Prevention of HIV Protease Inhibitor-Induced Dysregulation of Hepatic Lipid Metabolism by Raltegravir via Endoplasmic Reticulum Stress Signaling Pathways

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ABSTRACT

Hyperlipidemia associated with the HIV protease inhibitor (PI), the major component of highly active antiretroviral treatment (HAART) for HIV infection, has stimulated interest in developing new agents that minimize these side effects in the clinic. HIV integrase inhibitor is a new class of anti-HIV agents. Raltegravir is a first-in-its-class oral integrase inhibitor and has potent inhibitory activity against HIV-1 strains that are resistant to other antiretroviral regimens. Our previous studies have demonstrated that HIV PI-induced endoplasmic reticulum (ER) stress links to dysregulation of lipid metabolism. However, little information is available as to whether raltegravir would have similar effects as the HIV PIs. In this study, we examined the effect of raltegravir on lipid metabolism both in primary rat hepatocytes and in in vivo mouse models, and we further

determined whether the combination of raltegravir with existing HIV PIs would potentially exacerbate or prevent the previously observed development of dyslipidemia. The results indicated that raltegravir did not induce ER stress or disrupt lipid metabolism either in vitro or in vivo. However, HIV PI-induced ER stress and lipid accumulation were significantly inhibited by raltegravir both in in vitro primary rat hepatocytes and in in vivo mouse liver. High-performance liquid chromatography analysis further demonstrated that raltegravir did not affect the uptake and metabolism of HIV PIs in hepatocytes. Thus, raltegravir has less hepatic toxicity and could prevent HIV PI-induced dysregulation of lipid metabolism by inhibiting ER stress. These results suggest that incorporation of this HIV integrase inhibitor may reduce the side effects associated with current HAART.

HIV protease inhibitors (PIs) have been used successfully in highly active antiretroviral therapy (HAART) for HIV infection. Incorporation of HIV PIs in HAART causes profound and sustained suppression of viral replication, significantly reduces the morbidity and mortality, and prolongs the life span of patients with HIV infection. However, accumulating clinical evidence indicates that HAART has only

changed the clinical profile of HIV infection from a subacute lethal disease to a chronic ambulatory disease (Riddle et al., 2001; Moyle and Carr, 2002; Spector, 2006). Long-term administration of HAART eventually leads to severe drug-related adverse effects in patients and the selection of drug-resistant viral strains (Dayam et al., 2007). One of the most deleterious side effects of HIV PI therapy is development of dyslipidemia, a well established risk factor for the development of cardiovascular complications in these patients (Carr et al., 2001; Moyle and Carr, 2002). A rarely discussed but frequent observation is the development of nonalcoholic fatty liver disease (NAFLD) in patients under HAART.

Recent studies from our laboratory and other laboratories suggest that HIV PI-induced endoplasmic reticulum (ER)

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ABBREVIATIONS: PI, propidium iodide; HAART, highly active antiretroviral therapy; NAFLD, nonalcoholic fatty liver disease; ER, endoplasmic reticulum; UPR, unfolded protein response; CHOP, CCAAT/enhancer-binding protein homologous protein; ATF, activating transcription factor; XBP, X-box-binding protein; XBP-1s, spliced X-box-binding protein-1; SREBP, sterol regulatory element-binding protein; RPA, ribonuclease protection assay; LDL, low-density lipoprotein; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; CPT, carnitine palmitoyltransferase; CYP7A1, cholesterol 7- α -hydroxylase; ABC, ATP-binding cassette.

stress response and subsequent activation of the unfolded protein response (UPR) represent important cellular signaling mechanisms of HIV PI-induced metabolic syndromes (dyslipidemia, insulin resistance, and lipodystrophy/lipoatrophy) (Mallon et al., 2001; Moyle and Carr, 2002; Koster et al., 2003; Lee et al., 2007). The role of ER stress is a rapidly emerging field of interest in the pathogenesis of nearly all types of human liver diseases, including NAFLD and drug-induced liver injury (Kaplowitz et al., 2007). Our previous *in vitro* studies have demonstrated that most HIV PIs not only induce the accumulation of intracellular free cholesterol and lipid, activating the UPR in hepatocytes and macrophages, but also increase the release of inflammatory cytokines and promote foam cell formation in macrophages (Zhou et al., 2005, 2007). Of extreme interest is that similar patterns of dysregulation in key regulators of hepatocellular lipid homeostasis seen after the addition of HIV PIs to hepatocytes were also found in the livers of patients with NAFLD (Erickson, 2009).

