Cardiovascular, Pulmonary and Renal Pathology

Roles and Mechanisms of Human Immunodeficiency Virus Protease Inhibitor Ritonavir and Other Anti-Human Immunodeficiency Virus Drugs in Endothelial Dysfunction of Porcine Pulmonary Arteries and Human Pulmonary Artery Endothelial Cells

Xinwen Wang, Hong Chai, Peter H. Lin,
Qizhi Yao, and Changyi Chen

From the Molecular Surgeon Research Center, Division of Vascular Surgery and Endovascular Therapy, Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston; and the Michael E. DeBakey Veteran's Administration Medical Center, Houston, Texas

The objective of this study was to determine the effects of highly active antiretroviral therapy (HAART) drugs on pulmonary endothelial function. Porcine pulmonary arteries or human pulmonary arterial endothelial cells (HPAECs) were incubated with eight HAART drugs [ritonavir, indinavir, lopinavir, zidovudine (AZT), abacavir, stavudine, didanosine (ddI), and lamivudine] individually or in combination [three HAART drugs (3-plex; indinavir, stavudine, and ddI)] at their clinical plasma concentrations for 24 hours. Endothelium-dependent vasorelaxation in response to bradykinin was reduced significantly by the ritonavir in a concentration-dependent manner. Five other HAART drugs (indinavir, lamivudine, abacavir, AZT, and ddI) and the 3-plex significantly also impaired endothelium-dependent vasorelaxation in response to bradykinin. Five HAART drugs (ritonavir, indinavir, lamivudine, abacavir, and AZT) significantly decreased endothelial nitric oxide synthase (eNOS) expression and increased superoxide anion levels in both vessels and HPAECs. Furthermore, both ritonavir and AZT substantially activated ERK2 in HPAECs. Additionally, the antioxidants ginsenoside Rb1 and ginkgolide A effectively reversed HAART drug-induced vasomotor dysfunction and eNOS down-regulation. Inhibition of ERK1/2 also partially blocked ritonavir- and AZT-induced down-regulation of eNOS and vasomotor dysfunction. Thus, HAART drugs significantly impair endothelial functions of porcine pul-

monary arteries and HPAECs, which may be mediated by eNOS down-regulation, oxidative stress, and ERK1/2 activation. These findings suggest that HAART drugs may contribute to the high incidence of pulmonary artery hypertension in human immunodeficiency virus-infected patients. (Am J Pathol 2009, 174:771–781; DOI: 10.2353/ajpath.2009.080157)

Human immunodeficiency virus (HIV) infection remains a significant clinical problem globally. At the end of 2007, there were 33.2 million persons living with HIV infection, 2.5 million new infections, and 2.1 million deaths from HIV occurred during the year (Joint United Nations Programme on HIV/AIDS/World Health Organization: AIDS Epidemic Update; December 2007, <http://www.unaids.org>). Highly active antiretroviral therapy (HAART) is a combination of at least three antiretroviral drugs: a protease inhibitor and/or a non-nucleoside analog reverse transcriptase inhibitor and one or two nucleoside analog reverse transcriptase inhibitors. The ability of HAART regimens to suppress viral replication profoundly and to increase CD4⁺ T-cell counts has transformed HIV infection into a manageable chronic disease in many patients and has dramatically improved the prognosis of HIV-infected patients.¹ However, there is a growing concern regarding risk factors for cardiovascular disease observed in patients receiving HAART.^{2–5} Stein and colleagues⁵ reported

Supported in part by the National Institutes of Health (research grants DE15543 and AT003094 to Q.Y.; and HL65916, HL72716, EB-002436, and HL083471 to C.C.); the Michael E. DeBakey Department of Surgery, Baylor College of Medicine; and the Michael E. DeBakey Veteran's Administration Medical Center.

Accepted for publication November 11, 2008.

Address reprint requests to Changyi (Johnny) Chen, M.D., Ph.D., Michael E. DeBakey Department of Surgery (MARB413), Baylor College of Medicine, One Baylor Plaza, Mail Stop BCM390, Houston, TX 77030. E-mail: jchen@bcm.tmc.edu.

that HIV protease inhibitors impaired endothelial functions in humans by analysis of flow-mediated vasodilation of the brachial artery in patients with HIV-1 infection. Effects of other HAART drugs on the vascular system are primarily unknown.

The effects of HAART on the clinical course of HIV-associated pulmonary artery hypertension (HIV-PAH) are still debated. A retrospective study on the impact of antiretroviral therapy on cardiac involvement by Pugliese and colleagues⁶ analyzed a population of 1042 HIV-infected patients. They found that HIV-PAH was significantly more frequent in patients treated with HAART than in those treated only with nucleoside reverse transcriptase analogs. In another prospective study, patients with HIV-PAH treated with HAART drugs including protease inhibitors had an accelerated course of PAH and a worsening of pulmonary artery systolic pressure, even though the HIV viral load was low.⁷ Furthermore, Nunes and colleagues⁸ observed a higher mobility rate in HIV-PAH patients who received HAART. These studies indicated a potential connection between PAH and HAART. However, it lacks direct evidence that HAART drugs affect pulmonary artery cells and their functions. In addition, HIV and virus proteins may also contribute to HIV-PAH. For example, HIV gp120 proteins could induce apoptosis in primary human lung endothelial cells.⁹

