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Original Contribution

Comparative analysis of ER stress response into HIV protease inhibitors: Lopinavir but not darunavir induces potent ER stress response via ROS/JNK pathway

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ABSTRACT

HIV protease inhibitor (PI)-induced ER stress has been associated with adverse effects. Although it is a serious clinical problem for HIV/AIDS patients, comparative analyses of ER stress induction by clinically used PIs have rarely been done. Especially, there is no report on the differential ER stress response between lopinavir (LPV) and darunavir (DRV), although these PIs are the most clinically used PIs. We show here that LPV induces the most potent CHOP expression, ER stress marker, among the 9 Food and Drug Administration (FDA)-approved PIs in human peripheral blood mononuclear cells, several human epithelial cells, and mouse embryonic fibroblasts. LPV induced the most potent ROS production and JNK activation in 9 PIs. A comparison among the most clinically used PIs, ritonavir (RTV), LPV, and DRV, revealed that LPV potently and RTV moderately but not DRV induced ER stress via ROS-dependent JNK activation rather than proteasome inhibition. Finally, we analyzed ER stress induction in tissues of mice intraperitoneally injected with RTV, LPV, and DRV. RTV and LPV but not DRV showed ER stress induction in several mice tissues. In conclusion, we first identify LPV as the most potent ER stress inducing PI among 9 FDA-approved PIs in human cells, and although clinical verification is necessary, we show here that DRV has the advantage of less ROS and ER stress induction potential compared with LPV *in vitro* and *in vivo*.

Introduction

The combination antiretroviral therapy (cART) effectively improves HIV/AIDS patients' life prognosis [1]. The HIV-1 protease inhibitor (PI) is a component of this combination therapy based on its suppressive effect on HIV-1 protease and viral maturation to block viral proliferation [2]. Various PIs have been developed in recent years. Ritonavir (RTV)-boosted lopinavir (LPV) and darunavir (DRV) are the most clinically used PIs for cART because these drugs have high binding affinity to HIV-1 protease and are highly active to drug-resistant HIV-1 [3–5]. But despite rigorous drug development PIs do not completely cure HIV/AIDS, and HIV/AIDS patients are required to maintain long-term PI treatment, which causes serious side effects such as hyperlipidemia, diabetes, diarrhea, and atherosclerosis [6].

ER stress is a common molecular mechanisms for PI-induced side effects. Several PIs disrupt lipid metabolism in hepatocytes [7], induce apoptosis in pancreatic β cells to inhibit insulin secretion [8], activate the expression of inflammatory cytokines in macrophage [9], and disrupt the barrier integrity in intestinal epithelial cells [10] via ER stress induction as a common molecular mechanism. Although ER stress is thought to be important for the induction of several side effects by PIs, exhaustive comparison of clinically used PIs in terms of their potential to induce ER stress has not been done. Additionally, there is no report comparing the ER stress-inducing effects of LPV and DRV despite some clinical reports indicating the less adverse effects of DRV compared to those of LPV [11–15].

The accumulation of unfolded proteins, calcium disruption, and ROS production are well known triggers of ER stress [16]. Physiologically, ER stress correlates to several diseases such as diabetes,

Abbreviations: APV, Amprenavir; ARE, antioxidant response; ATV, Atazanavir; cART, Combination antiretroviral therapy; DRV, Darunavir; FDA, Food and Drug Administration; IDV, Indinavir; LDH, lactate dehydrogenase; LPV, Lopinavir; NAC, *N*-acetyl-L-cysteine; NFV, Nelfinavir; PBMC, Peripheral blood mononuclear cells; PI, Protease inhibitor; PRI, Propidium iodide; RTV, Ritonavir; SQV, Saquinavir; TPV, Tiprenavir

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hyperlipidemia, neurodegenerative disorder, autoimmune disease, and cancer [17]. Mammalian cells have a homeostasis response against ER stress, called ER stress response or unfolded protein response. ER stress activates several signaling molecules, PERK and IRE1, to activate the transcription factors XBP1, ATF4, and ATF6. These transcription factors regulate cellular transcription and translation to decide cellular responses including protein synthesis, cell cycle, and apoptosis regulation [16–19]. It was previously reported that several PIs induce ER stress via proteasome inhibition and or ROS production [20–22]. Therefore examining ER stress induced by PI is important for clarifying the mechanisms of PI-induced side effects.