Recently, a new class of drug, the HIV integrase inhibitor, has been developed. Raltegravir (also known as MK0518, Isentress) is a first-in-its-class oral integrase inhibitor and was approved by the Food and Drug Administration on October 12, 2007 (Nair and Chi, 2007; Cocohoba and Dong, 2008). Raltegravir prevents the integration of proviral DNA into host genome and has potent *in vitro* inhibitory activity against HIV-1 strains that are resistant to other antiretroviral regimens (Cocohoba and Dong, 2008). Limited clinical studies have shown that raltegravir has equal or greater therapeutic efficacy in treatment of both naive and experienced patients (2008; Steigbigel et al., 2008; Imaz et al., 2009). However, little information is available as to whether raltegravir would have similar dyslipidemia side effects as the HIV PIs. The current study was aimed at examining the effect of raltegravir on lipid metabolism in hepatocytes and further determining whether the combination of integrase inhibitor with the existing, most commonly used HIV PIs lopinavir and ritonavir would potentially exacerbate or prevent the previously observed development of dyslipidemia.

Materials and Methods

Materials. Antibodies against CCAAT/enhancer-binding protein homologous protein (CHOP), activating transcription factor (ATF)-4, X-box-binding protein (XBP)-1, lamin B, sterol regulatory element-binding protein (SREBP)-1, horseradish peroxidase-conjugated donkey anti-goat IgG, and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibody against β -actin was from Calbiochem (San Diego, CA). Bio-Rad protein assay reagent, Criterion XT Precast Gel, and Precision Plus Protein Kaleidoscope standards were obtained from Bio-Rad Laboratories (Hercules, CA). RNAqueous total RNA isolation kit and MAXIscript T7 and ribonuclease protection assay (RPA) II kits were from Ambion (Austin, TX). High-capacity cDNA Archive kit was from Applied Biosystems (Foster City, CA). All other chemical reagents were from Sigma-Aldrich (St. Louis, MO).

Isolation and Culture of Primary Hepatocytes. Primary hepatocytes were isolated from male Sprague-Dawley rats (250–300 g) and cultured as described previously (Zhou et al., 2006).

Animal Studies. Animal studies were approved by Institutional Animal Care and Use Committee of Virginia Commonwealth University and were conducted in accordance with the Declaration of Helsinki, the *Guide for the Care and Use of Animals* (National

Academies Press, Washington, DC, 1996), and all applicable regulations. To examine the effect of raltegravir on HIV PI-induced lipid accumulation in liver, male C57BL/6 mice (8 weeks old) were randomly assigned to four groups ($n = 8$): 1) control, 2) raltegravir, 3) lopinavir/ritonavir, and 4) raltegravir plus lopinavir/ritonavir. Mice were fed with a standard diet and gavaged with control solution, raltegravir (50 mg/kg), lopinavir/ritonavir (4:1; 50 mg/kg), or raltegravir plus lopinavir/ritonavir for 4 weeks. All mice were housed under identical conditions in an aseptic facility and given free access to water and food. Mice were weighed once a week to adjust drug intake. At the end of each time period, mice were fasted for 16 h and blood samples were collected. Serum total cholesterol, free cholesterol, triglyceride, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein-cholesterol, alanine aminotransferase, and aspartate aminotransferase were measured using standard enzymatic techniques in the clinical laboratory at Hunter Holmes McGuire Veterans Affairs Medical Center (Richmond, VA). Total RNA was isolated for real-time RT-PCR analysis of the key genes involved in lipid metabolism as described previously (Zhou et al., 2006).

Histopathology Analysis. The liver tissue sections were collected and fixed in 4% paraformaldehyde in 0.1 M PBS at room temperature overnight. The regions of the specimens were standardized for all mice. Paraffin-embedded tissue sections (6 μ m) were stained with hematoxylin and eosin according to standard techniques. Samples were examined in a blinded manner to evaluate the presence of steatosis, inflammation, and fibrosis. Lesions were evaluated semiquantitatively on a four-point scale (1, absent; 2, mild; 3, moderate; and 4, intense) for each damage as described by Brunt et al. (1999). The extent of lesions in each mouse is expressed as the average score of three separate specimens.

High-Performance Liquid Chromatography Analysis of Metabolism of HIV PIs and Raltegravir in Rat Primary Hepatocytes. Rat primary hepatocytes were treated with HIV PIs and raltegravir (25 μ M) for various times (0–24 h). The culture medium and total cell lysates of each time point were collected. The drugs in media and cells were extracted using solid phase C-18 cartridges as described previously (Zhou et al., 2006). A 1200 Series high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA) and a C-18 reverse phase column (5 μ m, 4.6 mm $\times$ 25 cm; Beckman Coulter, Fullerton, CA) were used to quantify the HIV PIs and raltegravir in cells and medium. The gradient mobile phase for raltegravir consisted of acetonitrile (A) and 0.01% triethylamine (B) adjusted to pH 3.0 by phosphoric acid. The starting mobile phase consisted of 40% A and 60% B (v/v) and was switched to 75% A and 25% B at 15 min. The mobile phase was delivered at 1 ml/min. The fluorescence detector was used to detect raltegravir using excitation/emission wavelengths of 299/396 nm (Poirier et al., 2008). The mobile phase for ritonavir and lopinavir was acetonitrile/20 mM sodium dihydrogen phosphate, pH 6.0 [60:40 (v/v)], and 0.025% triethylamine. The mobile phase was delivered at 1 ml/min. The peaks of ritonavir and lopinavir were detected at 210 nm.