Recently, we have demonstrated that HIV protease inhibitors including ritonavir, amprenavir, and saquinavir can directly cause endothelial dysfunction in porcine coronary arteries through the oxidative stress, the activation of mitogen-activated protein kinases (MAPKs), and the down-regulation of endothelial nitric oxide synthase (eNOS).^{10,11} Furthermore, endothelial dysfunction induced by HIV protease inhibitors can be effectively reversed by several antioxidants including ginsenosides. We also show that ginkgolide A (GA), a major constituent of *Ginkgo biloba*, effectively prevents homocysteine-induced endothelial dysfunction.¹² Kappert and colleagues¹³ reported that HAART [lopinavir, ritonavir, lamivudine, and zidovudine (AZT)] inhibited endothelial cell-mediated healing and promoted neointima formation after angioplasty in rats. More recently, a study showed that AZT or AZT plus indinavir treatments dramatically reduced endothelium-dependent vessel relaxation in aortic rings of rats and increased the reactive oxygen species (ROS) production in human umbilical vein endothelial cells.¹⁴

In the current study, we hypothesized that HIV protease inhibitor ritonavir and other HAART drugs may cause endothelial dysfunction in pulmonary arteries through unique molecular pathways, and ginseng and *Ginkgo biloba* compounds may effectively block these detrimental effects of HAART drugs on pulmonary arteries. We used both the porcine pulmonary artery model and human pulmonary artery endothelial cells (HPAECs) to elucidate the effects and potential molecular mechanisms of HAART drugs on pulmonary arteries. This study may provide valuable information for the development of effective strategies for the prevention and treatment of HIV-PAH.

Materials and Methods

Chemicals and Reagents

HAART drugs AZT, abacavir, stavudine (d4T), didanosine (ddI), lamivudine, indinavir, lopinavir, and ritonavir were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. All HAART drugs were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was adjusted to less than 0.1% (v/v), which was used in all controls. DMSO, thromboxane A₂ analogue U46619, bradykinin, sodium nitroprusside (SNP), ginsenoside Rb1, GA, and Tri-reagent kit were obtained from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium was obtained from Life Technologies, Inc. (Grand Island, NY). Lucigenin was obtained from Molecular Probes (Eugene, OR). Antibody against human eNOS was obtained from BD Transduction Laboratories (Lexington, KY). The biotinylated horse anti-mouse IgG and avidin-biotin complex kit were obtained from Vector Laboratories (Burlingame, CA).

Myograph Model

Fresh porcine lungs were harvested from young adult farm pigs (6 to 7 months old) at a local slaughterhouse, placed in a container filled with cold phosphate-buffered saline (PBS) solution, and immediately transported to the laboratory. The third division of porcine pulmonary arteries (3 to 4 mm in diameter) were isolated and cut into 5-mm rings. Several rings from each lung were allocated into groups: controls (DMSO); those treated with anti-HIV drugs including AZT, abacavir, d4T, ddI, lamivudine, indinavir, lopinavir, or ritonavir at their clinical plasma concentrations as shown in Table 1; those treated with combination of three HAART drugs (3-plex; 12.5 $\mu\text{mol/L}$ indinavir, 4 $\mu\text{mol/L}$ d4T, and 11 $\mu\text{mol/L}$ ddI); and those treated with HAART drugs plus Rb1 or GA. Experiments were performed at least in triplicate.

The myograph tension system used in our laboratory has been previously described.¹² Briefly, the rings were cultured in the medium for 24 hours and then were suspended between the wires of the organ bath chamber (Multi Myograph System 700MO; Myo Technology, Aarhus N, Denmark) in 6 ml of Krebs's solution. After equilibration, each ring was precontracted with U46619 (3×10^{-7} mol/L). After 30 to 60 minutes of contraction, the relaxation concentration-response curve was generated by adding six cumulative additions of the endothelium-dependent vasodilator bradykinin (from 10^{-11} to 10^{-6} mol/L) every 3 minutes. In addition, 10^{-6} mol/L SNP was added to the organ bath, and endothelium-independent vasorelaxation was recorded.

Cell Culture

HPAECs were purchased from Cambrex (San Diego, CA). The cells were used at passage 4 to 5. Once cells

Table 1. Maximal Plasma Concentrations of Anti-HIV Drugs in the Clinical Dose (Physician Desk Reference 2006)

Drug	Drug type	Brand name	Dose	Cmax*	Concentration
Ritonavir	PI	Norvir	600 mg, bid	11.2 ± 3.6 µg/ml	15.5 µmol/L
Indinavir	PI	Crixivan	800 mg, tid	8.9 µg/ml	12.5 µmol/L
Lopinavir	PI	Kaletra	400 mg, qd	9.6 ± 4.4 µg/ml	15 µmol/L
Zidovudine (AZT)	NRTI	Retrovir	300 mg, qd	1.06 µg/ml	4 µmol/L
Abacavir	NRTI	Ziagen	300 mg, bid	3.0 ± 0.89 µg/ml	10 µmol/L
Stavudine (d4T)	NRTI	Zerit	40 mg, bid	0.9 µg/ml	4 µmol/L
Didanosine (ddI)	NRTI	Videx	200 mg, bid	2.6 µg/ml	11 µmol/L
Lamivudine	NNRTI	Epivir	150 mg, bid	1.5 ± 0.5 µg/ml	6.5 µmol/L

*Cmax, the peak plasma concentration; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor.

grew to 80 to 90% confluence in six-well culture plates, they were treated with DMSO as a control or with HAART drugs for 24 hours at 37°C. Cells were then applied to studies of eNOS expression, superoxide anion production, mitochondrial membrane potential, and MAPK phosphorylation.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The porcine pulmonary artery endothelial cells were collected from cultured artery rings by scraping the luminal surface with surgical blades. Total cellular RNA was then extracted using a RNeasy-4PCR kit (Ambion, Austin, TX). Primers for eNOS were designed via the Beacon Designer 2.1 software (Bio-Rad, Hercules, CA). The sequences of the eNOS primers were: 5'-GCCAGAACACAGCCAGCTC-3' (sense) and 5'-CCCAGTTCTCACACGAGGGAAC-3' (antisense). The CD31 primer sequences were: 5'-AAGCAGAACTAAGTGCCTGGAG-3' (sense) and 5'-CCTGACCCTCAAGGCTTGAAC-3' (antisense). The iQ SYBR green supermix kit and iCycler iQ real-time PCR detection system (Bio-Rad) were used in real-time PCR. Relative mRNA levels of eNOS were presented as $2^{[Ct(CD31) - Ct(eNOS)]}$ as previously described.³

