Sertraline, an Antidepressant, Induces Apoptosis in Hepatic Cells Through the Mitogen-Activated Protein Kinase Pathway

Si Chen,⁎ Jiekun Xuan,⁎ Liqing Wan,⁎† Haixia Lin,‡ Letha Couch,⁎ Nan Mei,‡ Vasily N. Dobrovolsky,‡ and Lei Guo*†

⁎Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. FDA, Jefferson, AR 72079; †Shanghai Institute for Food and Drug Control, Shanghai 201203, PR China; and ‡Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, U.S. FDA, Jefferson, AR 72079

*To whom correspondence should be addressed at Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. FDA, 3900 NCTR Road, HFT-110, Jefferson, AR 72079. Fax: (870) 543-7136. E-mail: lei.guo@fda.hhs.gov.

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Sertraline is generally used for the treatment of depression and is also approved for the treatment of panic, obsessive-compulsive, and posttraumatic stress disorders. Previously, using rat primary hepatocytes and isolated mitochondria, we demonstrated that sertraline caused hepatic cytotoxicity and mitochondrial impairment. In the current study, we investigated and characterized molecular mechanisms of sertraline toxicity in human hepatoma HepG2 cells. Sertraline decreased cell viability and induced apoptosis in a dose- and time-dependent manner. Sertraline activated the intrinsic checkpoint protein caspase-9 and caused the release of cytochrome c from mitochondria to cytosol; this process was Bcl-2 family dependent because anti-apoptotic Bcl-2 family proteins were decreased. Pretreatment of the HepG2 cells with caspase-3, caspase-8, and caspase-9 inhibitors partially but significantly reduced the release of lactate dehydrogenase, indicating that sertraline-induced apoptosis is mediated by both intrinsic and extrinsic apoptotic pathways. Moreover, sertraline markedly increased the expression of tumor necrosis factor (TNF) and the phosphorylation of JNK, extracellular signal-regulated kinase (ERK1/2), and p38. In sertraline-treated cells, the induction of apoptosis and cell death was shown to be the result of activation of JNK, but not ERK1/2 or p38 in the mitogen-activated protein kinase (MAPK) pathway. Furthermore, silencing MAP4K4, the upstream kinase of JNK, attenuated both apoptosis and cell death caused by sertraline. Taken together, our findings suggest that sertraline induced apoptosis in HepG2 cells at least partially via activation of the TNF-MAP4K4-JNK cascade signaling pathway.

Key Words: sertraline; liver toxicity; mitochondrial dysfunction; apoptosis; cell death; MAPK pathway.

Sertraline (trade name Zoloft), a selective serotonin reuptake inhibitor (SSRI) class antidepressant, is the most prescribed psychiatric medication in the United States (Kaplan and Zhang, 2012). Acute liver failure due to sertraline use has been described, although the frequency is relatively low (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). Recently, we reported that sertraline induced cell death and the disrupted liver mitochondrial function in rat hepatocytes. Our mechanistic study suggested that mitochondrial dysfunction contributes to sertraline-associated liver toxicity (Li et al., 2012).

Mitochondria, the power house of the cell, contain proapoptotic molecules that are important in programmed cell death (apoptosis). Generally, 2 major pathways are recognized to be involved in apoptosis, namely, the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway (Danial and Korsmeyer, 2004; Kroemer et al., 2007; Taylor et al., 2008). The intrinsic pathway is characterized by the loss of mitochondrial membrane potential, mitochondrial dysfunction, and the subsequent release of cytochrome c. The Bcl-2 family of proteins is closely involved in permeabilization of mitochondrial membrane and the leakage of cytochrome c. The Bcl-2 family is composed of proapoptotic members (eg, Bax, Bak, Bid, Bik, Bim, and Puma) and antiapoptosis members (eg, Bcl-2 and Mcl-1) (Chao and Korsmeyer, 1998). Proapoptotic proteins suppress the protective effect of antiapoptotic proteins and promote the release the apoptogenic factor cytochrome c from the mitochondria into the cytosol (Marsden et al., 2002). Cytochrome c subsequently activates caspase-9, which causes the activation of caspase-3, the “executioner” of apoptosis, resulting in cell death (Degterev et al., 2003; Zimmermann and Green, 2001). The extrinsic apoptotic pathway is initiated by binding of proapoptotic ligands, such as tumor necrosis factor (TNF) and Fas ligand, to their specific receptors, tumor necrosis factor receptor 1 (TNFR1) and Fas, forming a death-inducing signaling complex (DISC). The formation of the DISC recruits procaspase-8 and leads to...
the activation of caspase-8 that eventually activates caspase-3 as well.