A standard curve of each drug was constructed using weighted linear regression of peak area ratio values of the calibration standards. The percentage of drug recovery after the solid phase extraction was determined by comparing the extracted internal standard.

Analysis of Apoptosis by Annexin V and Propidium Iodide Staining. Rat primary hepatocytes were treated with HIV PIs and raltegravir (25 μ M) for 24 h and stained with Annexin V-FITC and propidium iodide using an ApoAlert Annexin V kit (BD Biosciences, San Jose, CA), according to the protocol recommended by the manufacturer. Annexin V/propidium iodide-stained cells were visualized under fluorescence microscopy with a 40 $\times$ objective using a dual-filter set for FITC and rhodamine (Zhou et al., 2005, 2006). The relative fluorescence density was determined using IPLab 4.0 software (Improvision, Coventry, UK).

Western Blot Analysis. The nuclear and cytosolic proteins were prepared from rat primary hepatocytes or C57/BL6 mouse liver homogenate as described previously (Zhou et al., 2006). The protein

TABLE 1
Primer sequences for real-time PCR

Gene	NCBI RefSeq	Forward Primer (5'–3')	Reverse Primer (5'–3')
FAS	M76767	GACTACAGACAACAGCAACC	CTCAGACAGGCACTCAGG
ABCA1	NM_013454	ATTGCCAGACGGAGCCG	TGCCAAAGGGTGGCACA
ABCG1	NM_053502	CAAGTCCGTGTGCGTGTC	CCTCCGTGAAGTTGTTGTCC
ABCG5	AF312714	TGCGTCTCCTACCTCCTG	ACTGCCTCTACCTTCTTGTC
ABCG8	AF351785	GCCTCGCTCTGACATCTTC	GCCACCTCCTGTTCTTTGC
ACC-1	EF121986	AAGGCTATGTGAAGGATG	CTGTCTGAAGAGGTTAGG
ATF-4	NM_009716	CCTAGGTCTCTTAGATGACTATCTGGAGG	CCAGGTCATCCATTTCGAAACAGAGCATCG
CPT-1 α	NM_031559	AAGCCAGACGAAGAACATTG	ACCTTGACCATAGCCATCC
CHOP	NM_007837	AGGAGAAGGAGCAGGAGAAC	AGAGACAGACAGGAGGTGATG
XBP-1s	AF443192	TGAGTCCGCAGCAGGTG	GACAGGGTCCAACCTGT
β -Actin	NM_031144	TATCGCGCAATGAGCGTTTC	AGCACTGTGTGGCATAGAGG

NCBI, National Center for Biotechnology Information.

concentration was determined using Bio-Rad protein assay reagent (Bio-Rad Laboratories). The nuclear extracts (30 μ g of protein), cytosol extracts (70 μ g of protein), and total cell lysate proteins (80 μ g) were resolved on 10% or 7 or 12% Bis-Tris NuPAGE Novex gels or 10% Criterion XT precast gels, respectively, and transferred to nitrocellulose membranes. Immunoblots were blocked overnight at 4°C with 5% nonfat milk in Tris-buffered saline buffer and incubated with primary antibodies. Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibody and the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences, Boston, MA). The membranes were stripped with stripping buffer (62.5 mM Tris-HCl, pH 6.8, containing 100 mM β -mercaptoethanol and 2% SDS) and reprobed with antibody against lamin B or β -actin. The density of the immunoblot bands was analyzed using ImageJ computer software (National Institutes of Health, Bethesda, MD).

RNA Isolation and RPA. Total RNA was isolated from rat primary hepatocytes using the guanidine thiocyanate cesium chloride centrifugation method. All RPA probes for rat cholesterol 7- α -hydroxylase (CYP7A1), HMG-CoA-reductase, LDL-receptor, sterol 27-hydroxylase, and cyclophilin and details of RPA were described previously (Zhou et al., 2006). The density of bands was analyzed using ImageJ computer software (National Institutes of Health) and normalized to rat cyclophilin.

Real-Time Quantitative PCR. Total cellular RNA was isolated from rat primary hepatocytes after treatment with HIV PIs and raltegravir, or with vehicle control (dimethyl sulfoxide) for 24 h, using the SV Total RNA Isolation System (Promega, Madison, WI). Total RNA (2 μ g) was used for the first-strand cDNA synthesis using the High-Capacity cDNA Archive kit (Applied Biosystems). The mRNA levels of CHOP; XBP-1; ATF-4; fatty acid synthase (FAS); acetyl-CoA carboxylase (ACC)-1; carnitine palmitoyltransferase (CPT)-1 α ; and ATP-binding cassette (ABC) transporters A1, ABCG1, ABCG5, and ABCG8 were quantified using specific primers for each gene (Table 1). iQ SYBR Green Supermix (Bio-Rad Laboratories) was used as a fluorescent dye to detect the presence of double-stranded DNA. The mRNA values for each gene were normalized to internal control β -actin mRNA. The ratio of normalized mean value for each treatment group to vehicle control group was calculated.

