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ROS generation and JNK activation contribute to 4-methoxy-TEMPO-induced cytotoxicity, autophagy, and DNA damage in HepG2 cells

Zhuhong Zhang¹, Zhen Ren², Si Chen², Xiaoqing Guo¹, Fang Liu³, Lei Guo², and Nan Mei¹

¹Division of Molecular and Genetic Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA

²Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA

³Division of Neurotoxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA

Abstract

4-Methoxy-TEMPO, a derivative of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), is a stable nitroxide radical and is generally used in organic and pharmaceutical syntheses for the oxidation of alcohols. Previously, we reported the involvement of reactive oxygen species (ROS) and c-Jun N-terminal kinases (JNK) in TEMPO-induced apoptosis in mouse L5178Y cells. In this study, we investigated 4-methoxy-TEMPO induced toxicity in human HepG2 hepatoma cells and its underlying mechanisms. Treatments with 4-methoxy-TEMPO (0.5–5 mM for 2–6 h) caused oxidative stress as demonstrated by increased intensity of the ROS indicator H₂DCF-DA, decreased levels of glutathione. 4-Methoxy-TEMPO treatment also induced DNA damage as characterized by increased levels of DNA tail intensity in the Comet assay, increased phosphorylation of related proteins including γ -H2A.X, p-Chk1, and p-Chk2, and activation of MAPK signaling pathways. In addition, 4-methoxy-TEMPO also induced autophagy as demonstrated by the conversion of LC3B-I to II, decreased level of p62, and the appearance of GFP-LC3B punctae. To investigate the crosstalk between different signaling pathways, pretreatment of HepG2 with *N*-acetylcysteine, an ROS scavenger, attenuated 4-methoxy-TEMPO-induced DNA damage, suppressed JNK activation, and diminished autophagy induction. Furthermore, inhibiting JNK activation by a JNK-specific inhibitor, SP600125, decreased DNA damage levels induced by 4-methoxy-TEMPO. These results suggest that multiple mechanisms including ROS generation, DNA damage, and MAPK activation contribute to 4-methoxy-TEMPO-induced toxicity.

Correspondence to: Nan Mei.

Compliance with ethical standards

Conflict of interest

There was no conflict of interest declared.

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Keywords

Nitroxide; 4-Methoxy-TEMPO; Oxidative stress; DNA damage; MAPK pathway

Introduction

Nitroxides have a single unpaired electron delocalized between a nitrogen and an oxygen, and their important biological activities have been recognized for decades (Gallez et al. 2004; Guo et al. 2013). Piperidine nitroxides, a class of stable organic free radicals of low molecular weight, are lipophilic and permeable through cell membranes (Soule et al. 2007a; Wilcox and Pearlman 2008). The protective effects of piperidine nitroxides against oxidative stress in cells and tissues lead to their broad use in various therapeutic applications, such as cancer prevention and treatment, protection against ionizing radiation, as probes in studying intracellular redox metabolism using functional imaging, and as paramagnetic contrast agents in nuclear magnetic resonance imaging (Soule et al. 2007b). In addition, piperidine nitroxides are often utilized in catalytic systems to enable efficient oxidation of a broad range of primary alcohols in organic and pharmaceutical syntheses (Hoover and Stahl 2011; Sheldon et al. 2002).

However, the widespread use of piperidine nitroxides has also raised safety concerns. It has been reported that TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), one of the frequently used piperidine nitroxide catalysts, resulted in intercellular reactive oxygen species (ROS) generation and DNA damage in mammalian cells (Guo et al. 2015). Furthermore, TEMPO and several of its derivatives induced genotoxicity in the Ames test, the mouse lymphoma assay, and the in vitro micronucleus assay (Gallez et al. 1992; Guo et al. 2013; Sies and Mehlhorn 1986). One TEMPO derivative, 4-methoxy-TEMPO, is commonly employed for oxidation of alcohols, as well as in synthesis of 2-substituted benzoxazoles that are widely used as antibacterial, antifungal, antimicrobial, anti-proliferative, and antiviral agents (Chen et al. 2008; Demmer and Bunch 2015). In addition, one study reported that 4-methoxy-TEMPO induced apoptosis in the tumor cells of rats bearing Yoshida sarcoma (Metodiowa et al. 1999). To date, research on the safety and toxicity of 4-methoxy-TEMPO is lacking, and whether or not 4-methoxy-TEMPO exerts toxicity similar to TEMPO remains largely unknown.

Reactive oxygen species, which are the byproducts of aerobic metabolism, play an important role in cell fate depending on the cell type, cellular context, and the level of ROS (Matsuzawa and Ichijo 2008). Normal levels of ROS are required for the growth and survival of cells, whereas excessive ROS can result in oxidative stress, leading to DNA damage, apoptosis, and necrosis (Tasdogan et al. 2017). DNA damage, caused by chemical and physical factors, can induce a complex array of processes, such as DNA repair mechanisms, regulation of cell checkpoints, cell cycle arrest, gene transcription profile alterations, induction of autophagy or, induction of apoptosis to eliminate damaged cells (Czarny et al. 2015; Rodriguez-Rocha et al. 2011). The fate of cells with DNA damage depends on the capability of DNA repair pathways. An effective DNA repair process could restore DNA

integrity, whereas the unrepaired DNA damage might trigger cell death or damaged cells might evade apoptosis, potentially leading to cancer.

