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Misoprostol Reverse Hippocampal Neuron Cyclooxygenase-2 Downstream Signaling Imbalance in Aluminum-Overload Rats



Yuanxin Guo¹, Wenjuan Lei², Jianfeng Wang², Xinyue Hu², Yuling Wei², Chaonan Ji² and Junqing Yang^{2,*}

¹Department of Pharmacy, Chengdu Fifth People's Hospital, Chengdu, Sichuan 611130, China;

²Department of Pharmacology, Chongqing Medical University, Key Laboratory of Biochemistry and Molecular Pharmacology, Chongqing 400016, P.R. China



Junqing Yang

Abstract: Although COX-2 inhibition in animal models of neurodegenerative diseases has shown neuroprotection, recent studies have revealed some serious side effects (ulcers, bleeding, fatal cerebrovascular diseases etc.) and the limited benefits of COX-2 inhibitors. A more focused approach is necessary to explore the therapeutic effect of the COX downstream signaling pathway in neurological research. The aim of this study was to explore the alterations of the PGES-PGE₂-EP signal pathway and the effect of misoprostol on neurodegeneration by chronic aluminum-overload in rats. Adult rats were treated by intragastric administration of aluminum gluconate. The PGE₂ content and expression of PGES and EPs in the hippocampi of rats were detected using ELISA, q-PCR and Western blot analysis, respectively. The content of malondialdehyde (MDA) and the activity of superoxide dismutase (SOD) in the rat hippocampi were also detected. The misoprostol treatment dose-dependently improved spatial learning and memory function as well as healing after hippocampal neuron damage induced by chronic aluminum-overload in rats. Meanwhile, the administration of misoprostol resulted in a decrease in the PGE₂ level and down-regulation of the mPGES-1, EP₂ and EP₄ expression levels, while there was a dose-dependent up-regulation of EP₃ expression. These results suggest that misoprostol possesses a neuroprotective property, and the mechanism involves affecting the EP₃ level and reducing the endogenous production of PGE₂ through a negative feedback mechanism, increasing the EP₃ expression level, decreasing the EP₂ and EP₄ expression levels, and rebuilding the mPGES-1-PGE₂-EP_{1,4} signal pathway balance. In this way, misoprostol has a counteractive effect on oxidant stress and inflammation in the central nervous system. The PGES-PGE₂-EPs signaling pathway is a potential therapeutic strategy for treating neurodegeneration in patients.

Keywords: Alzheimer disease, neurodegeneration, misoprostol, PGE₂, PGES, EPs receptor.

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INTRODUCTION

Currently, there are increases of the global aging population and neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD), which have become major problems that seriously diminished elderly health and life quality [1-3].

Although the mechanisms of AD has not been completely elucidated, a large number of drug related epidemiological studies revealed that long-term use of nonsteroidal anti-inflammatory drug (NSAIDs) was able to inhibit cyclooxygenase 2 (COX-2) expression, and consequently decrease the risk of people suffering from AD [4-6].

Among the known subtypes of COX, COX-2, which is structurally different from COX-1, is inducible such that its expression level and catalytic activity rapidly increase after a variety of noxious stimulations [7, 8]. The use of selective

COX-2 inhibitor could down-regulate COX-2 expression, reducing the production of deleterious prostaglandins, and play a distinct role in nerve protection. This result demonstrates that COX-2 could be a drug target for preventing diseases such as AD [9-11]. However, many experiments found that although sustained administration of selective COX-2 inhibitor could prevent mild cognitive impairment caused by acute or chronic brain injury disease, it could not heal severe AD patients or reverse their cognitive defects. Additionally, the long-term use of these drugs increase the risk of bleeding, gastrointestinal ulcers, fatal heart and cerebrovascular side effects [12, 13]. Therefore, it was suggested that COX-2 downstream signaling needs to be further studied.

