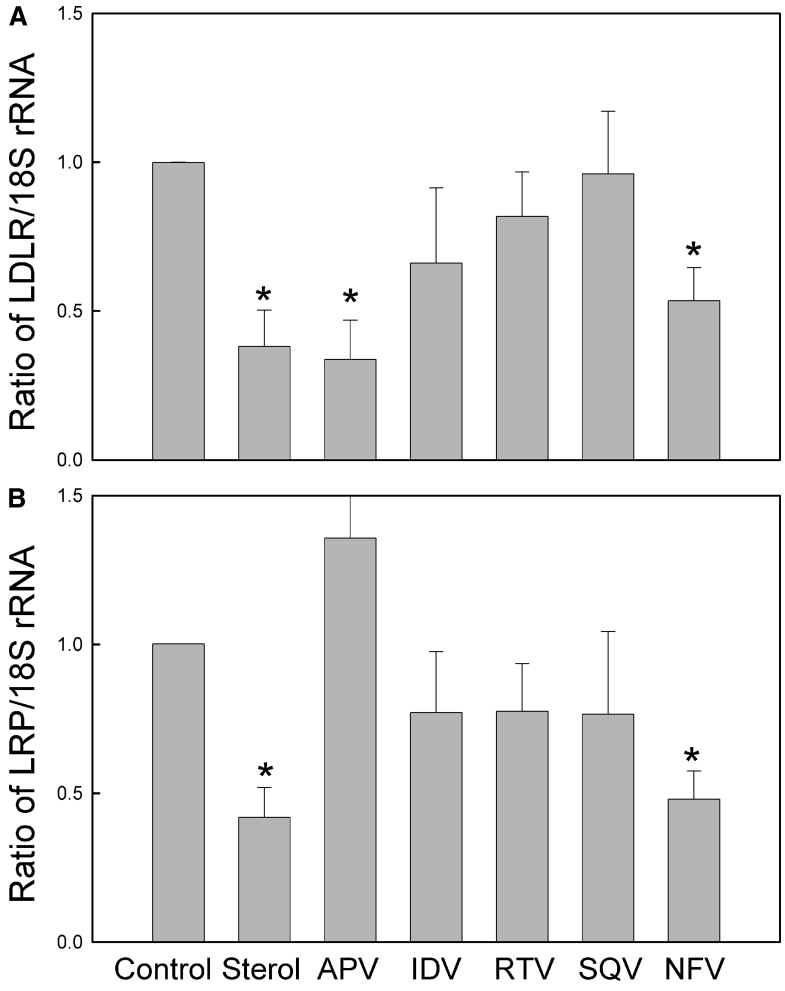

Fig. 5. Real-time quantitative PCR shows the effect of HIV protease inhibitors on LDLR and LRP mRNA levels. Total RNA was isolated from HepG2 cells treated with sterol (1 μg/ml of 25-hydroxycholesterol and 10 μg/ml of cholesterol in ethanol) or various protease inhibitors. All HIV protease inhibitors were at 20 μM, with the exception of Nelfinavir, which was at 15 μM. Traces of DNA were removed by treating RNA with DNase. Total RNA free of DNA was reversibly transcribed to cDNA using random hexamers. Real-time quantitative PCR was used for quantification of LDLR, LRP, and VLDLR mRNAs as described previously (41). The amount of LDLR, LRP, or VLDLR mRNA was determined by amplification of the cDNA target using corresponding primers and TaqMan probes designed from human LDLR and LRP gene sequences (see Experimental Procedures). PCR amplification of each LDLR and LRP gene and 18S rRNA were carried out in the same tube to minimize random errors. The amount of LDLR mRNA is present as the ratio of LDLR (in panel A) and LRP (panel B) mRNA to 18S RNA in each sample. The results are the average of two independent experiments, each performed in triplicate. * $P < 0.01$ versus control untreated cells. Error bars indicate SD.

cultured in the presence of sterol, and the results show that LDLR mRNA levels decrease under these conditions, consistent with previous studies (48, 49). Unexpectedly, LRP mRNA levels are also decreased when cells are cultured in the presence of sterol (Fig. 5B), results that are consistent with the effect of sterol on protein levels. Nelfinavir treatment significantly decreased mRNA levels of LRP (Fig. 5B), while other HIV protease inhibitors had little effect on LRP mRNA levels. Taken together with the previous experiments, these results demonstrate that Nelfinavir specifically down-regulates both LDLR and LRP mRNA and protein levels.