In this study, first we screened the ER stress induction potential of 9 Food and Drug Administration (FDA)-approved PIs (SQV, saquinavir; RTV, ritonavir; IDV, indinavir; NFV, nelfinavir; APV, amprenavir; LPV, lopinavir; TPV, tiprenavir; ATV, atazanavir; DRV, darunavir), and identified that LPV has the most potent ER stress induction potential among these PIs in human peripheral blood mononuclear cells, several human cell lines, and mouse embryonic fibroblasts. By a comparison between LPV and DRV, we clearly indicated that DRV does not induce ER stress and apoptosis. On the other hand, LPV induced ER stress and apoptosis, not by proteasome inhibition but by ROS-dependent JNK activation. Collectively, these results indicated that the most clinically used PIs, LPV and DRV, show clear differences in terms of ER stress and cytotoxicity induction potential.

Materials and methods

Reagents, plasmids, and antibodies

The reagents used are as follows: Nine PIs were obtained as previously described [23]. DCFH-DA was from Sekisui Medical (Tokyo, Japan). Amplex Red was from Invitrogen Japan (Tokyo, Japan). SP600125 and SB203580 were from Wako (Osaka, Japan). Q-VD-OPh was from R&D Systems (Minneapolis, MN, USA). Salubrinal (Sal) was from Calbiochem (San Diego, CA). Bortezomib (Bor) was from Selleck Chemicals LLC (Houston, TX). *N*-Acetyl-L-cysteine (NAC) was from Sigma-Aldrich Co. (St. Louis, MO). Plasmids used are as follows: pCAX-F-XBP1ΔDBD-venus, pCAX-HA-2xXBP1ΔDBD (anATG)-LUC-F, and pCAX-hATF4(1-285)-hRL-HA. The pCAX plasmids were described previously [24]. pGL4-ARE-reporter (pGL4.37) and pGL4-ATF6-reporter (pGL4.39) plasmids were from Promega (Madison, WI). Proteasome sensor vector was from Clontech (Palo Alto, CA). Antibodies used are as follows: Antibodies for XBP1, ATF4, ATF6, CHOP, γ-tubulin, and HRP-conjugated anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-12 and caspase-4 antibodies were from Millipore (Bedford, MA) and MBL (Nagoya, Japan), respectively. Ubiquitinated protein antibody was from AffinitiBioreagents (Golden, CO). eIF2α antibody was from Sigma-Aldrich Co. (St. Louis, MO). Antibodies for CHOP, PERK, IRE1, phoshorylated-eIF2α, JNK, phosphorylated-JNK, p38, phosphorylated-p38, ERK, phosphorylated-ERK, cleaved caspase-3, caspase-9, and HRP-conjugated anti-mouse or anti-rabbit IgG were from Cell Signaling Technology (Danvers, MA). HRP-conjugated anti-rat IgG was from DakoCytomation (Glostrup, Denmark).

