Analysis of endoplasmic reticulum stress in placentas of HIV-infected women treated with protease inhibitors

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Abstract

Combined antiretroviral therapy has proven efficacy in decreasing vertical HIV transmission. However, endoplasmic reticulum stress is a known side effect of HIV protease inhibitors. We investigated endoplasmic reticulum stress in placentas of HIV-infected and uninfected mothers by PCR-based splicing analysis of the specific endoplasmic reticulum stress marker XBP1 in post-delivery placental samples of uninfected mothers and in HIV-infected mothers taking antiretroviral therapy. No elevated XBP1 splicing could be detected in placentas of uninfected mothers and most of the mothers receiving combined anti-retroviral therapy. However, markedly elevated XBP1 splicing was found in the placentas of three individuals on combined antiviral therapy, all receiving lopinavir or atazanavir. In vitro experiments confirmed induction of endoplasmic reticulum stress by lopinavir and atazanavir in trophoblast-derived cell lines. Since endoplasmic reticulum stress occurred in selective patients only, individual differences in susceptibility of HIV-infected mothers to protease inhibitor induced endoplasmic reticulum stress can be postulated.

1. Introduction

Endoplasmic reticulum stress in various human tissues and cell types has been described as an adverse effect common to many HIV protease inhibitors [1–5]. The exact mechanism by which HIV protease inhibitors cause endoplasmic reticulum stress is not known. It is assumed that this is either mediated by their ability to interact with a yet unidentified endoplasmic reticulum-resident aspartic protease, with ensuing accumulation of incompletely processed proteins within the endoplasmic reticulum, or by their ability to induce oxidative stress [6], another well-known protein-damaging condition that leads to endoplasmic reticulum stress [7,8].

Accumulation of misfolded or incorrectly processed proteins within the endoplasmic reticulum leads to the recruitment of luminal endoplasmic reticulum chaperones and folding proteins such as BiP (binding protein) to these proteins in order to assure refolding of these proteins or to prevent formation of possible cytotoxic protein aggregates [7,8]. Recruitment of BiP to misfolded proteins is associated with its release from three endoplasmic reticulum membrane-bound signaling molecules, IRE1, PERK, and ATF6, which subsequently become activated and convey the endoplasmic reticulum stress signal. This includes signal transmission into the cytosol by inducing protein phosphorylation cascades and signal transmission into the nucleus by activation of transcription factors that subsequently transcribe cytoprotective molecular chaperones to cope with the cellular stress situation. However, long-term impairment of the endoplasmic reticulum homeostasis can result in severe disturbances of cellular metabolism and can lead to cell death resulting in various pathological conditions found in humans [7,8].

Excessive endoplasmic reticulum stress has also been associated with the induction of autophagy or reticulophagy, a mechanism by which damaged regions of the endoplasmic reticulum might be removed [9,10]. The endoplasmic reticulum membrane-resident IRE1 molecule also causes an unconventional cytoplasmic splicing of the XBP-1 (X-box binding protein 1) mRNA, which results in the expression of the active form of the XBP-1 transcription factor. XBP1 is one of those transcription factors which lead to the expression of the characteristic endoplasmic reticulum stress response genes, and can also be used as a specific endoplasmic reticulum stress marker since its splicing by IRE1 can easily be detected by means of an PCR assay [5]. Using this assay, we analyzed and characterized...
the occurrence of the endoplasmic reticulum stress reaction in placenta tissue samples of HIV-infected women receiving combined antiviral therapy, of HIV-uninfected women, and in trophoblast-derived cell lines exposed to antiretroviral drugs.