Studies have shown that mitogen-activated protein kinase (MAPK) pathway is involved in the apoptotic process (Chang and Karin, 2001). The activity of the MAPK superfamily members is regulated through reversible dual phosphorylation of threonine and tyrosine residues. Three major MAPKs have been classified: extracellular signal-regulated kinase (ERK1/2), JNK, and p38. The ERK signaling cascade activation is stimulated by growth factors, linking cell proliferation to survival. In contrast, JNK and p38 are activated by cellular damage signaling, linking the induction of apoptosis to cell death (Chang and Karin, 2001; Xia et al., 1995). JNK also plays a role in the release of cytochrome c. JNK’s activity can also be induced by proapoptotic ligand TNF (Dhanasekaran and Reddy, 2008); thus, JNK participates in both intrinsic and extrinsic apoptotic pathways.

In the present study, we investigated the involvement of MAPK signaling pathway in the toxic effect of sertraline in HepG2 cells. We demonstrate that sertraline induces apoptosis via both intrinsic and extrinsic caspase-dependent pathways. We also demonstrate that the TNF-initiated MAPK signaling pathway is involved in sertraline-induced apoptosis as well as cellular damage.

MATERIALS AND METHODS

Chemicals and reagents. Sertraline, William’s E medium, penicillin, streptomycin, propidium iodide, selective p38 MAPK inhibitor SB 239063, JNK inhibitor SP600125, ERK inhibitor PD184352, and dimethylsulfoxide (DMSO) were from Sigma-Aldrich (St Louis, Missouri). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, Georgia). RNase A was from Qiagen (Valencia, California). The general caspase inhibitor (Z-IETD-FMK), the caspase-3 inhibitor (Z-DEVD-FMK), the caspase-8 inhibitor (Z-LEHD-FMK), and the caspase-9 inhibitor (Z-LEHD-FMK) were from R&D systems (Minneapolis, Minnesota). Blasticidin S HCl was from Life Technologies (Grand Island, New York).

Cell culture. The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, Virginia). The cells were grown in William’s E medium supplemented with 10% FBS and antibiotics (50 U penicillin/ml and 50 μg streptomycin/ml) at 37°C in a humidified atmosphere with 5% CO₂. Unless otherwise specified, HepG2 cells were seeded at a concentration of 2–5 × 10⁵ cells/ml in volumes of 100 μl/well in 96-well 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 about 24 h prior to treatment with the indicated concentrations of sertraline or the vehicle control DMSO. The final concentration of DMSO was 0.1%.

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

Vector construction and stable cell lines establishment. A specific target site for silencing of human MAP4K4 was identified using RNAi Designer available at the Invitrogen’s Web site (http://maidesigner.invitrogen.com/maixeexpression/index.jsp). Small hairpin RNA (shRNA) sequences were designed according to the structure of a doxycycline (DOX)-inducible shRNA lentiviral expression vector (Biosettia Inc.). The resulting shRNA-encoding DNA oligo containing inner palindromic sequences was synthesized (Biosynthesis, Inc., Lewisville, Texas): sh1 (5'-AAAAGACCAACTCTGGCTTTGTATGGATCCAAATACGAGCAGGTTGTC-3') and the scramble shRNA (5'-AAAGCTATCATCTCGAGCAATTTGGATCCAAATTTTCTGCAGATGTAGC-3'), which did not contain significant homology to known genes, were used as control in silencing experiments. The generation of the lenti-shRNA vectors and the establishment of the stable cell lines were described previously (Chen et al., 2013). In total, 2 stable cell lines were generated, namely, scramble (SC) and sh1-MAP4K4.

Cellular ATP level measurement. ATP content was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation, Madison, Wisconsin) and measured with a Synergy 2 Microplate Reader (BioTek, Winonoski, Vermont). The cellular ATP content was calculated by comparing the intensities of luminescence of the treated cells to that of the DMSO controls.

Lactate dehydrogenase assay. The cytotoxicity of sertraline was assessed using the lactate dehydrogenase (LDH) assay as described previously (Li et al., 2012).

Mitochondria isolation. The separation of mitochondria from cytosolic components was performed using Pierce’s mitochondria isolation kit (ThermoScientific, Wilmington, Delaware) according to the manufacturer. Briefly, HepG2 cells (2 × 10⁵), treated with different concentrations of sertraline for 6h, were pelleted by centrifuge. The cells were lysed and the resultant lysates were collected and processed according to the Dounce homogenization protocol of the manufacturer.

Cell cycle analysis. The subdiploid DNA peaks of HepG2 cells after sertraline treatments were measured using cell cycle analysis as described previously (Chen et al., 2013).