Cell culture, treatment, and transfection

Human embryonic kidney cells, HEK293, human hepatoma cells, HepG2, lung adenocarcinoma cells, A549, human colorectal cells, HCT116, and mouse embryonic fibroblasts, MEF, were maintained as previously described [25]. All cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO_2. Treatment of cells with indicated doses of PIs was carried out for indicated times. For inhibition of caspase, JNK, p38, and eIF2α phosphatase, cells were pretreated with Q-VD-OPh, SP600125, SB203580, and salubrinal for 1 h before PI treatment, respectively. Transient transfections of plasmids were performed using Hilymax (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instruction. Stably transfected HEK293 cells were established by 500 μg/ml G418 treatment. Human peripheral mononuclear cells (PBMC) were collected from adult male donors after informed consent was obtained in accordance with the Declaration of Helsinki and based on a protocol approved by the Institutional Review Board of the Faculty of Medical and Pharmaceutical Sciences, Kumamoto University. PBMC was maintained in RPMI-1640 containing 10% FBS and antibiotics. Small interfering RNA (siRNA) for JNK1/2 (si-JNK1/2) was transfected into HEK293 cells using Trans-IT TKO (Mirus, Madison, WI) according to the manufacturer's instructions. A 50 nM JNK1/2 siRNA duplex was transfected into 70% confluent cells to knock down JNK1/2. GL2-luciferase (luc) siRNA duplex was used as a control. The cells were treated with HIV-PIs and harvested 48 h after transfection. The siRNA oligonucleotide sequences are as shown below. JNK1 siRNA sense, 5′-GACCAUUUCAGAAUCAGACUU-3′; JNK1 siRNA antisense, 5′-AAGUCUGAUUCUGAAAUGGUC-3′; JNK2 siRNA sense, 5′-GAUGCUAACUUAUGUCAGGUU-3′; JNK2 siRNA antisense, 5′-AACCUGACAUAAGUUAGCAUC-3′. The negative control siRNA (MISSION siRNA Universal Negative Control; Sigma-Aldrich, Tokyo, Japan) was also used (con-si).

RT-PCR analysis

Total RNA isolation and quantitative RT-PCR (Q-PCR) analyses for CHOP, XBP1s, and internal controls 18S ribosomal RNA (18SrRNA) were carried out as previously described [25]. The normalized gene expression values were expressed as the relative quantity of CHOP gene-specific messenger RNA (mRNA). The oligonucleotide primers used in quantitative RT-PCR are as shown below. Human CHOP-Fw, 5′-ATGGCAGCTGAGTCATTGCCTTTC-3′; human CHOP-Rv,5′-AGAAGCAGGGTCAAGAGTGGTGAA-3′; human XBP1s-Fw, CCGCAGCAGGTGCAGG; human XBP1s-Rv, GAGTCAATACCGCCAGAATCCA; human 18s-Fw,5′-CGGCTACCACATCCAAGGAA-3′; human 18s-Rv,5′-GCTGGAATTACCGCGGCT-3′; mouse CHOP-Fw, 5′-CATACACCACCACACCTGAAAG-3′; mouse CHOP-Rv, 5′-CCGTTTCCTAGTTCTTCCTTGC-3′; mouse 18s-Fw,5′-GTAACCCGTTGAACCCCATT-3′; mouse 18s-Rv, 5′-CCATCCAATCGGTAGTAGCG-3′.

To analyze the XBP-1 splicing, semi-RT-PCR analyses for the human spliced form and the unspliced form of XBP-1 mRNA and internal control human GAPDH were carried out as previously described [26]. The oligonucleotide primers used in quantitative RT-PCR are as shown below. XBP1-Fw,5′-TTACGAGAGAAAACTCATGGCC-3′; XBP1-Rv,5′-GGGTCCAAGTTGTCCAGAATGC-3′; GAPDH-Fw, 5′-CGGGAAGCTTGTGATCAATGG-3′; GAPDH-Rv,5′-GGCAGTGATGGCATGGACTG-3′.

Western blotting

For Western blotting analysis of XBP1s, ATF4, ATF6, and γ-tubulin, HEK293 cells were treated with PIs. Nuclear proteins were obtained as described previously [27]. For detection of XBP1s, a short form of the XBP1 band was separately detected from the full length of the XBP1 band by molecular size. For expression analysis of CHOP, cleaved caspase-3/4/9/12, Hsc70, or JNK, p38, ERK, and their phosphorylated form, whole proteins were recovered as described previously [28]. For examining CHOP and Hsc70 in mice tissues, tissue proteins were lysed in glycerol buffer, described previously [29]. Protein lysates were subjected to SDS-PAGE and Western blotting. Blots were probed with the indicated antibodies, and visualized using Chemi-Lumi One Super (Nakarai Tesk, Kyoto, Japan).