Neurotoxicology 75 (2019) 233–244

Contents lists available at ScienceDirect

Neurotoxicology

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

Full Length Article

Upregulation of CYP2E1 expression causes oxidative damage induced by 2-chloroethanol in primary cultured rat astrocytes

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ARTICLE INFO

Keywords:
1,2-Dichloroethane poisoning
Astrocytes
2-Chloroethanol
Cytochrome P450 2E1
Oxidative damage

ABSTRACT

Brain edema caused by subacute poisoning with 1,2-dichloroethane (1,2-DCE) has gained much attention during recent years, but its underlying mechanism is poorly understood. As an intermediate metabolite of 1,2-DCE *in vivo*, 2-chloroethanol (2-CE) can be transformed into chloroacetaldehyde and reactive oxygen species (ROS) through cytochrome P450 2E1 (CYP2E1) mediated metabolism. In previous studies, it was found that CYP2E1 expression is enhanced in the brain of mice treated with 1,2-DCE. This study was designed to verify the roles of CYP2E1 overexpression in 2-CE induced cytotoxicity in rat astrocytes, and the contribution of specific signaling molecules to the upregulation of CYP2E1 expression caused by 2-CE. The results of this study demonstrate that treatment with 2-CE can enhance CYP2E1 protein and mRNA levels, cause an increase in ROS and MDA levels, and higher percentages of apoptotic cells in rat astrocytes. Pretreatment with either diallyl sulfide or vitamin C, the inhibitor of CYP2E1 or scavenger of ROS, respectively, can suppress the levels of CYP2E1 expression, ROS and MDA, ameliorate cell apoptosis, and attenuate phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in these cells. Additionally, pretreatment with the inhibitor of either ERK1/2 or transcriptional factor specificity protein 1 (SP1) can suppress the CYP2E1 expression, and alleviate the oxidative damage caused to these cells. In conclusion, our findings demonstrate that CYP2E1 overexpression plays a crucial role in 2-CE induced oxidative damage of rat astrocytes, and that CYP2E1 expression is upregulated partially through the activation of the ERK1/2 and SP1 signaling pathways by ROS generated during CYP2E1-mediated 2-CE metabolism. This study provides novel information that can be used in elucidating the mechanism by which 1,2-DCE induces brain edema.

1. Introduction

1,2-Dichloroethane (1,2-DCE) is a synthetic halogenated hydrocarbon that is used as the monomer in the manufacture of polyvinyl chloride, and as an industrial solvent and adhesive thinner (Yu et al., 2013; Sun et al., 2016a). 1,2-DCE can quickly evaporate into the air and is inhaled by workmen in the workplace when it is used as a solvent. After absorption, 1,2-DCE is rapidly distributed into the body through systemic circulation and can easily pass through the blood brain barrier (BBB) and into the brain. During the past few decades, acute and subacute poisoning with 1,2-DCE of both workmen and laboratory animals have been reported (Hotchkiss et al., 2010; Liu et al., 2010; Wang et al., 2014; Zhou et al., 2015). Brain edema is the main pathological consequence of subacute poisoning with 1,2-DCE. However, its underlying mechanisms have been rarely reported (Yang et al., 2009; Wang et al., 2013; Chen et al., 2015).

Animal experiments have shown that 1,2-DCE is metabolized *in vivo* mainly with the aid of microsomal cytochrome P450 2E1 (CYP2E1) to produce 2-chloroethanol (2-CE), chloroacetaldehyde and chloroacetic acid (Guengerich et al., 1980; Sweeney et al., 2008). 2-CE can also be transformed into chloroacetaldehyde and chloroacetic acid through CYP2E1-mediated metabolism. Chloroacetaldehyde can directly interact with cellular components, and ultimately cause the oxidation of proteins, DNA and lipids. Moreover, in comparison with other P450 subtypes, CYP2E1 shows an apparently higher rate of oxidase activity and an ability to generate reactive oxygen species (ROS) through its

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https://doi.org/10.1016/j.neuro.2019.09.016
Received 8 August 2019; Received in revised form 29 September 2019; Accepted 30 September 2019
Available online 01 October 2019
0161-813X/

catalytic cycle (Liu et al., 2005). More importantly, as the inducing enzyme, CYP2E1 expression in both humans and animals can be up-regulated by its catalytic substrates during the metabolic process (Das et al., 2010; Zhang et al., 2015). It has been reported that the *in vivo* toxicities of acetaminophen, solvents and nitrosamines are enhanced as a result of CYP2E1 induction (Wargovich, 2006). Therefore, the metabolism of 1,2-DCE *in vivo*, which is regulated by CYP2E1 may enhance its cytotoxicity and contribute the formation of brain edema (Sun et al., 2016a,b; Jin et al., 2018).

Like other cytochrome P450 s, CYP2E1 is found mainly in hepatocytes, but recent reports have demonstrated its constitutive presence in both glial and neuronal cells of rat brain (Vichi et al., 2015; García-Suástegui et al., 2017). Using immunohistochemical staining, it has been found that CYP2E1 is heterogeneously distributed among brain regions, and the higher immunoreactivity was discovered in the frontal cortex, basal ganglia and hippocampus. Morphological and biochemical changes in brain regions as a result of alcohol consumption are significantly correlated with the expression level of CYP2E1 in animals (Ledesma et al., 2014; Toselli et al., 2015). These results suggest that CYP2E1 mediated intracerebral alcohol metabolism may lead to alcohol neurotoxicity. Therefore, it can be speculated that the induction of CYP2E1 in the brain may be related with cerebral edema caused by subacute poisoning of 1, 2-DCE in mice, and possibly show a similar relationship in humans as well.

Astrocytes, once thought to be little more than the glue that binds neurons in place, are now considered to take part in a variety of neuronal functions. In particular, they help to form a secure BBB, because their end-feet can adequately wrap a capillary. Thus, malfunction of astrocytes may disrupt BBB integrity, which results in vasogenic brain edema. In previous studies, it has been found that CYP2E1 expression in the brain of mice is upregulated upon treatment with 1,2-DCE (Jin et al., 2018). In this study, the role of CYP2E1 overexpression in 2-CE induced cytotoxicity in rat astrocytes and the involvement of specific signaling molecules involved in the upregulation of CYP2E1 expression caused by 2-CE, were further elucidated. For this, the inhibitors specific to CYP2E1 expression, ROS generation, extracellular signal-regulated kinase 1/2 (ERK1/2) and transcriptional factor specificity protein 1 (SP1) were individually applied before treatment of primary cultured rat astrocytes with 2-CE. Our study may provide information for elucidating the mechanisms that underlie 1,2-DCE induced brain edema.

2. Materials and methods

2.1. Reagents

2-Chloroethanol (> 99.0%) was purchased from Sinopharm Chemical Reagent Co., Ltd, China. Diallyl sulfide (DAS) and vitamin C (Vit C) were purchased from Tokyo Chemical Industry, Japan. SCH772984 (SCH), an inhibitor of ERK1/2 were obtained from MedChem Express, USA. Mithramycin A (MTMA), a DNA binding transcriptional inhibitor of SP1 was obtained from Tocris (Bristol, UK). The primary culture reagents used were purchased from Biological Industries, Israel. The JC-1 probe (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl- imidacarbocyanine iodide) was obtained from Sigma, USA, while the dihydroethidium (DHE) probe was obtained from KeyGEN BioTECH (Nanjing, China). Polyclonal primary antibodies against CYP2E1, SP-1 and glial fibrillary acid protein (GFAP) were obtained from Millipore, USA, while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used was the product of Proteintech (Wuhan, China). Primary antibodies against Bax, ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2) were the products of Cell Signaling Technology (Beverly, USA). Bcl-2 and phosphorylated SP1 (p-SP1) were purchased from Abcam (Cambridge, UK). Antibodies against nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and the catalytic subunit of γ-glutamylcysteine synthetase (γ-GCSc) were the products of Santa Cruz Biotechnology, USA. The BCA protein kit and enhanced ECL plus kit were obtained from Thermo Fisher Scientific, USA. The gene expression kit was purchased from Takara, Japan. The water used in this study was doubly distilled. Goat serum, RIPA Lysis Buffer, diamidino-phenylindole (DAPI) and secondary antibodies conjugated with Alexa Fluor 488 were obtained from the Beyotime Institute of Biotechnology (Shanghai, China). Malondialdehyde (MDA) levels assay kit was obtained from the Nanjing Jiancheng Bioengineering Institute, China.

Table 1
Primer sequences used for the amplification of each gene in this study.