2. Methods

2.1. Study participants

HIV-1-infected pregnant women who took an antiretroviral combination therapy were prospectively enrolled in the Department of Obstetrics at the University Hospital Munich. The control group consisted of HIV-uninfected healthy pregnant women who delivered during the study period at the same institution. Patients’ characteristics are summarized in Table 1. Delivery of HIV-infected and uninfected patients was all by Cesarean section. On the day of delivery, HIV drugs were taken by the patients in the same amount and frequency (twice per day) as during pregnancy. The study group consisted of women of various ethnicities, including Caucasian, African, and Asian. The protocol was approved by the local ethics committee. Written informed consent was obtained by all participants.

2.2. Cells and cell culture

The immortalized human 3A-subE placental cell line was purchased from ATCC (ATCC CRL-1584). JEG-3 human choriocarcinoma cells were kindly provided by Dr. Bernhard Ugele, University Hospital Munich. Both cell lines were cultured in fetal calf serum-containing RPMI1640 cell culture medium (Quantum 263, PAA, Pasching, Austria), supplemented with penicillin/streptomycin (PAA, Pasching, Austria) at 37 °C in a humidified atmosphere with 5% CO2. The 3A-subE placental cell line (IPA30-1) was derived from “term placental cells” transformed with a tsA mutant form of the simian virus 40 (SV40) [11] (http://www.lgcstandards-atcc.org/Products/All/CRL-1584.aspx). JEG-3 cells were derived from the Woods strain of the Erwin-Turner tumor [12] (http://www.lgcstandards-atcc.org/Products/All/HTB-36.aspx).

2.3. Tissue preparation

After delivery, placentas were transported on ice to the laboratory (maximum transportation time: 5 min), and pieces of placental tissue were excised and frozen at −80 °C. For RNA extraction, small tissue pieces were cut with a scalpel from the frozen placenta tissues and placed into a reaction vial placed in liquid nitrogen as previously described [5]. Frozen tissue samples were ground with a teflon pestle, thawed, and then dissolved in 1 ml Trizol reagent. Total RNA was extracted by the standard Trizol method as previously described [5]. mRNA was transcribed into cDNA with M-MLV reverse transcriptase (Promega, Mannheim, Germany) and oligo-DT as recommended by the supplier. RNA from placental cell lines was isolated with an RNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s recommendations. From each placenta, 2 different samples were taken from different regions of the placenta and prepared and analyzed separately. Final data were presented as mean values generated from the 2 samples collected.

2.4. Quantitative and semi-quantitative PCR analysis

RNA extracted from placental tissues obtained after delivery was analyzed by an XPB1 splicing-specific real-time PCR assay to detect the occurrence of ER stress. Semi-quantitative PCR amplification was performed with PCR master mix (Promega, Mannheim, Germany) at 26 cycles (β-actin) or 32 cycles (XPB1) in a 25 µl PCR volume. Primer pairs used were 5’-GGAGAGGAGCGGAGAAGAGG-3’ and 5’-GATGCCCAACAGGATAGC-3’ for XPB1, and 5’-GGAGAGGTCGGTCTAGTCG-3’ and 5’-GCCTGAGGGACGATGAT-5’ for β-actin amplification. Quantitative real-time PCR analysis was performed using the XPB1-specific primers described above in combination with the FAM-5’-TGCTGACTCCAGAGGCTGCA-3’-TAMRA probe and the β-actin-specific primers in combination with the probe FAM-5’-CCCTCTGGGACATGGTC-3’-TAMRA. Real-time PCR was performed with a 7500 Fast System real-time PCR cycler (Applied Biosystems, Darmstadt, Germany), using iTag Fast Supermix with ROX (BioRad, München, Germany). Relative expression analysis was calculated by the 2−∆∆CT method [13] using β-actin expression as a reference. Real-time PCR analysis was performed in duplicate on each placental tissue probe, resulting in 4 measured data for each patient. All primers were synthesized by biomers.net (Ulm, Germany).