The COX-2 signaling cascades consist of prostaglandin terminal synthase (PGS), prostaglandins (PGs) and receptors. The effector of COX-2 mediated neurotoxicity is prostaglandin E₂ (PGE₂), known to injure the hippocampus and cortical neurons *in vitro*. Paradoxically, PGE₂ has been shown to attenuate cultured neurons from β -amyloid and glutamate neurotoxicity as well as to protect cerebral excitotoxic or ischemic lesions *in vivo*. Therefore, PGE₂ may exert functionally opposing effects [14, 15]. PGE₂ is subsequently

*Address correspondence to this author at the Department of Pharmacology, Chongqing Medical University, Chongqing 400010, China; Tel: +86-23-68485161; Fax: +86-23-68485161; E-mail: 113927371@qq.com

catalyzed by the action of COX and prostaglandin E₂ synthase (PGES) from arachidonic acid. PGES is the terminal enzyme that catalyzes PGE₂ [6]. Currently, three subtypes have been found, including cPGES (cytosolic), mPGES-1 (microsomal) and mPGES-2. In general, cPGES and mPGES-2 are functionally coupled with COX-1 for mediating basal secretion of PGE₂ and ubiquitous expression that is stably expressed in the brain and other tissues. While mPGES-1 is inducible and functionally coupled with COX-2 in certain locations, its expression is up-regulated following a variety of cerebral insults.

The action of PGE₂ at four different prostaglandins receptors (EP₁₋₄) may explain its variable actions, such as inflammation, asthma, cancer, vasoconstriction and blood pressure regulation [15-18]. Ligation of EP₁ exerts a toxic effect in models of cerebral ischemia with phosphoinositide turnover and elevated intracellular [Ca²⁺]. Stimulation of the EP₂ and EP₄ rescued hippocampus and cortical neurons, in addition to *in vitro* and *in vivo* models of neurodegenerative disease and cerebral ischemia/reperfusion, is dependent on cAMP signaling. Activation of EP₃ attenuates motor neuron necrosis in the ALS model via PI3K/AKT. These results demonstrate the presence of a more complexity PGE₂ networks in the COX-2 signaling cascades, and these results were observed in different tissue sources, methodologies and animal models [16, 18].

The specific mechanism of brain injury and neuronal degeneration is not well understood because there have not been any reports about the correlation between biological properties and changes that damage features and (or) the protective prostaglandin pathway and brain damage and neurological degenerative disease [19]. Our interest is in knowing how misoprostol, an EP₃ agonist, affects the COX-2 downstream signaling cascades in aluminum-overload rats [20-23]. Furthermore, this study revealed that the COX-2 downstream prostaglandin signaling may be beneficial, including the modulation of a specific prostaglandin synthase or receptor for a superior therapeutic intervention compared with a generic block of the entire COX-2 signaling cascades [13, 14].

METHODS AND MATERIALS

Animals

This study was conducted in accordance with the Animal Laboratory Administrative Center and the Institutional Ethics Committee at Chongqing Medical University. Seventy-five Sprague Dawley male rats, weighing 200-250 g, (purchased from the Animal Laboratory Center of Chongqing Medical University), were randomly divided into five groups, including a control group, an aluminum-treated group (Al-overload group), and three misoprostol-treated groups (M-30, M-60 and M-120 for 30, 60 and 120 µg·kg⁻¹ misoprostol, respectively). Every group had fifteen rats.

Agents

Sodium gluconate (Chengdu Ke Long Chemical Technology Co., Ltd., China) and AlCl₃·6H₂O (Sinopharm Chemical Reagent Co., Ltd., China) were of analytical grade. Misoprostol (NPIL Pharmaceutical Co., Ltd, UK) was pre-

pared with 0.5% sodium carboxy methyl cellulose(CMC-Na) before use.

Establishment of Animal Models

The animals were first screened by the Morris Water Maze test to eliminate rats with outlining low scores. All groups were treated with intragastric administration once per day, five days per week for 20 continuous weeks. The Al-overload group received aluminum gluconate(Al³⁺ 200 mg/kg) intragastric administration, followed by an administration of 0.5% CMC-Na two hours later. The Al+M-30, Al+M-60 and Al+M-120 groups received intragastric administrations of 30, 60 and 120 µg·kg⁻¹ misoprostol, respectively, two hours after administration of aluminum gluconate. The control group received an equal volume of sodium gluconate followed by an equal volume of 0.5% CMC-Na two hours later [11, 21].