Nile Red Staining. Primary rat hepatocytes were treated with HIV PIs with or without raltegravir for 24 h. Cells were fixed in 3.7% formaldehyde and incubated with Nile red (100 ng/ml) in PBS for 5 min. After washing with PBS, the images were taken using a microscope (Olympus, Tokyo, Japan) equipped with an image recorder under 40 $\times$ lenses.

Measurement of Free Cholesterol, Total Cholesterol, and Triglyceride. Rat primary hepatocytes were plated on 60-mm plates overnight and then treated with control vehicle or HIV PIs with or without raltegravir for 24 h. The cells were collected and washed with PBS. Mice sera were collected at the end of the in vivo study. The triglyceride, total cholesterol, and free cholesterol were measured by using triglyceride, cholesterol E, and free cholesterol assay kits (Wako Bioproducts, Richmond, VA).

TABLE 2
Effect of HIV PIs on raltegravir pharmacokinetics

Group	Raltegravir (25 μ M)	RITV + Raltegravir (25 μ M)	Raltegravir (25 μ M)	LOPV + Raltegravir (25 μ M)
$T_{\max}$ (h)	1.00	1.00	1.00	1.00
$C_{\max}$ (μ g $\cdot$ h/mg)	0.10	0.08	0.14	0.12
AUC (μ g $\cdot$ h/mg)	1.42	1.37	2.13	1.87
Relate AUC	1.00	0.96	1.00	0.88

AUC, area under the curve.

TABLE 3
Effect of raltegravir on HIV PIs pharmacokinetics

Group	RITV (25 μ M)	RITV + Raltegravir (25 μ M)	LOPV (25 μ M)	LOPV + Raltegravir (25 μ M)
$T_{\max}$ (h)	1.00	1.00	0.50	0.50
$C_{\max}$ (μ g $\cdot$ h/mg)	3.60	3.53	2.68	2.13
AUC (μ g $\cdot$ h/mg)	68.21	65.44	12.07	23.56
Relate AUC	1.00	0.96	1.00	1.95

AUC, area under the curve.

Bile Acid Synthesis by Primary Hepatocytes. The conversion of [14 C]cholesterol into methanol-water-soluble material was determined as described previously (Zhou et al., 2006). Cells were pretreated with ritonavir, raltegravir, or both (25 μ M) for 12 h, media were replaced, and [14 C]cholesterol (6×10^5 cpm/plate) and ritonavir/raltegravir were added. After 48-h incubation, media were harvested and analyzed according to the Folch technique (Folch et al., 1957).

Statistical Analysis. All results were expressed as mean $\pm$ S.E.M. One-way analysis of variance and Student's *t* test were used to analyze the differences between different treatments. Statistics were performed using GraphPad Pro (GraphPad Software Inc., San Diego, CA). A probability (*p*) of less than 0.05 was considered statistically significant.

Results

Interaction of Raltegravir and HIV PIs in Rat Primary Hepatocytes. To study the effect of raltegravir on HIV PI-induced dysregulation of lipid metabolism in hepatocytes, we first examined whether drug-drug interaction affects the drug metabolism of raltegravir and HIV PIs in primary rat hepatocytes. The metabolic rates in rat primary hepatocytes of raltegravir with ritonavir and lopinavir, the most commonly used HIV PIs in the clinic, were determined by high-performance liquid chromatography analysis. The results indicated that ritonavir and lopinavir had no significant effect on the uptake and pharmacokinetics of raltegravir (Table 2); raltegravir also did not affect the metabolism of the HIV PIs (Table 3).

Effect of Raltegravir on HIV PI-Induced Apoptosis in Rat Primary Hepatocytes. To determine whether raltegravir has a similar effect to HIV PIs on induction of apoptosis in hepatocytes, we treated the primary rat hepatocytes with raltegravir with or without ritonavir and lopinavir. The apoptotic and necrotic cells were stained using Annexin V-

Fig. 1. Effect of raltegravir on HIV PI-induced apoptosis in rat primary hepatocytes. Rat primary hepatocytes were treated with vehicle control, raltegravir (25 μ M), ritonavir (RITV; 25 μ M), lopinavir (LOPV; 25 μ M), RITV + raltegravir, or LOPV + raltegravir for 24 h and then stained with Annexin V-FITC/propidium iodide. Images of Annexin V-FITC/propidium iodide-stained cells were visualized under fluorescence microscopy with a dual filter set for FITC and rhodamine. A, representative images of Annexin V-FITC/propidium iodide-stained cells. B, relative fluorescence intensity.