The mitogen-activated protein kinase (MAPK) signaling pathway consists of three branches, the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK), and the p38 MAPK, with all three having different cellular functions (Krifka et al. 2013). The activation of MAPKs plays critical and complex roles in various physiological processes, including cell proliferation, differentiation, apoptosis, autophagy, and DNA damage repair (Wada and Penninger 2004). In general, the activation of JNK and p38 mediates signal transduction leading to cell death, whereas the activation of ERK1/2 promotes cell survival (Chang and Karin 2001). A variety of stimuli can trigger MAPK activation by phosphorylating MAPK signaling cascades (Sui et al. 2014; Yang et al. 2013). It has been demonstrated that ROS can activate MAPK signaling, and that attenuation of ROS by ROS scavengers can deactivate MAPK signal transduction pathways (Guo et al. 2015; McCubrey et al. 2006; Son et al. 2013). In particular, it has been reported that ROS-induced DNA damage may activate the JNK pathway (Picco and Pages 2013).

Autophagy is a regulated cellular self-eating process which degrades and recycles dysfunctional cellular components (Petibone et al. 2017). Accumulating evidence suggests that the interplay of autophagy and oxidative stress is involved in many human diseases and toxicant/drug-induced cellular responses (Filomeni et al. 2015; Navarro-Yepes et al. 2014; Petibone et al. 2017). Increased oxidative stress can induce autophagy and apoptosis, and both oxidative stress and autophagy are important factors in determining cell fate (death/survival) (Pallichankandy et al. 2015; Zhang et al. 2015a).

Previously, we demonstrated that TEMPO triggered oxidative stress (Guo et al. 2013), induced apoptosis and DNA damage, and activated the JNK pathway (Guo et al. 2015). In the present study, we investigated the toxicity of 4-methoxy-TEMPO and the possible underlying mechanisms, focusing on the roles of ROS and DNA damage. We also determined the role of MAPK signaling pathway in the cellular effects of 4-methoxy-TEMPO. In addition, we explored the potential role of autophagy induction in response to 4-methoxy-TEMPO treatment.

Materials and methods

Chemicals and reagents

2,2,6,6-Tetramethylpiperidine 1-oxyl (TEMPO), 4-methoxy-2,2,6,6-tetramethyl-1-piperidinyloxy (4-methoxy-TEMPO), Williams' medium E, Dulbecco's Modified Eagle's Medium (DMEM), JNK inhibitor SP600125, ERK inhibitor U0126, p38 MAPK inhibitor SB203580, *N*-acetylcysteine (NAC), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2',7'-Dichlorofluorescein diacetate (H₂DCF-DA), and penicillin-streptomycin antibiotic were obtained from Life Technologies (Grand Island, NY, USA).

Cell culture and treatment with 4-methoxy-TEMPO

The HepG2 cell line was from the American Type Culture Collection (ATCC; Manassas, VA, USA), and was cultured in Williams' medium E supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere with 5% CO₂. Cells were seeded at a density of 2×10^5 cells/ml in volumes of 100 µl per well in 96-well plates for toxicity assays or in volumes of 5 ml in 60-mm tissue culture plates or 10 ml in 100-mm tissue culture plates for protein isolation and Western blot analysis. Cells were cultured overnight prior to treatment with TEMPO, 4-methoxy-TEMPO, other TEMPO-derivatives, or vehicle control (DMSO).

Cell viability assay

The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) using colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was performed to quantify cell viability and proliferation according to the manufacturer's instructions. Absorbance was measured at 490 nm with a Synergy H4 Hybrid multimode microplate reader (BioTek, Winooski, VT, USA).

Cellular ATP level measurement

Cellular ATP levels were determined using a CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation) according to the manufacturer's instructions. After CellTiter-Glo reagent was added to the cells, luminescence was recorded with a Synergy H4 Hybrid microplate reader (BioTek). The cellular ATP content was calculated by comparing the intensities of luminescence of the treated cells to that of the DMSO controls.

Measurement of ROS

Intracellular ROS production was measured as described previously (Chen et al. 2017). Briefly, cells were seeded in 96-well plates; after 12 h, cells were treated with 10 µM H₂DCF-DA for 45 min at 37 °C in the dark, and then they were washed with PBS to remove unincorporated dye. Cells were exposed to 4-methoxy-TEMPO at concentrations ranging 0.5–5 mM in phenol-red-free medium. The cells were continuously incubated at 37 °C with 5% CO₂ and the fluorescence intensities were measured at 0.5, 1, 2, 4, and 6 h time points with a Synergy H4 Microplate Reader (BioTek).