Gene		Primer Sequence (5'-3')
CYP2E1	Forward	5'-GCTACTGAACCACAAGAATGA-3'
	Reverse	5'-CTCCGCACATCCTTCCAT-3'
SP1	Forward	5'-GCTGCTCAACTGTCCTCCAT-3'
	Reverse	5'-CTCCACCTGCTGTCTCATCAT-3'
Nrf2	Forward	5'-CACAGCCAACACATTCTTCT-3'
	Reverse	5'- CAGGACTCACAGGTACTTCT-3'
HO-1	Forward	5'- GTCCTCAAGAAGATTGCTCAGAAG-3'
	Reverse	5'- GTCATCTCCAGAGTGTTCATTCG-3'
γ-GCSc	Forward	5'-CTGCCACGGAACTGAACTCT-3'
	Reverse	5'-AGCAAACTTGAGAGAGGGCG-3'
GAPDH	Forward	5'- GCAAGAGAGAGGCCCTCAG-3'
	Reverse	5'- TGTGAGGGAGATGCTCAGTG-3'

2.2. Cell culture

In brief, the cerebral cortexes of newborn Wistar rats (1–3 days postnatal), were obtained from the Experimental Animal Center of China Medical University, and were carefully separated for primary culture of astrocytes, as previously described. They were cut into pieces and then enzymatically digested using 0.125% trypsin for 20 min at 37 °C. The dissociated cells were seeded into petri dishes pre-coated with poly-L-lysine and kept in a humidified incubator with 5% CO_2 at 37 °C. In order to remove weakly adherent microglia and oligodendrocytes, the astrocytes in culture dish were vigorously shaken overnight (250 rpm at 37 °C) on an horizontal shaker, once the culture reached confluence. Following vibration, the remaining cells were identified using positive immunostaining for GFAP and the astrocytes with a purity of > 95% were used for subsequent experiments.

All experiments were carried out in accordance with the guidelines of the National Laboratory for Animal Protection of China and were approved by the Animal Protection and Use Committee of China Medical University (IACUC: NO.16101). The best effort was made to ensure that a minimal number of animals were used causing the least amount of pain to them.

2.3. Cell treatments

2-CE (1 mol/L) was prepared as a stock solution with double distilled water and diluted into a final concentration, before being used with Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS). Astrocytes are treated for 1, 2, 4, 8, 12, 24 and 48 h with 30 mM 2-CE and treated with 0, 7.5, 15 and 30 mM 2-CE for 24 h to determine the effect of different 2-CE concentrations and durations on the expression of CYP2E1. Astrocytes were pretreated with 100 μM DAS or 100 μM Vit C one hour before 24 h treatment with 30 mM 2-CE, to test the effects of CYP2E1 inhibition or ROS scavenging. In order to determine the role of the ERK1/2 and SP1 signaling pathways in the upregulation of CYP2E1 expression in 2-CE treated rat astrocytes, the cells were pretreated with either SCH (0.1, 1 and 10 μM) or MTMA (125, 250 and 500 nM), one hour before 24 h treatment with 30 mM 2-CE. In addition, an untreated control group and an inhibitor control group were also included. The original solution of each inhibitor was prepared in dimethyl sulfoxide (DMSO) and diluted in DMEM to ensure that the final DMSO concentration was not > 0.1%.

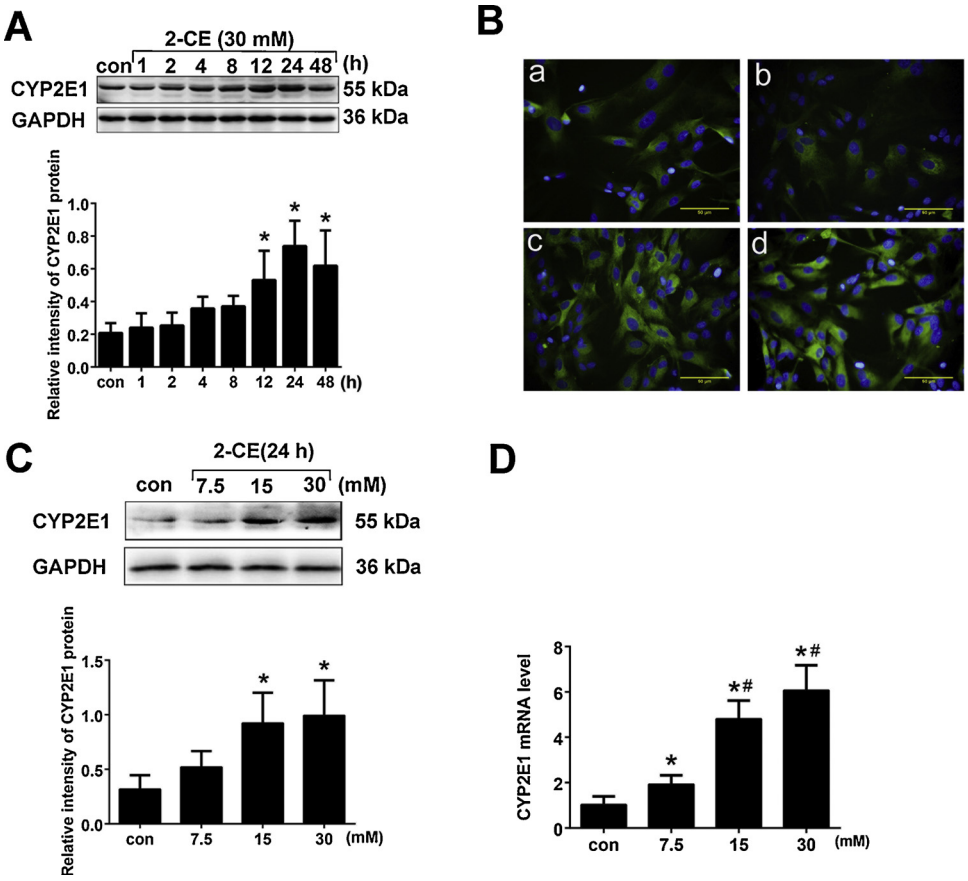


Fig. 1. Effects of 2-CE on CYP2E1 expression in primary cultured rat astrocytes.

(A) Western blotting analysis to determine the level of CYP2E1 protein in astrocytes treated with 30 mM 2-CE from 1 to 48 h. (B) Immunofluorescence staining to determine the expression of CYP2E1 protein in astrocytes treated with 0, 7.5, 15 and 30 mM 2-CE for 24 h (400×). (C) Western blotting analysis to determine the level of CYP2E1 protein in astrocytes treated with 0, 7.5, 15 and 30 mM 2-CE for 24 h. (D) Levels of CYP2E1 mRNA examined using real-time (RT)-PCR in astrocytes treated with 0, 7.5, 15 and 30 mM 2-CE for 24 h.

2-CE, 2-chloroethanol; CYP2E1, Cytochrome P450 2E1. (a to d) or control, 7.5 mM 2-CE, 15 mM 2-CE and 30 mM 2-CE represent cells treated with 0, 7.5, 15 and 30 mM 2-CE for 24 h, respectively.

Data are expressed as mean ± SD; n = 4; significance was determined using one-way ANOVA followed by SNK test. *, $p < 0.05$ versus the control group; #, $p < 0.05$ versus the 7.5 mM of 2-CE group.

2.4. Analysis

2.4.1. Immunofluorescence staining

In brief, astrocytes were fixed with 4% paraformaldehyde and permeabilized in PBS containing 0.2% Triton X-100 at room temperature. The cells were further blocked with 10% goat serum before being incubated with a primary antibody against CYP2E1, overnight at 4 °C. Subsequently, the labeled cells were incubated with a secondary antibody conjugated with Alexa Flour 488 at 37 °C. After counterstaining with DAPI, the stained cells were visualized under an Olympus IX71 fluorescence microscope, and fluorescence images were captured using an Olympus SC35 digital camera system. The primary antibody was omitted in the negative control.

2.4.2. Assessment of intracellular ROS levels

DHE, a cell-permeable blue fluorescent dye was used as a redox indicator to detect intracellular superoxide radicals. In brief, astrocytes were incubated in PBS with 10 μM DHE at 37 °C for 30 min. Subsequently, the relative fluorescence intensities of the cells were measured using a fluorescence microplate reader (BioTek Instruments, Inc. USA) at an excitation wavelength of 518 nm and a emission wavelength of 605 nm, and was visualized under a fluorescence microscope.

2.4.3. Assessment of lipid peroxides

The cells were collected in PBS to make a cell suspension at the end of the 2-CE exposure. After sonication, the cells were centrifuged at 4 °C, at 12,000 × g for 10 min, and then the lysates were allowed to react with thiobarbituric acid (TBA) at 95 °C for 30 min. The resulting dyed products were assessed by detecting their optical density (OD) at 532 nm. The amount of lipid peroxide in the lysates was evaluated using the standard solution prepared using MDA and was expressed as μmol MDA/gram cellular protein, according to the manufacturer's instructions.

2.4.4. Assessment of apoptosis

Cell apoptosis was detected using a flow cytometry assay. In brief, the cells were cultured in a 6-well culture dish and then treated with 2-CE, as mentioned above. The cells were suspended and diluted until 1×10^5 cells/ml for apoptosis detection, following enzymatic collection after 2-CE exposure. The staining processes with Annexin V-FITC and PI were performed in line with the manufacturer's instructions. The percentage of positive populations in the Q_2 (Annexin V+/PI-) and Q_3 (Annexin V+/PI+) quadrants were considered as the percentage of apoptotic cells.