Assessment of mitochondrial membrane potential. Changes in mitochondrial membrane potential were assessed by staining with JC-1 dye (5,50,6,6-tetra-chloro-1,10,10,30-tetraethylbenzimidazol-carbocyanine iodide) (Sigma). HepG2 cells, seeded in 96-well plates, were treated with sertraline at specified concentrations or DMSO for 2 and 6 h. After treatment, the plates were centrifuged at 400 × g for 5 min and the supernatant was aspirated. The cells were then incubated in 100 μl medium containing JC-1 dye (2.5 μg/ml) for 20 min at 37°C and subsequently washed with 100 μl PBS 3 times by centrifugation at 400 × g for 5 min. The ratio of red to green fluorescence was determined by a Synergy 2 Microplate Reader (BioTek).

Caspase-3/7 and caspase-9 activity measurement. The enzymatic activities of caspase-3/7 and caspase-9 were assayed using luminescent assay kits (Caspase-Glo 3/7 Assay Systems or Caspase-Glo 9 Assay Systems, Promega) according to the manufacturer’s instructions and measured with a Synergy 2 Microplate Reader (BioTek). The induction was calculated by comparing the luminescence of the treated cells to that of the DMSO controls.

RNA isolation. sh1-MAP4K4 and SC stable cell lines were seeded in 6-well plates and incubated with 1 μg/ml DOX for 4 days followed by continued culture for additional 24 h without DOX. Total RNA from cells was isolated using the RNeasy system (Qiagen). The yield of the extracted RNA was determined spectrophotometrically by measuring absorption at 260 nm using a Nanodrop 8000 (ThermoScientific).

Real-time PCR assay. The expression of TNF and MAP4K4 was determined using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California). Reactions were prepared according to the manufacturer’s instructions for FastStart Universal SYBR Green Master (Roche Applied Science, Indianapolis, Indiana) using the following primers: human TNF (5'-CAGAGGGCCGTGACCCCTC-3' and 5'-GGAAGCACCTTCCCTCAGATG-3'), human MAP4K4 (5'-AGAGGCTCCCAGAGGCGTTG-5' and 5'-CCACCTTGCCGGCCCTG-3'), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-AGAAGGCTTGGGCTCATTTG-3' and 5'-AGGGGCACCCACAGCTT-

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Assays were run with FastStart Universal SYBR Green Master Mix (2x) (ROX) under universal cycling conditions (10 min at 95°C; 15 s at 95°C, 1 min 60°C, 40 cycles). Data normalization and analysis were conducted as described previously (Guo et al., 2009, 2010).

**Western blot analysis.** Cells were grown and treated with sertraline in 60- or 100-mm tissue culture plates. Standard Western blots were performed using antibodies against caspase-3, caspase-9, cytochrome c, Bak, Bid, Bik, Bim, Bax, Bcl-2, Mcl-1, JNK, phospho-JNK (Thr 183/Thr 185), ERK1/2, phospho-ERK1/2 (Thr 202/Tyr 204), p38, and phospho-p38 (Thr 180/Tyr 182) (Cell Signaling Technology, Danvers, Massachusetts), and c-Jun, phospho-c-Jun (Ser 63), using GAPDH as internal control (Santa Cruz Biotechnology, Santa Cruz, California) followed by a secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology).

**Statistical analyses.** Data are presented as mean ± SD of at least 3 independent experiments. Analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, California). Statistical significance was determined by 1-way ANOVA followed by the Dunnett’s tests for pairwise comparisons or 2-way ANOVA followed by the Bonferroni’s post hoc test. The difference was considered statistically significant when $p < .05$.

**RESULTS**

**Sertraline Causes Cellular Damage and Mitochondrial Membrane Potential Depolarization in HepG2 Cells**

To determine whether sertraline induces cytotoxicity to HepG2 cells, cells were incubated in medium containing sertraline at concentrations from 6.25 to 50 μM, and an LDH release assay was performed at 2, 6, and 24 h. Figures 1A–C (lines) showed that LDH release increased in a time- and dose-dependent manner following the treatment with sertraline. Significant LDH release was observed with 37.5 μM sertraline treatment for 2 h, with about 16% of LDH being released into the culture medium, whereas only about 8% of LDH was released in DMSO control cells (Fig. 1A). At 6 and 24 h, sertraline caused significant cell damage, 25 μM sertraline increased LDH release to about 21% and 55%, respectively (Figs. 1B and 1C).

ATP level was measured in parallel to determine imbalances in energy metabolism in sertraline-treated cells. As shown in

![Graphs showing LDH release and ATP content](https://academic.oup.com/toxsci/article-abstract/137/2/404/1675598/406)
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Figures 1A–C (bars), at concentrations of 6.25 and 12.5 μM, sertraline significantly decreased ATP levels, whereas no obvious or much less cell death was detected as measured with LDH release at the same concentrations. These data indicate that in HepG2 cells, ATP depletion occurred prior to the disruption of cellular membrane integrity, which is in good agreement with our previous results with rat primary hepatocytes (Li et al., 2012).