Fig. 2. Effect of raltegravir on HIV PI-induced activation of caspase-9 in rat primary hepatocytes. A, representative immunoblots from three independent experiments for active caspase-9 from total protein lysates of rat primary hepatocytes treated with different amounts of raltegravir (0–25 μ M) in the absence or presence of lopinavir (LOPV)/ritonavir (RITV) (25 μ M) for 24 h. β -Actin was used as a loading control. B, relative protein level of active caspase-9. *, $p < 0.05$, statistical significance relative to vehicle control; #, $p < 0.05$, statistical significance relative to LOPV/RITV-treated group.

FITC and propidium iodide and detected by fluorescence microscopy as described previously (Zhou et al., 2005, 2006). The relative density of fluorescence staining was determined using IPLab 4.0 software. As shown in Fig. 1, raltegravir did not induce cell apoptosis, but it prevented HIV PI-induced cell apoptosis. We further examined the activation of caspases by Western blot analysis. As shown in Fig. 2, lopinavir/ritonavir-induced caspase-9 activation was inhibited by raltegravir.

Effect of Raltegravir on HIV PI-Induced ER Stress in Rat Primary Hepatocytes. To examine whether raltegravir has the similar effect as HIV PIs on the UPR activation, rat primary hepatocytes were treated with different amounts of raltegravir with or without lopinavir and ritonavir. The expression of UPR marker gene CHOP was determined by real-time RT-PCR. As shown in Fig. 3A, raltegravir did not induce CHOP mRNA expression, but it inhibited lopinavir/ritonavir-induced CHOP mRNA expression. To further confirm the effect of raltegravir on HIV PI-induced UPR activation, we examined the protein expression levels of CHOP and spliced XBP-1 (XBP-1s) using Western blot analysis. As shown in Fig. 3B, raltegravir did not induce CHOP and XBP-1s expression. Surprisingly, it inhibited HIV PI-induced CHOP and XBP-1s expression in primary rat hepatocytes.

Effect of Raltegravir on HIV PI-Induced Lipid Accumulation in Rat Primary Hepatocytes. To determine whether the protective effect of raltegravir on HIV PI-induced UPR activation is correlated to its effect on lipid homeostasis, we first examined the effect of raltegravir on lopinavir and ritonavir-induced lipid accumulation in hepatocytes. The intracellular lipid was stained using Nile red fluorescence dye as described previously (Wu et al., 2008). As shown in Fig. 4, raltegravir did not induce lipid accumulation, but it prevented HIV PI-induced lipid accumulation. We

Fig. 3. Effect of raltegravir on HIV PI-induced UPR activation in rat primary hepatocytes. **A**, rat primary hepatocytes were treated with different concentrations of raltegravir (0, 5, 15, or 25 μ M) in the absence or presence of lopinavir (LOPV)/ritonavir (RITV) (4:1; 25 μ M) for 24 h. The total cellular RNA was isolated and reverse-transcribed. The relative mRNA levels of CHOP were determined by real-time PCR as described under *Materials and Methods*. Values are mean $\pm$ S.E. of three independent experiments. *, $p < 0.05$, statistical significance relative to vehicle control. #, $p < 0.05$, statistical significance of the HIV PI-treated group and HIV PI + raltegravir group. **B**, representative immunoblots from three independent experiments for CHOP, XBP-1 and lamin B from nuclear extracts of rat primary hepatocytes treated with LOPV/RITV (4:1; 25 μ M) in the absence or presence of raltegravir (25 μ M) for 24 h. Lamin B was used as loading control.

further measured the intracellular levels of triglyceride, total cholesterol and free cholesterol using Wako kits. As shown in Fig. 5, lopinavir/ritonavir-induced increase of intracellular triglyceride, total cholesterol, and free cholesterol was also significantly inhibited by raltegravir.

Effect of Raltegravir and HIV PIs on the Key Genes Involved in Lipid Metabolism in Rat Primary Hepatocytes. To identify the mechanisms by which raltegravir prevents HIV PI-induced lipid accumulation in hepatocytes, we first examined the effect of raltegravir and HIV PIs on the protein levels of activated regulatory element-binding proteins SREBP-1 and SREBP-2. As shown in Fig. 6A, raltegravir had no effect on SREBP-1 and SREBP-2 expression, but significantly inhibited lopinavir/ritonavir-induced increase of protein levels of active SREBP-1. We also observed that raltegravir inhibited HIV PI-induced FAS expression (Fig. 6B). Real-time RT-PCR analysis indicated that lopinavir/ritonavir-induced decrease of mRNA levels of CPT-1 α , a key enzyme involved in fatty acid oxidation in mitochondria, was partially reversed by raltegravir (Fig. 6C).

