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Aquaporin-4 knockout enhances astrocyte toxicity induced by 1-methyl-4-phenylpyridinium ion and Lipopolysaccharide via increasing the expression of cytochrome P4502E1

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Abstract

The role of aquaporin-4 (AQP4) in the regulation of astrocytes function has been widely investigated. However, there is little information about its contribution to the drug metabolism enzymes such as Cytochrome P4502E1. In the present study, we investigated whether AQP4 is involved in the process of the cell damage caused by MPP^+ and LPS through regulating the expression of CYP2E1 in astrocytes. Compared to the wild-type, in primary astrocytes, AQP4 knockout increased the cell damage and the reactive oxygen species (ROS) production which were induced by MPP^+, LPS and ethanol. Notably, AQP4 knockout enhanced the up-regulation of the expression of CYP2E1 in astrocytes exposed to MPP^+, LPS and ethanol. Furthermore, Diallylsulphide (DAS), a CYP2E1 inhibitor, partially or almost abolished the cell injury and the ROS production of the astrocytes induced by MPP^+ and LPS. These findings indicate AQP4 protects astrocytes from the damage caused by MPP^+ and LPS through reducing the ROS production correlation to the diminished expression of CYP2E1.

Keywords

Aquaporin-4; astrocytes; CytochromeP4502E1; MPP^+; ROS

1. Introduction

Aquaporins (AQPs) are water channel proteins playing an important role in regulating water homeostasis under physiological and pathological conditions (Verkman, 2005). AQP4 is the most abundant isoform in adult brain (Badaut et al., 2002) and strongly expresses at borders between brain parenchyma and major fluid compartments, including astrocyte foot processes, glia limitans, ependyma, and subependymal cells (Badaut et al., 2002). It is involved in the regulation of brain volume homeostasis, the cerebrospinal fluid production, and the pathogenesis of brain edema.

Astrocytes are the most numerous non-neuronal cell-type in central nervous system and make up 50% of human brain volume. They perform several functions essential for normal

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neuronal activity, including producing trophic factors, regulating neurotransmitter and ion concentration, removing toxins and debris from the extracelluar space of CNS, and maintaining an extracellular milieu optimally suited for neuronal function. In addition, they are allowed by some specific enzyme systems to metabolize ammonia, glutamate, free radicals, xenobiotics, and metals, hence protecting the brain from the toxicity of these agents. On the other hand, evidence also showed that astrocytes play a vital part in the secondary amplification of cell injury in multiple neurodegenerative disorders such as Parkinson disease.

The cytochrome P450 enzymes are a superfamily of hemeproteins that serve as terminal oxidases in the mixed function oxidase system for metabolizing various endogenous substrates such as steroids, fatty acids, and xenobiotics including drugs and toxins (Guengerich et al., 1987). Multiple forms of CYPs are known to exist in hepatic and extrahepatic tissues, including brain (Walther et al., 1986). One of the isoforms that has received much attention during recent years is the ethanol-inducible CYP2E1, not only because of the capacity of this isoenzyme to metabolize ethanol to acetaldehyde, but also because of its role in the metabolic activation of a large number of toxicological compounds, including acetaminophen, various solvents, and nitrosamines (Ingelman-Sundberg et al., 1993). In addition, CYP2E1 has an apparently high rate of oxidase activity causing the formation of ROS during its catalytic cycle, which can initiate lipid peroxidation (Ekstrom and Ingelman-Sundberg, 1989) and damage cell membranes. Furthermore, the fundamental importance of CYP2E1 is suggested by its conservation from bacteria to plants, and to mammals and it does not exhibit the marked interindividual variation characteristic of other P450 enzymes. In the brain, CYP2E1 is constitutively expressed, e.g. in hippocampal pyramid neurons, cortical astrocytes, and endothelial cells (Hansson et al., 1990), and the enzyme has been found to be inducible and catalytically active in the brain (Montoliu et al., 1995; Tindberg et al., 1996; Warner and Gustafsson, 1994). It has been suggested that ethanol and LPS increased the expression of CYP2E1 and induced oxidative stress in astrocytes (Montoliu et al., 1995; Tindberg et al., 1996).

Increasing evidence showed that cytochrome P4502E1 is involved the MPTP-induced mouse model of PD (Pardini et al., 2008; Vaglini et al., 2004). However, whether MPTP influence the expression of CYP2E1 in astrocytes remains unclear. The neurotoxin MPP^+, a high-affinity inhibitor of mitochondrial complex I, is a metabolite of MPTP oxidation formed by monoamine oxidase-B in astrocytes (Tipton and Singer, 1993). And it is a common neurotoxin used to explore the alteration of astrocytic function (Tripanichkul et al., 2006). As a mitochondrial complex I inhibitor, it can produce astrocyte- and microglia-mediated neuroinflammation (Liss et al., 2005).