To determine whether sertraline causes mitochondrial disruption in HepG2 cells, membrane permeable JC-1 dye staining was performed. JC-1 dye exhibits potential-dependent aggregation in healthy mitochondria, signaled by a fluorescence emission shift from green to red. When mitochondrial membranes depolarize, JC-1-stained cells show a decrease in the ratio of red/green fluorescence. As shown in Figure 1D, there is a significant decrease in mitochondrial membrane potential in HepG2 cells exposed to sertraline starting from 12.5 μM. These data showed that sertraline initiated changes in the mitochondrial membrane potential, which could further result in disruption of active mitochondria and apoptosis.

Sertraline Induces Apoptosis in HepG2 Cells

Because the disruption of mitochondrial function is a distinctive characteristic of apoptosis, the above results encouraged us to test whether or not sertraline induces apoptosis. A set of apoptotic tests, including subdiploid DNA content characterization and caspase-9 and caspase-3/7 activation, were applied. Flow cytometric analysis of cell apoptosis showed that the ratio of cells with subdiploid nuclei formation increased significantly in a dose- and time-dependent manner following treatment with sertraline (Figs. 2A and 2B). At 6 h, 50 μM sertraline induced nearly 100% cell apoptosis, as all DNA peaks shifted to the sub-G1 phase.

Sequential activation of caspases plays a vital role in the apoptotic process. Accordingly, the enzymatic activities of caspase-9 and caspase-3/7 were measured. At 2 h, caspase-9 and caspase-3/7 were activated in response to sertraline treatment.

FIG. 2. Sertraline induced apoptosis in HepG2 cells. HepG2 cells were exposed to increasing concentrations (0, 6.25, 12.5, 25, 37.5, and 50 μM) of sertraline for 2 and 6 h. A, Flow cytometric analysis for the percentages of apoptotic cells with sub-G1 DNA content. Histograms shown are DNA content analyses for HepG2 cells treated with the indicated concentrations of sertraline for 2 and 6 h. Treated cells were stained with propidium iodide and processed for cell cycle analysis. Sub-G1 was defined as the cells undergoing apoptosis. B, The line graph depicts the mean percentage of sub-G1 ± SD from 3 independent experiments. *p < .05, **p < .01, and ***p < .001 versus DMSO-treated cells. C and D, Cellular caspase-9 and caspase-3/7 activity were expressed as fold change to DMSO control cells (*p < .05, **p < .01, and ***p < .001). Abbreviation: DMSO, dimethysulfoxide.
The effect started to be seen at 25μM, and the maximum induction appeared at 50μM. At 6h, the activities declined at the 37.5μM treatment (Figs. 2C and 2D), presumably due to significant necrotic cell death as measured with the LDH release assay (Fig. 1B).

To confirm further the caspase activation in sertraline-induced apoptosis, Western blot analysis was used to determine the cleavage of caspase-9 and caspase-3 and also the release of cytochrome c to the cytoplasm. The cleaved form of caspase-9 or caspase-3 was markedly increased at 50μM sertraline treatment, as demonstrated in Figure 3A. As indicated in Figure 3B, the cytosolic fraction of cytochrome c was significantly increased, accompanied by a decreased proportion in mitochondria.

The protective effects of general and specific caspase inhibitors on sertraline-induced cytotoxicity were assessed. As shown in Figure 3C, pretreatment (1 h) with 10μM Z-VAD-FMK (general caspase inhibitor), 10μM DEVD-FMK (caspase-3 inhibitor), 10μM IETD-FMK (caspase-8 inhibitor), or 10μM LEHD-FMK (caspase-9 inhibitor) significantly attenuated sertraline’s cytotoxicity, indicating the direct involvement of caspase-3, -8, and -9 in sertraline-associated apoptosis. It should note that although caspase inhibitors significantly attenuated sertraline-induced apoptosis statistically, the reserving effect was somehow partially (Fig. 3C), indicating other mechanisms may exist besides caspase-dependent pathway.

Collectively, the increase of sub-G1 percentage and the activation of caspase signaling pathway indicate that cellular damage and mitochondrial disruption (Fig. 1) resulted from apoptotic cell death in HepG2 cells (Figs. 2 and 3).

