Redox Biology 2 (2014) 599–609

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

Bypassing the compromised mitochondrial electron transport with methylene blue alleviates efavirenz/isoniazid-induced oxidant stress and mitochondria-mediated cell death in mouse hepatocytes

Kang Kwang Lee, Urs A. Boelsterli*

Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269, United States of America

ARTICLE INFO

Article history:
Received 10 March 2014
Accepted 11 March 2014

Keywords:
Efavirenz
Isoniazid
Mitochondria
Complex I
Drug-induced liver injury (DILI)
Hepatotoxicity
Methylene blue
Peroxynitrite

ABSTRACT

Efavirenz (EFV) is an anti-retroviral drug frequently combined with isoniazid (INH) to treat HIV-1/tuberculosis co-infected patients. Both drugs have been associated with idiosyncratic liver injury (DILI), but combined anti-retroviral and anti-tubercular therapy can increase the risk for DILI as compared to either drug class alone. Because both EFV and INH have been implicated in targeting mitochondria, we aimed at exploring whether the two drugs might cause synergistic effects on the electron transport chain. We found that EFV inhibited complex I activity in isolated mouse liver mitochondria (IC_{50} ~30 μM), whereas hydrazine, a major metabolite of INH generated by acylamidase-mediated hydrolytic cleavage, inhibited complex II activity (IC_{50} ~30 μM). Neither INH alone (≤1000 μM) nor EFV alone (≤30 μM) was able to induce cell injury in cultured mouse hepatocytes. However, combined EFV/INH exposure resulted in increased superoxide formation and peroxynitrite stress, leading to the opening of the cyclosporine A-insensitive mode of the mitochondrial permeability transition (mPT), and necrotic cell death. The peroxynitrite scavengers, CBA or Fe-TMPyP, protected against mPT induction and alleviated cell injury. The acylamidase inhibitor *bis-p*-nitrophenyl phosphate prevented cell injury, suggesting that hydrazine greatly contributed to the toxicity. Methylene blue, a redox-active alternative electron acceptor/donor that bypasses complex I/II, effectively protected against EFV/INH-induced toxicity. These data demonstrate that, in murine hepatocytes, the mitochondrial electron transport chain is a critical target of combined EFV/INH exposure, and that this drug combination can lead to peroxynitrite stress-induced mPT and hepatocellular necrosis. These results are compatible with the concept that underlying silent mitochondrial dysfunction may be a key susceptibility factor contributing to idiosyncratic drug-induced liver injury.

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Introduction

An increasing number of therapeutic drugs have been implicated in targeting mitochondria and causing mitochondrial dysfunction, which likely contributes to some of the adverse effects and organ toxicity associated with these drugs [1–4]. Specifically, inhibition of mitochondrial electron transport at one or several sites of the electron transport chain (ETC) is one common mechanism by which drugs can interfere with energy homeostasis and the redox balance in mitochondria [5]. Minor impairment of ETC function normally does not entail biologically significant effects, due to the large inherent reserve capacity of the mitochondrion, and because of certain functional threshold effects for respiratory complexes I through IV [6–8]. However, this may dramatically change in the presence of an inherited or acquired mitochondrial deficiency, which can greatly amplify superimposed drug effects and severely impair energy production and mitochondrial function [9]. For example, underlying pharmacologic or genetic complex I dysfunction has been implicated in augmenting and potentiating the mitochondrial and cellular toxicity of mitochondria-targeting drugs [10–13].

Consistent with this concept, we have recently demonstrated that selective inhibition of complex I with rotenone or piericidin A was able to trigger lethal cell injury induced by otherwise non-toxic concentrations of the anti-tubercular drug, isoniazid (INH) in cultured mouse hepatocytes [14]. The mechanism of this synergistic effect is

Abbreviations: 1-ABT, 1-aminobenzotriazole; BNPP, *bis-p*-nitrophenyl phosphate; CBA, coumarin-7-boronic acid; CYP, cytochrome P450; EFV, efavirenz; ETC, electron transport chain; Fe-TMPyP, 5,10,15,20-tetrakis(*N*-methyl-4′-pyridyl)porphyrinato iron(III); INH, isoniazid; MB, methylene blue (methylthioninium chloride, 3,7-bis(dimethylamino)phenazathionium chloride); NAT, *N*-acetyltransferase; ROS, reactive oxygen species.

* Correspondence to: Department of Pharmaceutical Sciences, University of Connecticut, School of Pharmacy, 69 North Eagleville Road, Unit 3092, Storrs, CT 06269, United States of America.

E-mail address: urs.boelsterli@uconn.edu (U.A. Boelsterli).

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http://dx.doi.org/10.1016/j.redox.2014.03.003

not completely clear, but we have shown that hydrazine, a major hydrolytic metabolite of INH, inhibited mitochondrial complex II and caused increased leakage of superoxide from the electron transport chain [14]. The resulting joint inhibition of complexes I and II caused massive ATP depletion and necrotic cell death in hepatocytes.