LDLR function in cells is impaired by Nelfinavir

To determine whether HIV protease inhibitors affect the ability of HepG2 cells to catabolize LDL, HepG2 cells were first treated with HIV protease inhibitors for 2–3 days prior to measuring their ability to internalize ^{125}I-LDL. As expected, cells cultured in the presence of sterol showed a significant decrease in LDLR functional activity, as assessed by their ability to mediate the internalization of ^{125}I-LDL (**Fig. 6A**). These results are in excellent agreement with previous studies (50, 51). Nelfinavir reduced the capacity of HepG2 cells to mediate the internalization of ^{125}I-LDL, inhibiting the amount of LDL internalized by 50% when compared with control cells (Fig. 6A), indicating that the LDLR function was significantly decreased. Thus, the ability of Nelfinavir to decrease LDLR mRNA and protein levels results in a significant decrease in LDLR functional activity. Other HIV protease inhibitors had no significant reproducible effect on LDLR activity.

We next examined the dose response of this effect in HepG2 cells. In these experiments, HepG2 cells cultured in lipoprotein-free serum were treated with increasing amounts of Nelfinavir or Saquinavir for 2 days. The cells where then incubated with ^{125}I-LDL, and the amount of ligand internalized was measured. As shown in Fig. 6B, Nelfinavir reduced the amount of ^{125}I-LDL internalized in a dose-dependent manner, whereas Saquinavir had little effect on LDLR function. Collectively, our results demonstrate that Nelfinavir inhibits the LDLR function in a dose-dependent manner by reducing receptor levels.

Catabolic function of LRP is also affected by Nelfinavir

To determine whether HIV protease inhibitors affect LRP function as well, HepG2 cells were first treated with various HIV protease inhibitors for 2 days prior to measuring their ability to internalize the LRP-specific ligand, ^{125}I-α_2M-trypsin. Treatment of cells with Nelfinavir resulted in a significant decrease in LRP activity, as assessed by the ability of the cells to internalize ^{125}I-labeled α_2M-trypsin, while Indinavir seemed to reproducibly enhance the

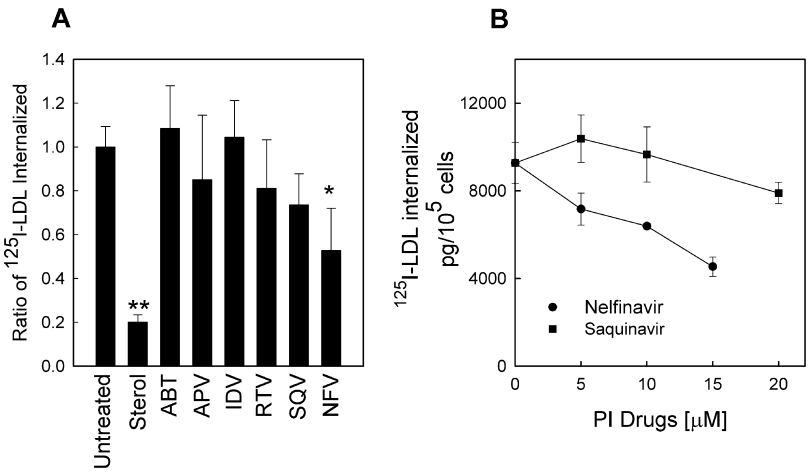

Fig. 6. Nelfinavir reduces functional activity of the LDL receptor in HepG2 cells. A: HepG2 cells were cultured in lipoprotein-free media (untreated), lipoprotein-free media containing sterol (1 μg/ml of 25-hydroxycholesterol and 10 μg/ml of cholesterol in ethanol), or indicated HIV protease inhibitors for 3 days. All HIV protease inhibitors were at 20 μM, with the exception of Nelfinavir, which was at 12.5 μM. Following this incubation, 5 μg/ml of ^{125}I-LDL was added and incubated with the cells for 3 h at 37°C. After incubation, the amount of internalized ^{125}I-LDL was determined as described in Experimental Procedures. The results shown are the average of four independent experiments, each performed in duplicate. * $P < 0.01$, ** $P < 0.005$ versus control untreated cells. B: HepG2 cells were cultured in lipoprotein-free media for 3 days with the indicated concentrations of Nelfinavir or Saquinavir. Following this incubation, 5 μg/ml of ^{125}I-LDL was added and incubated with the cells for 3 h at 37°C. After incubation, the amount of internalized ^{125}I-LDL was determined as described in Experimental Procedures. Error bars indicate SD.

amount of ^{125}I-labeled α_2M-trypsin internalized (**Fig. 7**). Because Indinavir does not increase LRP protein or mRNA levels, the mechanism of this effect is not known. Other protease inhibitors had no significant effect on LRP-mediated internalization of ^{125}I-labeled α_2M-trypsin. Interestingly, these experiments also revealed a significant decrease in LRP activity in the presence of sterol (Fig. 7), which is consistent with the lower level of LRP antigen (Fig. 4) and LRP mRNA (Fig. 5) observed in the presence of sterol.