**Antiapoptotic Bcl-2 Family Proteins Are Involved in Sertraline-Induced Apoptosis**

It is known that the balance between the activities of proapoptotic and antiapoptotic Bcl-2 family members in the intrinsic mitochondrial apoptosis pathway regulates caspase activation and determines cellular fate (Chao and Korsmeyer, 1998). The protein expression levels of proapoptotic Bax, Bid, Bik, Bim, Bak, Puma and antiapoptotic Bcl-2 and Mcl-1 were determined using Western blot analysis in sertraline-treated cells. As shown in Figure 4A, only the key antiapoptotic members Bcl-2 and Mcl-1 were notably decreased in a time- and dose-dependent manner, and there was no change in proapoptotic Bax, Bid, Bik, Bim, Bak, and Puma levels (Fig. 4B), indicating that antiapoptotic members rather than proapoptotic members contribute the most to sertraline-associated apoptosis.

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**FIG. 3.** Effects of sertraline on apoptosis related proteins in HepG2 cells. A, Total cellular proteins were extracted at 6h after sertraline treated, and levels of caspase-3 and caspase-9 were detected by Western blotting. GAPDH was used as a loading control. Similar results were obtained from 3 independent experiments. B, Mitochondrial and cytosolic proteins were extracted at 6h after sertraline treatment, and levels of cytochrome c were detected by Western blotting. GAPDH was used as a loading control. Similar results were obtained from 3 independent experiments. C, HepG2 cells were pretreated with 10μM Z-VAD-FMK (general caspase inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), and Z-LEHD-FMK (caspase-9 inhibitor) for 1 h prior to treatment of sertraline for 2h. Cell viability was assessed by LDH assay. The bar graphs are shown as mean ± SD of 3 experiments. *p < .05, **p < .01, and ***p < .001 compared with treatment of sertraline alone. Abbreviations: DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase.
Sertraline Alters MAPK Pathway in HepG2 Cells

Apoptosis also occurs when the extrinsic pathway is activated. The extrinsic apoptotic pathway is initiated by cell death receptors, such as TNFR and FAS located in the plasma membrane. Binding of ligands such as TNF or FASL to their receptors initiates the extrinsic pathway and leads to activation of caspases (Chu, 2013; Wajant, 2002).

To explore whether or not sertraline induces apoptosis through the extrinsic pathway, the expression level of the TNF gene was measured by quantitative real-time PCR. As indicated in Figure 5A, TNF expression was significantly increased in response to the treatment with sertraline. At 6h, treatment of 25μM sertraline increased TNF expression about 6-fold, whereas about 66-fold induction was observed at a concentration of 50μM compared with DMSO control.

Binding of TNF to its receptor triggers the MAPK signaling pathway, which is important in apoptotic process (Chang and Karin, 2001; Xia et al., 1995). In a preliminary study using gene expression microarrays, we observed that the MAPK pathway was significantly altered in HepG2 cells exposed to sertraline (Chen et al., unpublished data). Accordingly, we investigated the activation (phosphorylation) of 3 key members (JNK, p38, and ERK1/2) in the MAPK signaling pathway. Phosphorylation of JNK and p38 was substantially increased in a dose- and time-dependent manner after 2- and 6-h sertraline treatment (Fig. 5B). Phosphorylation of ERK1/2 was also increased at 2h in a dose-dependent manner; however, at 6h, the maximum phosphorylation occurred at 12.5μM and declined with 25 and 50μM sertraline (Fig. 5B). These results indicate that sertraline possibly regulates TNF-initiated JNK- and p38-MAPK signaling pathways.

MAP4K4-JNK Pathway Regulates Sertraline-Induced Apoptosis and Cellular Damage

To investigate further which members of MAPK pathway are involved in sertraline-induced cell death and determine the precise regulatory pathway, inhibitors targeting different