Western Blot Analysis

Cellular proteins of HPAECs were extracted. An equal amount of total proteins (50 µg) was loaded onto 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, fractionated by electrophoresis, and transferred to polyvinylidene difluoride membranes. The membrane was incubated with the monoclonal antibody against human eNOS (1:2000) at 4°C overnight. The membrane was incubated with anti-mouse (1:10,000) horseradish peroxidase-labeled antibodies for 40 minutes. Protein bands were visualized with enhanced chemiluminescence plus chemiluminescent substrate. Densitometric measurement was performed to quantify the relative expression of target proteins versus β-actin.

Flow Cytometry

HPAECs were treated with or without HAART drugs for 24 hours. The cells were detached with trypsin/ethylenedia-

minetetraacetic acid solution and then suspended in cold staining buffer (PBS containing 2% fetal bovine serum and 0.09% sodium azide). The cells were permeabilized with cytoperm/cytofix buffer in 4°C for 20 minutes. Cells (1×10^6) were immunostained with anti-human eNOS antibody at 4°C for 30 minutes. Mouse IgG1 was used as an isotype control. Phycoerythrin-conjugated anti-mouse IgG1 was used as secondary antibody for eNOS staining. The cells were washed, fixed with 2% paraformaldehyde, and analyzed by FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) with the CellQuest software. Data are presented as the percentage of positive cells corresponding to the gated cells and mean fluorescence intensity in each experiment.

For the detection of ROS generation, HPAECs were incubated with 0.5 ml of dihydroethidium (DHE, 3 µmol/L) for 20 minutes at room temperature. DHE is freely permeable to cells. In the presence of superoxide anion, DHE is oxidized to ethidium bromide (EtBr) with red fluorescence, and it is trapped by intercalating with the DNA. EtBr is excited at 488 nm with an emission spectrum of 610 nm. Thus, the amount of EtBr detected by fluorescence measurement instruments such as flow cytometer is well correlated to the level of cellular superoxide anion. Superoxide anion in the cells was then analyzed by FACScalibur flow cytometry and presented by the percentage of positively staining cells in HPAECs.

Lucigenin-Enhanced Chemiluminescence Assay

Levels of superoxide anion produced by endothelial cells of the porcine arteries were detected using the lucigenin-enhanced chemiluminescence method as previously described in our studies.¹¹ Briefly, the rings were cut open longitudinally and trimmed into 5 × 5-mm pieces. An assay tube was filled with 5 µmol/L lucigenin. Time-based reading with luminometer was recorded. The data, in relative light units per second (RLU/second) for each sample, were averaged between 5 and 10 minutes. Values of blank tubes containing the same reagents as the vessel ring samples were subtracted from their corresponding vessel samples. The area of each vessel segment was measured using a caliper and was used to normalize the data for each sample. Final data were represented as RLU/second/mm².

Figure 1. Effects of ritonavir, Rb1, and GA on vasomotor function in porcine pulmonary arteries. Porcine pulmonary artery rings were cultured with DMSO as a control or treated with anti-HIV drugs or antioxidants (Rb1 or GA) for 24 hours. **A:** Maximal contraction of the vessel rings in response to thromboxane A₂ analogue U46619 (3×10^{-7} mol/L). **B:** Precontracted vessels were tested for endothelium-dependent relaxation by adding a series of concentrations of bradykinin (10^{-11} to 10^{-6} mol/L). **C:** Endothelium-independent relaxation in response to SNP (10^{-6} mol/L). **D:** Fifteen μmol/L ritonavir showed significant impairments in vasorelaxation compared with the control, whereas both Rb1 and GA significantly improved vasorelaxation compared with ritonavir alone group. * $P < 0.05$ versus controls (DMSO only). $n = 3$. RVT, ritonavir; Rb1, ginsenoside Rb1.

Assessment of Mitochondrial Membrane Potential ($\Delta\psi_m$)

$\Delta\psi_m$ was assessed by using flow cytometry analysis of cells stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanide iodine (JC-1, MitoScreen kit; BD Biosciences, San Diego, CA). Mitochondria with a normal $\Delta\psi_m$ concentrate JC-1 into aggregates (red fluorescence). Cells (5×10^5) were incubated with 10 μg/ml of JC-1 for 15 minutes at 37°C and analyzed by FACS-Calibur flow cytometry.

Bio-Plex Immunoassay

HPAECs were treated with 15 μmol/L ritonavir or 8 μmol/L AZT for 0, 5, 10, 20, 30, 60, and 90 minutes. Cell lysates (25 μg) were prepared for the MAPK immunoassay by using Bio-Plex phosphoprotein assay and total target assay kits. The kits were applied on a Luminex multiplex system (Bio-Rad) following the manufacturer's instructions, which can detect the amount of phosphorylated and total ERK2, p38, and JNK in a small amount of cell lysates. Results were presented as the ratio of phosphorylated and total target proteins.

Statistical Analysis

All data are presented as the mean $\pm$ SEM. All data were corrected with Bonferroni posthoc test. Differences among three or more groups were analyzed using one-way analysis of variance. Student's *t*-test was used for comparison between two groups. A *P* value < 0.05 was regarded as significant.

