Toxicology 322 (2014) 78–88

Contents lists available at ScienceDirect

Toxicology

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

Sertraline induces endoplasmic reticulum stress in hepatic cells

Si Chen[a], Jiekun Xuan[a], Letha Couch[a], Advait Iyer[a,b], Yuanfeng Wu[a], Quan-Zhen Li[c], Lei Guo[a,*]

[a] Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. FDA, Jefferson, AR 72079, USA
[b] Biological Sciences, University of Maryland, Baltimore, MD 21250, USA
[c] Department of Immunology and Internal Medicine, Microarray Core Facility, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

ARTICLE INFO

Article history:
Received 18 March 2014
Received in revised form 8 May 2014
Accepted 16 May 2014
Available online 24 May 2014

Keywords:
Apoptosis
Endoplasmic reticulum stress
Drug-induced liver toxicity
Reporter gene assay
Sertraline
MAPK pathway

ABSTRACT

Sertraline is used for the treatment of depression, and is also used for the treatment of panic, obsessive-compulsive, and post-traumatic stress disorders. Previously, we have demonstrated that sertraline caused hepatic cytotoxicity, with mitochondrial dysfunction and apoptosis being underlying mechanisms. In this study, we used microarray and other biochemical and molecular analyses to identify endoplasmic reticulum (ER) stress as a novel molecular mechanism. HepG2 cells were exposed to sertraline and subjected to whole genome gene expression microarray analysis. Pathway analysis revealed that ER stress is among the significantly affected biological changes. We confirmed the increased expression of ER stress makers by real-time PCR and Western blots. The expression of typical ER stress markers such as PERK, IRE1α, and CHOP was significantly increased. To study better ER stress-mediated drug-induced liver toxicity; we established *in vitro* systems for monitoring ER stress quantitatively and efficiently, using *Gaussia* luciferase (Gluc) and secreted alkaline phosphatase (SEAP) as ER stress reporters. These *in vitro* systems were validated using well-known ER stress inducers. In these two reporter assays, sertraline inhibited the secretion of Gluc and SEAP. Moreover, we demonstrated that sertraline-induced apoptosis was coupled to ER stress and that the apoptotic effect was attenuated by 4-phenylbutyrate, a potent ER stress inhibitor. In addition, we showed that the MAP4K4-JNK signaling pathway contributed to the process of sertraline-induced ER stress. In summary, we demonstrated that ER stress is a mechanism of sertraline-induced liver toxicity.

Published by Elsevier Ireland Ltd.

1. Introduction

Sertraline, a selective serotonin reuptake inhibitor (SSRI) class antidepressant, is the most widely prescribed psychiatric medication in the United States (Kaplan and Zhang, 2012). Although generally considered safe, acute liver failure has been associated with the use of sertraline (Carvajal Garcia-Pando et al., 2002; Collados et al., 2010; Fartoux-Heymann et al., 2001; Galan Navarro, 2001; Hautekeete et al., 1998; Kim et al., 1999; Persky and Reinus, 2003; Tabak et al., 2009; Verrico et al., 2000).

Previously, we reported that sertraline disrupted liver mitochondrial function in rat hepatocytes (Li et al., 2012) and caused apoptosis in HepG2 cells (Chen et al., 2014b). We also demonstrated that the MAPK signaling pathway, in particular, the TNF-initiated-MAP4K4-JNK cascade was activated and involved in sertraline-induced apoptosis (Chen et al., 2014b).

In this study, using microarray analysis, we identified new molecular mechanisms of sertraline's toxicity. HepG2 cells were exposed to sertraline at various concentrations for 6 h and then subjected to whole genome gene expression microarray analysis. Pathway analysis revealed that endoplasmic reticulum (ER) stress and the MAPK signaling pathway are among the significantly affected biological changes.

Endoplasmic reticulum, a membranous organelle, performs numerous functions including protein folding, trafficking, and post-modulation, and regulation of the intracellular calcium homeostasis. Disturbances of ER function by stimuli such as altered cellular redox, oxidative stress, unbalanced calcium homeostasis, and energy deprivation lead to ER stress response and activate a specific signaling pathway, namely, unfolded protein response (UPR) as adaptive mechanisms. Excessive and prolonged ER stress causes apoptosis and eventually necrotic cell death. Three major UPR branches, PERK (PKR-like endoplasmic reticulum kinase), IRE1α (inositol-requiring enzyme 1α), and ATF6 (activation of

* Corresponding author at: 3900 NCTR Road, HFT-110, Jefferson, AR 72079, USA. Tel.: +1 870 543 7048; fax: +1 870 543 7136.
E-mail address: Lei.Guo@fda.hhs.gov (L. Guo).