Measurement of glutathione level

The intracellular reduced glutathione (GSH) levels were measured as described previously (Guo et al. 2015). Total glutathione, including GSH and oxidized glutathione (GSSG), and GSH/GSSG ratios in 4-methoxy-TEMPO and DMSO treated cells were assessed using the GSH/GSSG-Glo Assay kit (Promega) according to the manufacturer's suggestion.

Comet assay

The alkaline Comet assay was performed as described previously (Zhang et al. 2015b). Briefly, HepG2 cells were seeded into 6-well plates and cultured overnight. The cells were

exposed to different concentrations of 4-methoxy-TEMPO (0.5–5 mM) for 4 h. After the treatment, DNA single- and double-strand breaks were assessed using the Comet Assay reagent kit for single cell gel electrophoresis assay (Trevigen, Gaithersburg, MD, USA). In each experiment, two slides were used for each concentration and 100 cells were randomly scored per concentration. The experiment was repeated twice and in total, 300 cells per concentration from three independent exposures were analyzed. The percentage of DNA in Comet tails was calculated using the Comet Assay IV digital analysis system and software (Perceptive Instruments; Edmunds, UK).

Western blot analysis

Cells were cultured and treated with 4-methoxy-TEMPO or DMSO in 60-mm tissue culture plates. The following primary antibodies were used: γ -H2A.X, p-Chk1 (Ser-345), p-Chk2 (Thr-68), JNK, phospho-JNK (Thr183/Tyr185), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), p38, and phospho-p38 (Thr180/Tyr182) (Cell Signaling Technology; Danvers, MA, USA), LC3B and GFP (Sigma-Aldrich). GAPDH (Santa Cruz Biotechnology; Dallas, TX, USA) was used as the internal control followed by a secondary antibody conjugated with horseradish peroxidase (HRP; Santa Cruz). Western blots were performed for each treatment from three or four independent experiments. The intensity of each band was quantified with Image J (Version 1.51p, NIH).

Confocal microscopic analysis

Confocal microscopic analysis was performed as described previously (Chen et al. 2014a). Briefly, HepG2-GFP-LC3B cells, that stably express GFP-LC3B, were seeded on coverslips in 24-well plates at 1×10^5 cells/well. After treatment with 3 or 5 mM 4-methoxy-TEMPO or DMSO for 4 h, cells were washed and fixed. Then, the presence of GFP-LC3B punctae was evaluated using an Olympus FV1000 confocal microscope (Olympus; Center Valley, PA, USA) by observing GFP fluorescence on coverslips mounted onto slides using Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories; Burlingame, CA, USA).

Statistical analysis

Data were presented as the mean $\pm$ standard deviation (SD) of at least three independent experiments. Analyses were performed using GraphPad Prism 6 (GraphPad Software; La Jolla, CA, USA). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test for comparisons between different concentrations to vehicle control, or between the two treatment-groups at the same concentration of 4-methoxy-TEMPO. The difference was considered statistically significant when p was less than 0.05.

Results

4-Methoxy-TEMPO induces cytotoxicity in HepG2 cells

In our previous study, we showed that TEMPO induced cytotoxicity in mouse L5178Y cells (Guo et al. 2015). In this study, using the HepG2 human hepatic cell line, we compared the cytotoxicity of TEMPO and its six derivatives, including 4-acetamido-TEMPO, 4-amino-

TEMPO, 4-hydroxy-TEMPO, 4-hydroxy-TEMPO benzoate, 4-methoxy-TEMPO, and 4-oxo-TEMPO. Cytotoxicity was assessed after a 4-h treatment using the MTS viability assay. As shown in Fig. 1a, HepG2 cells showed the highest sensitivity to TEMPO, followed by 4-methoxy-TEMPO. Although 4-methoxy-TEMPO is a widely used TEMPO derivative, research into its cytotoxicity is limited. Therefore, in the current study, we focused the mechanistic studies on 4-methoxy-TEMPO. Using two different parameters, the MTS viability assay and ATP content measurement, we further evaluated the cytotoxicity of 4-methoxy-TEMPO. HepG2 cells were treated with 4-methoxy-TEMPO at concentrations of 0.5–5 mM for 2, 4, and 6 h. Figure 1 shows that 4-methoxy-TEMPO induced significant time- and concentration-dependent growth inhibition (Fig. 1b) and decreases in ATP content (Fig. 1c), beginning at the 1 mM concentration and 2-h exposure interval.

4-Methoxy-TEMPO exhibits pro-oxidant activities

Exposure to TEMPO resulted in ROS production and GSH depletion, two indicators of oxidative stress, in mouse L5178Y cells (Guo et al. 2015). The intracellular ROS levels in all treatments were sharply increased after adding TEMPO to the media, reached the peak levels at up to 4 h, then gradually reduced after the peak, and returned to the background level at 24 h (Guo et al. 2013). It is of interest to investigate whether or not 4-methoxy-TEMPO induces similar effects in human cells. Therefore, HepG2 cells were treated with 4-methoxy-TEMPO at concentrations ranging between 0.5 and 5 mM, and ROS production was monitored at 0.5, 1, 2, 4, and 6 h (Fig. 2a).