FIG. 4. Changes of Bcl-2 family members in response to sertraline treatment. Total cellular proteins were extracted at 2 and 6h after exposure to sertraline. The expression level of proapoptosis Bcl-2 family members including Bak, Bid, Bik, Bim, Bax, and Puma (A) and antiapoptosis proteins including Bcl-2 and Mcl-1 (B) was examined by Western blotting. GAPDH was used as a loading control. The figure is representative of 3 independent experiments. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIG. 5. Effects of sertraline on extrinsic apoptotic pathway in HepG2 cells. A, Total RNAs were isolated at 6h after sertraline treatment. The expression levels of TNF were determined by real-time PCR. Values were means ± SD of 3 separate experiments. **p < .01 and ***p < .001 versus the DMSO controls. B, Total cellular proteins were extracted at 2 and 6h after exposure to sertraline. The expression levels of activated JNK (phospho-JNK), p38 (phospho-p38), and ERK1/2 (phospho-ERK1/2) were detected by Western blot analyses. GAPDH was loaded as internal control. Data are typical of 3 experiments. Abbreviations: DMSO, dimethylsulfoxide; ERK1/2, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumor necrosis factor.
members of the MAPK pathway were used. SP600125 (a specific inhibitor of JNK), SB239063 (a potent p38 inhibitor), and PD184352 (a highly selective inhibitor of ERK1/2) were added to cell cultures 2 h prior to treatment with sertraline. As shown in Figure 6A, pretreatment of 10 μM SP600125, 10 μM SB239063, or 2 μM PD184352 notably suppressed the activation (phosphorylation) of JNK, p38, or ERK1/2, respectively, elicited by 25 μM sertraline. As for the endpoint of apoptosis (ie, caspase-3/7), pretreatment with 10 μM SP600125 significantly inhibited caspase-3/7 activation induced by 25 μM sertraline (Fig. 6B). Although there was a trend of inhibition of caspase-3/7 with SB239063 and PD184352, the changes were not statistically significant (Fig. 6B). Furthermore, 10 μM SP600125 significantly reduced cell death induced by 25 and 37.5 μM sertraline (as measured by LDH release) (Fig. 6C); however, did not reduce cell death induced by 50 μM. The JNK inhibitor SP600125 likely blocks the early stage of cell death, that is, apoptosis (Fig. 6B), a significant necrotic response occurred at 50 μM so that the JNK inhibitor was not able to rescue cell death. Pretreatment with SP600125 also significantly diminished cell death induced by 24-h treatment of 12.5 and 25 μM sertraline, again had no effect on higher doses (37.5 and 50 μM) (Supplementary Figure 1). These data indicated that JNK rather than ERK1/2 or p38 is the major player in sertraline-induced cellular damage and apoptosis.

We next examined c-Jun, a transcriptional factor participating in apoptosis and proliferation that is downstream of JNK (Wisdom et al., 1999; Zhou and Thompson, 1996). Western blotting results showed that the levels of c-Jun and phosphorylated c-Jun were both upregulated following the treatment with sertraline and that the upregulation was time and dose dependent (Fig. 6D).

MAP4K4 is an enzyme that specifically activates JNK and mediates the TNF signaling pathway (Yao et al., 1999). To investigate the involvement of events upstream of JNK in apoptosis induced by sertraline, MAP4K4 was silenced in HepG2...
cells using a DOX-induced lentivirus system. shRNA targeting MAP4K4 was designed and shown to downregulate the transcription of MAP4K4, with efficiency of 88% (Fig. 7A). As shown in Figure 7B, silencing of MAP4K4 decreased the activation of caspase-3/7 induced by 25 and 37.5 μM sertraline. Silencing of MAP4K4 also prevented the decline of ATP levels and also decreased the LDH release induced by sertraline starting at 37.5 μM for 2-h (Figs. 7C and 7D), 25 μM for 6-h (Supplementary Figures 2A and 2C), and 12.5 μM for 24-h treatments (Supplementary Figures 2B and 2D). These data indicate that the MAP4K4-JNK pathway is critical for sertraline-induced apoptosis as well as for cellular damage.

**DISCUSSION**

Previously, we have studied the hepatotoxicity of sertraline using rat primary hepatocytes and isolated mitochondria. We demonstrated that sertraline induced mitochondrial membrane permeability and inhibited and uncoupled mitochondrial oxidative phosphorylation. The liver mitochondrion is an important target through which sertraline exerts its toxicity (Li et al., 2012).

Mitochondria play important roles in activating apoptosis in mammalian cells. One of 2 major apoptotic pathways is named the “mitochondrial pathway” (Wang and Youle, 2009). The results of our previous study prompted us to determine the role of apoptosis in sertraline’s toxicity.

The convergence of intrinsic and extrinsic apoptotic pathways occurs ultimately at the activation of caspase-3/7, the key “executioner” of downstream events in apoptosis (Degterev et al., 2003). Therefore, caspase-3/7 was used as the primary endpoint to examine the possibility of sertraline-induced apoptosis, and 3 different cell lines—rat primary hepatocytes, the human hepatic cell line HepG2, and the murine hepatic cell line Hepa1c1c7—were tested in our initial study. Treatment with 50 μM sertraline for 2 h increased