Previous studies focused on the role of AQP4 in the acute regulation of brain water homeostasis and there was little information regarding whether AQP4 participate in the regulation of drug metabolism enzymes. In the present study, we demonstrated that AQP4 knockout enhanced MPP^+ and LPS-induced astrocyte injury by up-regulating the expression of CYP2E1.

2. Materials and methods

2.1. Materials

MPP^+ and LPS were purchased from Sigma-Aldrich (St.Louis, MO, USA); ethanol was from Sinopharm (Shanghai, China). Antibody to CYP2E1 was obtained from Abcam (Cambridge, UK). Antibody to GFAP, rabbit TRITC and mouse FITC were obtained from Chemicon (Temecula, CA , USA). Trypase and MTT were purchased from Amresco (Solon, OH, USA). Fetal bovine serum was purchased from Sijiqing (Hangzhou, China). Trizol was

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from Invitrogenlife (Carlsbad, CA, USA). MLV and RNase inhibitor were purchased from Promega (Madison, WI, USA). DCFH-DA was purchased from Biyuntian (Shanghai, China), LDH diagnostic Kit was from JianCheng(Nanjing, China) and the other chemicals were all obtained from Sigma unless otherwise stated.

2.2. Generation of AQP4$^{-/-}$ mice

AQP4-knockout mice were generated as previously described (Fan et al., 2005). Mice were kept under environmentally controlled conditions (ambient temperature, 22±1°C; humidity, 40%) on a 12 hour light-dark cycle with food and water adlibitum. All the experiments were approved by IACUC (Institutional Animal Care and Use Committee) of Nanjing Medical University. And efforts were made to minimize animal suffering and to reduce the number of animals used for the experiments.

2.3. Immunofluorescence

After animals were perfused with 4% paraformaldehyde, and brains were dissected out and maintained in 4% paraformaldehyde overnight. Brains were cryopreserved in 30% sucrose in phosphate buffered solution and then stored at −70°C until used. Thirty micrometers sections were incubated overnight with rabbit anti-mouse CYP2E1 antibody (1:400), or rat anti-mouse GFAP (a special protein of astrocyte) antibody (1:300) at 4°C overnight, and then incubated in another cocktail solution containing goat anti-rabbit FITC (1:200) and goat anti-rat TRITC (1:200) antibodies at room temperature for 1 h. Specimens were observed under a confocal microscope (Zeiss Axiovert LSM510 ×250) for visualization and photography.

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2.4. Primary astrocyte culture

Confluent primary astrocyte culture were prepared from cerebral cortex of newborn of WT (AQP4$^{+/+}$) and KO (AQP4$^{-/-}$) mice and homozygous mutant mouse as described previously (Ivanova and Beyer, 2003), with minor modifications. Briefly, postnatal (P1-P2) mice were killed by rapid decapitation, the cerebral cortices were removed and separated from meninges and basal ganglia, and tissue was dissociated with 0.25% trypase at 37°C and terminated by Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS) and penicillin/streptomycin. After centrifugation at 1500 r.p.m. for 5 min, the cell pellets were resuspended and seeded on poly-lysine-coated flask. The cultures were maintained at 37°C in a humidified 5% CO2-95% air atmosphere. Culture medium was replaced 24 h later and then changed every 2-3 days. Before experiments, astrocytes were replated on poly-lysine-coated 6- or 24-well plates. Immunocytochemistry showed that about 98% of the cells stained positively for the astrocytic marker glial fibrillary acid protein (GFAP).

2.5. Cell viability and morphology

Cell viability was detected by MTT assay. Astrocytes were seeded in poly-lysine coated 96-well plates and treated with MPP^{+}, LPS, ethanol or the same volume of solvent for 48 h with or without pretreating the diallylsulphide (DAS) for 24 h. Then 20μl of 5 mg/ml MTT were added to cells, and cells were incubated at 37°C for another 4 h. The culture medium was discarded and 0.1 ml DMSO was used to dissolve the precipitate. The absorbance was measured at 570 nm using an Automated Microplated Reader ELx800 (BioTek). The cytotoxicity assessment was exhibited by cytotoxicity index, which was calculated as previously described (Sipe et al., 1998). The morphological changes were observed before adding MTT and images were taken under an inverted light microscope (Nikon, Japan, 22°C).

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2.6. Lactate dehydrogenase release

Astrocytes were seeded in poly-lysine pre-coated 24-well plates and treated with MPP^+, LPS or ethanol for 48 h, the lactate dehydrogenase (LDH) release in culture medium of $AQP4^{+/+}$ and $AQP4^{-/-}$ astrocytes was measured by using a LDH diagnostic Kit according to manufacturer's instructions. LDH activity was calculated by measuring absorbance at 490nm. The data were represented as a percentage of LDH release of control cells.