Reactive oxygen species induction was observed as early as the 0.5-h treatment with 0.5 mM 4-methoxy-TEMPO. The maximum ROS induction was 4.4-fold that of the DMSO control at 2 h and 5 mM 4-methoxy-TEMPO treatment. At 4 and 6 h, although ROS production remained significantly elevated compared to the corresponding DMSO control, the levels were less prominent compared to levels at the earlier time-points. For example, the ROS production was 3.5-fold higher at 2 h for 4 mM 4-methoxy-TEMPO, whereas ROS levels were 2.9-fold higher and 2.3-fold higher at 4 and 6 h, respectively, for the same 4 mM dosage. The reduced ROS levels were presumably due, in part, to cellular antioxidant response mechanisms and decreased cell viability (Fig. 1). Glutathione is present in the cells in both reduced (GSH) and oxidized (GSSG) states, and the harmful effect of ROS is counterbalanced by antioxidants such as GSH. Therefore, to evaluate the role of antioxidants in 4-methoxy-TEMPO induced ROS, the levels of GSH and GSSG were measured at 2 and 4 h after treatment to calculate the GSH/GSSG ratios. As shown in Fig. 2b, at 2 h, GSH/GSSG ratios significantly decreased in the cells treated with 2 mM and higher concentrations of 4-methoxy-TEMPO. The reduction in GSH/GSSG ratios was more prominent at the 4-h time point, and starting from 1 mM 4-methoxy-TEMPO. Taken together, increased intensity of the ROS indicator and decreased level of GSH/GSSG ratios imply that oxidative stress resulted from 4-methoxy-TEMPO treatment.

To explore further the role of ROS generation in the cytotoxicity of 4-methoxy-TEMPO, we employed a ROS scavenger, NAC, to suppress intracellular ROS levels. Pretreating HepG2 cells with 10 mM NAC for 1 h, prior to exposures of 0.5–5 mM 4-methoxy-TEMPO for 4 h, significantly attenuated ROS induction and confirmed the effectiveness of NAC pretreatment

(Fig. 2c). Importantly, the NAC pretreatment alleviated 4-methoxy-TEMPO cytotoxicity, as evidenced by smaller reductions in both cellular ATP content (Fig. 2d) and cell viability (Fig. 2e) in NAC pretreated samples. These results showing that NAC partially rescued the HepG2 cells from ROS damage stemming from 4-methoxy-TEMPO treatment confirmed that 4-methoxy-TEMPO cytotoxicity was mediated by ROS generation.

4-Methoxy-TEMPO induces ROS-mediated DNA damage

Next, we investigated whether ROS overproduction, induced by 4-methoxy-TEMPO exposure in HepG2 cells, might result in DNA damage. The Comet assay, a gel electrophoresis procedure with single-cell resolution of DNA damage, assessed DNA strand breaks in HepG2 cells treated with 0.5–5 mM 4-methoxy-TEMPO for 4 h. As shown in Fig. 3a, treatment with 4-methoxy-TEMPO resulted in a significant concentration-dependent increase in Comet tail intensity beginning at the 2 mM concentration. DNA damage following 4-methoxy-TEMPO treatment was confirmed further by measuring histone H2A.X phosphorylation (γ -H2A.X), which is a hallmark of double-strand DNA breakage (Rogakou et al. 1998). Additionally, the DNA damage responsive proteins phosphorylated-Chk1 (p-Chk1) and phosphorylated-Chk2 (p-Chk2) were also assessed. HepG2 cells exposed to 4-methoxy-TEMPO exhibited concentration- and time-dependent increases in expression of γ -H2A.X, p-Chk1, and p-Chk2 (Fig. 3b). Elevation in the p-Chk1 and p-Chk2 was observed as early as 2 h for 3–5 mM 4-methoxy-TEMPO treatments. Slight induction of γ -H2A.X was observed in cells treated with 5 mM 4-methoxy-TEMPO at 2 h, but was more prominent at lower 4-methoxy-TEMPO concentrations with longer (4 and 6 h) exposures. Collectively, the increased intensity in the Comet tail, the induction of γ -H2A.X, and the increased expression of p-Chk1 and p-Chk2 indicate that DNA damage was induced in response to 4-methoxy-TEMPO treatment.

To investigate whether a causal relationship exists between 4-methoxy-TEMPO-induced ROS and DNA damage, we inhibited ROS generation by pretreating HepG2 cells with 10 mM NAC prior to a 4-h treatment with 4 mM 4-methoxy-TEMPO. The presence of NAC significantly reduced the tail intensity in the Comet assay, in comparison to 4 mM 4-methoxy-TEMPO treatment alone (Fig. 3c), suggesting that NAC attenuated 4-methoxy-TEMPO induced DNA damage. Supporting this finding, a similar effect was obtained by Western blot, where the induction of γ -H2A.X by 4 mM 4-methoxy-TEMPO was partially blocked by NAC treatment (Fig. 3d). These findings support the assumption that DNA damage might result from ROS overproduction following 4-methoxy-TEMPO treatment.