Results

Effects of HIV Protease Inhibitor Ritonavir and Other Anti-HIV Drugs on Vasomotor Functions in Porcine Pulmonary Arteries

We first tested the effects of anti-HIV drugs on vasomotor functions in porcine pulmonary arteries by a myograph system including vascular contraction (U46619), endothelium-dependent (bradykinin), and endothelium-independent (SNP) relaxation assays. Both the contraction and endothelium-dependent relaxation of the vessel rings were significantly reduced by ritonavir in a concentration-dependent manner (Figure 1, A and B). At 15 and 30 μmol/L ritonavir, the endothelium-dependent relaxation of vessel rings in response to 10^{-6} mol/L bradykinin was significantly reduced by 39% and 63%, respectively, compared with controls ($P < 0.05$). In response to 10^{-6} mol/L SNP, the vessel rings treated with 30 μmol/L ritonavir revealed a significant decrease of the endothelium-independent relaxation by 57% compared with controls (Figure 1C, $P < 0.05$). Furthermore, antioxidants GA or Rb1 at 10 μmol/L effectively blocked ritonavir-induced vasomotor dysfunction in porcine pulmonary arteries (Figure 1D, $P < 0.05$).

In addition to ritonavir, endothelium-dependent relaxation of the vessel rings was significantly reduced by five other HAART drugs (indinavir, lamivudine, abacavir, AZT, and ddI) at their clinical plasma concentrations, respectively, compared with controls (Figure 2A, $P < 0.05$). However, both lopinavir- and d4T-treated vessels did not show significant changes of vasorelaxation in response to 10^{-8} mol/L bradykinin (Figure 2A). In separate experiments, both indinavir and AZT significantly reduced en-

Figure 2. Effects of other HAART drugs on vasomotor function in porcine pulmonary arteries. Porcine pulmonary artery rings were treated with HAART drugs or with DMSO as a control for 24 hours. **A:** In response to 10^{-8} bradykinin, endothelium-dependent relaxation of vessels was significantly reduced by indinavir, lamivudine, abacavir, and AZT at their clinical plasma concentrations, respectively, compared with controls. **B:** Endothelium-dependent relaxation in response to bradykinin (10^{-11} to 10^{-6} mol/L) was significantly reduced by AZT in a concentration-dependent manner compared with controls. **C:** Endothelium-dependent relaxation in response to bradykinin (10^{-8} mol/L) was significantly impaired by indinavir, ddi, or 3-plex, as compared with controls, whereas the Rb1 plus 3-plex group demonstrated a significant improvement in vasorelaxation compared with the 3-plex group (** $P < 0.05$, $n = 3$). * $P < 0.05$ versus controls (DMSO only). $n = 3$. Rb1, ginsenoside Rb1; IDV, indinavir.

endothelium-dependent relaxation of the vessel rings in a concentration-dependent manner (Figure 2, B and C). In response to 10^{-8} mol/L bradykinin, indinavir at 12.5 μ mol/L and AZT at 8 μ mol/L significantly reduced endothelium-dependent relaxation of the vessel rings by 24% and 19%, respectively, compared with controls ($P < 0.05$). Furthermore, we evaluated the effect of HAART 3-plex (indinavir, d4T, and ddi) on the porcine pulmonary artery rings, and showed that 3-plex significantly impaired endothelium-dependent relaxation compared with controls. Consequently, ginsenoside Rb1 at 10 μ mol/L abolished 3-plex-induced vasomotor dysfunction (Figure 2C, $P < 0.05$).

Effects of HIV Protease Inhibitor Ritonavir and Other Anti-HIV Drugs on eNOS Expression in Both Porcine Pulmonary Arteries and HPAECs

To investigate whether eNOS could be involved in the anti-HIV drug-induced vasomotor dysfunction in porcine pulmonary arteries, eNOS expression in both treated arterial rings and HPAECs was analyzed by real-time PCR and flow cytometry. The effects of HAART drugs and antioxidants (Rb1 and GA) on the eNOS mRNA expression in the treated arterial rings are shown in Figure 3, A and B. Significant decreases of eNOS mRNA were observed in a concentration-dependent manner in response to ritonavir treatment. At 15 μ mol/L of ritonavir, eNOS mRNA in arterial rings showed a significant decrease by 58% compared with controls (Figure 3A, $P < 0.05$). In addition, co-treatment with ritonavir and Rb1 or GA significantly increased the eNOS mRNA levels in the vessel rings compared with ritonavir only group (Figure 3A, $P < 0.05$). Consistent with the mRNA level, ritonavir treatment also decreased the eNOS protein level in HPAECs. Rb1 or GA effectively blocked the ritonavir-induced decrease in eNOS protein levels (Figure 3C).

Expression of eNOS mRNA in the arterial rings was significantly reduced by four other HAART drugs (indinavir, lamivudine, abacavir, and AZT) at their clinical plasma concentrations, respectively, compared with con-

trols (Figure 3B, $P < 0.05$). Flow cytometry analysis showed similar decreases of eNOS protein in HPAECs when treated by these HAART drugs. However, the other three drugs (lopinavir, ddi, and d4T) had no effect on eNOS expression. Furthermore, we evaluated the effect of antioxidants on AZT-induced down-regulation of eNOS in HPAECs, and we found that both Rb1 and GA co-treatments improved eNOS protein levels compared with AZT-only group (Figure 3C). In addition to flow cytometry analysis, Western blot analysis also demonstrated a similar pattern of eNOS expression in HPAECs treated with these HAART drugs, and antioxidant Rb1 partially blocked the down-regulation of eNOS induced by AZT and ritonavir in HPAECs (Figure 3D).