2.4.5. Assessment of mitochondrial membrane potential

Mitochondrial damage was evaluated through the dissipation of mitochondrial membrane potential. The cells were washed with PBS and incubated with a total of 0.5 ml JC-1 staining working solution in the dark, at 37 °C for 30 min, after 2-CE exposure. For fluorescence imaging, the labeled cells were captured using an Olympus IX70 inverted phase-contrast fluorescent microscope equipped with a digital camera. A red fluorescence indicates a potential JC-1 dependent aggregation in mitochondria, while a green fluorescence in the cytoplasm indicates the presence of the monomeric form of JC-1.

2.4.6. Western blotting analysis

In brief, astrocytes were harvested by scraping at the end of the exposure. The cells were suspended and homogenized in an iced RIPA buffer. Total protein was extracted after centrifugation (12,000 × *g* for 10 min) at 4 °C, and protein concentrations of samples were determined using a BCA kit. Equal amounts of proteins were fractionated using 10% SDS-PAGE and then transferred onto PVDF membranes (Millipore,

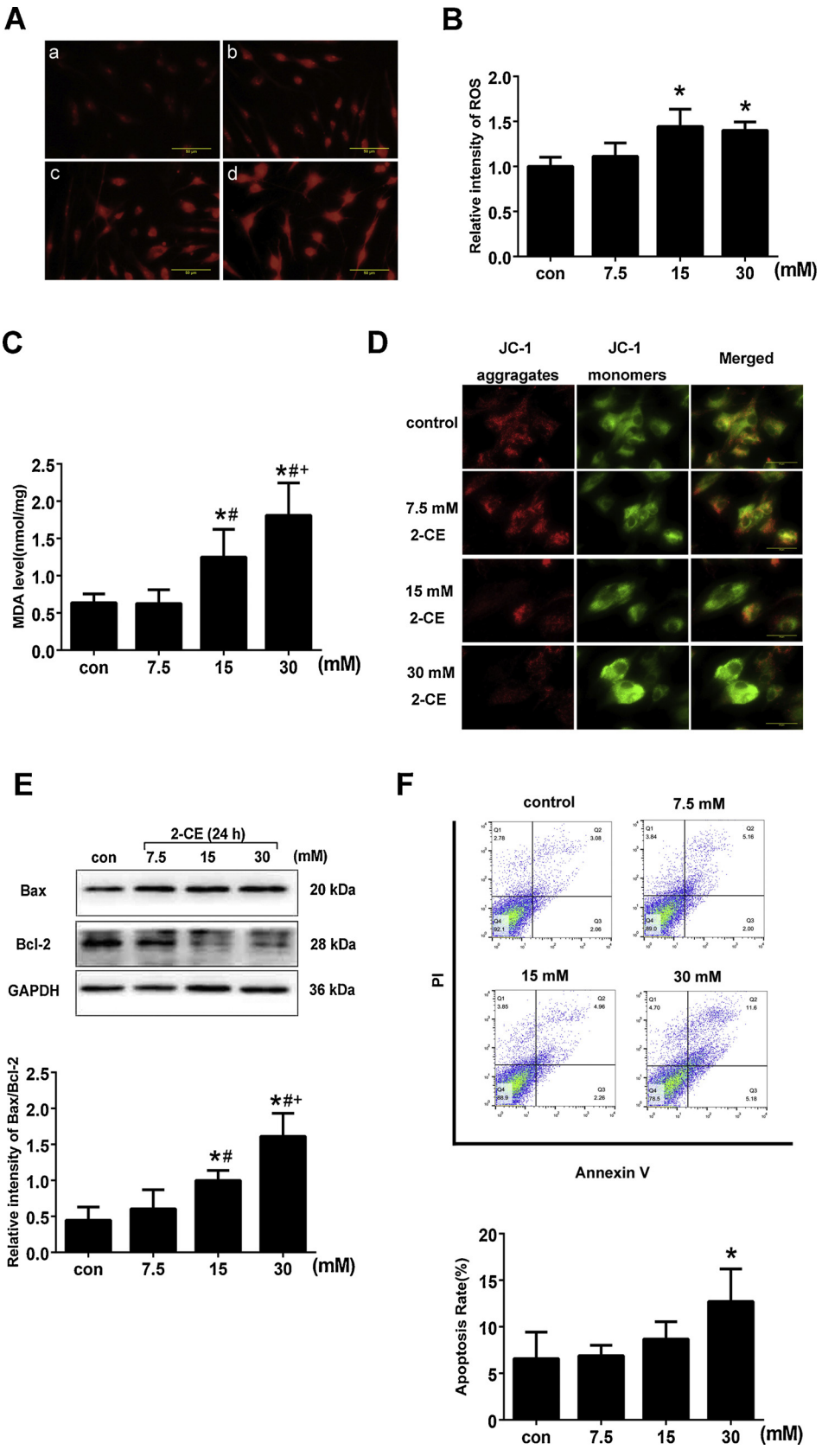


Fig. 2. 2-CE induced oxidative damage of astrocytes.

(A and B) ROS determined using Dihydroethidium on 2-CE exposed astrocytes, which were visualized using fluorescent microscopy (400×), and the fluorescence intensity was quantified. (C) Levels of lipid peroxide in 2-CE exposed astrocytes represented by MDA. (D) Images of mitochondrial membrane potential determined by JC-1 (400×). The red color indicates the potential-dependent aggregation of JC-1 in the mitochondria, while green color indicates the monomeric form of JC-1 in the cytosol. (E) Western blotting analysis of the protein levels of Bax and Bcl-2 in 2-CE exposed astrocytes. (F) Cell apoptosis determined using flow cytometry. The cells in the Q2 + Q3 quadrants were considered as the apoptotic cells.

ROS, reactive oxygen species; MDA, malondialdehyde. (a to d) or control, 7.5 mM 2-CE, 15 mM 2-CE and 30 mM 2-CE represent cells treated with 0, 7.5, 15 and 30 mM 2-CE for 24 h, respectively.

Data are expressed as mean ± SD; n = 4; significance was determined using one-way ANOVA followed by SNK test. *, $p < 0.05$ versus the control group; #, $p < 0.05$ versus the 7.5 mM of 2-CE; +, $p < 0.05$ versus 15 mM of 2-CE group.

Bedford, MA, USA). Thereafter, the membranes were incubated with primary antibodies against CYP2E1, Nrf2, HO-1, γ-GCSc, Bax, Bcl-2, p-ERK1/2, ERK1/2, p-SP1, SP1 or GAPDH at 4 °C overnight, after blocking with 5% non-fat milk at room temperature for 2 h. On the following day, they were washed and reacted with secondary antibodies and visualized using an ECL plus kit. Each protein band was quantified through densitometry using image analysis software (Gel-Pro analyzer v4.0) and the relative protein expression levels were normalized with