4-Methoxy-TEMPO activates MAPK signaling

Previously, we have demonstrated that TEMPO can activate the MAPK signaling pathway (Guo et al. 2015). To determine whether 4-methoxy-TEMPO triggers MAPK signaling, we first examined the expression and phosphorylation of MAPK signaling proteins by Western blot. Following 4-methoxy-TEMPO treatment for 4 h, JNK, ERK1/2, and p38, three members of the MAPK signaling pathway, all exhibited activation as demonstrated by their increased phosphorylation (Fig. 4a, b), while little change was observed in the total levels of these proteins.

To determine the biological significance of MAPK phosphorylation in 4-methoxy-TEMPO-induced cytotoxicity, HepG2 cells were pretreated with specific inhibitors targeting JNK (SP600125), ERK1/2 (U0126), and p38 (SB203580) for 2 h, followed by treatment with 4-methoxy-TEMPO for 4 h. Pretreatment with 10 μ M SP600125 partially rescued the decrease in ATP content (Fig. 4d) and cell viability (Fig. 4e) caused by 4-methoxy-TEMPO, whereas pretreatment with SB203580 or U0126 did not show significant effect in ATP content and cell viability compared with the 4-methoxy-TEMPO treatment only group (Supplementary Fig. 1). Western blot further confirmed the efficiency of SP600125, U0126, and SB203580 to inhibit JNK (Fig. 4c), ERK1/2 (Supplementary Fig. 1a), and p38 (Supplementary Fig. 1d), respectively, with no effect on each total protein level. These data suggest that although these three MAPK signaling proteins are phosphorylated; only JNK contributes to 4-methoxy-TEMPO-induced cytotoxicity.

As described above, 4-methoxy-TEMPO-induced ROS generation resulted in DNA damage. We next investigated whether JNK activation is also associated with DNA damage. Cells were pretreated with 10 μ M SP600125 for 2 h to inhibit JNK, and then treated with 4-methoxy-TEMPO for 4 h. In the SP600125 treatment group, lower γ -H2A.X levels suggest that JNK phosphorylation might promote 4-methoxy-TEMPO-induced DNA damage (Fig. 4c).

4-Methoxy-TEMPO-induced ROS results in JNK activation

Reactive oxygen species overproduction leads to activation of many cell-signaling pathways. To investigate whether ROS generation impacts the MAPK signaling pathway, HepG2 cells were pretreated with 10 mM NAC for 1 h prior to the treatment with 4-methoxy-TEMPO for 4 h. The results indicated that NAC partially reversed the JNK phosphorylation status but did not alter phosphorylation of ERK1/2 or p38 (Fig. 4f), suggesting that JNK activation following 4-methoxy-TEMPO treatment was triggered, at least in part, by ROS generation.

4-Methoxy-TEMPO induces autophagy in HepG2 cells

A variety of stressors including ROS can stimulate autophagy (Pallichankandy et al. 2015; Zhang et al. 2015a). We first assessed whether or not autophagy is induced by 4-methoxy-TEMPO. Generally, autophagy induction can be evaluated by monitoring autophagosome formation and autophagic flux, which is a dynamic process of autophagosome synthesis and degradation. As demonstrated in Fig. 5a, 4-methoxy-TEMPO induced LC3B conversion from cytosolic LC3B-I to autophagosomal membrane-bound LC3B-II. Because LC3B conversion is a hallmark of autophagosome formation, the time- and concentration-dependent LC3B conversion suggested that 4-methoxy-TEMPO induced the autophagosome formation. Moreover, 4-methoxy-TEMPO decreased the levels of p62 protein, an autophagy-specific substrate (Fig. 5a), and an additional indication of autophagic flux.

We previously generated a cell line, HepG2-GFP-LC3B, that stably expresses a GFP-LC3B fusion protein that is used to quantitatively measure both autophagosome formation (punctae formation) and autophagic flux (generation of free GFP fragment) (Chen et al. 2014a). Treatment of HepG2-GFP-LC3B cells with 4-methoxy-TEMPO of 3 and 5 mM for 4 h caused significant increase in the number of GFP-LC3B punctae (Fig. 5b) and in generation

of free GFP (Fig. 5c). Taken together, the induction of autophagosome formation (LC3B conversion and punctae formation) and progression of autophagic flux (reduction of p62 and generation of free GFP fragments) demonstrate that autophagy was induced by 4-methoxy-TEMPO treatment.

Results from the current study showed that the production of ROS is an early event following treatment with 4-methoxy-TEMPO. To investigate the potential role of ROS in 4-methoxy-TEMPO-induced autophagy in HepG2 cells, we pretreated HepG2 cells with NAC before exposure to 4-methoxy-TEMPO. NAC partially counteracted LC3B conversion, p62 reduction (Fig. 5d), and autophagosome punctae formation (Fig. 5e) induced by 4-methoxy-TEMPO alone. These data suggest that autophagy induction could be a downstream response activated by ROS generation.