Our previous studies have shown that HIV PIs induce intracellular cholesterol accumulation in hepatocytes through inhibition of bile acid synthesis, which is one of the major cholesterol output pathways (Zhou et al., 2006). Raltegravir had no

Fig. 4. Effect of raltegravir on HIV PI-induced lipid accumulation in rat primary hepatocytes. Rat primary hepatocytes were treated with 25 μ M lopinavir (LOPV), ritonavir (RITV), or LOPV/RITV (4:1) with or without raltegravir (25 μ M) for 24 h. The intracellular lipid was stained with Nile red dye and visualized under a fluorescence microscope as described under *Materials and Methods*. Representative images from three independent experiments are shown.

effect on the expression of CYP7A1, the key enzyme involved in bile acid synthesis; it also did not reverse HIV PI-induced inhibition of CYP7A1 and bile acid formation (Fig. 7). However, raltegravir prevented lopinavir/ritonavir-induced inhibition of mRNA expression of ATP-binding cassette transporters such as ABCA1, ABCG5, and ABCG8, which mediate the efflux of intracellular cholesterol (Fig. 8; Wang and Tall, 2003).

Effect of Raltegravir on HIV PI-Induced ER Stress and Dyslipidemia In Vivo. To determine whether the protective effect of raltegravir on HIV PI-induced UPR activation and dysregulation of lipid metabolism in vitro primary hepatocytes occurs in vivo, C57/BL6 male mice were treated with lopinavir/ritonavir with or without raltegravir for 4 weeks. The serum lipid profile was determined. As shown in Fig. 9, lopinavir/ritonavir significantly increased serum triglyceride and cholesterol levels, which were inhibited by raltegravir cotreatment. But they had no effect on high-density lipoprotein-cholesterol and LDL-cholesterol levels (data not shown). Similarly, HIV PI-induced lipid accumulation in liver was also reduced by raltegravir (Fig. 10, A and B). The Western blot and real-time RT-PCR further indicated that HIV PI-induced increase of SREBP-1 and FAS expression was inhibited by raltegravir (Fig. 10, C and D). In addition, HIV PI-induced UPR activation was also inhibited by raltegravir (Fig. 11).

Discussion

The development of HIV PIs has been one of the most significant advances of the past decade in controlling HIV

Fig. 5. Effect of raltegravir and HIV PIs on intracellular lipids in primary rat hepatocytes. Rat primary hepatocytes were treated with different concentrations of raltegravir (0, 5, 15, or 25 μM) in the absence or presence of lopinavir (LOPV)/ritonavir (RITV) (4:1; 25 μM) for 24 h. The intracellular triglyceride, total cholesterol, and free cholesterol levels were determined using Wako kits as described under *Materials and Methods*. Values are mean $\pm$ S.E. of three independent experiments. *, $p < 0.05$ and ***, $p < 0.001$, statistical significance relative to vehicle control. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$, statistical significance of HIV PI + raltegravir-treated group relative to HIV PI-treated group.

infection. Incorporation of HIV PIs in HAART has markedly decreased mortality and morbidity of patients with HIV infection. Despite the clinical successes of HIV PIs, accumulating clinical evidence suggests that treatment with HIV PIs is implicated in the pathogenesis of metabolic syndrome, including insulin resistance, dyslipidemia, and lipodystrophy, and potentially contributes to cardiovascular complications associated with HAART (Mallon et al., 2001; Moyle and Carr, 2002; Koster et al., 2003; Spector, 2006). Lipid abnormalities and inflammation associated with HIV PIs are two important

Fig. 6. Effect of raltegravir and HIV PIs on the expression of key genes involved in lipid metabolism in primary rat hepatocytes. A, representative immunoblots from three independent experiments for SREBP-1 and lamin B from nuclear extracts of rat primary hepatocytes treated with different concentrations of raltegravir (0, 5, 15, or 25 μM) in the absence or presence of lopinavir (LOPV)/ritonavir (RITV) (4:1; 25 μM) for 24 h. Lamin B was used as loading control. B, representative immunoblots from three independent experiments for FAS and β -actin from cytoplasmic proteins of rat primary hepatocytes treated with different concentrations of raltegravir (0, 5, 15, or 25 μM) in the absence or presence of LOPV/RITV (4:1; 25 μM) for 24 h. β -Actin was used as loading control. C, rat primary hepatocytes were treated with different concentrations of raltegravir (0, 5, 15, or 25 μM) in the absence or presence of LOPV/RITV (4:1; 25 μM) for 24 h. The total cellular RNA was isolated and reverse-transcribed. The relative mRNA levels of CPT1 α were determined by real-time PCR as described under *Materials and Methods*. Values are mean $\pm$ S.E. of three independent experiments. *, $p < 0.05$, statistical significance relative to vehicle control. #, $p < 0.05$ and ##, $p < 0.01$, statistical significance of HIV PI + raltegravir-treated group relative to HIV PI-treated group.

risk factors for cardiovascular disease, which is becoming the leading cause of morbidity and mortality in HIV-1-infected patients (Moyle and Carr, 2002). At present, the relationship between HAART and atherosclerosis and the underlying mechanisms behind HIV PI-associated serious metabolic abnormality in HIV-infected patients are still unknown. Therapeutic and preventive strategies have so far been of only limited success. Studies from our group and other groups suggest that HIV PI-induced ER stress represents an important cellular mechanism underlying HIV PI-induced dyslipidemia, insulin resistance, and atherosclerosis (Carr et al., 2001; Zhou et al., 2005, 2006, 2007; Spector, 2006). It also has been reported that HIV PIs disrupt glucose metabolism by