2.7. Measurement of reactive oxygen species generation

ROS production was monitored by flow cytometry using DCFH-DA. Cells were collected after different treatment for 48 h and washed with cold PBS, then resuspended in PBS and incubated for 30min at 37°C with 5mM DCFH-DA added. The fluorescence intensity was measured at 488 nm excitation and 525 nm emissions using a flow cytometer FACSCalibur. The data were analyzed using MODFIT and CELLQUEST software.

2.8. Reverse transcription PCR

Total RNA was prepared from astrocytes using Trizol reagent. The first-strand cDNA was synthesized with the mixture of total RNA, oligo (dT), MLV reverse transcription, RNase inhibitor, and dNTP in a final volume of 20 μl. The primers used for mouse CYP2E1 and GAPDH were as follows: CYP2E1 forward, GAGACACCGTGTTCCGAGGAT and reverse, TCCAGAAGACAGAGTCAGATCTCGATA; GAPDH forward, GTATGTCGTGGAGTCTACTGGTGTC and reverse, GGTGCAGGATGCATTGCTGACATTC. The thermal cycling conditions of the PCR were 94°C for 5 min, followed by 23-35 cycles for 20s at 94°C, 20s at 64°C, 1 min at 72°C, and a final extension at 72°C for 7 min. The amplified products were separated by electrophoresis in 0.5×Tris - acetate EDTA buffer with a 1.5% agarose gel containing 0.1μg/ml of ethidium bromide (6μl). The DNA bands were visualized and analyzed by Biosens sc 810 Gel Electrophoresis Image analytic system (Shanghai, China). The ratio of CYP2E1 mRNA and GAPDH mRNA was calculated.

2.9. Immunocytochemistry

Astrocytes were propagated in flasks for 13-15 days and were plated in poly-lysine coated 24-well plates at a density of 5×10^4 cells/well in growth medium. After adhesion for 24h, cells were incubated with MPP^+, LPS and ethanol for another 48h. For immunocytochemistry, the cells were fixed with 4% paraformaldehyde for 30 minutes and then permeabilized with 0.02% TritonX-100 containing 0.1% BSA for 1h. Immune complexes were formed by incubating cells with primary antibody CYP2E1 (1:400) overnight at 4°C followed by incubation with anti-rabbit secondary antibody (1:800) for 1h at 37°C and visualized by DAB. Cell morphology was monitored using an inverted light microscope (Nikon, Japan, 22°C) (Acquisition software: ACT-1). Control staining was performed without the primary antibodies.

After immunocytochemical staining, the astrocytes were captured at a magnification of ×250. Three visions from each well were randomly selected in order to analyze immunoreactive productions of CYP2E1 by using Leica Image Processing and Analysis System (Zhou et al., 2008). The mean optical intensity of the astrocytes was measured.

2.10. Statistical analysis

All data were presented as the mean ± SEM. Statistical analysis between $AQP4^{+/+}$ and $AQP4^{-/-}$ mice was performed with two-tailed indirect Student tests, using SPSS 10.0 for Windows (SPSS Inc, Chicago, IL, USA). And statistical analysis for multiple comparisons

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was performed by a one-way ANOVA test with Bonferroni's corrections. The level of statistical significance is defined as $P < 0.05$.

3. Results

3.1. AQP4 knockout increased cytotoxicity and the ROS production induced by MPP^+, LPS and ethanol in astrocytes

It has been reported that exposure to MPP^+, LPS and ethanol potentially result in cell damage or death, and all of them can increase the ROS production in astrocytes. We therefore investigated whether knockout AQP4 impact the cytotoxicity and ROS production in astrocytes induced by MPP^+, LPS and ethanol. The primary cultured astrocytes of $AQP4^{+/+}$ and $AQP4^{-/-}$ mice were treated with MPP^+ (100μM), LPS (1μg/ml) and ethanol (200mM) respectively for 48 h. Cell viability was measured by MTT analysis, the release of LDH into the cell medium measured by LDH diagnostic Kit and the level of ROS was detected using DCFH-DA by flow cytometer. The results showed that the cell viability induced by MPP^+, LPS or ethanol was significantly lower in $AQP4^{-/-}$ astrocytes than that in $AQP4^{+/+}$ genotype ($P < 0.05$) (Fig.1A $n = 4$), and the LDH release induced by MPP^+, LPS was significantly higher in $AQP4^{-/-}$ astrocytes than that in $AQP4^{+/+}$ genotype ($P<0.05$) (Fig.1B, n=3). Similarly, the ROS production in both $AQP4^{+/+}$ and $AQP4^{-/-}$ astrocytes exposed to MPP^+ (100μM), LPS (1μg/ml) or ethanol (200mM) were increased. In $AQP4^{+/+}$ astrocytes, MPP^+, LPS or ethanol increased the ROS production to 222% ($P < 0.05$) , 159% and 160% compared to the control group, while in $AQP4^{-/-}$ ones, they increased the ROS production to 585%, 291%, and 416% respectively ($P<0.01$) (Fig.1C, $n = 3$).

