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Endoplasmic reticulum stress and MAPK signaling pathway activation underlie leflunomide-induced toxicity in HepG2 Cells

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ABSTRACT

Leflunomide, used for the treatment of rheumatoid arthritis, has been reported to cause severe liver problems and liver failure; however, the underlying mechanisms are not clear. In this study, we used multiple approaches including genomic analysis to investigate and characterize the possible molecular mechanisms of the cytotoxicity of leflunomide in hepatic cells. We found that leflunomide caused endoplasmic reticulum (ER) stress and activated an unfolded protein response, as evidenced by increased expression of related genes including CHOP and GADD34; and elevated protein levels of typical ER stress markers including CHOP, ATF-4, p-eIF2α, and spliced XBP1. The secretion of *Gaussia* luciferase was suppressed in cells treated with leflunomide in an ER stress reporter assay. Inhibition of ER stress with an ER stress inhibitor 4-phenylbutyrate, and knockdown of ATF-4 and CHOP genes partially protected cells upon leflunomide exposure. In addition, both genomic and biochemical analyses revealed that JNK and ERK1/2 of MAPK signaling pathways were activated, and both contributed to the leflunomide-induced cytotoxicity. Inhibiting JNK activation using a JNK inhibitor attenuated the ER stress and cytotoxicity of leflunomide, whereas inhibiting ERK1/2 using an ERK1/2 inhibitor or ERK1/2 siRNA increased the adverse effect caused by leflunomide, suggesting opposite roles for the two pathways. In summary, our data indicate that both ER stress and the activation of JNK and ERK1/2 contribute to leflunomide-induced cytotoxicity.

1. Introduction

Leflunomide (originally branded as ARAVA®) is used to treat active moderate-to-severe rheumatoid arthritis and psoriatic arthritis. It is a pyrimidine synthesis inhibitor and belongs to immunosuppressive disease-modifying antirheumatic drug (DMARD) category (Sanders and Harisdangkul, 2002). During post approval usage, cases of liver injury and liver failure caused by leflunomide alone or in combination with other drugs have been reported. Forty-nine cases of severe liver injury, of which 14 resulted in fatal liver failure, were documented between 2002 and 2009 due to treatment of leflunomide (Alcorn et al., 2009). In 2010, the U.S. Food and Drug Administration (FDA) added a "black box" warning regarding severe liver injury for leflunomide, and the drug is contraindicated in certain patients with preexisting liver conditions. Moreover, due to reported abnormal elevation of liver enzymes caused by leflunomide (van Roon et al., 2004), the level of liver enzyme alanine transaminase in patients is recommended to be monitored during drug treatment. Despite these adverse effects, little information is available on the underlying mechanisms of the observed hepatotoxicity of leflunomide.

Multiple mechanisms such as mitochondrial dysfunction, chemically reactive metabolites, apoptotic and necrotic toxicity, lysosomal dysfunction, bile transport inhibition, and immune-mediated mechanisms make substantial contributions to the pathogenesis of drug-induced liver toxicity (Dragovic et al., 2016). Endoplasmic reticulum (ER) stress, which received less attention in the past, has been recently described as an important mechanism for drug-induced liver toxicity (Chen et al., 2014c, 2015; Ren et al., 2016; Uzi et al., 2013). Disruption of ER function by external stimuli can result in ER stress, a condition involving accumulation of unfolded proteins in the ER lumen. This perturbation activates an unfolded protein response (UPR), to re-establish the homeostasis in the ER (Chen et al., 2014b). UPR is mainly composed by three branches: PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor

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6 (ATF6); each of the branches regulates the expression of corresponding genes through various transcriptional factors. Upon the activation of PERK, eukaryotic initiation factor 2α (eIF2α) is phosphorylated and activated, which increases the translation of transcriptional factors, including transcription factor 4 (ATF4), and reduces overall protein synthesis to decrease the load on the ER. Activation of IRE1α by phosphorylation triggers the splicing of X-box binding protein 1 (XBP1) mRNA. Spliced XBP1 then enters the nucleus and regulates gene expression as a transcription factor. For the ATF6 branch, ER stress results in the cleavage of ATF6 in Golgi, and in turn promotes the expression of related downstream genes. Although UPR aims to promote cell survival, it can also result in cell death under excessive ER stress (Chen et al., 2014b; Iurlaro and Munoz-Pinedo 2016).

The mitogen-activated protein kinase (MAPK) signaling cascade is composed of a large network of kinases and regulates numerous cellular processes, including cell proliferation, survival, and death (Plotnikov et al., 2011). MAPK has been shown to have a critical role in drug-induced liver toxicities due to its broad involvement in cellular functions and its interaction with various signal transduction pathways (Darling and Cook, 2014). C-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1 and 2 (ERK1/2), and p38 are the three major pathways of the MAPK network. Generally, JNK and p38 mediate signal transduction leading to cell death, whereas ERK1/2 activation plays a protective role and promotes survival (Chang and Karin, 2001).

In the current study, we explored the mechanisms underlying the cytotoxicity of leflunomide. Using multiple approaches, we studied the role of ER stress in leflunomide-induced liver toxicity. We also investigated the role of MAPK signaling cascade, particularly, JNK and ERK1/2, in the side effect of leflunomide.