Effects of HIV Protease Inhibitor Ritonavir and Other Anti-HIV Drugs on Superoxide Anion Production in Both Porcine Pulmonary Arteries and HPAECs

To investigate whether oxidative stress could play a role in the anti-HIV drug-induced vasomotor dysfunction in porcine pulmonary arteries, superoxide anion production from the arterial rings and HPAECs was analyzed by using lucigenin-enhanced chemiluminescence and flow cytometric measurement of DHE staining, respectively. Superoxide anion from the arterial rings was significantly increased in a concentration-dependent manner in response to ritonavir treatment. Ritonavir (15 μ mol/L) treatment significantly increased chemiluminescence signal (4.6 ± 1.1 RLU/second/ mm^2 , $n = 3$) than controls (2.79 ± 0.15 RLU/second/ mm^2 , $n = 3$) (Figure 4A, $P < 0.05$), indicating a 65% increase in ritonavir treatment compared with controls. In addition, superoxide anion in the arterial rings was significantly increased by four other HAART drugs (indinavir, lamivudine, abacavir, and AZT) at their clinical plasma concentrations, compared with controls (Figure 4B, $P < 0.05$). The increases of superoxide anion in the arterial rings induced by above anti-HIV drugs

Figure 3. Effects of HIV protease inhibitor ritonavir and other HAART drugs on eNOS expression in both porcine pulmonary arteries and HPAECs. Porcine pulmonary artery rings were cultured with or without HAART drugs for 24 hours, and the total mRNA was purified from endothelial layers of the rings. The eNOS mRNA was measured with real-time PCR. The eNOS mRNA level in each sample was normalized to CD31. Relative mRNA level was presented as $2^{-[\Delta(\Delta Ct)]}$. **A:** Ritonavir significantly decreased the porcine eNOS mRNA expression in a concentration-dependent manner compared with controls. **B:** The porcine eNOS mRNA was significantly reduced by indinavir, lamivudine, abacavir, and AZT at their clinical plasma concentrations, respectively, compared with controls. **C:** The eNOS protein levels in HPAECs were measured by flow cytometry. Consistent with mRNA levels, the treatments of the above drugs also induced decreases of eNOS protein levels in HPAECs (**e, f, i, j, k, l, D**). Rb1 or GA blocked the decrease of eNOS protein levels induced by RTV and AZT (**g, h, m, n**). x axis: eNOS expression fluorescence intensity; y axis: cell number. Individual percentage value represents the percentage of positive staining cells in the counted HPAECs from the different treatments. Figure 3C. **a.** No staining (control); **b.** Ab staining only; **c.** Rb1 (10 μM) only; **d.** GA (10 μM) only; **e.** RTV (15 μM); **f.** RTV (30 μM); **g.** RTV (15 μM) plus Rb1; **h.** RTV (15 μM) plus GA; **i.** Indinavir; **j.** Lamivudine; **k.** Abacavir; **l.** AZT; **m.** plus Rb1; and **n.** AZT plus GA. **D:** eNOS protein levels in HPAECs were demonstrated by Western blot. Quantitation of the band density was performed. * $P < 0.05$ versus controls (DMSO only). ** $P < 0.01$ versus RTV (15 μM) treatment alone. $n = 3$. Rb1, ginsenoside Rb1; RTV, ritonavir; IDV, indinavir.

were also confirmed in HPAECs by flow cytometric measurement of DHE staining (Figure 4C). Furthermore, treatment of HAART 3-plex showed a significant increase of superoxide anion in arterial rings compared with controls. The increase of superoxide anion was significantly abolished by Rb1 co-treatment compared with the 3-plex treatment group (Figure 4D, $P < 0.05$). Rb1 alone did not show any effect on the superoxide anion production.

Effects of HIV Protease Inhibitor Ritonavir and Other Anti-HIV Drugs on Mitochondrial Membrane Potential in HPAECs

Mitochondrial dysfunction could result in the increase of superoxide anion production. To determine whether the mitochondria could be a possible source of anti-HIV

Figure 4. Effects of the HIV protease inhibitor ritonavir and other anti-HIV drugs on superoxide anion production in both porcine pulmonary arteries and HPAECs. Superoxide anion levels in the endothelial layer of porcine pulmonary arteries were tested with lucigenin-enhanced chemiluminescence assay. **A:** Ritonavir significantly increased the superoxide anion levels of the vessel rings in a concentration-dependent manner compared with controls. **B:** Superoxide anion levels of the vessel rings were significantly reduced by indinavir, lamivudine, abacavir, and AZT at their clinical plasma concentrations, respectively, compared with controls. **C:** Superoxide anion levels in HPAECs were stained with DHE and analyzed by FACS Calibur flow cytometry. Similarly, superoxide anion levels in HPAECs were reduced by ritonavir (**c, d**), indinavir (**e**), lamivudine (**f**), abacavir (**g**), and AZT (**h**). Individual percentage value represents the percentage of positively staining cells in the counted HPAECs from different treatments. Figure 4C. **a.** No DHE (negative control); **b.** DHE only (staining control); **c.** RTV (15 μM); **d.** RTV (30 μM); **e.** Indinavir; **f.** Lamivudine; **g.** Abacavir; and **h.** AZT. **D:** Superoxide anion levels of the vessel rings were significantly impaired by 3-plex, whereas Rb1 significantly inhibited the increased levels of superoxide anion induced by 3-plex (** $P < 0.05$). * $P < 0.05$ versus controls (DMSO only). $n = 3$. Rb1, ginsenoside Rb1; RTV, ritonavir; IDV, indinavir.