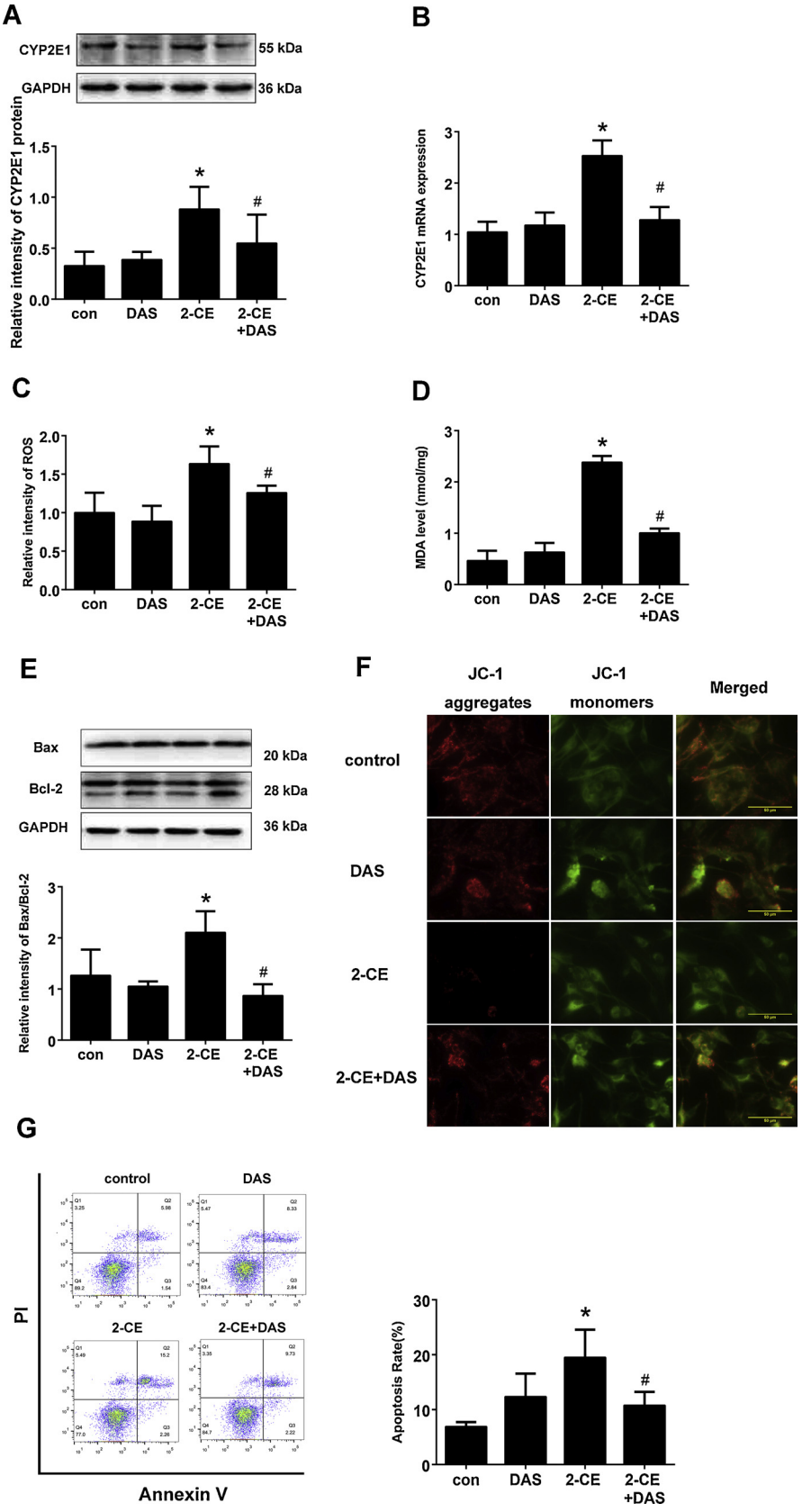


Fig. 3. Involvement of CYP2E1 in 2-CE induced oxidative damage of primary cultured rat astrocytes.
(A) Levels of CYP2E1 protein in astrocytes analyzed using western blotting. (B) Levels of CYP2E1 mRNA in astrocytes analyzed using real-time (RT)-PCR. (C) ROS levels in astrocytes determined using Dihydroethidium, and the fluorescence intensity was quantified. (D) Levels of lipid peroxide in astrocytes expressed by MDA. (E) Images of mitochondrial membrane potential (400×). (F) Levels of Bax and Bcl-2 proteins in astrocyte analyzed using western blotting. (G) Cell apoptosis determined using flow cytometry. The cells in Q2 + Q3 quadrants were considered as the apoptotic cells.
DAS, 2-CE, and 2-CE + DAS represent cells treated with 100 μM DAS for 25 h, 30 mM 2-CE for 24 h, and 100 μM DAS for 1 h followed by treatment with 30 mM 2-CE for 24 h, respectively; Control, untreated cells.
Data are expressed as mean ± SD; n = 4; significance was determined using one-way ANOVA followed by SNK test. *, $p < 0.05$ versus the control group; #, $p < 0.05$ versus the 30 mM of 2-CE group.

GAPDH from the same blot. The results are indicated as the relative intensity of target protein in the cells.

2.4.7. Quantitative real-time RT-PCR analysis

In brief, astrocytes were harvested by scraping at the end of the exposure, and total RNA was isolated using TRIzol Reagent. The first strand of cDNA was reverse transcribed using a PrimeScript RT reagent

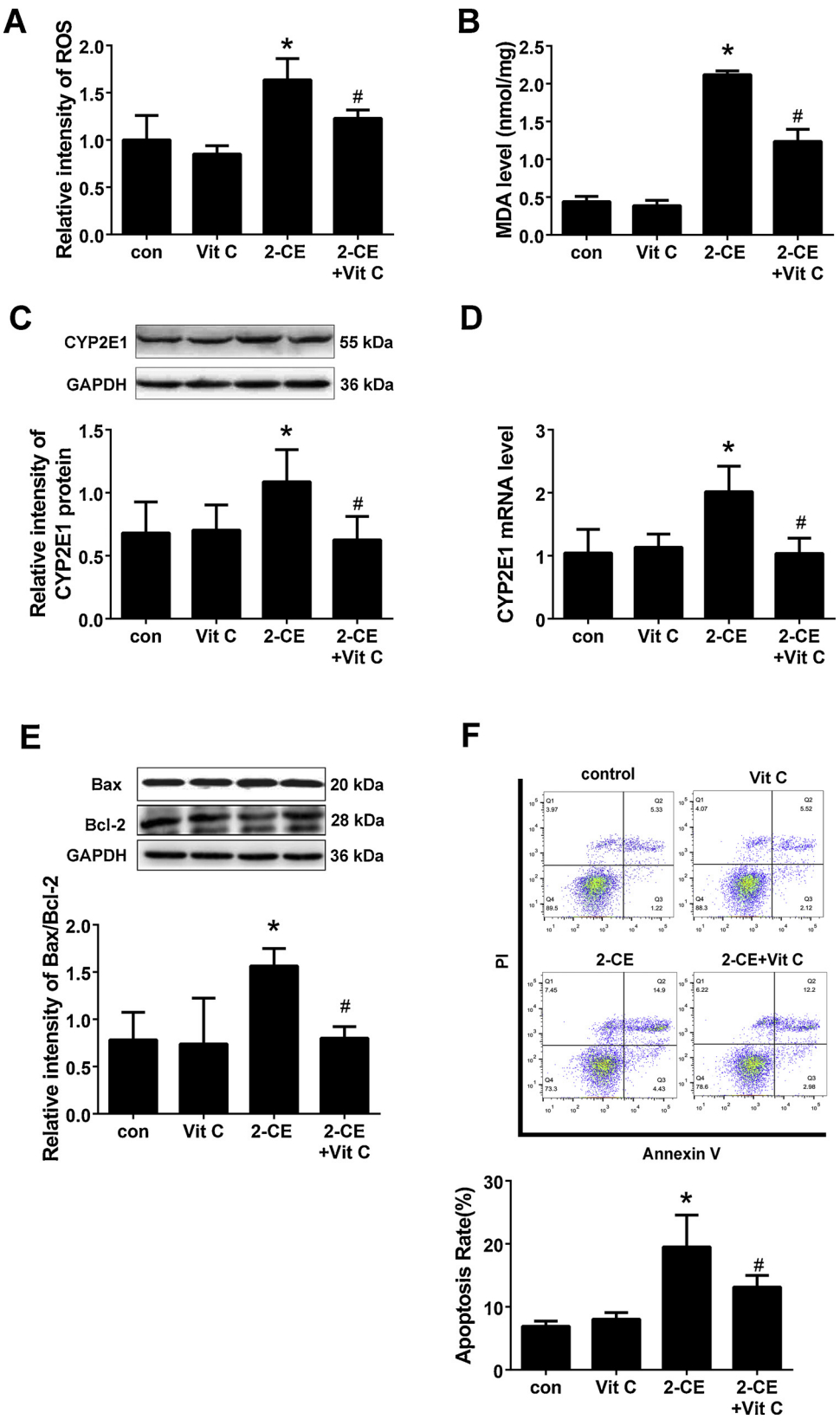


Fig. 4. Role of ROS in 2-CE induced CYP2E1 overexpression in primary cultured rat astrocytes.

(A) ROS levels in astrocytes determined using Dihydroethidium, and the fluorescence intensity was quantified. (B) Levels of lipid peroxide in astrocytes expressed by MDA. (C, D) Levels of CYP2E1 protein in astrocytes by Western blotting. (E, F) Levels of Bax and Bcl-2 proteins in astrocytes analyzed using western blotting. (G, H) Cell apoptosis determined using flow cytometry. The cells in Q2 + Q3 quadrants were considered as the apoptotic cells.

Vit C, 2-CE, and 2-CE + Vit C represent cells treated with 100 μM Vit C for 25 h, 30 mM 2-CE for 24 h, and 100 μM Vit C for 1 h followed by a treatment with 30 mM 2-CE for 24 h, respectively; Control, untreated cells.

Data are expressed as mean ± SD; n = 4; significance was determined using one-way ANOVA followed by SNK test. *, $p < 0.05$ versus the control group; #, $p < 0.05$ versus the 30 mM of 2-CE group.

kit and random primers. The primer pairs, detailed in Table 1, were used to amplify the fragments of CYP2E1, Nrf2, HO-1, γ-GCSc, SP1 and GAPDH. The reaction was conducted for 40 cycles at 95 °C for 5 s and 60 °C for 34 s using a SYBR Premix Ex Taq II and Quant Studio 6 Flex Real-Time PCR System (Life Technologies, USA). We used the comparative Ct method (ΔΔCt) and the $2^{-\Delta\Delta Ct}$ formula for relative quantification of the desired genes and GAPDH mRNA was used as an internal control.