Fig. 7. Effect of raltegravir and ritonavir on CYP7A1 and HMG-CoA-reductase mRNA expression and bile acid formation in rat primary hepatocytes. A, rat primary hepatocytes were treated with raltegravir (25 μ M) in the absence or presence of ritonavir (25 μ M) for 24 h. Total RNA was isolated. The mRNA levels of cholesterol 7 α -hydroxylase (CYP7A1) and HMG-CoA-reductase were determined by RPA analysis. Rat cyclophilin mRNA was used as loading control. B, rat primary hepatocytes were treated with different amounts of raltegravir (0–25 μ M) in the absence or presence of ritonavir (25 μ M) for 48 h. Bile acid biosynthesis was measured as conversion of [14 C]cholesterol to 14 C-labeled bile acids. Labeled bile acids were extracted from media by using the Folch method. Data are expressed as a percentage of vehicle control group. The values are means $\pm$ S.E. of three independent experiments. *, $p < 0.05$, statistical significance relative to vehicle control.

affecting glucose transporter 4, interrupting insulin signaling pathways, and impairing insulin-mediated glucose disposal (Hruz et al., 2002; Murata et al., 2002; Koster et al., 2003; Lee et al., 2007; Djedaini et al., 2009).

The ability of HIV to mutate and become resistant to the HIV PIs and reverse transcriptase inhibitors leads to an increasing proportion of the HIV-1-infected patients harboring treatment-resistant virus. As a key enzyme involved in viral replication, integrase presents a potential chemotherapeutic target for the treatment of HIV infection. Raltegravir is the first agent in a new class of antiretrovirals, HIV integrase inhibitors. It has a demonstrated potent efficacy against multidrug-resistant HIV-1 and was initially approved by Food and Drug Administration in 2007 to treat treatment-experienced HIV-1-infected patients (Cocohoba and Dong, 2008; Steigbigel et al., 2008; Elbasha et al., 2009; Hughes et al., 2009). Based on the most recent guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents released by the United States Department of Health and Human Services in December 2009, raltegravir also has been listed as one of the preferred regimens recommended for treatment-naïve HIV-1-infected patients. Clinical studies have shown that raltegravir was well tolerated and had fewer side effects compared with other classes of antiretrovirals, such as HIV PIs and reverse transcriptase inhibitors (Hughes et

Fig. 8. Effect of raltegravir and HIV PIs on ABC transporter mRNA expression in rat primary hepatocytes. Rat primary hepatocytes were treated with different concentrations of raltegravir (0–15 μ M) in the absence or presence of lopinavir (LOPV)/ritonavir (RITV) (4:1; 25 mM) for 24 h. The total cellular RNA was isolated and reverse transcribed. The relative mRNA levels of ABCA1, ABCG5, and ABCG8 were determined by real-time PCR as described under *Materials and Methods*. The values are means $\pm$ S.E. of three independent experiments. *, $p < 0.05$, statistical significance relative to vehicle control. #, $p < 0.05$, statistical significance of LOPV/RITV + raltegravir-treated group relative to LOPV/RITV-treated group.

al., 2009). The peak plasma concentration ranges from 10.63 to 24.67 μ M with 400- to 800-mg/day doses (Iwamoto et al., 2008c).

In the present study, we examined the effects of raltegravir on HIV PI-induced UPR activation, apoptosis, and dysregulation of lipid metabolism in vitro rat primary hepatocytes and in vivo C57/BL6 mice. The results demonstrated for the first time that raltegravir has minimal hepatic toxicity and could prevent HIV PI-induced apoptosis and dysregulation of lipid metabolism both in primary hepatocytes and in in vivo mouse livers.

Fig. 9. Effect of raltegravir and HIV PIs on serum lipid levels in vivo. Male C57BL/6 mice (8 weeks old) were treated with lopinavir (LOPV)/ritonavir (RITV) with or without raltegravir for 4 weeks. The serum triglyceride and total cholesterol levels were determined using Wako kits as described under *Materials and Methods*. Values are mean $\pm$ S.E. of five animals ($n = 5$). *, $p < 0.05$, statistical significance relative to vehicle control. #, $p < 0.05$, statistical significance of HIV PI + raltegravir-treated group relative to HIV PI-treated group.

Raltegravir is primarily metabolized in the liver by uridine diphosphate glucuronosyltransferase 1A1-mediated glucuronidation (Kassahun et al., 2007). Ritonavir is an inhibitor of CYP3A, which is recommended to be used as a boosting agent to increase the plasma levels of other HIV PIs (Hughes et al., 2009). Several studies have reported that there are no significant drug-drug interactions between HIV PIs and raltegravir (Iwamoto et al., 2008a,b; Imaz et al., 2009). Our pharmacokinetic studies also demonstrated that raltegravir had no significant effect on the uptake and metabolism of the most commonly used HIV PIs (ritonavir and lopinavir) in rat primary hepatocytes (Table 2). Similarly, HIV PIs also did not affect the uptake and metabolism of raltegravir (Table 3) in hepatocytes. Therefore, the protective effect of raltegravir on HIV PI-induced ER stress and apoptosis was not due to the decrease in intracellular concentration of HIV PIs in hepatocytes.