Figure 5. Effects of HIV protease inhibitor ritonavir and other anti-HIV drugs on mitochondrial membrane potential in HPAECs. HPAECs were treated with ritonavir, indinavir, lamivudine, abacavir, and AZT at their clinical plasma concentrations, respectively, for 24 hours. **A:** Mitochondrial membrane potential was assessed with JC-1 staining and flow cytometry analysis. DMSO treatment was used as a control (**a**). Normal potential (red) in HPAECs was decreased in the treatment with ritonavir (**b**), indinavir (**c**), lamivudine (**d**), abacavir (**e**), and AZT (**f**). Individual percentage value represents the percentage of positive cells with mitochondrial JC-1 staining in HPAECs. **B:** Quantitative analysis of mitochondrial membrane potential in HPAECs. * $P < 0.05$ versus controls (DMSO only). $n = 3$. RTV, ritonavir.

drug-induced superoxide anion production, the mitochondrial function was analyzed by flow cytometric measurement of JC-1 staining. As shown in Figure 5, A and B, at 15 μ M of ritonavir, aggregates of JC-1 (red fluorescence) were significantly reduced compared with controls ($P < 0.05$), indicating reduced mitochondrial membrane potential in ritonavir-treated HPAECs. Similarly, mitochondrial membrane potential in HPAECs was also reduced by three other HAART drugs (indinavir, abacavir, and AZT), compared with controls at their clinical plasma concentrations ($P < 0.05$ for all). Meanwhile, lamivudine also showed the trend to decrease the mitochondrial membrane potential in HPAECs.

Effects of HIV Protease Inhibitor Ritonavir and Other Anti-HIV Drugs on MAPK Phosphorylation and Endothelial Dysfunction in HPAECs and Porcine Pulmonary Artery Rings

To determine whether MAPK could be involved in signal transduction pathways of anti-HIV drug-induced endo-

thelial dysfunction, the activation status of three major MAPKs (ERK1/2, JNK, and p38) in HPAECs was determined by Bio-Plex immunoassay. Two HAART drugs, ritonavir (a protease inhibitor) and AZT (a nucleoside reverse transcriptase inhibitor), were tested. Ritonavir treatment (15 μ M/L) for 20 minutes substantially increased the phosphorylation of ERK2 in HPAECs by 3.5-fold compared with controls (Figure 6A). The phosphorylation of JNK and p38 in HPAECs was increased by 2.2-fold and 1.8-fold after 90 minutes treatment of ritonavir, respectively, compared with controls (Figure 6A). AZT treatment (8 μ M/L) for 30 minutes markedly increased the phosphorylation of ERK2 in HPAECs by 2.8-fold compared with controls. However, there were no substantial changes of phosphorylation of JNK and p38 MAPK in response to AZT treatment (Figure 6B). To explore whether HAART-induced ERK1/2 activation could be linked to their functional effects on endothelial dysfunction, eNOS expression and superoxide production in HPAECs were further evaluated by Western blot and DHE staining, respectively, in the presence or absence of MEK/ERK inhibitor, PD98059 (20 μ M/L). As shown in Figure 6C, MEK/ERK inhibitor partially blocked ritonavir- or AZT-induced decreases in eNOS protein levels. However, MEK/ERK inhibitor did not block HAART-induced increase in superoxide anion production (Figure 6D), indicating that ritonavir- or AZT-induced increase in superoxide anion production may occur at the upstream of ERK1/2 activation or independent to ERK1/2 activation during the action of HAART drugs in HPAECs. Finally, in a separate experiment, MEK/ERK inhibitor partially blocked ritonavir- or AZT-induced decrease in endothelium-dependent vasorelaxation in response to bradykinin (10^{-6} mol/L) in porcine pulmonary artery rings (Figure 6E), suggesting that ERK1/2 activation is involved in the HAART drug-induced endothelial dysfunction in the vascular system.

Discussion

In the current study, we demonstrate that individual HAART drugs (ritonavir, indinavir, lamivudine, ddI, abacavir, and AZT) and combination of three drugs (indinavir, ddI, and d4T) at their clinical plasma concentrations can cause endothelial dysfunction through eNOS down-regulation and superoxide anion production in porcine pulmonary artery rings and HPAECs. Consequently, antioxidant therapy with ginseng and *Ginkgo biloba* compounds effectively prevents the effects of HAART drugs on these models. This study is particularly important in the development of evidence-based strategies to control HIV-PAH.

PAH is a prominent feature of HIV infection and HAART.^{2,15} However, the pathogenesis of this disorder is unclear. It is well known that vasomotor dysfunction is the most important mechanism of PAH.¹⁶ For example, a hallmark of hypoxia-induced PAH is an increase in vasomotor tone.¹⁷ *In vivo*, pulmonary arterial smooth muscle cell contraction is influenced by vasoconstrictor and vasodilator factors secreted from the endothelium. In the

Figure 6. Roles of MARK in HAART drug-induced endothelial dysfunction in HPAECs and in porcine pulmonary artery rings. HPAECs were treated with ritonavir (15 μ mol/L) or AZT (8 μ mol/L). The phosphorylated and total ERK2, JNK, and p38 proteins were detected by Bio-Plex immunoassay. **A:** Ritonavir treatment increased the ratios of phosphorylated and total ERK2, JNK, and p38 proteins at 20 (**a**), 90 (**b**), and 60 (**c**) minutes, respectively. **B:** AZT treatment increased the ratios of phosphorylated and total ERK2 proteins at 30 (**a**) minutes. However, there were no substantial changes of phosphorylation of JNK (**b**) and p38 (**c**) in response to AZT treatment. **C:** The blockage effect of MEK/ERK inhibitor (ERKi) on reduced expression of eNOS in HPAECs induced by AZT and RTV was demonstrated by Western blot. **D:** Superoxide anion levels in HPAECs were stained with DHE and analyzed by FACS-calibur flow cytometry. MEK/ERK inhibitor (ERKi) did not show any significant effect on the increased superoxide anion in HPAECs induced by AZT and RTV. **E:** MEK/ERK inhibitor (ERKi) partially blocked AZT- or RTV-induced decrease in endothelium-dependent vasorelaxation in response to bradykinin (10⁻⁶ mol/L) in porcine pulmonary artery rings. RTV, ritonavir; ERKi, PD98059.