http://dx.doi.org/10.1016/j.tox.2014.05.007
0300-483X/Published by Elsevier Ireland Ltd.

transcription factor 6) have been described to promote cell survival by preventing and/or removing misfolded proteins. These three UPR transducers are activated by phosphorylating PERK and IRE1α or by translocating ATF6 to the Golgi when the ER is stressed. Subsequently, a cascade of reactions follows, activating numerous ER stress molecules that serve as measurable mechanism-based ER stress markers. For example, phosphorylation of eIF2α (eukaryotic initiation factor 2) and activation of CHOP (C/EBP CCAAT/enhancer binding protein homologous protein) by increasing the translation of ATF4 (activation of transcription factor 4) lead to attenuation of protein synthesis. A transcriptional factor, XBP (X-box binding protein 1), is activated by IRE1α through splicing of XBP mRNA, resulting in increased expression of genes involved in restoring protein folding or degrading unfolded proteins (Dara et al., 2011; Xu et al., 2005).

Based on the microarray results in this study, further experiments were focused on ER stress using biochemical and molecular approaches. The commonly studied markers (CHOP, p-eIF2a, p-PERK, ATF-4, and PDI) of ER stress were examined by Western blot, the expression of ER stress related genes was measured by real-time PCR, and the splicing of XBP mRNA was determined by PCR amplification. In order to efficiently monitor ER stress-mediated liver toxicity, we established two *in vitro* reporter assays in HepG2 cells. The establishment and utility of the assays are described and discussed.

2. Materials and methods

2.1. Chemicals and reagents

Sertraline, Williams' medium E, penicillin, streptomycin, dimethysulfoxide (DMSO), brefeldin A, and thapsigargin were from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). 4-Phenylbutyrate (4-PBA) was from BioVision (Milpitas, CA). Blasticidin S HCl and puromycin dihydrochloride were from Life Technologies (Grand Island, NY). Luciferase cell lysis buffer was from New England Biolabs (Ipswich, MA).

2.2. Cell culture

HepG2 cells were grown in Williams' medium E as described previously (Chen et al., 2013). Unless otherwise specified, HepG2 cells were seeded at a concentration of 2–5 × 10^5 cells/ml in volumes of 100 μl in the wells of 96-well tissue culture plates, in volumes of 5 ml in 60 mm tissue culture plates, or in volumes of 10 ml in 100 mm tissue culture plates. Cells were cultured for approximately 24 h prior to treatment with the indicated concentrations of sertraline or the vehicle control, DMSO (final concentration 0.1%).

The 293T cell line used for lentivirus packaging was purchased from Biosettia (San Diego, CA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS in the presence of 1 mM sodium pyruvate and non-essential amino acids.

2.3. Vector construction and stable cell line establishment

The gene expression lentiviral vector pLv-EF1α-MCS-IRES-Puro and the lentiviral labeling plasmid plv-EF1α-Firefly luciferase (Fluc)-IRES-Bsd were purchased from Biosettia (San Diego, CA). CMV-SEAP plasmid carrying secreted embryonic alkaline phosphatase (SEAP) (as template to amplify SEAP) was purchased from Addgene (Cambridge, MA) and pSV40-Gluc plasmid carrying secreted *Gaussia* luciferase (Gluc) (as template to amplify Gluc) was purchased from New England Biolabs. The cDNAs of SEAP and Gluc were amplified by PCR and subcloned into BamHI and NheI restriction sites of pLv-EF1α-MCS-IRES-Puro expression vector following the manufacturer's instructions. The generated lentiviral vectors and viral packaging plasmids (pMDL-G, pRSV-REV, and pVSV-G) were co-transfected into 293T cells to produce lentiviral stocks. The titrations of lentivirus stocks were measured with a functional lentivirus titering kit from Biosettia. HepG2 cells were infected with lentivirus carrying Fluc at a multiplicity of infection (MOI) of 10. Infected cells were selected with 6 μg/ml blasticidin (Bsd) to generate a polyclone of HepG2 cells with stable expression of firefly luciferase (Fluc). Subsequently, the cells with Fluc gene integrated were infected with lentivirus carrying either SEAP or Gluc at a MOI of 10. Infected cells were purified with 2 μg/ml puromycin. Thus, two stable cell lines (HepG2-Fluc-Gluc and HepG2-Fluc-SEAP) were generated.