DISCUSSION

Treatment of HIV patients with inhibitors of the HIV aspartyl protease has resulted in an effective therapy for HIV patients. However, these protease inhibitors do not appear to be precise in their cellular targets, and therapy is associated with a variety of metabolic disorders, which include hypercholesterolemia, hypertriglyceridemia, insulin resistance, and a peripheral lipodystrophy syndrome (2, 5, 7, 52–54). Molecular mechanisms associated with these metabolic alterations are currently uncertain, and it does not appear that one central mechanism accounts for all of the effects (13–15).

The objective of the current investigation was to test the hypothesis that HIV protease inhibitors alter normal regulatory mechanisms that control the function of lipoprotein receptors, one pathway by which plasma lipoprotein levels can be altered. An important site for the removal of LDL and apoE-rich chylomicron remnants is the liver, and the two hepatic lipoprotein receptors responsible for their uptake are the LDLR and LRP. In the present study, we tested the ability of six different HIV protease inhibitors used to treat patients to modulate the function of three receptors known to play important roles in lipoprotein metabolism. We found that one HIV protease inhibitor, Nelfinavir, specifically decreases mRNA and protein levels of the LDLR and LRP, which in turn decreases the functional activity of these two receptors. A decrease in LDLR and LRP function would be expected to result in increased plasma levels of LDL and chylomicron remnants, two atherogenic lipoproteins seen in patients receiving this HIV protease inhibitor, and may provide a molecular basis for the increased lipoprotein levels seen in patients receiving this HIV protease inhibitor. Further, as LRP has recently been shown to play an important role in protecting the vascular wall by modulating PDGF receptor activity (30–32), a reduction in its level might increase the risk of vascular disease.

Nelfinavir seems to modulate LDLR and LRP levels by altering the processing of the transcription factor SREBP. SREBP associates with another protein called SREBP cleavage activating protein (SCAP), which is an integral ER membrane protein that functions to anchor SREBP in the ER membrane (55, 56). SCAP is the sterol sensor molecule in the ER, and in the presence of sterols, interacts with INSIG-1, a membrane protein that facilitates retention of SREBP in the ER (57). In the sterol-depleted condition, SCAP/SREBP complexes are transported from the ER to the Golgi, where SREBP is cleaved by a protease, termed site-1 protease (58, 59). This cleavage produces two intermediate SREBP fragments that remain associated with membrane. The newly generated N-terminal

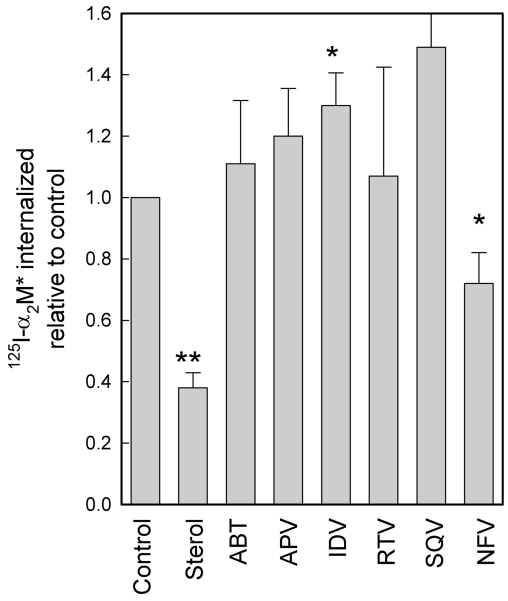

Fig. 7. Nelfinavir and sterol reduce functional activity of LRP in HepG2 cells. HepG2 cells were cultured in lipoprotein-free media (untreated), lipoprotein-free media containing sterol (1 μg/ml of 25-hydroxycholesterol and 10 μg/ml of cholesterol in ethanol), or various HIV protease inhibitors for 3 days. All HIV protease inhibitors were at 20 μM, with the exception of Nelfinavir, which was at 15 μM. The cells were then incubated with 10 nM of ^{125}I-α_2M-trypsin for 3 h at 37°C. After incubation, the amount of internalized ^{125}I-α_2M-trypsin was determined as described in Experimental Procedures. The results shown are the average of four independent experiments, each performed in duplicate. * $P < 0.01$, ** $P < 0.005$ versus control untreated cells. Error bars indicate SD.