3.2. Expression of CYP2E1 in astrocytes in vivo

The expression of CYP2E1 in astrocytes of brain sections was detected by double immunofluorescence procedures. Brain sections stained with anti-CYP2E1 and anti-GFAP primary antibodies and the secondary antibodies labeled with FITC and TRITC showed positive staining for CYP2E1 (Fig.2A, 2D) and GFAP (Fig.2B, 2E). As shown in Fig.2 (C, F) astrocytes exhibit staining with anti-GFAP (red-TRITC) as well as CYP2E1 (green-FITC). This result suggested CYP2E1 express in astrocytes.

3.3. AQP4 knockout enhanced the up-regulation of the CYP2E1 expression after exposure to MPP^+, LPS and ethanol

The level of CYP2E1 mRNA was determined by densitometry as shown in Fig.3A. It showed that MPP^+, LPS and ethanol treatment produced a significant increase in CYP2E1 mRNA level in $AQP4^{-/-}$ astrocytes compared to $AQP4^{+/+}$ astrocytes ($P < 0.05$, $n = 4$).

Immunocytochemistry combined with semiquantitative analysis was carried out to investigate whether AQP4 knockout increased the CYP2E1 protein expression in astrocytes. As shown in Fig.3 (B, top), CYP2E1 immunostaining in $AQP4^{-/-}$ astrocytes was much stronger than that in $AQP4^{+/+}$ controls after exposure to MPP^+ (100μM), LPS (1μg/ml) and ethanol (200mM) respectively ($P < 0.05$ $n = 4$). Semiquatitative analysis showed that the mean optical density of CYP2E1 in $AQP4^{-/-}$ astrocytes after exposure to MPP^+ (100μM), LPS (1μg/ml) and ethanol (200mM) were increased to 125%, 121%, 149% respectively, which was higher than that in $AQP4^{+/+}$ astrocytes (115%, 114%, 131%) Fig.3 (B, bottom) ($p < 0.05$ $n = 4$).

3.4. CYP2E1 inhibitor ameliorated the cell damage and the ROS production of the astrocytes induced by MPP^+ and LPS

To investigate the role of CYP2E1 in the cytotoxicity and the ROS elevation of astrocytes induced by MPP^+ and LPS, the diallylsulphide (DAS), a CYP2E1 inhibitor, was used. After

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preincubation with DAS for 24 h, the primary astrocytes were treated with MPP^+ (100μM), LPS (1μg/ml) and ethanol (200mM) for additional 48 h. As shown in Fig.4 (A, top), the astrocytes in PBS group possessed mainly rounded or oval somata and exhibited extensive neuritic outgrowth. After exposure to MPP^+, LPS and ethanol for 48 h, the somata of astrocytes became more asperous with shrunken morohologies, and their neuritic outgrowths were greatly reduced in size and decreased in number. The cytotoxicity of astrocytes was measured by MTT assay. As shown in Figure.4 (A, bottom), the number of the primary astrocytes treated with MPP^+, LPS and ethanol also decreased, which consisted with the morphological changes. Moreover, the morphological changes and the decreased cell number in $AQP4^{-/-}$ astrocytes were more significant than those in $AQP4^{+/+}$ astrocytes. When astrocytes were pretreated with DAS, the cell damage and the decreased cell number induced by MPP^+, LPS and ethanol were ameliorated (Fig.4A, n=4), and the ROS production induced by MPP^+ and LPS was attenuated as well (Fig.4B, n=3). It should be noticed that astrocytes treated with DAS alone, neither the cell damage nor the ROS generation changed. The data imply that DAS can partially or almost abolish the ROS production and the cell damage in astrocytes induced by MPP^+, LPS and ethanol.