**FIG. 7.** Effect of silencing MAP4K4 on sertraline-induced apoptosis and cell death in HepG2 cells. A, HepG2 cell lines stably express DOX-inducible sh1-MAP4K4 or a SC. These 2 cell lines were incubated with DOX for 4 days followed by continued culture for another 24 h without DOX; then the decrease in MAP4K4 mRNA was assessed by real-time PCR. Values are means ± SD of 3 separate experiments. ***p < .001 versus the SCs. B–D, sh1-MAP4K4 and SC cells were incubated with DOX for 4 days and then treated with sertraline at indicated concentration for another 2 h without DOX. Treated cells were then analyzed for caspase-3/7 activity, ATP content, and LDH release. The bar graphs show the mean ± SD of 3 experiments. *p < .05, **p < .01, and ***p < .001 compared with SCs. Abbreviations: DOX, doxycycline; LDH, lactate dehydrogenase; SC, scramble control.
caspase-3/7 activity about 1.5-fold, 21-fold, and 8-fold in rat primary hepatocytes, HepG2, and Hepa1c1c7, respectively (Fig. 2C and Supplementary Figure 3). Because maximum apoptotic induction was observed in HepG2 cells, subsequent studies were conducted in these cells. Human primary hepatocytes might be more relevant for studying of pharmaceuticals; however, high variability, limited availability, and inability to perform genetic modification (eg, overexpressing or silencing genes of interest) limit their use (Guo et al., 2011; Ning et al., 2008). On the other hand, HepG2 cells have proven value for toxicological studies in general (Bova et al., 2005; Dykens et al., 2008; Felsler et al., 2013; Greer et al., 2010; Guo et al., 2006; Juan-Garcia et al., 2013; Marroquin et al., 2007; Nguyen et al., 2013; Seeland et al., 2013) and for studying drug-induced apoptosis (Guo et al., 2006; Juan-Garcia et al., 2013; Nguyen et al., 2013). The lack of some drug-metabolizing genes in HepG2 cells may be problematic for studies focusing on metabolism; however, the ability to perform genetic modifications (ie, silencing of genes of interest, as in this study) is advantageous for the in-depth study of mechanisms of toxicity at the molecular level.

Clinically, the maximum plasmid concentration is around 0.3 or 0.74μM following a single oral dose of 200 or 400 mg sertraline administered (Mandrioli et al., 2013; Saletu et al., 1986). Concentrations of sertraline used in our study were higher than reported plasma concentrations. It is known that many variables including genetic variability, race, sex, age, metabolic capacity, drug-drug interactions, and preexisting diseases contribute to individual susceptibility of idiosyncratic drugs (Lee, 2003). To identify an idiosyncratic hepatotoxic drug, it is suggested that a dose that is equivalent to 100-fold of the C_{max} should be tested (Xu et al., 2008). Because the concentrations of sertraline in our study were 6.25–50μM, which is within the range of 100 times of reported C_{max} (30 or 74μM), the concentrations used in our study were meaningful.

Permeabilization of the mitochondrial membrane and release of cytochrome c into the cytosol are key determinants of mitochondria-mediated intrinsic apoptotic signaling pathway (Danial and Korsmeyer, 2004). The release of cytochrome c, in turn, activates caspase-9 and caspase-3/7 (Boehning et al., 2003). In our study, cytochrome c release (Fig. 3B) and caspase-9 and caspase-3/7 activation (Figs. 2C, 2D, and 3A) were accompanied by the decrease in mitochondrial membrane potential suggesting that sertraline induces apoptosis via the mitochondria-mediated intrinsic apoptotic signaling pathway.

The Bcl-2 protein family members are critical intracellular regulators of the intrinsic apoptotic pathway that controls permeability of the outer mitochondrial membrane and the release of cytochrome c (Rodriguez et al., 2011; Sharpe et al., 2004). In the present study, the Bcl-2 proapoptotic proteins Bax, Bid, Bik, Bim, Bak, and Puma showed no changes following the treatment with sertraline, whereas antiapoptosis proteins Bcl-2 and Mcl-1 were inhibited, implying that the inhibition of antiapoptosis proteins is responsible for the subsequent cytochrome c release and apoptosis (Fig. 4).

To uncover in-depth the molecular mechanisms of sertraline-induced toxicity, we performed microarray analysis to examine the global changes of gene expression in sertraline- and DMSO-treated HepG2 cells. The MAPK signaling pathway was one of the most significantly altered pathways, in addition to the cell cycle and apoptosis pathways (unpublished data). We also observed significant changes in expression of MAPK-associated genes, for example, MAP4K4, TNF, and c-Jun in sertraline-treated cells, which may help provide a better understanding of the mechanism of apoptosis induction in vitro.

MAPKs are involved in regulating intracellular signals in response to a wide array of extracellular stimulations. MAPK family members (ERK1/2, JNK, and p38) participate in cell proliferation, differentiation, survival, and apoptosis (Pearson et al., 2001). It has been reported that the balance between the growth signal-activated ERK1/2 and stress signal-activated JNK-p38 pathways governs whether or not cells survive or undergo apoptosis and necrosis (Xia et al., 1995).