2.5. Statistics

Data were expressed as mean ± SD, and were analyzed using the

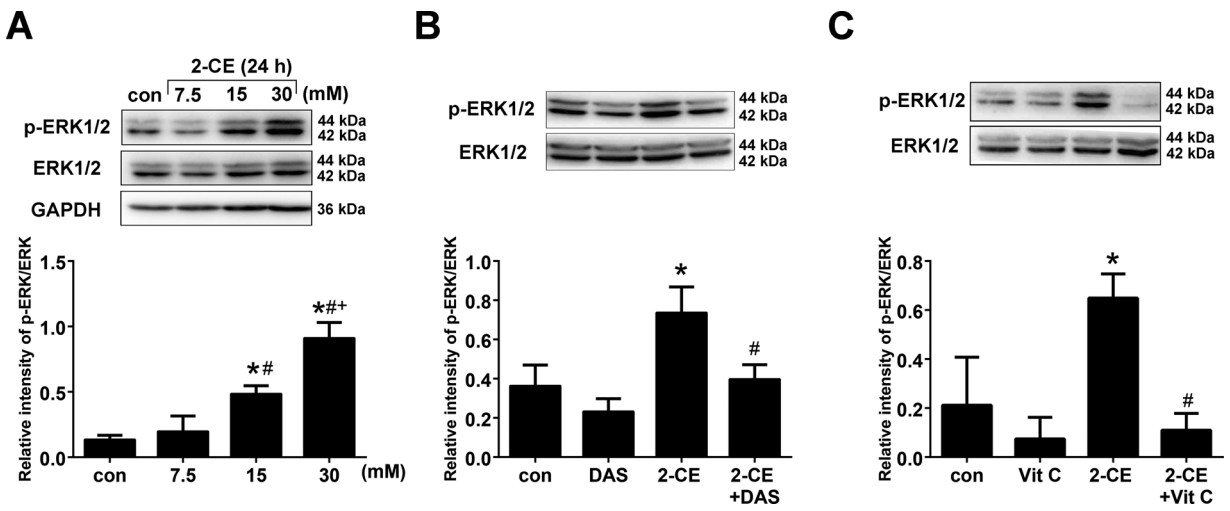


Fig. 5. Association of ERK phosphorylation with 2-CE treatment, CYP2E1 overexpression and ROS generation in primary cultured rat astrocytes.
(A) Western blotting analysis to determine levels of p-ERK1/2 and ERK1/2 proteins in astrocytes treated with different 2-CE concentrations. (B) Western blotting analysis to determine levels of p-ERK1/2 and ERK1/2 protein in 2-CE exposed astrocytes affected by pretreatment with DAS. (C) Western blotting analysis to determine levels of p-ERK1/2 and ERK1/2 proteins in 2-CE exposed astrocytes affected by pretreatment with Vit C.
7.5 mM 2-CE, 15 mM 2-CE and 30 mM 2-CE represent cells treated with 7.5, 15 and 30 mM 2-CE for 24 h, respectively; DAS, 2-CE, and 2-CE + DAS represent cells treated with 100 μM DAS for 25 h, 30 mM 2-CE for 24 h, and 100 μM DAS for 1 h followed by treatment with 30 mM 2-CE for 24 h, respectively; Vit C, 2-CE, and 2-CE + Vit C represent cells treated with 100 μM Vit C for 25 h, 30 mM 2-CE for 24 h, and 100 μM Vit C for 1 h followed by a treatment with 30 mM 2-CE for 24 h, respectively; Control, untreated cells.
Data are expressed as mean ± SD; n = 4; significance was determined using one-way ANOVA followed by SNK test. *, $p < 0.05$ versus the control group; #, $p < 0.05$ versus the 30 mM of 2-CE group.

analysis of variance test (one-way ANOVA), followed by the Student-Newman-Keuls (SNK) test. The analyses were conducted using SPSS for Windows v20.0 (SPSS Inc., USA) software. The data of each group for every indicator were derived from at least four independent experiments, in which the cells originated from different animals. A p value of < 0.05 is considered to indicate statistical significance.

3. Results

3.1. The relationship between the expression of CYP2E1 in 2-CE treated rat astrocytes and exposure duration and concentration

Although we previously reported that the expression of CYP2E1 increases in the brain of mice upon treatment with 1,2-DCE, the induction of CYP2E1 expression in 2-CE treated astrocytes is unclear. Therefore, the expression profiles of CYP2E1 were tracked in astrocytes treated with 30 mM 2-CE for 1–48 h, or 7.5, 15 and 30 mM 2-CE for 24 h. The level of CYP2E1 protein was found to increase significantly within 12 h of 2-CE exposure, peaked at 24 h, and showed a sustained higher level, compared with that of the control even after 48 h (Fig. 1A). Thus, for the following experiments, rat astrocytes were treated with 2-CE for 24 h. Moreover, levels of CYP2E1 protein and mRNA in the 15 and 30 mM 2-CE exposure groups increased significantly, compared with that of the control group (Fig. 1B–D). The CYP2E1 mRNA level in the 7.5 mM 2-CE treated group was also significantly higher than that in the control group. The CYP2E1 mRNA level in the 15 and 30 mM 2-CE treated group was significantly higher than that of the 7.5 mM 2-CE treated group.

3.2. Oxidative damage in rat astrocytes induced by 2-CE treatment

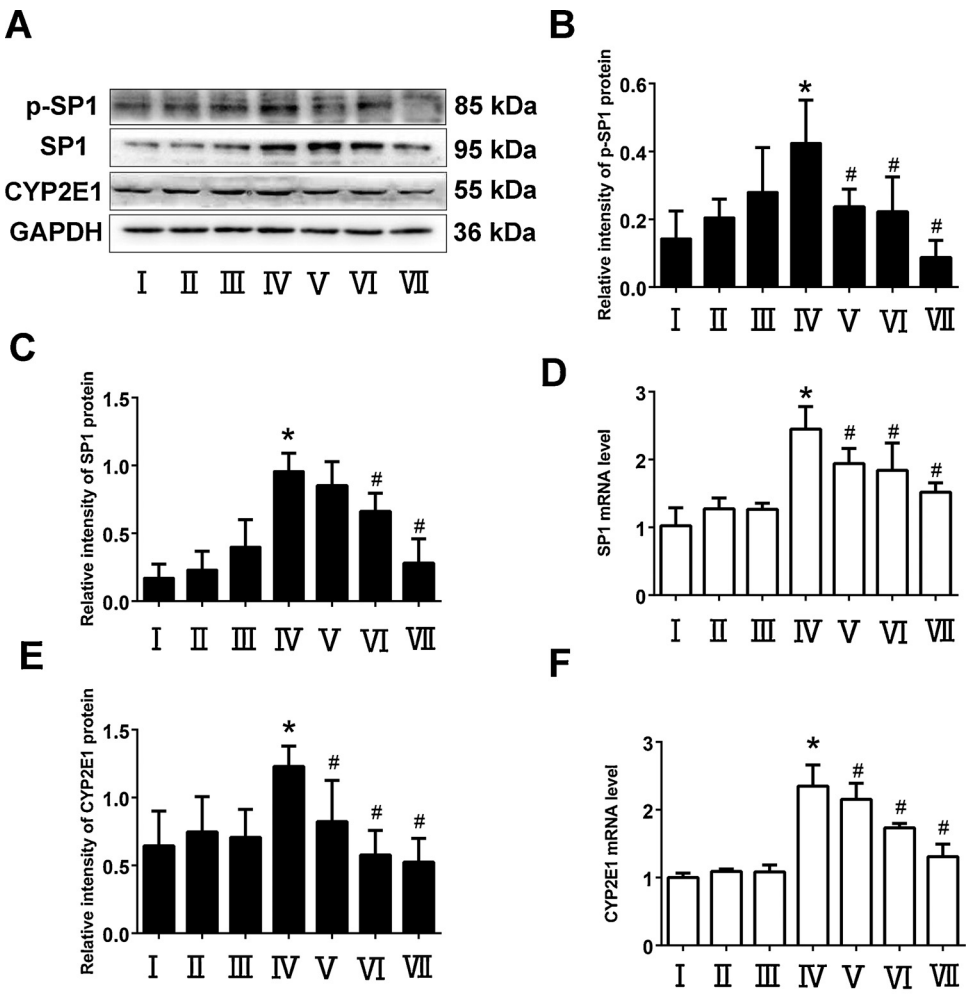
In this study, oxidative damage in 2-CE treated astrocytes was evaluated using levels of ROS and lipid peroxides (as indicated using MDA), percentages of apoptotic cells, ratios of Bax and Bcl-2 protein levels (Bax/Bcl-2), and mitochondrial membrane potential. A significant increase in ROS levels in rat astrocytes was found after 24 h of treatment with 15 and 30 mM 2-CE (Fig. 2A and B). Correspondingly, MDA levels in cells upon treatment with 15 and 30 mM 2-CE increased markedly in a dose-dependent manner (Fig. 2C). Moreover, the Bax/Bcl-2 levels increased significantly in a dose dependent manner, whereas mitochondrial membrane potential decreased markedly in astrocytes upon treatment with 15 and 30 mM 2-CE (Fig. 2D–F). Additionally, the percentage of apoptotic cells increased significantly upon treatment with 30 mM 2-CE (Fig. 2G and H). Collectively, these data indicate that ROS overgeneration in 2-CE treated rat astrocytes that may cause lipid peroxidation, and further disrupt the mitochondrial membrane, leading to cell apoptosis.