4. Discussion

AQP4 is one of the most predominant water channel proteins in the central nervous system, and it mainly locates on the astrocytes membrane. Astrocyte plays a crucial role in maintaining a proper environment for neuronal activity, and participates in the course of the drug metabolism and the detoxification of exogenous substances. Our previous studies have shown that AQP4 plays a vital role in modulating astrocytic function (Fan et al., 2005; Zeng et al., 2007; Fan et al., 2007; kong et al., 2008; Li et al., 2009). In the present study, we showed that the cell viability of astrocytes treated with MPP^+, LPS and ethanol is significantly lower in $AOP^{-/-}$ than that in $AQP4^{+/+}$ and the LDH release of astrocyte treated with MPP^+, LPS was significantly higher in $AOP^{-/-}$ than that in $AQP4^{+/+}$, which suggested AQP4 knockout increased the cytotoxicity of these toxins (Fig.1). It has been reported that ethanol, LPS and MPP^+ can increase the ROS production in astrocytes (Montoliu et al., 1995; Sun et al., 2008; Tindberg et al., 1996). Therefore, we investigated the difference of ROS production induced by MPP^+, LPS and ethanol between $AQP4^{+/+}$ and $AQP4^{-/-}$ astrocytes. The results showed that AQP4 knockout enhanced the ROS production in astrocytes induced by MPP^+, LPS and ethanol treatment (Fig.1B). These results suggested AQP4 can protect astrocytes against the damage induced by MPP^+, LPS and ethanol via reducing the ROS production.

CYP2E1 was the first isoform of cytochrome P450 enzymes identified as the microsomal ethanol oxidizing system (Lieber, 1990), and it is an effective generator of reactive oxygen species such as the superoxide anion radical and hydrogen peroxide (Lu and Cederbaum, 2008; Montoliu et al., 1995). Ethanol induction of CYP2E1 has been shown to result in the increased oxygen radical formation, the oxidative stress and the lipid peroxidation in rat brain and the cultured astrocytes. So we hypothesize that the enhancement of the cell damage caused by MPP^+, LPS and ethanol in $AQP4^{-/-}$ astrocytes is due to the up-regulation of CYP2E1 which further increase the production of ROS. Next we provided the evidence of the presence of CYP2E1 in astrocytes in brain slices, which have supported the earlier studies in indicating widespread immunoreactivity in the different cell types of the brain (Hansson et al., 1990), and the occurrence of CYP2E1 in cultured astrocytes (Montoliu et al., 1995; Tindberg, 2003; Tindberg et al., 1996).

Several fold increase in the protein expression of CYP2E1 and the enzyme activity in cultured neuronal and glial cells following treatment of ethanol have shown that the responsiveness to ethanol is retained in the primary cultures. Montoliu et al. (Montoliu et al.,

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1995) have also shown the induction of CYP2E1 in primary cultures of astrocytes. And our results showed knockout AQP4 enhanced the induction of CYP2E1 induced by ethanol in primary astrocytes (Fig.3A, B). The data suggest AQP4 is involved in the metabolism of the ethanol in the microsomal ethanol oxidizing system in astrocytes.

Astrocytes are the site of the bioactivation of the parkinsonism-inducing agent MPTP into its toxic MPP^+ (Denton and Howard, 1987). In this study, we found MPP^+ increased the expression of CYP2E1 in primary asrtocytes, which suggests CYP2E1 is likely to be involved in the astrocyte damage caused by MPP^+. It has been reported LPS can induce the CYP2E1 expression in rat and gerbil astrocytes, which suggested CYP2E1 may play a role in astrocytes during inflammation in the brain. We investigated the effect of AQP4 on the expression of CYP2E1 in astrocytes treated with LPS and MPP^+, and the results indicated that a significant increase in mRNA and protein expression of CYP2E1 in $AQP4^{-/-}$ astrocytes compared to the corresponding $AQP4^{+/+}$ astrocytes (Fig.3A, 3B). And the ethanol was used as a positive control to test the induction of CYP2E1 in astrocytes. These results indicted that the increase of cell damage induced by MPP^+, LPS and ethanol in $AQP4^{-/-}$ astrocyte may be partially due to the increased expression of CYP2E1.

CYP2E1 is well known for its roles in chemical detoxification/activation, fatty acid metabolism, metabolism of acetone to gluconeogenic intermediates, and free radical production. This enzyme is inhibited by several compounds including DDC, aldehydes, DAS and PIC (Lieber, 1997). In order to determine whether the enhanced cell damage in the $AQP4^{-/-}$ astrocytes is due to the increased expression of CYP2E1 which can produce the ROS in the presence of some substrates, DAS, a CYP2E1 inhibitor, was used. As expected, DAS decreased the cell injury and ROS generation caused by MPP^+ and LPS in both $AQP4^{+/+}$ and $AQP4^{-/-}$ astrocytes (Fig.4A, 4B).

In summary, this study leads to several important conclusions. First, we report for the first time that MPP^+ induces the CYP2E1 expression in the primary astrocytes. Therefore, CYP2E1 in astrocytes is likely to be involved in the metabolism of the endogenous and exogenous compounds, which can cause neurodegenerative diseases such as Parkinson disease. Second, AQP4 knockout enhances the cell damage in astrocytes mediated by MPP^+ and LPS through inducing the CYP2E1 expression. Taken together, this study provides new insight into the understanding of AQP4 in the drug metabolism enzymes in the neuroinflammation and the neurodegenerative diseases.