Morris Water Maze Tests

After terminating 20 weeks administration of aluminum-gluconate, we used the Morris water maze (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing) to evaluate spatial learning and memory (SLM) function alterations. Procedural details were previously reported [11, 21]. Briefly, the each group rats were permitted to study how to navigate the water maze for four consecutive days to measure the rats learning function. From each of four starting positions randomly, rats were put into the water facing the maze wall. The latency was recorded and the test was terminated when the rats found the platform in 180 s. On the 5th day, the rats memory function was tested. The animals in each group had a probe trial. The exploring time that animals passed through the previously platform location was considered the time for latency [11, 21].

HISTOPATHOLOGICAL EXAMINATION

After completing the behavioural assessments, tissue specimens were taken from the brain for histopathological examination. Four rats from each group were anesthetized and perfused with 10% neutral buffer formalin in phosphate buffered. The cerebral tissue was harvested and stored in the 4% paraformaldehyde. After embedding, 4-5µm sections were obtained, which were stained by Hematoxylin-Eosin. Morphologic alteration of hippocampal nerve cells were examined by light microscopy. The dead neurons and injured nerve cells were characterized by their nucleoli disappearance, pyknotic nuclei and acidophilic(eosinophilic) cytoplasm under light microscopy [24]. For neuron counting, ten consecutive fields were selected from the CA1 subfield of hippocampal neuron. Neurons with a distinct nucleolus were regarded as intact neurons. Neurons counting were under the microscope at 400× magnification. The extent of neuron death was calculated [11, 21].

ENDOGENOUS OXIDANTS AND ANTIOXIDANTS ASSAY

After completing the behavioural assessments, four rats from each group were anaesthetized and decapitated. The hippocampus were dissected out and homogenized with

normal saline at 4 °C, then centrifuged at 10,000× g for 10 mins. The supernatant was used for MDA and SOD assays in accordance to the method instruction of assay kit(Jiancheng Bioengineering Ltd, Nanjing, China).

PROSTAGLANDIN E₂ ASSAYS

After completing the behavioural assessments, four rats from each group were anaesthetized and decapitated. The hippocampus were isolated and weighed and quickly frozen with liquid nitrogen, then added with homogenization buffer (0.1 M PBS containing 10µM indometacin and 1mM EDTA) and centrifuged at 8,000× g at 4°C for 20 mins. The supernatant was collected and diluted. The concentration of dissected hippocampus was estimated in accordance to the manual of the ELISA kit (Cayman Chemical, Ann Arbor, MI). To avoid the production of other prostanoids from oxidation, the indometacin, a COX-1/2 nonselective inhibitor, was added in accordance to the instructions of the ELISA kit.

RNA Extraction and Real-Time PCR (qRT-PCR)

qRT-PCR was performed using the Real-time PCR CFX system (Bio-rad, USA). Total RNA from the hippocampus of each group was extracted using an RNAiso Plus isolation kit(TAKARA Bio CO., LTD, Dalian) in accordance to manufacturer's protocol. The information of forward and reverse primers are show in (Table 1). $\Delta\Delta C_T$ method was employed to count their expression with GAPDH as the endogenous control($\Delta\Delta C_T = \Delta C_T$ (Al-treated group)- ΔC_T (control group)). The amplification conditions were 3 min at 95 °C, followed by 40 cycles of 10 sec at 95 °C, 15sec at Annealing Temperature (Table 1) and 5 sec at 65 °C. All primers used were synthesized by ShanghaiSangon Company.

Western Blot Analysis

The hippocampus were dissected out and added by homogenization in tissue lysate solution (Beyotime Institute Biotechnology, Shanghai), followed by sonication and cen-

trifugation at 14,000×g at 4°C for 10 mins. The protein concentration of the supernatants was detected using the BCA method in accordance to the manual of the protein detection kit (Beyotime Institute Biotechnology, Shanghai). Forty micrograms of protein were separated by SDS-PAGE in 10% separating gels and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The blots were incubated at 4°C with primary antibodies measured against mPGES-1(1:400), EP₂ (1:400), EP₄(1:400) , EP₃(1:300), (Cayman Chemical, Ann Arbor, MI) and β -actin(1:1000) (Zhongshan Goldbridge Technology Co., Ltd., Beijing) overnight, then incubated with horseradish peroxidase-conjugated secondary antibody. The immunoreactive protein bands were visualized with ECL Western blot detection reagents (Pierce).The optical density of bands were detected by BioRad gel imaging and Image J software. The protein levels of mPGES-1 and EPs were estimated as ratios of the corresponding β -actin.