The role of ER stress is a rapidly emerging field of interest in the pathogenesis of various human diseases, including nearly all types of human liver diseases such as NAFLD, alcoholic liver disease, chronic viral hepatitis, and drug-induced fatty liver (Feng et al., 2003; Parker et al., 2005; Szegezdi et al., 2006; Zhang et al., 2006; Kaplowitz et al., 2007; Chinta et al., 2009). Our most recent studies demonstrated that activation of the ER stress also plays a critical role in HIV PI-induced disruption of intestinal barrier integrity (Wu et al., 2010). Both the in vitro and in vivo results obtained from this current study suggest that raltegravir could prevent HIV PI-induced apoptosis by inhibiting ER stress in rat primary hepatocytes and in liver. Consistent with our previous findings, we also found that inhibition of HIV PI-induced ER stress by raltegravir was correlated to its ability to reduce HIV PI-induced intracellular lipid accumulation and cholesterol levels both in vitro and in vivo. How-

Fig. 10. Effect of raltegravir on HIV PI-induced lipid accumulation and expression of key genes involved in lipid metabolism in mice. A, male C57BL/6 mice (8 weeks old) were treated with lopinavir (LOPV)/ritonavir (RITV) with or without raltegravir for 4 weeks. Photomicrographs of hematoxylin- and eosin-stained liver sections representing each of the four treatment groups, respectively, are shown. a, control group. b, LOPV/RITV group. c, raltegravir group. d, LOPV/RITV + raltegravir group. B, relative histologic score of hematoxylin- and eosin-stained liver sections. C, effect of raltegravir on HIV PI-induced SREBP-1 expression. The protein levels of the nuclear SREBP-1 (nSREBP-1) were determined by Western blot analysis and normalized to the protein levels of nuclear protein loading control of lamin B. The relative density was shown for each treatment group. Values are mean $\pm$ S.E. of five animals ($n = 5$). *, $p < 0.05$, statistical significance relative to vehicle control. #, $p < 0.05$, statistical significance of HIV PI + raltegravir-treated group relative to HIV PI-treated group. D, effect of raltegravir on HIV PI-induced FAS and ACC-1 expression. The mRNA levels of FAS and ACC-1 in liver tissues of each treatment group were determined using real-time RT-PCR as described under *Materials and Methods*. Values are mean $\pm$ S.E. of five animals ($n = 5$). *, $p < 0.05$, statistical significance relative to vehicle control. #, $p < 0.05$, statistical significance of HIV PI + raltegravir-treated group relative to HIV PI-treated group.

Fig. 11. Effect of raltegravir on HIV PI-induced UPR activation in vivo. Male C57BL/6 mice (8 weeks old) were treated with lopinavir (LOPV)/ritonavir (RITV) with or without raltegravir for 4 weeks. The mRNA levels of CHOP and XBP-1 in liver tissues of each treatment group were determined using real-time RT-PCR as described under *Materials and Methods*. Values are mean $\pm$ S.E. of five animals ($n = 5$). *, $p < 0.05$, statistical significance relative to vehicle control. #, $p < 0.05$, statistical significance of HIV PI + raltegravir-treated group relative to HIV PI-treated group.

ever, the relationship of ER stress activation and hepatic lipid accumulation remains to be further determined.

The pathways of cholesterol and fatty acid metabolism are woven together tightly. Lipid overloading of hepatocytes is a prerequisite for the sequence of events that lead to liver injury and fibrogenesis. Accumulation of hepatic lipid results when lipid influx and de novo synthesis exceeds hepatic lipid export and use. Further analysis of the effect of raltegravir and HIV PIs on the expression of key genes involved in lipid metabolism in liver indicated that raltegravir inhibited HIV PI-induced up-regulation of SREBP-1, FAS, and ACC-1 and down-regulation of CPT-1 α (Fig. 6). Although raltegravir had no effect on HIV PI-induced inhibition of bile acid formation (Fig. 7), it up-regulated the expression of ABCA1, ABCG5, and ABCG8, which mediate the efflux of cellular cholesterol (Fig. 8). Further studies are needed to uncover the molecular mechanisms underlying the potential protective effects of raltegravir against HIV PI-associated dyslipidemia.

In summary, the addition of a HIV integrase inhibitor and second generation PIs has significantly increased the number of available agents to treat multidrug-resistant HIV and reduce the potential adverse effects associated with commonly used anti-HIV agents. The preliminary observations presented in this study suggest that HIV integrase inhibitors may be useful compounds not only for inhibiting HIV but also for treating NAFLD.

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