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Abbreviation

AQP4	Aquaporin-4
CYP2E1	cytochrome P4502E1
ROS	reactive oxygen species
LPS	Lipopolysaccharide

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MPP^+	1-methyl-4-phenylpyridinium ion
MAO-B	monoamine oxidase-B
GFAP	glial fibrillary acid protein
DAS	diallylsulphide
MTT	3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide
LDH	Lactate dehydrogenase
DDC	diethyldithiocarbamate
PIC	phenylethylisothiocyanate
PD	Parkinson's disease
DMEM	Dulbecco's modified Eagle's medium
DAB	Diaminobenzidine

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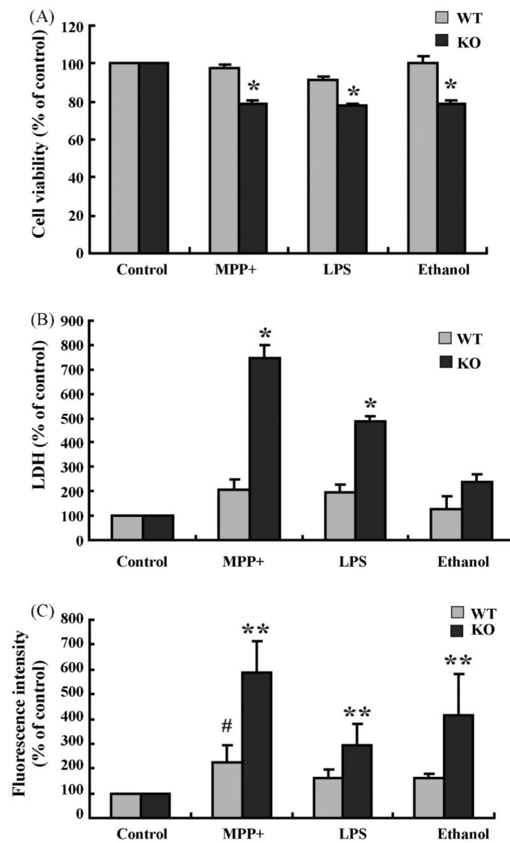

Fig.1.

Effects of MPP^+, LPS and ethanol on the cell injure and ROS production in primary astrocytes of WT ($AQP4^{+/+}$) and KO ($AQP4^{-/-}$). (A) Cell viability of WT ($AQP4^{+/+}$) and KO ($AQP4^{-/-}$) astrocytes when exposed to MPP^+ (100μM), LPS (1μg/ml) and ethanol (200mM) for 48h. Values are mean ± SEM from four experiments performed. * $P < 0.05$ vs astrocytes from $AQP4^{-/-}$ controls (black column), n = 4. (B) LDH release in the culture medium of $AQP4^{+/+}$ and $AQP4^{-/-}$ astrocytes after exposure to MPP^+ (100μM), LPS (1μg/ml) and ethanol (200mM) for 48h. * $P < 0.05$ vs astrocytes from $AQP4^{-/-}$ controls (black column), n = 3. (C) ROS production of WT ($AQP4^{+/+}$) and KO ($AQP4^{-/-}$) astrocytes after incubation with MPP^+ (100μM), LPS (1μg/ml) and ethanol (200mM) for 48h. ** $P < 0.01$ vs astrocytes from $AQP4^{-/-}$ mice controls (black column); # $P < 0.05$ vs astrocytes from $AQP4^{+/+}$ mice controls (grey column), n = 3.

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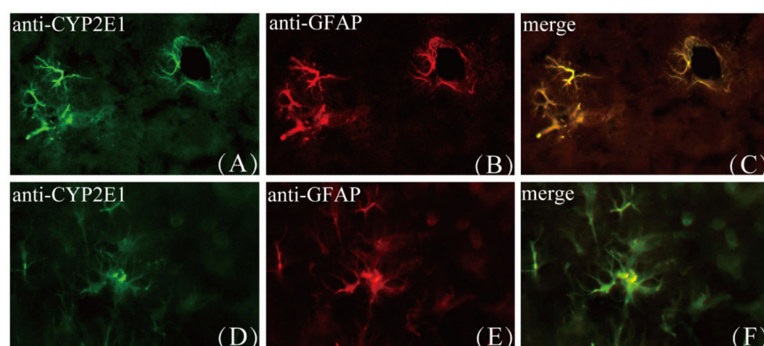

Fig.2.
Double immunofluorescence of GFAP and CYP2E1 of brain sections. (A) (D) showed that the cells are positive for CYP2E1 (green-FITC). (B) (E) showed immunoreactivity in same cells with anti-GFAP (red-TRITC), a astrocyte marker, and (C) (F) represented an overlay of the two images(250×).