2.5. Drugs and drug treatment

All antiretroviral medications for patients, including Kaletra (lopinavir + ritonavir), Reyataz (atazanavir), Norvir (ritonavir), Vimuran (nevirapine), Truvada (tenofovir + emtricitabine), Viread (tenofovir), Combivir (lamivudine + azidothymidine), Epivir (lamivudine), and Zidovudine (azidothymidine) were provided by the University Hospital Pharmacy. For in vitro experiments, the tablets of fixed dose combination lopinavir plus ritonavir (Kaletra® 200 mg + 50 mg) were ground and extracted with dimethyl sulfoxide (DMSO) to reach a final concentration of 10 mg/ml, corresponding to 8 mg/ml (12.72 µM) lopinavir (MW 628.81) plus 2 mg/ml (2.77 µM) ritonavir (MW 721). Chemically pure atazanavir sulfate (MW 802.93), as used for in vitro experiments, was purchased from Sellecchem (Munich, Germany) and kept as a 10 mg/ml (12.45 mM) stock solution after direct solution in 100% DMSO. In cell culture experiments, except for the sample with the highest atazanavir concentration, additional DMSO was added to achieve a similar concentration of DMSO in samples and controls (0.2% DMSO in case that 20 µg/ml atazanavir was used as the highest concentration, and 0.15% in case that 15 µg/ml atazanavir was used as the highest experimental concentration). Similar supplementations were made for experiments with Kaletra, for which the highest concentration of DMSO used was 0.2%.

2.6. Autophagy staining

Autophagy was microscopically visualized with the fluorescent autophagy detection marker monodansylcadaverine (Singa, Munich, Germany). For this, JEG3 cells were seeded on glass chamber slides 24 h prior to a further 24 h incubation of the cells with the corresponding protease inhibitors. All incubations were performed under standard cell culture conditions. After 24 h incubation with protease inhibitors, JEG3 cells incubated for further 30 min with 1 µg/ml of monodansylcadaverine (1 mg/ml stock solution in DMSO) in cell culture medium. After washing with PBS, slides were subjected to fluorescence microscopy using a DAPI-suited blue-cyan filter (365 nm excitation filter; 420–470 nm emission filter). Cells were then photographed as viable cells by means of a Zeiss Axiosphore fluorescent microscope (Zeiss, Munich, Germany). For autophagy detection by FACScan analysis, JEG3 cells were seeded in 6-well cell culture plates (2 × 105 cells/well) and allowed to grow for 24 h under cell culture conditions before application of HIV protease inhibitors for further 24 h. Then, cells were incubated with a 1:1000 dilution of an autophagy detection dye (Cyto-ID Autophagy Detection Kit; Enzo Life Sciences, Lörrach, Germany) for 1 h, collected by trypsinization, and subjected to FACScan analysis (Beckman Coulter Epics XL), using a 488 nm excitation filter and a 525 nm emission filter.
### Table 1

Patients’ characteristics.

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<th>Body length (cm)</th>
<th>Body weight (kg)</th>
<th>CDC Class</th>
<th>CD4+ cells/μl</th>
<th>Viral load (copies/ml)</th>
<th>Other diseases</th>
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<td>–</td>
<td>–</td>
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**Abbreviations:** cART, combined antiretroviral therapy; AZT, zidovudine; 3TC, lamivudine; TDF, tenofovir; FTC, emtricitabine; NVP, nevirapine; LPV, lopinavir; ATV, atazanavir; r, ritonavir (boosting dose).
2.7. Statistical analysis

Statistical analysis was performed by the IBM SPSS Statistics 22 program, using the Mann–Whitney U-test for independent samples. All data are presented by mean value ± standard deviation (SD). Significance was assumed by p values ≤0.05.

3. Results

3.1. Patient population and sample collection

Placental tissue samples derived from 15 HIV-infected women receiving combined anti-retroviral therapy were collected and compared with placental tissues samples from 6 non-infected women. No vertical transmission occurred in our study group.