current study, vasomotor function was studied by myograph analysis, which has been used in our previous studies for many years.^{10–12,18} The vessel contractility was determined by vessel tension changes in response to thromboxane A₂ analogue U46619, which contracts vascular smooth muscle by binding to specific Gq/11 protein-coupled receptors (TP receptors) and leads to an increase in intracellular Ca²⁺ concentration and sensitization of the contractile proteins to Ca²⁺.¹⁹ Endothelium-dependent vasorelaxation was studied with bradykinin, which is a potent vasodilator that acts through the endothelial B2 kinin receptor to stimulate the release of NO through eNOS activation.²⁰ Endothelium-independent vasorelaxation was studied with SNP, which is widely used as an endothelium-independent NO donor.²¹ Our data showed that the contraction, endothelium-dependent, and endothelium-independent relaxation of the vessel rings were reduced by ritonavir treatment, and endothelium-dependent relaxation was also reduced by five other HAART drugs (indinavir, lamivudine, ddI, abacavir, and AZT) and three drugs combination of indinavir, ddI, d4T. However, lopinavir, an analog of ritonavir, did not show significantly detrimental effects on vasorelaxation in porcine pulmonary arteries in response to bradykinin. The concentrations of HAART drugs used in this study are clinically relevant maximal plasma concentrations (C_{max}) (Physicians' Desk Reference 2006). The HAART combination used in this study is based on clinical trials.²²

Consistent with current results, our previous data from porcine coronary arteries and human coronary arterial

endothelial cells (HCAECs) also showed that the HIV protease inhibitors ritonavir, amprenavir, and saquinavir have detrimental effects on endothelium-dependent relaxation.¹¹ The effect of HIV protease inhibitors on endothelium-dependent vasorelaxation was suggested by the finding of a clinical cross-sectional case-control study measured flow-mediated vasodilation of the brachial artery in HIV patients who using HIV protease inhibitors. Stein and colleagues⁵ found a significant reduction of flow-mediated vasodilation in HIV patients receiving protease inhibitors compared with the patients without protease inhibitors treatment. To better delineate the role of the drug versus the disease in protease inhibitor-associated endothelial dysfunction, the study of the effects of the HIV protease inhibitors on endothelial function in healthy HIV-negative patients was performed. Shankar and Dube²³ showed that in healthy volunteers, indinavir caused impaired endothelium-dependent vasodilation after 4 weeks. Most recently, an *in vivo* study reported that AZT or AZT plus indinavir treatments dramatically reduced endothelium-dependent vessel relaxation in aortic rings of rats.²⁴ Our current study demonstrated, for the first time, several individual HAART drugs and the combination of three drugs could induce vasomotor dysfunction in porcine pulmonary arteries, which may suggest a mechanism of HIV-PAH.

The eNOS-derived NO is an endogenous vasodilatory gas that continually regulates the diameter of blood vessels and maintains an anti-proliferative and anti-apoptotic environment in the vessel wall.²⁵ The phenotype of the

eNOS knockout mouse is characterized by pulmonary artery hypertension, which is reversed when the eNOS gene is delivered back to the lung.²⁶ Thus, any factors that impair the eNOS system may contribute to the formation of pulmonary artery hypertension. The current study showed that eNOS expression in both porcine pulmonary artery rings and HPAECs was significantly reduced by HAART drugs including indinavir, lamivudine, abacavir, and AZT, whereas the other three drugs (lopinavir, d4T, and ddI) had no effects on eNOS expression. Combined with myograph data described above, these data indicate that these HAART drugs caused endothelial dysfunction by impairing the eNOS system in porcine pulmonary arteries. At least at this point from both vasomotor reactivity and eNOS expression data in the current study, lopinavir shows less detrimental effects, suggesting its advantage compared with other HIV protease inhibitors such as ritonavir. In addition, these data are consistent with more recent studies from our laboratory, which demonstrated that the HIV protease inhibitors ritonavir, amprenavir and saquinavir markedly reduced eNOS expression from porcine coronary arteries and HCAECs.¹² An *in vivo* study of the effect of indinavir on vascular dysfunction showed that excretion of urinary nitrite, a stable degradation product of NO, decreased in indinavir-treated rats.²⁷ Additionally, the current study showed that two HAART drugs ritonavir and AZT substantially increased the phosphorylation of ERK2 in HPAECs, and MEK/ERK inhibitor effectively blocked ritonavir- or AZT-induced decreases in eNOS protein levels in HPAECs, indicating the involvement of MAPK signal transduction pathway in the process of endothelial dysfunction induced by HAART drugs. Furthermore, the MEK/ERK inhibitor partially blocked ritonavir- or AZT-induced decrease in endothelium-dependent vasorelaxation in response to bradykinin (10^{-6} mol/L) in porcine pulmonary artery rings, which could be associated with eNOS levels in these treatment groups. Thus, activation of ERK1/2 in pulmonary artery endothelial cells is one of possible mechanisms of endothelial dysfunction induced by HAART drugs in the pulmonary artery system.