Discussion

The present study aimed to evaluate the toxicity of 4-methoxy-TEMPO, and to investigate the mechanisms underlying its toxicity. We previously studied the cytotoxicity and genotoxicity of TEMPO, an important chemical in the class of piperidine nitroxides, in L5178Y mouse lymphoma cells and TK6 human lymphoblastoid cells (Guo et al. 2013, 2015). In both cell lines, TEMPO exposure induced cytotoxicity and genotoxicity that was augmented by the presence of metabolic activation (S9), indicating metabolism might be important in TEMPO's toxicity. Using a panel of human and rodent tumor and non-tumor cell lines, Gariboldi et al. (1998) reported that liver cell lines were more resistant to the cytotoxic effects of another TEMPO derivative, 4-hydroxy-TEMPO, when compared to breast, colon, and ovary cell lines. These results suggest hepatic metabolism of TEMPO and its derivatives may play a role in their toxicity (Gariboldi et al. 1998). Therefore, given the association between hepatic metabolism and the toxicity of TEMPO and its derivatives, we used HepG2 human hepatoma cells in this mechanistic study.

Previous reports indicate that nitroxides have biphasic oxidative properties. For example, TEMPO and 4-hydroxy-TEMPO at low micromolar concentrations act as antioxidants, whereas at higher millimolar concentrations they exert a pro-oxidant effect that results in glutathione depletion, impaired ATP production, and cytotoxicity (Israeli et al. 2005; Offer et al. 2000; Simonsen et al. 2009). In addition, TEMPO and six of its derivatives at the concentrations up to 500 μ M showed no significant cytotoxicity to human breast adenocarcinoma (MCF-7) cells, whereas two other derivatives (out of 8), 4-nonylamino-TEMPO at 250 μ M and 4-cyano-TEMPO at 500 μ M, did produce cytotoxicity (Sadowska-Bartosz et al. 2015). In this study, we focused on the toxic effects of TEMPO and its derivatives rather than on their pharmacological effects. It has been reported that nitroxides may accumulate in some tissues at great concentrations. For example, when C3H mice were injected with 5 μ l/g body weight of 150 mM 4-hydroxy-TEMPO in the tail vein for nitroxide-based redox imaging, the maximum blood and tissue concentrations were 6.1–8.1 mM (Davis et al. 2011). In addition, high concentrations of 4-hydroxy-TEMPO (5 or 30 mM) in drinking water were also administered to the rats with amyotrophic lateral sclerosis (a neurodegenerative disease) and it moderately extended the survival of these animals

(Linares et al. 2013). Therefore, the dose-range concentrations selected in this study were at millimolar levels starting from 0.5 mM to assess fully their toxic potential.

All six tested derivatives produced different magnitudes of cytotoxicity in HepG2 cells. Among which, 4-methoxy-TEMPO had similar cytotoxicity to TEMPO (Fig. 1a), so the subsequent toxicity and mechanistic studies focused on 4-methoxy-TEMPO. Furthermore, we found that 4-methoxy-TEMPO induced cytotoxicity in HepG2 cells could be attenuated by the antioxidant NAC (Figs. 1, 2). This finding is in good agreement with observations from TEMPO and 4-hydroxy-TEMPO studies, where cellular damage was attributed to their pro-oxidant effects (Guo et al. 2015; Simonsen et al. 2009).

In addition to oxidative stress, our study demonstrated that many cellular stress responses, including DNA damage, JNK activation, and autophagy induction, were resulted from 4-methoxy-TEMPO-induced toxicity. It is likely that ROS overproduction is the upstream event triggering the multiple cellular stress responses, which eventually lead to cell death. Evidence supporting this assertion is threefold: (1) detection of ROS overproduction occurred at a lower concentration and an earlier time point than did detection of decreased cell viability (Fig. 2a). (2) cellular ATP depletion (Fig. 1c), glutathione reduction (Fig. 2b), DNA damage (Fig. 3a), JNK activation (Fig. 4a), and autophagy induction (Fig. 5a–c) followed 4-methoxy-TEMPO exposure, and (3) inhibition of ROS production by NAC significantly diminished those cellular responses induced by 4-methoxy-TEMPO, including DNA damage (Fig. 3c, d), JNK activation (Fig. 4f), autophagy induction (Fig. 5d, e), ATP decreases (Fig. 2d), and cell viability decreases (Fig. 2e). Additionally, we found that JNK activation is not only a consequence of ROS overproduction, but also a positive regulator of DNA damage, as evidenced by lower induction of γ -H2A.X when p-JNK function was inhibited, further suggesting crosstalk between the different mechanisms (Fig. 4c). It is worth noting that NAC only showed modest protective effect on 4-methoxy-TEMPO-induced cell viability reduction (Fig. 2e) and DNA damage (Fig. 3c, d); therefore, 4-methoxy-TEMPO-induced ROS may not be the only cause of cell death.

