HIV Protease Inhibitor Ritonavir Induces Cytotoxicity of Human Endothelial Cells

Dian-sheng Zhong, Xiang-huai Lu, Brian S. Conklin, Peter H. Lin, Alan B. Lumsden, Qizhi Yao, Changyi Chen

Objective—Although HIV protease inhibitors have been successfully used against HIV infection, many metabolic side effects and premature cardiovascular diseases are often associated with this therapy. The mechanisms of these complications are not clear. In this study, we investigated the effect of the HIV protease inhibitor ritonavir on human endothelial cell cultures.

Methods and Results—By using nonradioactive cell proliferation and cytotoxicity assays, human endothelial cells treated with ritonavir showed a significant decrease in cell viability and an increase in cytotoxicity in a time- and dose-dependent fashion. Mitochondrial DNA was also substantially damaged with ritonavir treatment by long polymerase chain reaction analysis. In contrast, ritonavir had a very limited effect on endothelial apoptosis, as assessed by analyses of DNA fragmentation and cellular caspase-3 activity.

Conclusions—These data demonstrate, for the first time, that the HIV protease inhibitor ritonavir at concentrations near clinical plasma levels is able to directly cause endothelial mitochondrial DNA damage and cell death mainly through necrosis pathways but not through apoptosis. This study suggests that HIV protease inhibitor–mediated endothelial injury may contribute to its cardiovascular complications. (Arterioscler Thromb Vasc Biol. 2002;22:1560-1566.)

Key Words: HIV protease inhibitor ■ ritonavir ■ cytotoxicity ■ endothelial cells ■ cardiovascular disease

Human immunodeficiency virus protease is an important virally encoded enzyme that cleaves the gag and gag-pol protein precursors to produce mature and infectious virus particles. Thus, HIV protease has been the target of antiviral therapy. HIV protease inhibitors were introduced in 1995 for the treatment of HIV-infected patients. HIV protease inhibitors, including saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir, rapidly and profoundly reduce the viral load, as indicated by a decline in plasma HIV RNA concentrations within a few days after the start of treatment.1,2 Reductions in the viral load are paralleled by mean increases in the CD4 count of 100 to 150 cells/mm3.3,4 However, HIV protease inhibitors are often associated with a number of metabolic side effects and cardiovascular complications. The major concern raised by protease inhibitor–associated elevation in plasma levels of cholesterol and triglycerides relates to the risk of premature development of atherosclerosis.5-7 Indeed, rapidly evolving plaques may be particularly unstable and thus prone to rupture, generating an acute coronary event in HIV protease inhibitor–treated patients.8 However, the mechanisms of HIV protease inhibitor–associated cardiovascular disease are not known.

Normal endothelial cell function and integrity are crucial in preventing vascular disease formation and thrombosis. We hypothesized that HIV protease inhibitors could cause endothelial injury or dysfunction by either direct cytotoxic effect or indirect effect of protease inhibitor–related metabolic changes on endothelial cell growth and function. In the present study, we investigated the effects of ritonavir, one of the HIV protease inhibitors, on cell viability, cytotoxicity, mitochondrial DNA damage, and apoptosis in human endothelial cell cultures. The present study may provide insight into the understanding of cardiovascular complications associated with the use of HIV protease inhibitors.

Methods

Cells and Reagents

Immortalized human dermal microvascular endothelial cells (HMECs)9 were obtained from Dr Wright S. Caughman, Department of Dermatology, Emory University School of Medicine, Atlanta, Ga. HMECs were grown in MCDB 131 medium supplemented with human recombinant epidermal growth factor (10 ng/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mmol/L, GIBCO-BRL), hydrocortisone (2.0 μg/mL, Sigma Chemical Co), and FBS (10%, Cellgro Co). For all experiments, HMECs were seeded on culture flasks or plates and cultured in humidified 95% air/5% CO2 at 37°C overnight. The medium was then removed, and the cells were made quiescent by incubation with serum-free MCDB 131 medium for 24 hours before the experiments. The cells were used at passages 30 to 35. Human umbilical vein endothelial cells...
The endothelial cells (HUVECs, Clonetics) were grown in endothelial growth medium (Clonetics), including endothelial basal medium, bovine brain extract (12 μg/mL), human recombinant epidermal growth factor (10 ng/mL), hydrocortisone (1 μg/mL), gentamycin and amphotericin B (GA-1000, 50 μg/mL), and 2% FBS. The cells were used at passages 3 to 5. Pure ritonavir powder was generously provided by Abbott Laboratories, North Chicago, Ill. It was then dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the experiments was adjusted to <1% (vol/vol), which showed no cytotoxicity to endothelial cell cultures, as demonstrated by our pretesting (data not shown) and by another study. N-Acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), an inhibitor of caspase-3, was purchased from Oncogene Co.