Statistical Analysis

The data were reported as mean \pm SD. Statistical analysis was performed on SPSS 16.0 (SPSS Inc. Chicago, US). Within-group variances were evaluated by Dunnett's t-test. The data with P < 0.05 were considered to be statistically significant.

RESULTS

Spatial Learning and Memory Function Alteration

Throughout the twenty continuous weeks of experiments, ten rats died(two rats in control group, four rats in Al-overload group, two rats in M-30 group, one rat in M-60 group and one rat in M-120 group). However, autopsy results from the dead rats showed no obvious abnormality in the organs or peripheral tissue.

The Morris water maze demonstrated that during the acquisition trials, there was no discrepancy in behavior between the groups. However, in contrast to the control group, there was a change in the spatial learning function test from d3 to d4, and a latency for Al-overload group to learn to

Table 1. Primer sequences of mPGES-1, EP₂, EP₃ and EP₄ mRNA for Real-time PCR analysis.

Gene	Primer Sequence (5' to 3')	Amplicon Length	Annealing Temperature
GADPH	F: ACAGCAACAGGGTGGTGGAC R: TTTGAGGGTGCAGCGAACTT	250 bp	63.5 °C
mPGES-1	F: GTGATGGAGAACAGCCAGGT R: CAAGGAAGAGGAAGGGGTAGAT	241 bp	63.5 °C
EP2	F: CGGACACCCTTACTTCTACAGG R: AGAGCAAGGAGACCCCATAGAT	86 bp	57.9 °C
EP3	F: GTGTACTGTCCGTCTGCTGGTC R: TCCGCTTCAGTTGTTTCATC	167 bp	62.3 °C
EP4	F: CATTGTTGGTAAGCCAGTGAC R: CCGAAGAAAAGTAGGATGAAGGT	103 bp	62.3 °C

Table 2. Effects of misoprostol on spatial learning and memory function in aluminum overload rats ($\bar{x} \pm s$).

	N	Latency (s)				
		d 1	d 2	d 3	d 4	d 5
Control group	13	63.7±15.2	39.3±12.6	27.0±13.6	18.3±10.8	10.9±7.6
Al-overload group	11	65.8±16.2	54.1±14.5	42.6±17.8 [#]	31.9±12.8 [#]	29.5±16.9 ^{##}
Misoprostol- 30 group	13	53.7±7.54	38.3±16.2	27.4±8.6	20.7±9.2 [*]	15.23±8.1 [*]
Misoprostol- 60 group	14	60.8±17.6	39.7±15.1	24.6±12.1 [*]	19.82±6.37 ^{**}	12.9±8.0 ^{**}
Misoprostol- 120 group	14	65.9±11.9	38.0±12.4 [*]	22.6±8.7 ^{**}	17.4±7.7 ^{**}	8.7±3.9 ^{**}

The rats in Al-overload group spent longer latency to explore the platform than the rats in control group during the five days. The rats in the Misoprostol group exhibited a significantly shorter latency compared with Al-overload group during the five days. [#]P < 0.05 and ^{##}P < 0.01 compared with control group, ^{*}P < 0.05 and ^{**}P < 0.01 compared with Al-overload group (n=11-14, Datas are reported as mean ± SD.)

navigate the platform significantly increased ($P < 0.05$); on d5, the spatial memory function test and latency were significantly longer ($P < 0.01$). Administration of misoprostol dose-dependently reduced the latency in the aluminum-overload rats (Table 2).

ALTERATIONS OF NEURONAL PATHOMORPHOLOGY

In the control group, the hippocampus neurons were regularly structured and closely arranged, and the morphological structure was intact and clear. By comparison, the Al-overload group showed significant injuries with remarkable karyopyknosis and cell loss. Administration of misoprostol dose-dependently reduced the neuronal pathomorphology in the aluminum-overload rats (Fig. 1).