The MAPK signaling pathway promotes diverse cellular responses, and it can be activated by various extracellular and intracellular stimuli. Many studies have demonstrated that ROS production in cells can activate the three members of MAPK signaling, ERK1/2, JNK, and p38 (Krifka et al. 2013). Additionally, activation of MAPK signaling can either promote or suppress cell proliferation, depending on the type and extent of the stimuli and cellular conditions (Tormos et al. 2013). The three members of the MAPK pathway can respond to ROS production with distinct effects on cell survival and death, and can regulate each other to modulate MAPK signaling (Wagner and Nebreda 2009). In the present study, JNK activation was ROS-mediated, as NAC treatment attenuated a 4-methoxy-TEMPO-induced increase of p-JNK (Fig. 4f). The application of a JNK inhibitor also demonstrated that JNK played an important role in 4-methoxy-TEMPO-induced cell death (Fig. 4d, e). However, although p38 and ERK1/2 were phosphorylated upon 4-methoxy-TEMPO exposure, these processes may not directly relate to ROS, as NAC did not affect their activation (Fig. 4f). Furthermore, pretreatment with p38 or ERK1/2 inhibitors did not alter cell viability compared to treatment with 4-methoxy-TEMPO alone (Supplementary Fig. 1). Thus, the roles of phosphorylated ERK1/2 and p38 remain unclear, but it seems they play only minor

roles in DNA damage or cell death caused by 4-methoxy-TEMPO. However, these observations bring up new questions. What stimuli triggered the activation of ERK1/2 and p38? What pathways/networks regulate these MAPK members? What are the consequences of ERK1/2 and p38 activation? It would be of interest to answer these questions in further investigation into the effect 4-methoxy-TEMPO exposure on hepatic cells.

Studies have shown that the production of intercellular ROS is essential for autophagosome formation under certain stress conditions such as cell starvation. Meanwhile, ROS can also promote autophagy under pathological conditions such as brain injury, ischemia/reperfusion, and chemical-induced toxicity (Chen et al. 2014b; Gong et al. 2012; Jacobson 1996; Li et al. 2015; Xie et al. 2011). ROS formation regulates autophagy induction via complex transcriptional and post-transcriptional mechanisms. Autophagy induction, in turn, alleviates cellular oxidative damage by degrading oxidized substances and eliminating other unwanted substances (Li et al. 2015). However, this so-called “pro-survival” effect of autophagy was not observed in our study, as modifying autophagy status had no influence on cytotoxicity. In particular, inhibiting autophagy by knocking down Atg7 gene function or by chemical inhibitors (3-methyladenine and chloroquine) did not aggravate 4-methoxy-TEMPO toxicity (Supplementary Fig. 2). The reason for this unexpected outcome is not clear; possibly, because autophagy induction is not sufficient to overcome the toxic effects induced by other mechanisms. The regulation of cell survival and death is a complicated process with the involvement of multiple organelles and an array of signaling pathways. Prior to toxic insults, adaptive responses are initiated to counteract the toxicity within cells. Autophagy is considered one of these adaptive responses and may reduce the level of ROS to protect cells from damage. However, the toxicity caused by prolonged stress may exceed cellular adaptive capacity and eventually overcome the defensive effect of autophagy.

In summary, the current study suggests that multiple mechanisms, including ROS overproduction, DNA damage, autophagy, and JNK pathway activation, participate in the toxicity of 4-methoxy-TEMPO in human hepatic cells. ROS overproduction seems to play a critical role in these cellular responses. Our findings provide new insights into the mechanisms of 4-methoxy-TEMPO toxicity, and improve our understanding of potential hazards associated with the use of 4-methoxy-TEMPO. However, the current study did not emphasize the role of metabolic effects on TEMPO-derivative toxicity. For a better understanding of the contribution of metabolism on TEMPO-derivative toxicity, additional studies using metabolically active cells such as primary human hepatocytes and HepaRG cells are needed.

Supplementary Material

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Fig. 1.

4-Methoxy-TEMPO induced cytotoxicity in HepG2 cells. **a** HepG2 cells were exposed to various concentrations (0.5–8 mM) of seven piperidine nitroxides for 4 h, and cell viability was measured using the MTS assay. HepG2 cells were exposed to various concentrations (0.5–5 mM) of 4-methoxy-TEMPO for 2, 4, and 6 h before **b** cytotoxicity determined using the MTS assay and **c** measurements of ATP content. The data points represent the mean $\pm$ SD from three independent experiments with 4 parallel samples per concentration in each experiment. *Indicates $p < 0.05$ compared to the vehicle controls

Fig. 2.