2. Material and methods

2.1. Chemicals and reagents

Williams' Medium E, 4-phenylbutyrate acid (4-PBA), and dimethysulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Antibiotic-antimycotic was obtained from Life Technologies (Grand Island, NY). PureCol Bovine Collagen Solution was from Advanced BioMatrix (San Diego, CA). Leflunomide was purchased from Enzo Life Sciences (Farmingdale, NY). SP600125 (JNK inhibitor) and PD184352 (ERK1/2 inhibitor) were from LC laboratories (Woburn, MA).

2.2. Cell culture and drug treatment

HepG2 human hepatic cells were cultured in Williams' Medium E supplemented with 10% FBS and 1 × antibiotic-antimycotic, as described previously (Chen et al., 2014a, 2015). The passage number did not exceed 10. Depending on the specific assay, cells were either seeded at a density of 3×10^5 cells/ml in volumes of 100 µl in the wells of 96-well tissue culture plates, or in volumes of 5 ml in 60 mm plates. Unless otherwise specified, cells were maintained in growth medium for approximately 24 h before treatment with the indicated concentrations of leflunomide and/or inhibitors, or the vehicle DMSO control (the final concentration did not exceed 0.1%). For drug treatment, leflunomide was added at the final concentrations of 50 to 300 µM as indicated in the text, to cells in 96-well plates for toxicity assays or 60 mm plates for biochemical assays. The ER stress inhibitor 4-PBA (1 mM) was added 2 h prior to drug treatment and remained in the medium during the treatment. The JNK inhibitor SP600125 was added 2 h prior to drug treatment at a final concentration of 20 µM, and was reduced to 10 µM in the medium during the treatment. The ERK1/2 inhibitor PD184352 (2 µM) was added 2 h prior to the treatment and then removed before exposure to leflunomide. The stable cell line for the *Gaussia* Luciferase assay (HepG2-Fluc-Gluc) was established previously (Chen et al., 2014c) and grown in Williams' Medium E. The preparation and drug treatment conditions for this cell line were the same as described for the HepG2 cells.

HepaRG cells (terminally differentiated hepatic cells) were obtained from Life Technologies. Terminally differentiated HepaRG cells were cultured in Williams' Medium E supplemented with the Thaw, Plate, & General Purpose Medium Supplement (Life Technologies) for one day, and then maintained in Williams' Medium E supplemented with the Maintenance/Metabolism medium supplement for additional 7 days. Differentiated cells were then seeded at a density of 3.5×10^5 cells/ml in volumes of 100 µl in the wells of 96-well tissue culture and incubated for another 2 days for drug treatment. Cells were exposed to leflunomide for 6 h at the concentrations from 50 to 300 µM, and the ATP contents were measured.

Three samples of primary human hepatocytes, HH1051 (single donor, 23 year-old male Caucasian), HH1083 (single donor, 45 year-old female Caucasian), and PHH8007A (pooled from 5 donors) (In Vitro ADMET Laboratories LLC, Columbia, MD) were thawed and recovered using Universal Cryopreserved Recovery Medium (UCRM, IVAL Inc., Columbia, MD). The cells were then suspended in hepatocyte induction medium (HIM, IVAL Inc) and plated in bovine collagen-coated 96-well tissue culture plates at a density of 3×10^4 cells/100 µl/per well. The cells were incubated in a humidified 95% balanced air/5% CO_2 atmosphere in a 37 °C incubator for 16 h to facilitate attachment. After attachment, the medium was removed and the cells were washed once to remove unattached cells before the treatment.

2.3. Cellular ATP level measurement

Cellular ATP contents were quantified using a CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation, Madison, WI). After the specified length of drug exposure, the supernatants were aspirated and 10 µl ATP assay reagent with 90 µl of serum-free culture medium was added to each well of the 96-well plates. Luminescence was immediately measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). The cellular ATP content of treated cells was calculated as the percentage of the DMSO controls.

2.4. MTS cell viability assay

Cell viability was quantified using a tetrazolium reduction-based assay (MTS, Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega) as previously described (Li et al., 2011).

2.5. Next-generation sequencing analysis (RNA-seq)

RNA-seq was performed at the Microarray Core Facility of the University of Texas Southwestern Medical Center (http://microarray.swmed.edu). RNA-seq libraries were constructed and sequenced using the TruSeq protocol on an Illumina HiSeq 2000 platform. Data analysis was as described previously (Chen et al., 2017). The read counts for each sample were normalized as reads per million mapped reads (RPM) and transformed to $\log_2$ scale for further analysis. For each gene, a value of 1 was added to the read count values to avoid infinite values before $\log_2$ transformation. A differentially expressed gene was identified with a fold change (FC) greater than 1.5 (both up-and down-regulation) and a nonstringent p cutoff of 0.05 in comparison to the DMSO control group. Principal component analysis (PCA) was performed using Array Track software developed by NCTR/FDA (version 3.5.0) (http://www.fda.gov/nctr/science/centers/toxicoinformatics/ArrayTrack/).

2.6. RNA isolation and taqman quantitative real-time PCR

HepG2 cells were seeded on 60 mm plates approximately 24 h before exposure to various concentrations of leflunomide. Total RNA was

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