In an attempt to translate these findings into a clinically more relevant situation, we have exposed hepatocytes to a combination of efavirenz (EFV) and INH. Efavirenz is an anti-retroviral drug frequently combined with INH to treat HIV-1/tuberculosis co-infected patients [15,16]. Efavirenz has recently been associated with liver injury in susceptible patients, and a series of elegant mechanistic studies have revealed that EFV causes mitochondrial stress in murine hepatocytes [17–20]. On the other hand, INH has been used therapeutically for many decades, and the risk for inducing liver injury in susceptible patients has been well known [21,22], but the susceptibility factors are largely unknown. Interestingly, combined anti-retroviral/anti-tubercular therapy significantly increases the risk for developing liver injury as compared to anti-retroviral therapy alone [23], but it is not known whether mitochondrial dysfunction may be involved. The aim of this study was to explore whether EFV in combination with INH will precipitate cell injury in mouse hepatocytes via joint inhibition of the respiratory complexes I and II. Furthermore, we sought to explore whether pharmacologic intervention with methylene blue, an alternative electron carrier that can bypass the proximal ETC, can prevent the energy crisis and protect against lethal cell injury associated with the mitochondria-targeting drugs.

Materials and methods

Chemicals

Isoniazid (INH), efavirenz (EFV), and hydrazine (HzN) were purchased from Sigma (St. Louis, MO). All chemicals were obtained at the highest grade available.

Animals

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Connecticut. Young adult male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Prior to use, the mice were acclimatized for >1 week and kept on a 14/10-h light/dark cycle under controlled environmental conditions. They had free access to mouse chow (Teklad Global Rodent Diet; Harlan Laboratories, Boston, MA) and water.

Isolation of hepatic mitochondria and complexes I and II activity measurement

Mitochondria were isolated from untreated mice according to standard procedures as previously described [14]. Protein content was determined with the BCR protein assay using albumin as the reference protein. The mitochondria-enriched fractions were kept at −80 °C until analysis. Complexes I and II activities were determined in freeze–thawed (2×) mitochondria according to standard methods [24]. Briefly, complex I was measured as NADH: ubiquinone oxidoreductase activity in 25 mM potassium phosphate buffer containing 5 mM $MgCl_2$, pH 7.2, and 2.5 mg/ml BSA, 0.13 mM NADH, 2 μg/ml antimycin A, and 65 μM ubiquinone (Q1). NADH oxidation was monitored as decrease in absorbance at 340 nm. Complex II was measured as succinate: ubiquinone oxidoreductase activity, linked to the artificial electron acceptor, 2,6-dichlorophenolindophenol (DCPIP), in phosphate buffer without BSA, containing 20 mM sodium succinate, 50 μM DCPIP, 2 μg/ml antimycin A, 2 μg/ml rotenone, and 65 μM Q1. DCPIP reduction was monitored at 600 nm.

Primary mouse hepatocyte culture and exposure to drugs

Hepatocytes were isolated from mice by retrograde collagenase perfusion, and subsequently cultured in supplemented Williams' Medium E as described [14]. Briefly, the cells were plated in 48-well plates (8.0×10^4 cells per well) coated with 50 μg/ml rat tail collagen. The hepatocytes were allowed to attach for 3 h in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Subsequently, the cells were washed and then incubated in the same medium. After overnight preculture, the medium was replaced by fresh serum- and antibiotic-free medium to which the drugs were added from stock solutions. DMSO was used as solvent for EFV and other lipophilic compounds (final concentrations not exceeding 0.1%), and culture medium was used to dissolve INH. In some experiments, the cells were post-treated with methylene blue (MB) 20 min after exposure to EFV and/or INH.

Determination of cell injury

Release of cytosolic lactate dehydrogenase (LDH) into the extracellular medium (CytoTox-One Homogeneous Membrane Integrity Assay, Promega, Madison, WI) was used as an indicator of cytotoxicity. The data were expressed as percentage of activity present in the medium as compared to the total intra- and extracellular LDH activity. Total cellular ATP content was measured by luminescence techniques (Cell Titer-Glo Luminescent Cell Viability Assay, Promega). Chemiluminescence was determined in black 96-well plates, and ATP content was calculated from a standard curve. INH, EFV, or MB did not interfere with the luciferin/luciferase reaction. The nuclear fluorescence of propidium iodide, which can permeate into cells with compromised plasma membrane only, was taken as an indicator of necrotic cell death.

Assessment of the mitochondrial permeability transition

To demonstrate the opening of the mitochondrial permeability transition (mPT) pore, we used the fluorogenic marker, calcein acetoxymethylester (AM). To selectively label mitochondria with the probe, the cells were loaded with 1 μM calcein-AM in the presence of $CoCl_2$ (1 mM) for 15 min at 4 °C, followed by 6 h incubation at 37 °C [25]. By loading the cells in the cold, the calcein-AM can cross both the cell membrane and the mitochondrial membranes, because the cytosolic esterases are not active. Upon recovery at 37 °C, calcein-AM in mitochondria is cleaved and calcein remains trapped. Because the added Co^{2+}, which is a high-affinity ligand for calcein, quenches the fluorescence upon binding, the cytosol readily loses its fluorescence, whereas intact mitochondria retain their bright fluorescence because Co^{2+} is not readily taken up by mitochondria. However, following drug-induced opening of the mPT pore, the intramitochondrial calcein (Mr = 622) will leak into the cytosol, and the mitochondrial staining will be lost. The green fluorescence was imaged with an Olympus Bx51 fluorescence microscope (40× objective).

Assessment of mitochondrial transmembrane potential in hepatocytes

The mitochondrial inner transmembrane potential ($\Delta\psi_m$) was measured with tetramethyl rhodamine methylester (TMRM, Molecular Probes/Invitrogen). Hepatocytes were loaded with TMRM (100 nM; non-quenching mode) [26] for 20 min at 37 °C, and drug-induced changes in fluorescence were recorded with an Olympus Bx51 fluorescence microscope.

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