4-Methoxy-TEMPO induced ROS generation and glutathione depletion. **a** HepG2 cells were treated with various concentrations (0.5–5 mM) of 4-methoxy-TEMPO for 0.5, 1, 2, 4, and 6 h, before the ROS values were measured using H_2DCF -DA staining. **b** Relative GSH/GSSG ratios were assessed after 2- and 4-h treatments with 4-methoxy-TEMPO. HepG2 cells were pretreated with 10 mM NAC for 1 h prior to an additional 4-h treatment with 4-methoxy-TEMPO before measuring **c** intracellular ROS levels, **d** ATP content, and **e** cell viability. The data points represent the mean $\pm$ SD from at least three independent experiments. * and # indicate $p < 0.05$ compared to the vehicle control without or with NAC pretreatment, respectively. & Indicates $p < 0.05$ between the treatments with and without NAC pretreatment at the same concentration of 4-methoxy-TEMPO

Fig. 3.

4-Methoxy-TEMPO induced ROS-mediated DNA damage. **a** HepG2 cells were exposed to different concentrations (0.5–5 mM) of 4-methoxy-TEMPO for 4 h and DNA damage was determined using the alkaline Comet assay. **b** Total cellular proteins from HepG2 cells were extracted after 2, 4, and 6 h of 4-methoxy-TEMPO treatments and the levels of γ -H2A.X, p-Chk1, and p-Chk2 were detected by Western blot. After 1-h pretreatment with or without 10 mM NAC, HepG2 cells were exposed to 4-methoxy-TEMPO for 4 h. **c** DNA damage was determined using the alkaline Comet assay. **d** Total cellular proteins were extracted and the levels of γ -H2A.X was detected by Western blot. GAPDH was used as a loading control (**b**, **d**). The data points in bar graphs (**a**, **c**) represent the mean $\pm$ SD from at least three independent experiments. * Indicates $p < 0.05$ between the treatment with 4-methoxy-TEMPO and the control (**a**), and & indicates $p < 0.05$ between the treatments with and without NAC pretreatment at the same concentration of 4-methoxy-TEMPO (**c**)

Fig. 4.

4-Methoxy-TEMPO activated the MAPK signaling pathway. **a** HepG2 cells were treated with the indicated concentrations of 4-methoxy-TEMPO for 4 h. Total cellular proteins were extracted and the levels of JNK, p-JNK, ERK1/2, p-ERK1/2, p38, and p-p38 were determined by Western blot. **b** The densitometric analysis of (a) was quantified from three independent experiments. Intensities of bands were normalized to the amount of GAPDH. * Indicates $p < 0.05$ between the treatment with 4-methoxy-TEMPO and the control. **c–e** HepG2 cells were pretreated with 10 μ M SP600125 (JNK inhibitor) for 2 h prior to a 4-h treatment with 4-methoxy-TEMPO. The levels of p-JNK, JNK, and γ -H2A.X were detected by Western blot (c). ATP content was measured by CellTiter-Glo Luminescent assay (d). Cell viability was determined using the MTS assay (e). The data points (d, e) represent the mean $\pm$ SD from at least three independent experiments. * and # indicate $p < 0.05$ compared to the vehicle control without or with SP600125 pretreatment. & Indicates $p < 0.05$ between the treatments with and without SP600125 pretreatment at the same concentration of 4-methoxy-TEMPO. **f** HepG2 cells were pretreated with 10 mM NAC for 1 h before treating with 4 mM 4-methoxy-TEMPO for additional 4 h. The levels of p-JNK, JNK, ERK1/2, p-ERK1/2, p38, and p-p38 were determined by Western blot. GAPDH was used as a loading control (a, c, f).

Fig. 5.

4-Methoxy-TEMPO induced autophagy in HepG2 cells. **a** Total cellular proteins were extracted from HepG2 cells exposed to different concentrations of 4-methoxy-TEMPO for 2, 4, and 6 h. The levels of LC3B and p62 were detected by Western blot. **b** HepG2-GFP-LC3B cells were treated with the indicated concentrations of 4-methoxy-TEMPO for 4 h. The GFP-LC3B foci (arrow) were recorded using a confocal laser microscope. Representative images were from three independent experiments (left panel) and the bar graph represents the percentage of cells with punctae (right panel). * Indicates $p < 0.05$ compared with the vehicle control. **c** HepG2-GFP-LC3B cells were incubated with different concentrations of 4-methoxy-TEMPO for 2, 4, and 6 h. Total cellular proteins were extracted and the induction of free GFP was detected by Western blot. **d** After 1 h pretreatment with or without 10 mM NAC, cells were exposed to 4-methoxy-TEMPO for 4 h without NAC. Western blot analysis was performed with antibodies against LC3B and p62 (left panel) and the densitometric analysis was quantified from three independent experiments (right panel). Intensities of bands were normalized to the amount of GAPDH. **e** The GFP-LC3B punctuation (arrow) was observed by a confocal laser microscope. Representative images were from three independent experiments (left panel) and the bars represent the percentage of cells with punctae (right panel). GAPDH was used as a loading control (**a**, **c**, **d**). The data points in bar graphs (**b**, **d**, **e**) represent the mean $\pm$ SD from three individual experiments. & Indicates $p < 0.05$ between the treatments with and without 10 mM of NAC pretreatment at the same concentration of 4-methoxy-TEMPO (**d**, **e**)