3.3. Involvement of CYP2E1 in 2-CE induced oxidative damage in rat astrocytes

In order to assess the possible role of CYP2E1 expression in 2-CE-induced cytotoxicity, the cells were pretreated with 100 μM DAS (a selective inhibitor of CYP2E1), one hour before 24 h treatment with 2-CE. As expected, pretreatment of 2-CE treated astrocytes with DAS significantly suppresses the increase in levels of CYP2E1 protein and mRNA (Fig. 3A–C). Meanwhile, pretreatment with DAS can significantly attenuate the increase in levels of ROS and MDA in 2-CE treated astrocytes (Fig. 3D and E). Moreover, pretreatment with DAS could also attenuate changes in mitochondrial membrane potential, Bax/Bcl-2, and cell apoptosis in 2-CE treated astrocytes (Fig. 3F–J). These results suggested that the induction of CYP2E1 in 2-CE treated rat astrocytes may result in ROS generation, and is further involved in the oxidative damage of these cells.

3.4. Role of ROS generation in the upregulated expression of CYP2E1 in 2-CE treated rat astrocytes

The cells were pretreated with 100 μM Vit C, a scavenger of ROS, 1 h before 2-CE exposure. Similar to the results achieved using DAS, pretreatment with Vit C could also markedly attenuate the increase in levels of ROS, MDA, Bax/Bcl-2 and CYP2E1 protein, as well as apoptosis rate in 2-CE treated rat astrocytes (Fig. 4A–H). In addition, treatment of rat astrocytes with DAS or Vit C alone did not markedly alter CYP2E1



	I	II	III	IV	V	VI	VII
30 mM 2-CE	-	-	-	+	+	+	+
SCH (μM)	-	-	10	-	0.1	1	10
DMSO	-	+	-	-	-	-	-

Fig. 6. Involvement of the ERK signaling pathway in 2-CE induced upregulation of CYP2E1 in primary cultured rat astrocytes.
(A) Images of western blotting analysis to determine levels of CYP2E1, SP1 and p-SP1 proteins in astrocytes. (B) Quantification of western blotting analysis to determine the level of p-SP1 protein in astrocytes. (C) Quantification of western blotting analysis to determine the level of SP1 protein in astrocytes. (D) Level of SP1 mRNA analyzed using real-time (RT)-PCR in astrocytes. (E) Quantification of western blotting analysis to determine the level of CYP2E1 protein in astrocytes. (F) Level of SP1 mRNA analyzed using real-time (RT)-PCR in astrocytes.
Cells in the exposure group and intervention groups were exposed to 30 mM 2-CE for 24 h. Cells in the intervention groups were pre-exposed with SCH 1 h followed by a treatment with 30 mM 2-CE for 24 h; SCH, SCH772984, a specific inhibitor of ERK1 or ERK2; p-SP1, phosphorylated SP1; Control, untreated cells.
Data are expressed as mean ± SD; n = 4; significance was determined using one-way ANOVA followed by SNK test. *, $p < 0.05$ versus the control group; #, $p < 0.05$ versus the 30 mM of 2-CE group.

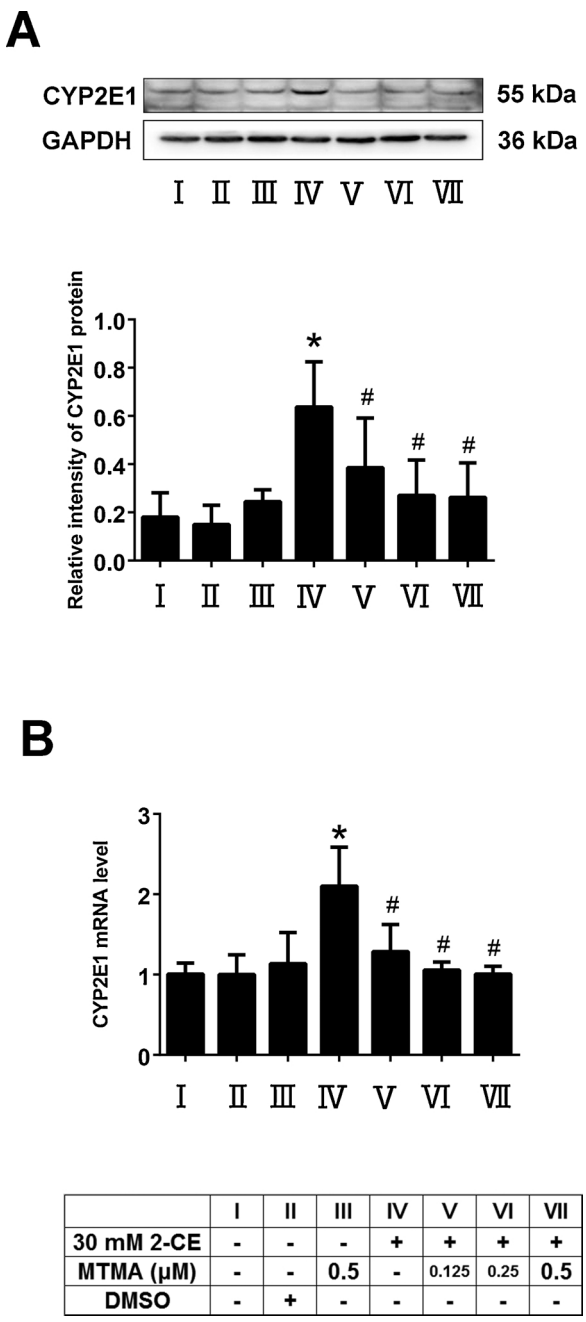
expression. Taken together, our findings demonstrate that the induction of CYP2E1 in 2-CE treated astrocytes is most probably mediated by ROS generation during the metabolism of 2-CE.

3.5. Involvement of the ERK1/2 and SP1 signaling pathways in the upregulation of CYP2E1 expression in 2-CE treated rat astrocytes

First, the effect of 2-CE on ERK1/2 phosphorylation in astrocytes was examined. Compared with the control group, the ratios of p-ERK1/2 and ERK1/2 protein levels (p-ERK/ERK) in cells upon treatment with 15 and 30 mM 2-CE increased obviously in a dose dependent manner, indicating that the ERK1/2 signaling pathway in rat astrocytes can be activated as a result of 2-CE exposure (Fig. 5A and B). Moreover, pretreatment with either DAS or Vit C was able to significantly suppress the increase of p-ERK/ERK in 2-CE treated astrocytes (Fig. 5C–F). Furthermore, pretreatment with SCH, a specific inhibitor of ERK1/2, significantly suppresses the increase in protein and mRNA levels of both CYP2E1 and SP1, and p-SP1 levels in 2-CE treated astrocytes (Fig. 6A–F). In addition, pretreatment with MTMA, a specific inhibitor of SP1, could also significantly suppress the increase in CYP2E1 mRNA and protein levels of 2-CE treated astrocytes (Fig. 7A and B).

3.6. Activation of the Nrf2 signaling pathway in 2-CE treated rat astrocytes

On the other hand, as an essential transcription factor, Nrf2 mediates the expression of antioxidant enzyme genes in response to ROS generation. Thus, the upregulated expression of Nrf2 and its target proteins may be an indicator of potential oxidative stress in the cells. In this study, we also probed the expression of Nrf2, HO-1 and γ-GCSc in 2-CE treated rat astrocytes. First, the expression profiles of Nrf2 and HO-1 protein were tracked in the cells upon treatment with 30 mM 2-CE at various points in time. Levels of both Nrf2 and HO-1 protein increased significantly within 24 h of 2-CE exposure, and a sustained higher level, compared with that of the control level, was observed even after 48 h (Fig. 8A and B). Additionally, compared with that of the control group, Nrf2 and HO-1 protein and mRNA levels and γ-GCSc mRNA levels were found to significantly increase in cells treated with 15 and 30 mM 2-CE (Fig. 8C–E). Meanwhile, the level of Nrf2 protein in 2-CE treated astrocytes increased in a dose-dependent manner, and the level of Nrf2 mRNA in the 7.5 mM 2-CE treated group was also significantly higher than that of the control group. The levels of γ-GCSc protein in the cells upon treatment with 30 mM 2-CE were also significantly elevated. Moreover, pretreatment with either DAS or Vit C



	I	II	III	IV	V	VI	VII
30 mM 2-CE	-	-	-	+	+	+	+
MTMA (μM)	-	-	0.5	-	0.125	0.25	0.5
DMSO	-	+	-	-	-	-	-

Fig. 7. Involvement of the SP1 signaling pathway in 2-CE induced upregulation of CYP2E1 in primary cultured rat astrocytes.