intermediate fragment of SREBP becomes a substrate for a second protease, designated site-2 protease, which cleaves within the transmembrane-spanning domain of N-terminal SREBP (60). Following cleavage, the newly generated N-terminal fragment of SREBP is released from the membrane and diffuses to the nucleus to enhance transcription of genes containing sterol-regulatory elements (20).

In mammals, three SREBP isoforms have been identified, and are termed SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides (21). The roles of SREBP-1c and SREBP-2 are more restrictive than that of SREBP-1a (21). SREBP-1c preferentially enhances transcription of genes required for fatty acid synthesis, while SREBP-2 preferentially activates genes involved in cholesterol synthesis (20). Our data reveal that activation of both SREBP-1 and SREBP-2 is decreased upon Nelfinavir treatment and increased upon treatment with Saquinavir and Ritonavir. These results demonstrate that various HIV protease inhibitors have drastically different effects on cells. Thus, while Ritonavir, Saquinavir, and Nelfinavir, all increase plasma LDL in patients receiving these drugs, they appear to do so by completely different mechanisms. Ritonavir and Saquinavir increase levels of active forms of SREBP in the nucleus, thereby increasing lipid biosynthesis, whereas Nelfinavir decreases levels of active SREBP in the nucleus, resulting in decreased lipid clearance and turnover. Differential effects of HIV protease inhibitors on SREBP activation also explain the apparent contradictions in the literature (15, 18).

The mechanism by which Nelfinavir exerts its effect is not clear at this time, and we considered the possibility that Nelfinavir might inhibit site-1 and/or site-2 proteases. However, we found that induction of ER stress by tunicamycin or thapsigargin, which requires site-1 and site-2 protease activity (40), was not inhibited by Nelfinavir, implying that this drug does not block site-1 or site-2 protease activity. Interestingly, our studies revealed that Nelfinavir itself was able to significantly increase production of GRP78 and GRP94, indicating that this drug induces ER stress. The ER is responsible for the folding and processing of proteins, and it has become clear that it is a site of a robust degradation pathway that recognizes and destroys unfolded proteins (61, 62). A number of situations increase levels of unfolded proteins in the ER lumen, including diminished protein glycosylation or treatment with drugs such as tunicamycin. The net effect is a state of stress in which the burden of unfolded proteins exceeds the folding capacity of the ER machinery. The ER responds to this stress by initiating the unfolded protein response (UPR), which includes activation of the transcription factor ATF6 (63) that initiates production of chaperones, such as GRP94 and GRP78, which are involved in protein folding. A relationship between ER stress and SREBP-1 protein and antigen levels has been described by Werstuck et al. (64), who found that homocysteine-induced ER stress activated the UPR and increased SREBP-1 levels. Transfection of cells with GRP78 reduced the homocysteine-induced increase in SREBP-1 levels. These studies suggest that homocysteine-induced ER stress enhances lipid biosynthesis and uptake via increases in SREBP levels. However, in our study, Nelfinavir-induced ER stress had no noticeable effect on total SREBP levels, but rather reduced the levels of active SREBP in the nucleus.

In the present study, we also noted a prominent effect of sterol on LRP mRNA and protein levels. This resulted in a substantial decrease in LRP functional activity in the presence of excess sterol. While earlier work in fibroblasts suggested that LRP levels are not regulated by sterol (28), the results in the present study are consistent with recent reports (65) indicating that LRP function is decreased in mouse macrophage-like J774A.1 cells when the cells are cultured in the presence of sterol. Interestingly, an SRE-1 binding site was identified in the 5′-untranslated flanking region of the LRP gene (66) that may be responsible for sterol sensitivity. A similar region in the promoter of the LDLR is used for control of expression by cholesterol.

In summary, our studies have clearly demonstrated that Nelfinavir reduces LDLR and LRP mRNA and protein levels, thereby reducing their catabolism activity. We show that this HIV protease inhibitor exerts its effect by reducing levels of active SREBP in the nucleus. These findings

represent a mechanism by which Nelfinavir may induce hyperlipidemia and hypercholesterolemia in HIV patients undergoing therapy with this drug.

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