**Cell Viability Assay**

Cell viability was assessed by a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS), assay kit (Promega Co). The assay procedures were followed as instructed in the kit.

**Cytotoxicity Assay**

Lactate dehydrogenase (LDH) release from cells was determined by using an LDH detection kit (Promega) as an index of cytotoxicity or necrosis. The assay procedure was performed according to the instructions included in the kit.

**Mitochondrial DNA Damage Detection**

The effect of ritonavir on mitochondrial DNA damage was determined by long polymerase chain reaction (PCR) and calculation of DNA lesion frequencies as previously described.

**Flow Cytometry Analysis**

A terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) apoptosis detection kit (Oncogene Co) was used in the present study. The procedures were provided in the kit. In brief, the cells (1x10⁶) were incubated in the absence or presence of 15 and 30 μmol/L concentrations of ritonavir for 24 hours for HUVECs or 48 hours for HMECs. The cells were pelleted and resuspended in 4% formaldehyde for 10 minutes and permeabilized with protease K solution for 4 minutes. The cells were then resuspended in 60 μL of terminal deoxynucleotidyl transferase labeling reaction solution and incubated for 90 minutes at 37°C in the dark. Finally, the samples were analyzed with a FACSScan flow cytometer (Becton Dickson Co).

**Caspase-3 Activity Assay**

Caspase-3 activity was detected by the Caspase-3 Activity Assay Kit (Oncogene Co) according to the manufacturer’s assay procedures.

**Statistical Analysis**

All experiments were performed 4 times at the same conditions. The data were expressed as mean±SE. The data from the treated groups were compared with those from the control (untreated) groups by using the paired Student t test (2-tailed). A value of P<0.05 was considered significant.

**Results**

**Effect of Ritonavir on Endothelial Cell Viability**

The effect of ritonavir on cell viability was determined by MTS assay. Initially, HMECs were treated with ritonavir (30 μmol/L) for different periods of time. Cell viability rates of HMECs were 76.79±3.12%, 67.75±3.63%, 52.01±4.17%, and 43.7±2.96% at 8, 24, 48, and 72 hours, respectively, showing a significant decline at all time points compared with controls (100%, P<0.001; Figure 1A). With establishment of the kinetics of cellular injury, a dose-dependent experiment was performed to determine the concentrations of ritonavir that effectively decreased cell viability. HMECs were cultured with different doses of ritonavir for 48 hours. Significant decreases of cell viability were observed with ≥15 μmol/L ritonavir but not with ≤10 μmol/L ritonavir compared with control (n=4 for each group). *P<0.05 and **P<0.001.

**Effect of Ritonavir on LDH Release From Endothelial Cells**

The cytotoxicity of ritonavir in human endothelial cells was further evaluated by LDH release assay. After treatment with
ritonavir, HMECs and HUVECs significantly increased LDH release. In HMECs treated with 7.5, 15, and 30 μmol/L ritonavir for 48 hours, the cytotoxicity index was increased to 10.11±1.17%, 23.19±5.0%, and 57.43±10.78%, respectively, showing a significant increase with ritonavir treatment at 15 and 30 μmol/L (P<0.05) but not at 7.5 μmol/L (Figure 2B). Furthermore, treatment with 15 and 30 μmol/L ritonavir for 24 hours significantly increased HUVEC cytotoxicity to 23.82±8.4% and 70.43±11.01%, respectively (P<0.05), but treatment with 7.5 μmol/L ritonavir produced no significant increase (9.25±6.65%, Figure 2B). Thus, ritonavir had a significant cytotoxic effect in HMECs and in HUVECs.