(A) Western blotting analysis to determine the level of CYP2E1 protein in astrocytes. (B) The level of CYP2E1 mRNA in astrocytes analyzed using real-time (RT)-PCR.

Cells in the exposure group and intervention groups were exposed to 30 mM 2-CE for 24 h. Cells in the intervention groups were pre-exposed with MTMA 1 h followed by a treatment with 30 mM 2-CE for 24 h; MTMA, Mithramycin A, a specific inhibitor of SP1; Control, untreated cells.

Data are expressed as mean ± SD; n = 4; significance was determined using one-way ANOVA followed by SNK test. *, $p < 0.05$ versus the control group; #, $p < 0.05$ versus the 30 mM of 2-CE group.

could significantly suppress the increase in protein and mRNA expression of Nrf2, HO-1 and γ-GCSc in 2-CE treated astrocytes. These results confirm the hypothesis that ROS could be generated during CYP2E1-mediated 2-CE metabolism, which may cause cellular oxidative stress, thereby activating the Nrf2 signaling pathway.

4. Discussion

Due to tight association with microvasculature, astrocytes may be the first site of 2-CE accumulation, and the primary target of 2-CE cytotoxicity in the brain. Brain edema is commonly classified into vasogenic and cytotoxic edema resulting from disruption in BBB and brain cells. Our previous studies have demonstrated that treatment with 2-CE could disturb mitochondrial function and glutamate metabolism in rat astrocytes, which might cause a collapse in maintenance of the ionic gradients across the cell membrane and clearance of glutamate in the extracellular space, leading to calcium overload and water excess in both glial cells and neurons (Sun et al., 2016a,b; Wang et al., 2018a,b). On the other hand, findings from our previous studies also demonstrated that the expression of matrix metalloproteinase-2 and -9 could be upregulated in 2-CE treated astrocytes, leading to disruption of BBB integrity (Sun et al., 2016a,b; Wang et al., 2017; Jin et al., 2019). Thus, understanding the role of CYP2E1 overexpression in 2-CE induced astrocyte cytotoxicity may contribute to uncover the mechanisms by which 1,2-DCE induces brain edema.

Current data indicate that the expression of CYP2E1 in 2-CE treated astrocytes are transcriptionally upregulated in a time and dose dependent manner. Thus, our findings are in line with that of previous reports, which indicate that CYP2E1 is readily available and inducible in astrocytes following mechanical or ischemic injury in rat brain or in cultured rat astrocytes, in response to treatment with ethanol or lipopolysaccharide (LPS) (Montoliu et al., 1995; Tindberg et al., 1996). It was recently reported that CYP2E1 can be induced in astrocytes, but not in microglia, in response to treatment with acrylonitrile (Caito et al., 2014). Additionally, a study by Tindberg (2003) found that CYP2E1 induction in astrocytes is observed within 12 h of treatment with phorbol ester, with a peak reached at 24 h, which decreases at 48 h, and returns to control level after 60–72 h. In contrast, CYP2E1 induction in these cells increases within 5 h, and peaks at 8–24 h in response to LPS exposure (Kelicen and Tindberg, 2004). These results indicate that the induction patterns of CYP2E1 in rat astrocytes are different based on the treatment agent used, among them LPS appears to cause the rapid and potent induction of CYP2E1.

Moreover, accumulating evidence indicates that CYP2E1 induction in ethanol-treated astrocytes are of toxicological interest, since CYP2E1-mediated ethanol metabolism may result in the production of acetaldehyde and ROS (Haorah et al., 2005; Zimatkin et al., 2006; Deng and Deitrich, 2008). In this study, it was found that treatment with 2-CE could increase the generation of ROS and lipid peroxides, decrease mitochondrial membrane potential, and induce the apoptosis of rat astrocytes. In our previous studies, treatment with 7.5–120 mM 2-CE was found to lead to the dose-dependent decrease in cell viability, mitochondrial membrane potential and non-protein sulfhydryl levels, which coincides with the increase of ROS levels in these cells. Taken together, our findings suggest that oxidative damage may play an essential role in the 2-CE-induced cytotoxicity of rat astrocytes. Furthermore, the results of this study indicate that ROS generation in 2-CE treated astrocytes seems to be dependent on CYP2E1 expression, since pretreatment with DAS, the inhibitor of CYP2E1 could suppress levels of ROS and lipid peroxides in these cells. Similar results have been reported in astrocytes treated with ethanol or LPS (Montoliu et al., 1995; Tindberg et al., 1996; Hao et al., 2010).

Another significant finding of this study is that the induction of CYP2E1 in 2-CE treated astrocytes may be dependent on ROS generation, since pretreatment with Vit C, a scavenger of ROS, can prevent the induction of CYP2E1 in these cells. Our data suggest that the CYP2E1-mediated metabolism of 2-CE may result in ROS generation that furthers CYP2E1 induction, finally lead to oxidative damage and cell apoptosis. Thus, ROS generation is not only a consequence of CYP2E1 induction in 2-CE treated astrocytes, but is also a mediator of 2-CE induced CYP2E1 upregulation. Our results are line with previous observations that pretreatment with vitamin C abrogates ethanol-

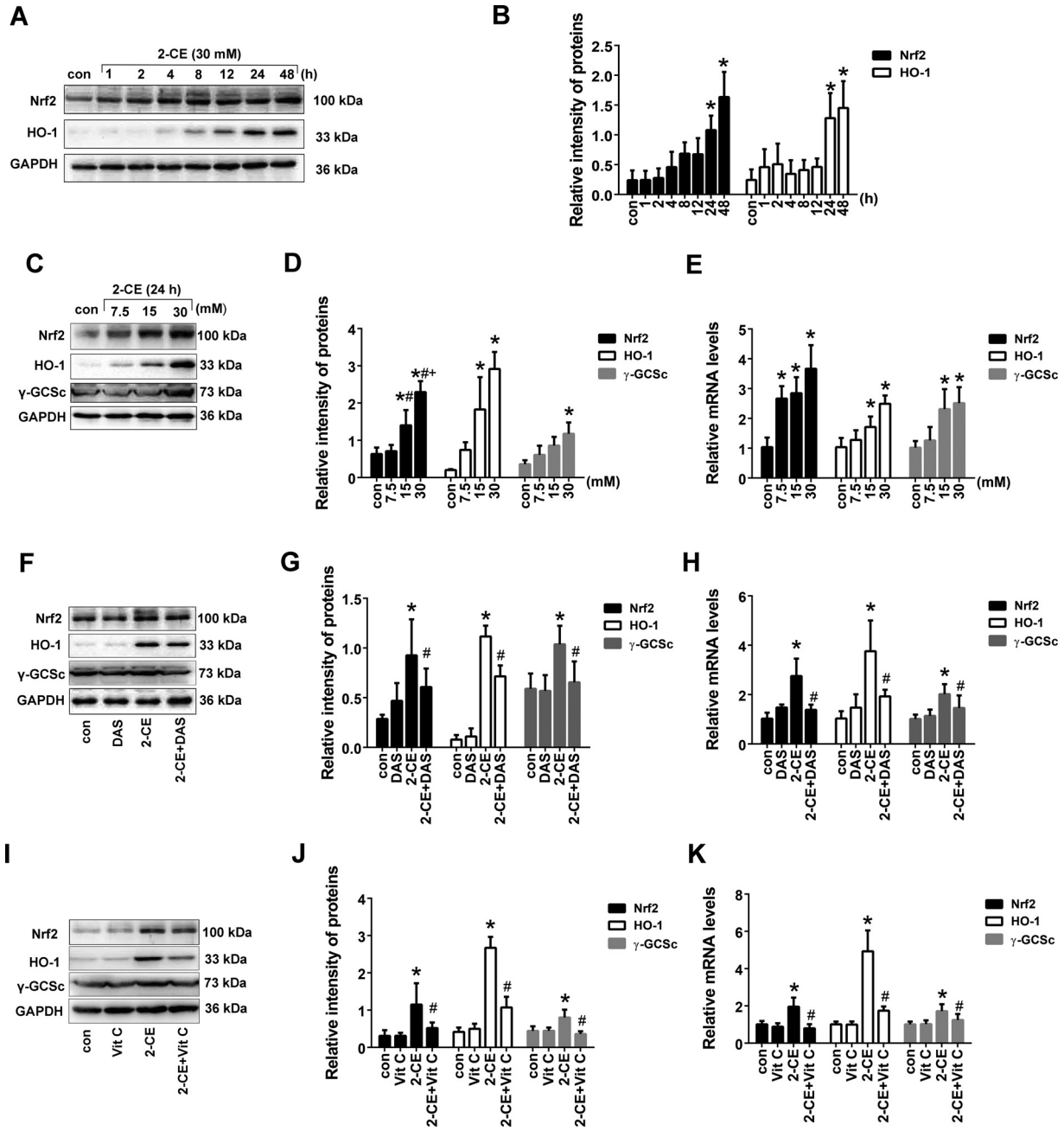


Fig. 8. Effects of 2-CE on the Nrf2 signaling pathway in primary cultured rat astrocytes.