Oxidative stress plays a critical role in pulmonary artery hypertension. For example, ROS levels were elevated in the pulmonary arteries in experimental animals and humans with pulmonary artery hypertension.²⁸ ROS could mediate increased endothelin-1 production and down-regulation of eNOS expression in an ovine model of persistent pulmonary artery hypertension.²⁹ The current study demonstrated that some HAART drugs significantly increased superoxide anion production in porcine pulmonary arteries and HPAECs, while decreased mitochondrial membrane potential simultaneously in HPAECs. HAART drug-induced mitochondrial dysfunction may be responsible for the superoxide anion production.

ROS are generated mainly from normal metabolism such as mitochondrial respiratory chain wherein excess electrons are donated to molecular oxygen to generate superoxide anion. In addition to mitochondrial dysfunction, other major sources of ROS generation include the synthesis by dedicated enzymes, such as NADPH oxidase (p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox}, and p91^{phox}),

which is best characterized as a major superoxide-generating enzyme in phagocytes. Indeed, our previous studies have indicated that ritonavir also increased NADPH oxidase in porcine arteries³⁰ and THP1-derived macrophages.³ Several other studies also showed the involvement of ROS in the treatment of HAART drugs. Exposure of either indinavir or nelfinavir combined with AZT and efavirenz increases ROS formation in human aortic endothelial cells.³¹ HAART drugs AZT and indinavir disrupt endothelial cell junctions and mitochondria and cause vascular damage.³² The recent studies from our laboratory demonstrated that the superoxide anion levels were significantly increased in porcine coronary arteries treated with ritonavir and amprenavir.^{33,34} New findings in the current study further demonstrate the role of ROS system in HAART drug-treated porcine pulmonary arteries and HPAECs. Superoxide dismutase (SOD) is an antioxidant enzyme involved in the defense system against ROS. SOD catalyzes the dismutation reaction of superoxide radical anion to hydrogen peroxide, which is then catalyzed to innocuous O₂ and H₂O by glutathione peroxidase and catalase, however this conversion is not 100% efficient, and residual peroxides persist in the cell. Previously, we reported that reducing superoxide anion by MnTBAP, a SOD mimic, effectively abolished the CRP-induced decrease in cholesterol efflux.³⁵ One of limitations of the current study is that SOD was not used to confirm the superoxide anion production in the endothelial cells. Thus, the effect of SOD on reversing HAART-induced endothelial dysfunction warrants further investigation.

Although HAART drugs could induce ERK1/2 activation, MEK/ERK inhibitor did not block HAART-induced increase in superoxide anion production, which may indicate that HAART-induced increase in superoxide anion production may occur at the upstream of ERK1/2 activation or may be independent to ERK1/2 activation during the action of HAART drugs in HPAECs. This hypothesis is supported by many other studies. For example, ROS modulates cardiomyocyte response to ischemia/reperfusion through MAPK activation.³⁶ ROS scavengers inhibited the activities of ERK1/2 and p38 in alveolar epithelial cells.^{37,38} Romeis and colleagues³⁹ reported that ROS production is induced by an independent mechanism of MAPK activation.

Ginseng and *Ginkgo biloba* compounds are the most widely recognized herbal antioxidants. Ginsenosides have protective roles in atherosclerotic plaque formation and various vascular injuries attributable in part to preventing free radical injury.^{40,41} Ginsenoside Rb1 is able to counteract the action of ROS induced by xanthine.⁴² *Ginkgo biloba* extract contains ginkgolides and other compounds. The antioxidative activity of *Ginkgo biloba* compounds contributes to the protective effects in multiple areas including visual, cardiovascular, pulmonary, and central nervous systems.¹⁰ Because HAART drugs may contribute to endothelial dysfunction induced by oxidative stress, we tested whether such antioxidants could reverse the endothelial dysfunction induced by oxidative stress. Indeed, the current study shows Rb1 effectively abolished the oxidative stress induced by the

HAART combination in porcine pulmonary arteries. Both Rb1 and GA effectively improved HAART drug-induced down-regulation of eNOS and inhibition of endothelium-dependent vasorelaxation in porcine pulmonary arteries. In porcine coronary arteries, our previous studies also showed that Rb1 effectively blocked ritonavir-induced endothelial dysfunction, eNOS down-regulation and superoxide anion production.^{33,34} These studies indicate an important future therapeutic strategy of using antioxidant-based therapy in counteracting the adverse effects of HAART drugs in the vascular system.

Currently, there are 24 approved antiretroviral drugs targeting various viral proteins or critical points in the virus-host life cycle. Clinical complications are mainly studied on the first generation of antiretroviral drugs or relative old antiretroviral drugs including ritonavir, indinavir, lopinavir, AZT, abacavir, d4T, ddI, and lamivudine. Integrase inhibitors (ie, raltegravir), emcitabine, and efavirenz were recently approved, and little is known about their general vascular complications or endothelial-specific toxicities. In the current study, we selected some HAART drugs with already demonstrated clinical complications and investigated their potential mechanisms of endothelial dysfunction induced by these drugs in the *in vitro* systems. Such effects of more current HAART drugs including tenofovir, emcitabine, efavirenz, atazanavir, and fosamprenavir warrant further studies in both clinical trials and *in vitro* systems.

In summary, the current study showed that several HAART drugs including ritonavir, indinavir, lamivudine, abacavir, and AZT can cause endothelial dysfunction through decreasing eNOS expression and increasing oxidative stress in porcine pulmonary arteries and HPAECs. Consequently, reducing oxidative stress by selected antioxidants may be able to prevent HAART drug-associated PAH. Thus, the current study provides many new and significant aspects in the field of HIV and HAART research.

Acknowledgments

We thank the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, for the HAART drugs including AZT, abacavir, d4T, ddI, lamivudine, indinavir, lopinavir, and ritonavir.

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