**Effect of Ritonavir on HMEC Mitochondrial DNA Damage**

HMECs were treated with ritonavir (7.5, 15, and 30 μmol/L) for 24 hours. Mitochondrial DNA damage was analyzed with long PCR and calculation of DNA lesion frequencies. The highest dose of ritonavir treatment (30 μmol/L) resulted in less long PCR product yield, indicating that mitochondrial DNA damage occurred (Figure 3A). After normalization with control data, the mitochondrial DNA lesion frequencies were 2.91, 12.62, and 40.78 lesions per 10 kb for treatment with 7.5, 15, and 30 μmol/L ritonavir, respectively (Figure 3B). Thus, ritonavir caused mitochondrial DNA damage in human endothelial cells in a dose-dependent manner.

**Effect of Ritonavir on Endothelial Cell Apoptosis**

To test whether ritonavir could cause endothelial apoptosis in addition to necrosis, TUNEL flow cytometry analysis was performed. HMECs incubated in either the absence (control) or presence of ritonavir (15 and 30 μmol/L) for 48 hours showed apoptosis rates of 7.1%, 7.2%, and 18.5%, respectively (Figure 4A). After treatment with 0 (control), 15, and 30 μmol/L ritonavir for 24 hours, HUVECs showed apoptosis rates of 7.1%,
7.3%, and 14.6%, respectively (Figure 4B). These data indicated that ritonavir at the 15 μmol/L concentration had no effect on apoptosis in HMECs or in HUVECs, whereas treatment with 30 μmol/L ritonavir showed a limited increase of endothelial cell apoptosis compared with no treatment. Furthermore, endothelial apoptosis after treatment of ritonavir was also studied by caspase-3 activity assay. HMECs were treated with ritonavir at 0 (control), 15, and 30 μmol/L concentrations for 48 hours, and caspase-3 activity was 117.2.04, 120.3.60 and 120.75.3.40 relative fluorescence unit (RFU), respectively, showing no significant differences between control and treated groups (Figure 5). Thus, ritonavir is able to cause endothelial death that is mainly due to cell necrosis mechanisms but not apoptosis.

Effects of Caspase-3 Inhibitor on Ritonavir-Mediated Cell Death of HMEC Cultures
To further distinguish the effect of ritonavir on cell necrosis and apoptosis, MTS and LDH release assays were performed with a caspase-3 inhibitor, Ac-DEVD-CHO (100 μmol/L), which inhibits only apoptosis but not necrosis. HMECs treated with 30 μmol/L ritonavir for 48 hours significantly decreased cell viability (down to 56.20±1.03%, P<0.01), whereas treatment with 30 μmol/L ritonavir and 100 μmol/L Ac-DEVD-CHO showed no differences in cell viability (51.65±4.17%) compared with ritonavir treatment alone (Figure 6A). Furthermore, treatment with 30 μmol/L ritonavir significantly increased LDH release from HMECs and showed 40.53±6.24% cytotoxicity (P<0.01), whereas the treatment including Ac-DEVD-CHO (100 μmol/L) did not significantly change ritonavir-induced cytotoxicity (Figure 6B).

Figure 4. Effect of ritonavir on apoptosis of HMECs and HUVECs. TUNEL flow cytometry analysis was performed to determine cell apoptosis. HMECs (A) and HUVECs (B) were cultured for 48 and 24 hours, respectively, in either the absence (a) or presence of 15 μmol/L (b) and 30 μmol/L (c) ritonavir. Ritonavir treatment at 15 μmol/L showed no effects on apoptosis for either HMECs or HUVECs, whereas treatment with 30 μmol/L ritonavir showed a limited increase of endothelial cell apoptosis compared with no treatment.