<table>
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<tr>
<th>Patient</th>
<th>Rel. XBP1 values</th>
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<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>2.82 (±3.47)</td>
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<td>3</td>
<td>0.1 (±0.13)</td>
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<td>4</td>
<td>1.5 (±2.1)</td>
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<td>5</td>
<td>1.25 (±0.6)</td>
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<td>6</td>
<td>8.88 (±12.6)</td>
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<td>73.2 (±7.88)</td>
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</tr>
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<td>7.36 (±2.95)</td>
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Table 2: XBP1 splicing activity in placental tissues of HIV drug-treated women.

Abbreviations: cART, combined antiretroviral therapy; AZT, zidovudine; 3TC, epivir; TDF, tenofovir; FTC, emtricitabine; NVP, nevirapine; LPV, lopinavir; ATV, atazanavir; r, ritonavir (boosting dose).

No malformations or significant complications were observed in the newborns (Table 1). The birth weight did not differ significantly in the study group and the control group (2871 ± 433 g vs. 2953 ± 400 g; p = 0.613 (mean ± SD)). Except for one case in the study group all deliveries were at term (≥37 + 0 weeks of gestation).

3.2. XBP1 splicing in placenta tissues of HIV-infected women treated with antiretroviral drugs

In all placental tissues of the 6 non-infected mothers tested, only minimal levels of XBP1 splicing could be detected (mean relative XBP1/β-actin mRNA level: 1.71 ± 0.46; Fig. 1; Table 2). In mothers receiving combined anti-retroviral therapy, the relative XBP1/β-actin mRNA level was found to be significantly increased (mean relative XBP1/β-actin mRNA level: 42.19 ± 106.18; Fig. 1; Table 2: p = 0.023; patients with the same ordinal numbers in Tables 1 and 2 represent identical persons). Individual analysis, however, revealed a strong inter-individual variation among the mothers tested. In particular, two individual samples from HIV-positive mothers revealed highly elevated levels of XBP1 splicing (relative XBP1/β-actin mRNA levels of 243, and 442, respectively: Fig. 1; Table 2). An analysis of the drug constituents revealed that these mothers received a drug combination of either Kaletra plus...
Truvada (lopinavir, ritonavir, tenofovir, and emtricitabine), or a combination of Kaletra plus Viread and Viramune (lopinavir, ritonavir, tenofovir, and emtricitabine; refer to the legend, Fig. 1). An elevated XBP1 splicing was also observed for a combination treatment with Reyataz plus Truvada and Norvir (atazanavir, ritonavir, tenofovir, and emtricitabine; reln of XBP1/β-actin mRNA level of 73; Fig. 1; Table 2). Since HIV protease inhibitors had already been identified as endoplasmic reticulum stress-inducing agents, and because the patients with the highest levels of XBP1 splicing received either Kaletra or Reyataz, we tested the sensitivity of placenta-derived cell lines (JEG3 and 3a-Sub E) to lopinavir plus ritonavir, the active components of Kaletra, and to atazanavir, the active compound of Reyataz. In both the immortalized 3a-Sub E trophoblast cell line and the JEG3 chorionic carcinoma cell line, a marked lopinavir/ritonavir-induced XBP1 splicing could be observed by real-time PCR (Fig. 2A) and semiquantitative PCR analysis (Fig. 2B). XBP1 splicing reached its maximum level at already 10 μg/ml of Kaletra (lopinavir plus ritonavir, 4:1, w/w), a concentration that has been described to be within the pharmacological range in patients receiving this medication [14,15].

Intermediate levels of XBP1 splicing could be observed at concentrations as low as 5 μg/ml of lopinavir/ritonavir, corresponding to 4 μg/ml lopinavir plus 1 μg/ml ritonavir. Similar results were observed with atazanavir (Fig. 2C), although the induction of XBP1 splicing required higher concentrations of at most 10 μg/ml of atazanavir (Fig. 2C). This concentration of atazanavir necessary to induce endoplasmic reticulum stress in vitro was higher than the concentrations detected in atazanavir/ritonavir-treated pregnant women, in whom maximum serum concentrations of atazanavir were described to drop during pregnancy to concentrations as low as 3.3 μg/ml (mean value) in the third trimester, before being again elevated to concentrations of up to 5.6 μg/ml (mean value) post-partum [16].