ALTERATIONS OF MDA CONTENT AND SOD ACTIVITY

The MDA content in aluminum-overload group significantly elevated in comparison with the the control group. Misoprostol administration distinctly decreased the MDA content induced by the aluminum-overload treatment (Table 3).

The SOD activity in aluminum-overload group significantly decreased in comparison to the control group. Misoprostol administration distinctly increased the SOD activity induced by the aluminum-overload treatment (Table 3).

THE CONTENT OF PROSTAGLANDIN E₂ ALTERATIONS

The content of PGE₂ in the aluminum-overload group significantly elevated in comparison to the control group. The administration of misoprostol distinctly blunted the elevated of PGE₂ content caused by aluminum-overload (Table 4).

Alterations in mPGES-1, EP₂, EP₃ and EP₄ mRNA Expression

The qPCR results demonstrated that mPGES-1, EP₂ and EP₄ mRNA remained at low levels in the control group, whereas the aluminum-overload treatment significantly up-

regulated the expression of mPGES-1, EP₂ and EP₄ mRNA in the hippocampus. Administration of misoprostol dose-dependently down-regulated the expression of mPGES-1, EP₂ and EP₄ mRNA (Fig. 2).

The qPCR results demonstrated that EP₃ mRNA remained at a high level in the control group, whereas the aluminum-overload treatment significantly down-regulated the expression of EP₃ mRNA in the hippocampus. Administration of misoprostol dose-dependently up-regulated the expression of EP₃ mRNA (Figs. 2).

Alterations in mPGES-1, EP₂, EP₃ and EP₄ Protein Expression

Similar to mPGES-1, EP₂ and EP₄ mRNA expression, mPGES-1, EP₂ and EP₄ protein expression in the hippocampus of the aluminum-overload group increased significantly in comparison to the control group. Administration of misoprostol dose-dependently down-regulated the expression of mPGES-1, EP₂ and EP₄ protein (Fig. 3).

Similar to EP₃ mRNA expression, EP₃ protein expression in the hippocampus of the aluminum-overload group decreased in comparison to the control group. Administration of misoprostol dose-dependently up-regulated the expression of the EP₃ protein (Fig. 3).

DISCUSSION

In the development process of central nervous system (CNS) diseases, inflammation and oxidative stress play equally vital roles [5, 25, 26]. There was a correlation between COX-2 alterations and the severity of brain injury and neuronal degeneration. Our previous study found that hippocampal COX-2 mRNA and protein expression were significantly elevated in chronic aluminum overload rats, however, COX-2 inhibitors (meloxicam) blocked COX-2 activity, which is a potential mechanism to suppress the generation of PGs [10, 11, 27]. These results suggested that COX-2 could be used as a potential drug target for the cure of a variety of CNS diseases. However, the beneficial effects are limited to some NSAIDs because many clinical trials have demonstrated that the administration of naproxen / celecoxib could not reverse AD or prevent cognitive function loss in AD pa-