Consistent with gene expression data, 25μM sertraline treatment for 6h markedly increased phosphorylation and activation of ERK1/2, JNK, and p38 (Fig. 5B). This was paralleled by ATP depletion (Fig. 1B) and significant cell apoptosis (Figs. 2A and 2B). The involvement of JNK activation in sertraline-induced cell death (apoptosis and necrosis) was further confirmed by pretreating HepG2 cells with a specific inhibitor of JNK (SP600125) (Figs. 6B and 6C).

JNK plays an important role in both intrinsic and extrinsic apoptotic pathways. JNK promotes intrinsic apoptosis by modulating the activities of pro- and antiapoptotic Bcl-2 family proteins (Dhanasekaran and Reddy, 2008). It is possible that the decrease in the levels of antiapoptotic proteins Bcl-2 and Mcl-1 (Fig. 4B) may be regulated by JNK. In addition, JNK activation is required for TNFα-mediated caspase-8 cleavage, which is the initiation event in death receptor apoptotic signaling pathway (Deng et al., 2003). In the TNFα-activated JNK signaling pathway, MAP4K4 is a specific upstream kinase in the MAP4K4→MAP3K7→MAP2K4, MAP2K7→JNK kinase cascade (Yao et al., 1999). In the present study, we demonstrated that sertraline upregulates TNFα expression and JNK activation. Moreover, blocking JNK activation with a specific inhibitor (Fig. 6) or decreasing the upstream kinase MAP4K4 by silencing its gene expression (Fig. 7) attenuated sertraline-induced apoptosis and necrosis.

Antidepressants are classified as SSRI, tricyclic antidepressants, tetracyclic antidepressants, monoamine oxidase inhibitors, or serotonin/noradrenergic reuptake inhibitors based on their pharmacological effects (Carrasco and Sandner, 2005; Holtzheimer and Nemeroff, 2006). A number of in vitro studies have reported that some antidepressants induced apoptosis (Chang et al., 2008; Jan et al., 2013; Levkovitz et al., 2005; Xia et al., 1996, 1998; Zhang et al., 2013). Although the molecular mechanism is not clearly elucidated, it was
reported that the tricyclic antidepressants clomipramine, imipramine, and citalopram had apoptotic effects in human peripheral lymphocytes, as determined by DNA gel electrophoresis and flow cytometric analysis (Xia et al., 1996, 1998). The SSRI antidepressants paroxetine and fluoxetine also have been shown to possess apoptotic activity in glia and neural cell lines, as quantified by flow cytometric analysis, cytochrome c release, and the activation of caspase-3. Activation of c-Jun was also demonstrated in the study, indicating the involvement of JNK pathway because c-Jun is a major end-product of JNK pathway activation (Levkovitz et al., 2005). In human osteosarcoma cells, paroxetine evoked apoptosis via the p38 MAPK-dependent signaling pathway but not the ERK- or JNK-dependent pathways (Chou et al., 2007). In another study, in Neuro-2a cells treated with maprotolite, a tetracyclic antidepressant, there was an induction of apoptosis and increased caspase-3 activation. Maprotolite-induced apoptosis was mediated through the activation of JNK and the inactivation of ERK (Jan et al., 2013). Desipramine, another tricyclic antidepressant, displayed apoptotic activity in human PC3 prostate cancer cells and activated the phosphorylation of JNK, but not ERK or p38 in the MAPK signaling pathway (Chang et al., 2008). Due to nonspecific mode of action, it is not surprising that the apoptotic activity of antidepressants eventually induced cell death in various types of cells. Although it is likely that antidepressant-induced apoptosis is mediated by MAPK signaling pathway, there was discrepancy as to which signaling cascade was activated. Sertraline was found for the first time in the present study to induce apoptosis via MAPK signaling pathway. The activation of 3 key protein kinases ERK, p38, and JNK depends on the cell type, metabolic state, and environment of the cell and the stimulus (Raman et al., 2007). It is possible that different classes of antidepressants trigger distinct MAPK pathway in different cell types by activating various kinases.

Besides MAPK pathway, some other signaling pathways also have been shown to be involved in sertraline-induced cell death (Reddy et al., 2008; Tzadok et al., 2010). For instance, sertraline inhibited the phosphorylation of Akt and caused cell death in human melanoma cells (Reddy et al., 2008), suggesting a role of Akt in sertraline-induced cell proliferation and apoptosis and also a complex mode of action in sertraline’s toxicity.

In summary, sertraline induces apoptosis in HepG2 cells in a dose- and time-dependent manner. Both intrinsic and extrinsic apoptotic pathways play roles. Although 3 key molecules (JNK, p38, and ERK1/2) in MAPK pathway were altered, TNFα-MAP4K4-JNK may be the most important pathway responsible for sertraline-induced apoptosis.

**SUPPLEMENTARY DATA**

Supplementary data are available online at [http://toxsci.oxfordjournals.org/](http://toxsci.oxfordjournals.org/).

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