(A, B) Western blotting analysis to determine the levels of Nrf2 and HO-1 proteins in astrocytes treated with 30 mM 2-CE for 1–48 h. (C, D) Western blotting analysis to determine the levels of Nrf2, HO-1 and γ-GCSc proteins in astrocytes exposed to 0, 7.5, 15, and 30 mM 2-CE for 24 h. (E) Levels of Nrf2, HO-1 and γ-GCSc mRNA in astrocytes exposed to 0, 7.5, 15, and 30 mM 2-CE for 24 h analyzed using real-time (RT)-PCR. (F, G) Western blotting analysis to determine the levels of Nrf2, HO-1 and γ-GCSc proteins in 2-CE exposed astrocytes affected by pretreatment with DAS. (H) Levels of Nrf2, HO-1 and γ-GCSc mRNA in astrocytes affected by pretreatment with DAS analyzed using real-time (RT)-PCR. (I, J) Western blotting analysis to determine the levels of Nrf2, HO-1 and γ-GCSc proteins in 2-CE exposed astrocytes affected by pretreatment with Vit C. (K) Levels of Nrf2, HO-1 and γ-GCSc mRNA in astrocytes affected by pretreatment with Vit C analyzed using real-time (RT)-PCR. 7.5 mM 2-CE, 15 mM 2-CE and 30 mM 2-CE represent cells treated with 7.5, 15 and 30 mM 2-CE for 24 h, respectively; DAS, 2-CE, and 2-CE + DAS represent cells treated with 100 μM DAS for 25 h, 30 mM 2-CE for 24 h, and 100 μM DAS for 1 h followed by treatment with 30 mM 2-CE for 24 h, respectively; Vit C, 2-CE, and 2-CE + Vit C represent cells treated with 100 μM Vit C for 25 h, 30 mM 2-CE for 24 h, and 100 μM Vit C for 1 h followed by a treatment with 30 mM 2-CE for 24 h, respectively; Control, untreated cells.

Data are expressed as mean ± SD; n = 4; significance was determined using one-way ANOVA followed by SNK test. *, $p < 0.05$ versus the control group; #, $p < 0.05$ versus the 30 mM of 2-CE group.

mediated cell toxicity and apoptosis, as well as CYP2E1 induction in astrocytes. Many reports indicate that CYP2E1 induction can be regulated through transcriptional, post-transcriptional, translational, and post-translational mechanisms (Novak and Woodcroft, 2000). Up to date, no information has been published on the mechanism of CYP2E1 induction in 2-CE treated astrocytes, and the current study further investigates the contribution of specific signaling molecules to its induction.

It is well accepted that ROS is one of the primary activators or amplifiers of cellular signaling pathways. Previous reports have shown

that ethanol can induce CYP2E1 expression by activating multiple signaling pathways and transcription factors (Vallés et al., 2004). A study by Tindberg (2003) has indicated that the phorbol ester-induced CYP2E1 expression in astrocytes relies on the protein kinase C (PKC) signaling pathway and tyrosine kinase activity. It has also been reported that LPS-mediated CYP2E1 induction in astrocytes is related to the activation of MEK3 and C/EBP-β (Kelicen and Tindberg, 2004). Thereafter, Jin et al. (2013) reported that the PKC/JNK/SP1 signaling pathway is involved in ethanol-mediated CYP2E1 induction in astrocytes. Consistent with these reports, our previous studies clearly demonstrate that pretreatment of n-acetyl-l-cysteine (NAC), an effective antioxidant, could decrease the phosphorylation of p38 MAPK, JNK1/2 and ERK1/2 in 2-CE treated astrocytes, suggesting that mitogen-associated protein kinase (MAPK) signaling pathways can be activated by ROS in these cells (Wang et al., 2017). Further, with regard to transcription factors involved in CYP2E1 induction, our previous results also indicate that NF-κB and AP-1 can be activated by 2-CE *via* the p38 MAPK signaling pathway in astrocytes (Wang et al., 2018a,b).

The results of this study reveal that the ERK1/2 and SP1 signaling pathway plays an essential role in CYP2E1 induction of 2-CE treated astrocytes. The levels of p-ERK1/2 and p-SP1, and the expression of SP1 increase dramatically upon treatment with 2-CE in rat astrocytes, while pretreatment with either DAS or Vit C could markedly suppress these changes. More importantly, pretreatment with the inhibitor of either ERK1/2 or SP1 could attenuate the increase in the expression of CYP2E1 mRNA and protein levels in 2-CE treated astrocytes. Taken together, our findings demonstrate that CYP2E1 expression is transcriptionally upregulated by ROS-mediated activation of the ERK1/2 and SP1 signaling pathways in 2-CE treated astrocytes. ERK1/2 acts as a pro-apoptotic signaling molecule that responds to DNA damage stimuli and is an upstream factor of SP1 (Qureshi et al., 2005; Lu and Xu, 2006). SP1 is an ubiquitously expressed transcription factor that can bind to the promoter of CYP2E1 and control the expression of CYP2E1 (Peng et al., 2000; Li et al., 2019). Na et al. (2017) recently reported that the upregulation of CYP2E1 by ethanol is mediated by the p38 MAPK and ERK1/2 signaling pathways, rather than by the JNK pathway in SH-SY5Y cells, a human dopaminergic neuron. However, Jin et al. (2013) reported that the JNK signaling pathway is involved in the regulation of CYP2E1 mRNA by ethanol in SVGA astrocytes. It has been reported that the p38 MAPK signaling pathway is involved in the regulation of CYP2E1 mRNA in response to LPS in astrocytes (Kelicen and Tindberg, 2004). Thus, it can be assumed that the signaling molecules involved in the regulation of CYP2E1 expression in different types of cells may be diverse.

One possible consequence of ROS generation is the activation of the Nrf2 signaling pathway, which in turn promotes the transcription of its downstream genes involved in the antioxidative response. In this study, we found that the expression of Nrf2, HO-1 and γ-GCSc in 2-CE treated astrocytes are transcriptionally upregulated, while pretreatment of these cells with either DAS or Vit C can markedly suppress these changes, indicating that the Nrf2 signaling pathway is activated by CYP2E1-mediated ROS generation. Further, our results indicate that the Nrf2 signaling pathway is sensitive to the intracellular status of oxidative stress, and can be used as an indicator of the adaptive response to oxidative stress.

Nrf2, a nuclear transcription factor, is confined to the cytoplasm by binding with Keap1 in the quiescent cells. Meanwhile, Keap1 is a cysteine-rich protein that is sensitive to ROS generation in cells. Consequently, the combination of Nrf2 and Keap1 is disrupted, while Nrf2 is released and translocated into the nucleus. Once in the nucleus, Nrf2 may initiate the transcription of antioxidant genes, and play a key role in the defense of oxidative damage (Jaiswal, 2004). HO-1 is the rate-limiting enzyme that is involved in the conversion of heme into biliverdin, carbon monoxide and free iron. Thus, HO-1 and its metabolic products are involved in the maintenance of cellular redox homeostasis during oxidative stress. GSH, the most important nonenzymatic antioxidant in cells, can be replenished by γ-GCSc mediated synthesis. Many studies have demonstrated that both HO-1 and γ-GCSc are the main effectors of the Nrf2 signaling pathway. Although the Nrf2 signaling pathway is activated in 2-CE treated rat astrocytes, the results of the present study indicate that normal compensatory mechanisms that combat oxidative stress appear to be insufficient in protecting the cells from injury.

Taken together, the results of this study indicate that the induction of CYP2E1 expression may play an important role in 2-CE induced oxidative damage of rat astrocytes, and that the generation of ROS during CYP2E1-mediated metabolism of 2-CE could result in CYP2E1 induction by activating the ERK1/2 and SP1 signaling pathways. This study provides novel evidence for clarifying the mechanisms that underlie 1,2-DCE associated brain edema. However, due to the variety of possibilities and insufficient data concerning the mechanism by which 2-CE treatment and CYP2E1 expression relate to oxidative stress, more research is needed to elucidate its exact pathogenesis.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (NSFC 81872573, 81573105, 81172644), Program for Liaoning Excellent Talents in University (LR2013038) and the Program for Liaoning Excellent Talents in University (LR2013038).

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