Figure 5. Effects of ritonavir on caspase-3 activity of HMECs. HMECs were treated with ritonavir at 0 (control), 15, and 30 μmol/L concentrations for 48 hours. Caspase-3 activity was not significantly different between control and treated groups (P>0.05, n=4 for each group).
Cell necrosis normally results from a severe cellular insult. Internal organelle and plasma membrane integrity is lost, resulting in the spilling of cytosolic and organelle contents into the surrounding environment. In the present study, we have demonstrated by MTS assay that ritonavir significantly reduces the viability of human endothelial cells in a time- and dose-dependent fashion. A cytotoxicity assay was also used in the present study to quantitatively measure LDH, a stable cytosolic enzyme that is released on cell lysis, and we demonstrated that ritonavir significantly increases human endothelial cell cytotoxicity. In HMECs treated with 15 and 30 μmol/L ritonavir for 48 hours, the cytotoxicity index was significantly increased to 23.19±5.0% and 57.43±10.78%, respectively. Furthermore, HUVECs treated with 15 and 30 μmol/L ritonavir for 24 hours showed a significant increase of cytotoxicity to 23.82±8.4% and 70.43±11.01%, respectively. Thus, primary cultures of macrovascular endothelial cells, HUVECs, are more sensitive to ritonavir treatment than those of immortalized microvascular endothelial cells, HMECs.

It is well known that mitochondrial injury is one of the most common reasons for toxin-induced cell death.11–13 Mitochondrial injury is reflected by DNA damage, changes in gene expression, protein synthesis, and redox function. In the present study, we tested the hypothesis that ritonavir could induce mitochondrial DNA damage in human endothelial cells, which was determined by analysis of DNA lesion frequency based on the PCR of long DNA fragments. HMECs treated with ritonavir (7.5, 15, and 30 μmol/L) for 24 hours showed mitochondrial DNA lesion frequencies of 2.91, 12.62, and 40.78 lesions per 10 kb, respectively. These data may suggest that ritonavir-induced mitochondrial DNA damage is one of the mechanisms of ritonavir-induced cell death because mitochondrial DNA damage could inactivate the electron transport complex or inhibit mtDNA transcription, thereby altering normal mitochondrial functions. In our previous study,14 ritonavir increased superoxide anion (O$_2^-$) production in cell cultures and in monkey arteries. Human aortic endothelial cells were treated with ritonavir (15 μmol/L) for 4 hours, and the O$_2^-$ chemiluminescence signal was significantly increased by 32% in the treated cells compared with the control cells. Iliac arteries were harvested from 2 healthy rhesus macaque monkeys and were cut into 5-mm rings, which were treated with ritonavir (15 μmol/L) for 2 hours; the O$_2^-$ chemiluminescence signal was significantly increased (by 43% to 55%) in these cells (2722 counts/s) compared with control cells (1252 to 1538 counts/s). In addition, ritonavir-induced endothelial injury was also observed in monkey arteries. The arterial rings were treated with ritonavir (15 or 30 μmol/L) for 24 hours and then subjected to myograph analysis. Compared with the control condition, treatment with ritonavir significantly reduced endothelium-dependent vasorelaxation in response to a gradient of acetylcholine.

Apoptosis, another form of cell death, is a genetically programmed event with DNA fragmentation and occurs without membrane lysis, thereby avoiding subsequent...
surrounding tissue injury and limiting the consequences of cell death.\textsuperscript{15} However, endothelial cell apoptosis has also been implicated in the development of increased vascular permeability and capillary leak syndrome during systemic inflammatory response syndrome.\textsuperscript{16,17} The present study was designed to investigate whether pretreatment of endothelial cells with ritonavir would lead to endothelial cell apoptosis by TUNEL flow cytometry analysis, which detects DNA fragmentation. We found that ritonavir at a concentration of 15 μmol/L had no effects on apoptosis in either HMECs or HUVECs, whereas treatment with 30 μmol/L ritonavir showed a limited increase of endothelial cell apoptosis compared with no treatment. Thus, compared with cell necrosis, apoptosis has a much smaller impact on ritonavir-induced cell death. To further confirm this observation, the effect of ritonavir on caspase activity was determined. Caspases are cysteolic aspartate-specific proteases that are responsible for the deliberate disassembly of cells into apoptotic bodies. Caspases are present as inactive proenzymes, most of which are activated by proteolytic cleavage. Caspase-8, caspase-9, and caspase-3 are situated at pivotal junctions in apoptotic pathways. In the present study, the effect of ritonavir on caspase-3 activity was investigated. Consistent with TUNEL flow cytometry data, ritonavir did not affect caspase-3 activity in human endothelial cell cultures. Moreover, to distinguish the effect of ritonavir on cell necrosis and apoptosis, additional experiments were performed with a caspase-3 inhibitor, Ac-DEVD-CHO, which inhibits only apoptosis but not necrosis. Compared with cells treated with ritonavir alone, human endothelial cells treated with ritonavir plus Ac-DEVD-CHO showed no differences in MTS and LDH release assays. These data demonstrate that ritonavir-induced endothelial cell injury is mainly due to the cell necrosis pathway, although high-dose (30 μmol/L) treatment with ritonavir results in a limited level of apoptosis.