2.4. Luciferase activity

Gluc activity was measured by adding 50 μl *Gaussia* luciferase assay reagent (Nanolight Technology, Pinetop, AZ) to 5 μl of the conditioned cell-free medium and immediately determined the bioluminescence using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). For the measurement of Fluc activity, the enzyme was first extracted from treated cells by adding 20 μl luciferase cell lysis buffer (New England Biolabs) to each well and then incubated at room temperature for 15 min on an orbital shaker. Then, 50 μl of the firefly luciferase assay reagent (Nanolight Technology) was added directly to the cell lysate in a 96 well plate, mixed well, and read immediately in the Synergy 2 Microplate Reader (BioTek).

2.5. Secreted alkaline phosphatase (SEAP) assay

The SEAP detection assay was performed by using the phospha-Light-EXP SEAP reporter gene assay system (Life Technologies) according to the manufacturer's instructions. In brief, an aliquot of 5 μl cell free conditioned medium was mixed with 50 μl of assay buffer in a white 96-well assay plate and incubated at 65 °C for 5 min. Then, 50 μl of reaction buffer was added and the mixture was incubated at room temperature for 20 min. The chemiluminescence was determined by the Synergy 2 Microplate Reader (BioTek).

2.6. Caspase-3/7 activity measurement

The enzymatic activity of caspase-3/7 was measured using a luminescent assay kit (Caspase-Glo® 3/7 Assay Systems, Promega) using a Synergy 2 Microplate Reader (BioTek). The induction of activity was calculated by comparing the luminescent of the treated cells to that of the DMSO controls.

2.7. RNA isolation

HepG2 cells at a density of 2 × 10^5 ml^{-1} were seeded on 60 mm plates as described under *Cell culture* and treated with various concentrations of sertraline. Total RNA was isolated using the mini RNeasy system (Qiagen, Germantown, MD). The yield of the extracted RNA was determined spectrophotometrically by measuring absorption at 260 nm using a NanoDrop 8000 (Thermo Scientific, Wilmington, DE). The purity and quality of RNA were evaluated using a RNA 6000 LabChip and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). High quality RNA with RNA integrity numbers (RINs) greater than 9.0 was used for microarray experiments and PCR assays.

2.8. Quantitative real-time PCR (qPCR) assay

qPCR was used to determine the mRNA expression of the genes involved in ER stress (PERK, IRE1α, HERPUD1, GADD34, and CHOP) with the expression of GAPDH as the endogenous control. Primers used for qPCR are as shown in Table 1.

qPCR reactions were carried out using SsoAdvanced™ SYBR Green Supermix in a Bio-Rad CFX96™ Real-Time PCR Detection System under universal cycling conditions (10 min at 95 °C; 15 s at 95 °C, 1 min 60 °C, 40 cycles) according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Data normalization and analysis were conducted as described previously (Guo et al., 2009, 2010).

2.9. X-box binding protein1 (XBP1) mRNA splicing assay

The cDNAs were prepared from total RNAs (1 μg) using high capacity cDNA reverse transcription kits (Life Technologies) according to the manufacturer's protocol. To amplify XBP1 mRNA, the following primers were used: Forward 5′-TTACGAGAGAAAACTCATGGCC-3′ and Reverse 5′-GGGTCCAAGTTGTCCAGAATGC-3′. cDNA obtained with GAPDH primers (Forward 5′-AGAAGGCTGGGGCTCATTTG' and Reverse 5′-AGGGGCCATCCACAGTCTTC-3′) was used as gel loading control. PCR was conducted for 35 cycles (95 °C for 30 s; 55 °C for 1 min; and 72 °C for 1 min) on GeneAmp® PCR system 9700 (Life Technologies). The spliced (263 bp), unspliced (289 bp) XBP1 mRNA, and GAPDH mRNA were analyzed by electrophoresis on 4% (w/v) agarose gels, stained with 0.5 μg/ml ethidium bromide solution and visualized under UV light (ProteinSimple, Santa Clara, CA).

2.10. Microarray analysis

Microarray gene expression analysis was performed at the Microarray Core Facility of University of Texas Southwestern Medical Center (http://microarray.swmed.edu). Illumina Human HT-12 expression BeadChip arrays were used. Microarray data were extracted using BeadStudio v3.1 software, background-subtracted, and normalized using a cubic spline algorithm. Genes differentially expressed between groups were identified using the Illumina custom error model implemented in BeadStudio. A gene was considered significantly differentially expressed when a P value was less than 0.05 and the change was greater than 1.5-fold. Clustering analysis was conducted within ArrayTrack, a software system developed by the FDA's National Center for Toxicological Research for the management, analysis, visualization and interpretation of microarray data (http://www.fda.gov/nctr/science/centers/toxicoinformatics/ArrayTrack/).