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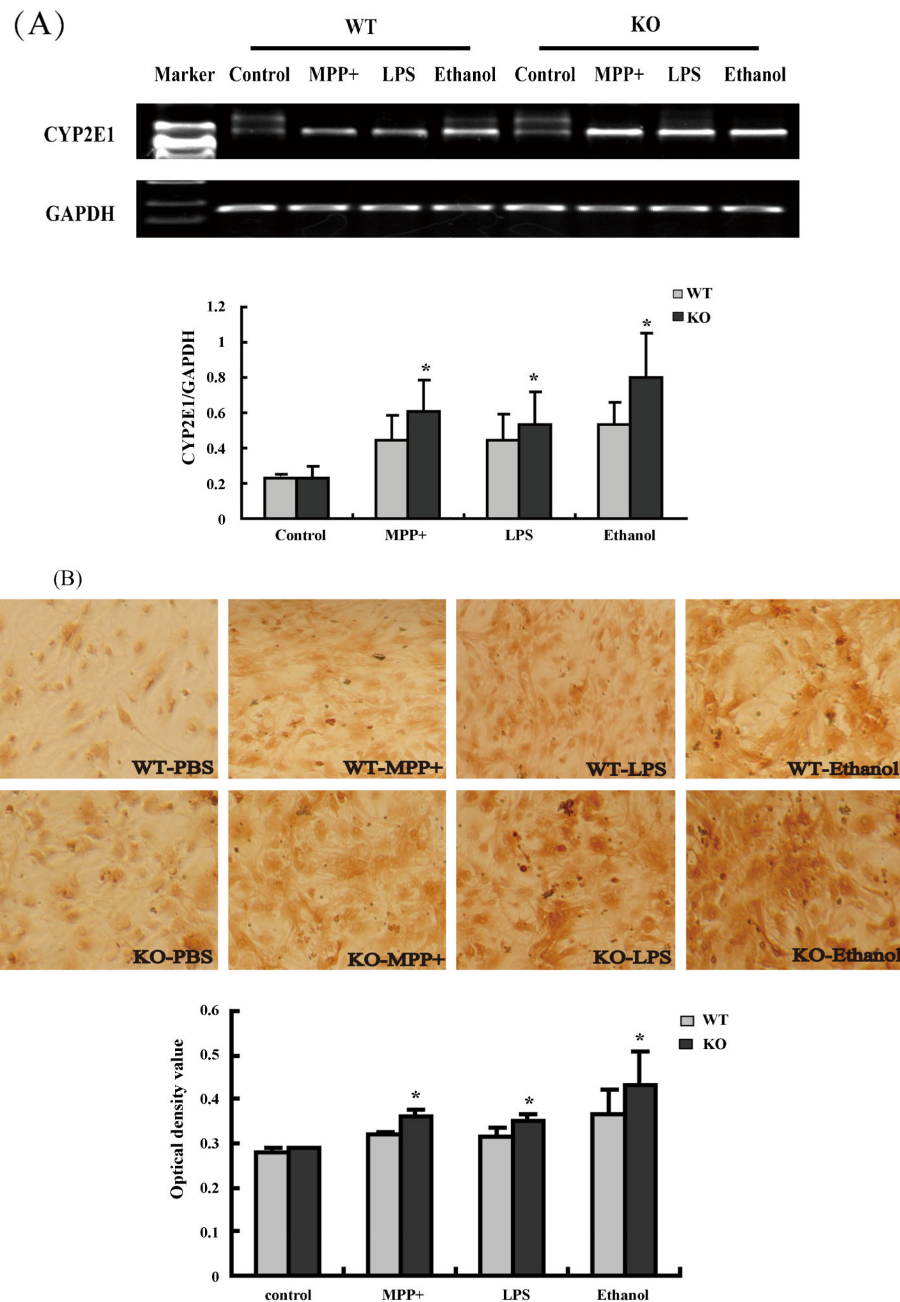

Fig.3.

Effects of MPP^+, LPS and ethanol on the CYP2E1 expression in primary astrocytes of WT ($AQP4^{+/+}$) and KO ($AQP4^{-/-}$). (A) CYP2E1 mRNA expression after exposure to MPP^+ (100μM), LPS (1μg/ml) and ethanol (200mM) for 48h in WT ($AQP4^{+/+}$) and KO ($AQP4^{-/-}$) astrocytes. The CYP2E1 mRNA level was normalized based on GAPDH, and the data are mean ± SEM from four independent experiments. * $P < 0.05$ vs astrocytes from $AQP4^{-/-}$ mice controls (black column), n = 4. (B) CYP2E1 protein expression after exposure to MPP^+ (100μM), LPS (1μg/ml) and ethanol (200mM) for 48h in WT ($AQP4^{+/+}$) and KO ($AQP4^{-/-}$) astrocytes. Semi-quantitative analysis showed mean optical density of CYP2E1-immunoreactive products in $AQP4^{+/+}$ and $AQP4^{-/-}$ astrocytes. And the data are mean ±

SEM from four independent experiments. * $P < 0.05$ vs astrocytes from $AQP4^{-/-}$ mice controls (black column), $n = 4$.