Although our data were generated from cell cultures, they may have clinical implications. During the drug development, the studies that measured the cytotoxicity of ritonavir on several cell lines showed that >20 μmol/L was required to inhibit cellular growth by 50%.\textsuperscript{18} However, the smaller doses of ritonavir, such as 15 μmol/L, resulted in 23% endothelial cytotoxicity in the present study. At the recommended dose of 600 mg ritonavir every 12 hours (for human use), the maximal plasma concentration is 8 to 15 μmol/L.\textsuperscript{18,19} In our in vitro study, treatment with 15 μmol/L ritonavir resulted in significant endothelial cell injury, which was demonstrated by a decrease of cell viability and an increase of cytotoxicity. In the patient, the duration of ritonavir treatment is usually >1 year, and cumulative damage of endothelial cells from ritonavir might occur. It is well known that endothelial injury or dysfunction is a major cause of atherosclerotic lesion formation and progression. Therefore, ritonavir-induced endothelial injury may contribute to its vascular complications.

The molecular mechanisms by which ritonavir is able to induce cytotoxicity and mitochondrial DNA damage in human endothelial cell cultures have not yet been elucidated. Ritonavir is able to inhibit the cytochrome P-450 system.\textsuperscript{20} Catalytic region of HIV protease has been reported to have limited homology with the LDL receptor–related protein and the cytoplasmic retinoic acid-binding protein type 1 and ritonavir does not have homology.\textsuperscript{21} Ritonavir is metabolized in vivo by the cytochrome P-450 3A4 enzyme system and is exported by P-glycoproteins.\textsuperscript{22} However, none of these properties satisfactorily explains the action of ritonavir on endothelial cells. Because the present study was limited to in vitro conditions, further studies in vivo are needed to deduce the mechanisms of HIV protease inhibitor–induced endothelial injury in animal models or in human subjects. The in vitro data may not account for many factors, including active metabolites, pharmacokinetic parameters, environmental factors, and genetic predisposition, which may influence the development of cardiovascular disease in the clinic. Additionally, these in vitro treatments may not account for the effect of serum protein binding and drug-drug interaction on the activities of the HIV protease inhibitors within the patient.

It now appears clear that clinical use of HIV protease inhibitors, including ritonavir, is associated with a potentially serious syndrome of metabolic abnormalities characterized by peripheral fat wasting, central adiposity, hypertriglyceridemia, hypercholesterolemia, and insulin resistance.\textsuperscript{23–25} Endothelial dysfunction of the brachial artery and endothelial dysfunction of the coronary artery are correlated with each other and predict future adverse cardiovascular events.\textsuperscript{26–30} In a recent study, patients receiving HIV protease inhibitors had markedly impaired endothelial function, as measured by endothelium-dependent vasorelaxation in brachial arteries.\textsuperscript{30} Thus, the direct cytotoxicity of the HIV protease inhibitors and the high levels of atherogenic lipoproteins resulting from its clinical use could be responsible for endothelial dysfunction in vivo.\textsuperscript{31,32} As survival of individuals with HIV infection increases, atherosclerotic vascular disease could become an important HIV-related complication. In fact, severe premature cardiovascular diseases have been reported in the patients who receive these medications.\textsuperscript{6,33–35} On one hand, we could study the molecular mechanisms to prevent and correct these adverse effects of current HIV protease inhibitors. On the other hand, future drug development may be necessary to design new compounds that maintain the efficacy in the management of HIV infection but that also minimize the side effects observed in the present study and in many other investigations.

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