Endoplasmic reticulum stress is often associated with autophagy to remove superfluous or damaged regions of the endoplasmic reticulum. Cell staining with the blue-green fluorescent autophagy detection marker monodansylcadaverine [17] revealed an increase in autophagic vesicles in JEG cells treated with either lopinavir/ritonavir or atazanavir (Fig. 3A). FACScan with a green fluorescent autophagy detection reagent analysis further confirmed induction of autophagy by atazanavir and lopinavir/ritonavir at pharmacologically relevant concentrations (Fig. 3B).

4. Discussion

With the use of combination antiretroviral therapy during pregnancy, the prevention of mother-to-child transmission has become highly effective, and transmission rates of <1% can be achieved. Nevertheless, possible short- and long-term adverse effects of these medications in both the mother and the fetus should be considered [18,19]. The use of protease inhibitors during pregnancy has been associated with an increase in premature deliveries and low birth weight [20,21]. A better understanding of the possible mechanisms may help to ensure the safety of HIV-infected pregnant women and their children.

ER stress primarily functions as a physiological and protective cell response mechanism to a variety of stimuli (such as insulin or glucose) or pathological conditions (such as ischemia,
hyoxia, or oxidative stress). The ability to respond to physiological
capital endoplasmic reticulum stress is essential for normal placental
and embryonic development [22]. However, excessive endoplasmic reticulum
stress as well as drug-induced endoplasmic reticulum
stress can cause major tissue damage and, in adults, has
been associated with diabetes, steatosis, systemic inflammation,
atherosclerosis, and several neurodegenerative disorders [23,7,24].

Animal studies have shown that ER stress has a detrimental
 effect on the development of both placenta and embryo [25].
In humans, elevated endoplasmic reticulum stress was shown to
play an important role in the development of human intrauterine
growth restriction (IUGR), and a marked increase in XBP1 splicing
and phosphorylation of eIF2a was found in placental tissues from
pregnancies complicated by IUGR [25]. The effect was even more
pronounced if both IUGR and preeclampsia occurred during the
pregnancy [26], and it has been shown that endoplasmic reticulum
stress leads to cell cycle arrest and reduced protein biosynthesis in
placental cells [26].

In our study the protease inhibitors lopinavir (in combination
with ritonavir as a booster) and atazanavir were associated with
elevated endoplasmic reticulum stress in placental tissue of HIV-
infected pregnant women. We were also able to induce excessive
endoplasmic reticulum stress in trophoblast derived cell lines by
exposing them to increasing levels of these compounds. The concentra-
tions of lopinavir/ritonavir necessary to induce endoplasmic reticulum
stress in vitro corresponded well to the concentrations observed in pregnant women treated with these drugs [14,15]. By contrast, the concentrations of atazanavir necessary to induce pro-
nounced endoplasmic reticulum stress in vitro were slightly higher
than those observed in pregnant women [16]. However, plasma
concentrations of atazanavir were also described to be highly vari-
able among pregnant women and were also shown to depend on
the total amount of atazanavir (plus ritonavir) taken [16].

Although experiments with immortalized cell lines in cell cul-
ture do not necessarily reflect the in vivo situation, which is
confirmed by the fact that not all HIV protease inhibitor-receiving
patients revealed elevated levels of endoplasmic reticulum stress, it
confirms the ability of lopinavir and atazanavir to induce endo-
plasmic reticulum stress in cell lines which originally have been
derived from trophoblastic tissue.