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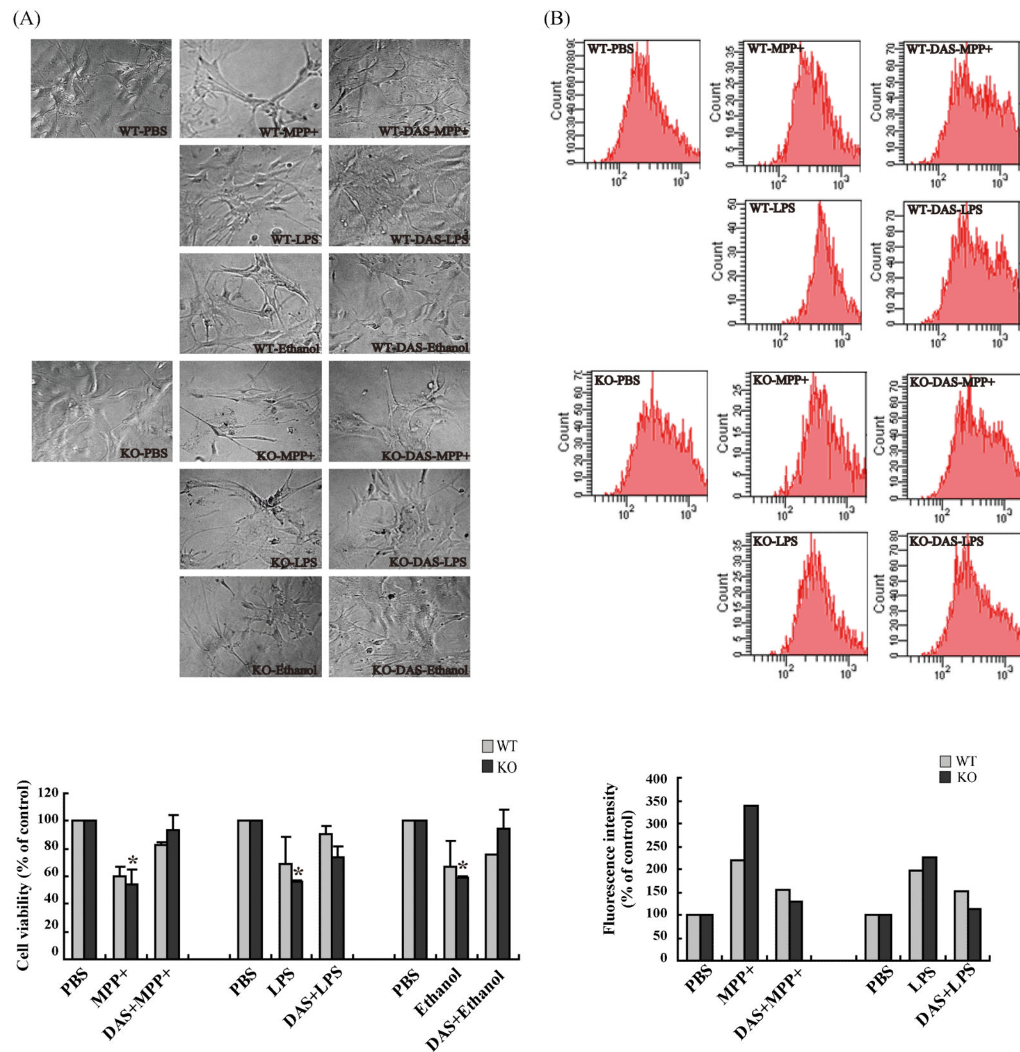

Fig.4.

DAS ameliorated the cell damage and the ROS production in primary astrocytes of WT ($AQP4^{+/+}$) and KO ($AQP4^{-/-}$). (A) DAS ameliorated the cell damage of the astrocytes induced by MPP^+ (100μM) and LPS (1μg/ml), the morphological analyse was taken under bright field (250×, top) and the cell viability was taken by MTT assay (bottom). * $P < 0.05$ vs astrocytes from $AQP4^{-/-}$ mice controls (black column), n = 4. (B) DAS reduced the ROS production induced by MPP^+ (100μM) and LPS (1μg/ml), we have done three independence experiments, and each result presents the same trend.