At this time it is not possible to ascertain the extent to which
this induction of endoplasmic reticulum stress by the protease
inhibitors lopinavir/ritonavir and atazanavir has a clinical signif-
ificance. Nevertheless, data from the French perinatal cohort have
linked per- and postnatal exposure to ritonavir-boosted lopinavir
(Kaletra®) to adrenal dysfunction in children [27], and, in the same
cohort, ritonavir has been associated with preterm deliveries [28].

Even though our findings indicate that endoplasmic reticu-
lim stress can be induced by exposure to lopinavir/ritonavir and
atazanavir, no detrimental pregnancy outcome was observed in our
study. This is reassuring, but – given the small number of patients
included – not conclusive, and more data are needed. Furthermore,
the levels of endoplasmic reticulum stress in the placental tissues
varied significantly between individuals and we could not show
consistently elevated levels of endoplasmic reticulum stress in all
patients who had received protease inhibitors during pregnancy.
In a previous study, we observed a high inter-individual variability
of maternal lopinavir concentrations in pregnant women at term
[14]. A study on HIV-infected women in Thailand treated with
a standard dose of lopinavir/ritonavir showed maximal maternal
plasma concentrations of lopinavir at 72 h postpartum to be around
11 μg/ml [15] and we previously reported maximal lopinavir levels
in European-resident women at delivery of approximately 8 μg/ml
[14]. In the present study, in which we used a comparable concentra-
tion range for in vitro studies on trophoblast-derived cell lines,
these concentrations were found to cause endoplasmic reticulum
stress in the in vitro model, confirming the notion that pharmaco-
logically achievable concentrations of HIV protease inhibitors may
cause endoplasmic reticulum stress in human placental cells.

The pharmacokinetics of protease inhibitors in pregnant women
may therefore vary significantly, and this may influence the degree
of endoplasmic reticulum stress induced. Because in the current
study we have no maternal pharmacokinetic data, this needs
further investigation. Several studies were able to show that pro-
tease inhibitors induce endoplasmic reticulum stress in various
tissues and cell types at pharmacologically achievable concentra-
tions. Lopinavir either as a single drug or boosted by ritonavir
was found to increase endoplasmic reticulum stress in intestinal
epithelial cells [4], macrophages [29], adipocytes [30], and to aggra-
vote alcohol-induced endoplasmic reticulum stress in hepatocytes
[31]. However, similar to our in vitro experiments on trophoblast-
derived cell lines, it is not clear whether these in vitro studies or
animal experiments can be transferred to the conditions found in
humans. Therefore, large scale analyses that also include pharma-
cological data, not only including serum levels but also the extent
of placenta tissue penetration by these drugs, are needed.

Protease inhibitor inducible insulin resistance is another
adverse effect known to be caused by HIV medications [32] and may
further aggravate the phenomenon of gestational diabetes that can
occur in pregnant women [33]. Since elevated glucose levels are
also known to promote endoplasmic reticulum stress [34], it may
be of interest to include blood glucose and lipid levels in further
studies for generation of a more comprehensive overview of fac-
tors that may contribute to placental endoplasmic reticulum stress
in HIV-infected mothers treated with protease inhibitors.

Since endoplasmic reticulum stress is of importance in both the
physiology and pathophysiology of the placenta, improved knowl-
dge about the influence of HIV protease inhibitors on placental
endoplasmic reticulum stress may be helpful for understanding
possible placenta-damaging effects and possible adverse effects for
the pregnancy and on the embryo. Because of the importance of HIV
drug combination therapy to prevent mother-to-child transmis-
sion, there is currently no alternative to the application of these
drugs to HIV-infected mothers. However, knowledge about possible
adverse effects such as endoplasmic reticulum stress and
medical conditions that might aggravate or predispose to such side
effects may help to identify patients who should attain a closer
monitoring during pregnancy.

Conflicts of interest

A.G. received payment for lectures from MSD Sharp & Dohme
GmbH and Bristol Myers Squibb GmbH & Co KG, and for meeting
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other authors have no conflict of interest to declare.

Transparency document

The Transparency document associated with this article can be
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