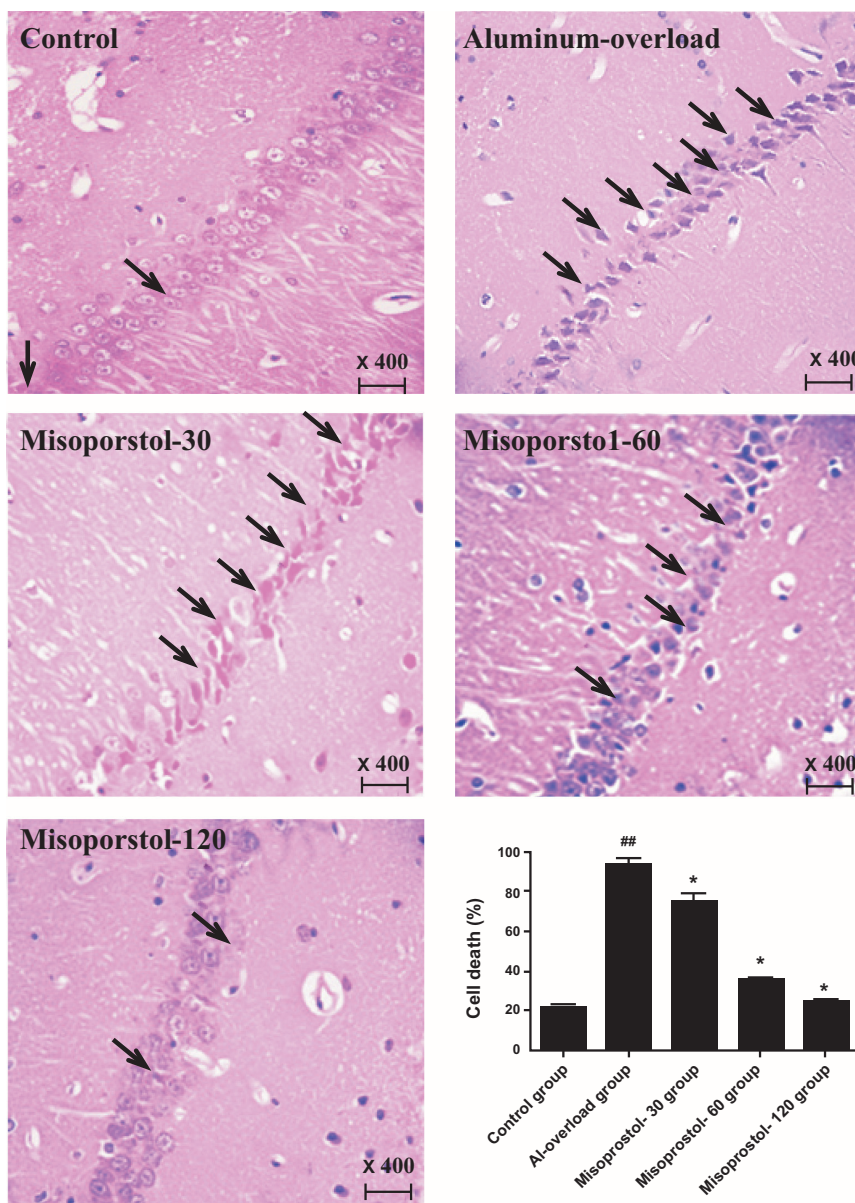


Fig. (1). Protection of hippocampus neurons from aluminum-overload injury by misoprostol in rats. Arrow indicates karyopyknosis of nerve cells. Dead nerve cell is identified by eosinophilic change including neuron became a red and deep cell with nucleoli disappear under light microscope. #P < 0.05 and ##P < 0.01 compared with control group, *P < 0.05 and **P < 0.01 compared with Al-overload group (Data are reported as mean ± SD. n=4).

Table 3. Effects of misoprostol on change of MDA content and SOD activity induced by aluminum-overload in rat hippocampus ($\bar{x} \pm s$, n=4).

	MDA (nmol/mg protein)	SOD (U/mg protein)
Control group	0.933±0.113	14.633±1.846
Al-overload group	4.887±1.368 ^{##}	7.866±1.369 ^{##}
Misoprostol- 30 group	2.785±0.374 [*]	10.145±2.983
Misoprostol- 60 group	2.103±0.403 ^{**}	12.875±3.093 [*]
Misoprostol- 120 group	1.654±0.244 ^{**}	13.069±2.754 [*]

Data are reported as mean ± SD. ^{##}P < 0.01 compared with control group, ^{*}P < 0.05 and ^{**}P < 0.01 compared with Al-overload group

Table 4. Effects of misoprostol on alterations of PGE₂ content induced by aluminum overload in rat hippocampus ($\bar{x} \pm s, n=4$).

	PGE ₂ (ng/g tissue)
Control group	61.30±17.80
Al-overload group	119.86±15.28 [#]
Misoprostol- 30 group	88.20±14.23 [*]
Misoprostol- 60 group	52.50±10.40 ^{**}
Misoprostol- 120 group	54.66±21.92 [*]

Data are reported as mean ± SD. [#]P < 0.05 compared with control group, ^{*}P < 0.05 and ^{**}P < 0.01 compared with Al-overload group

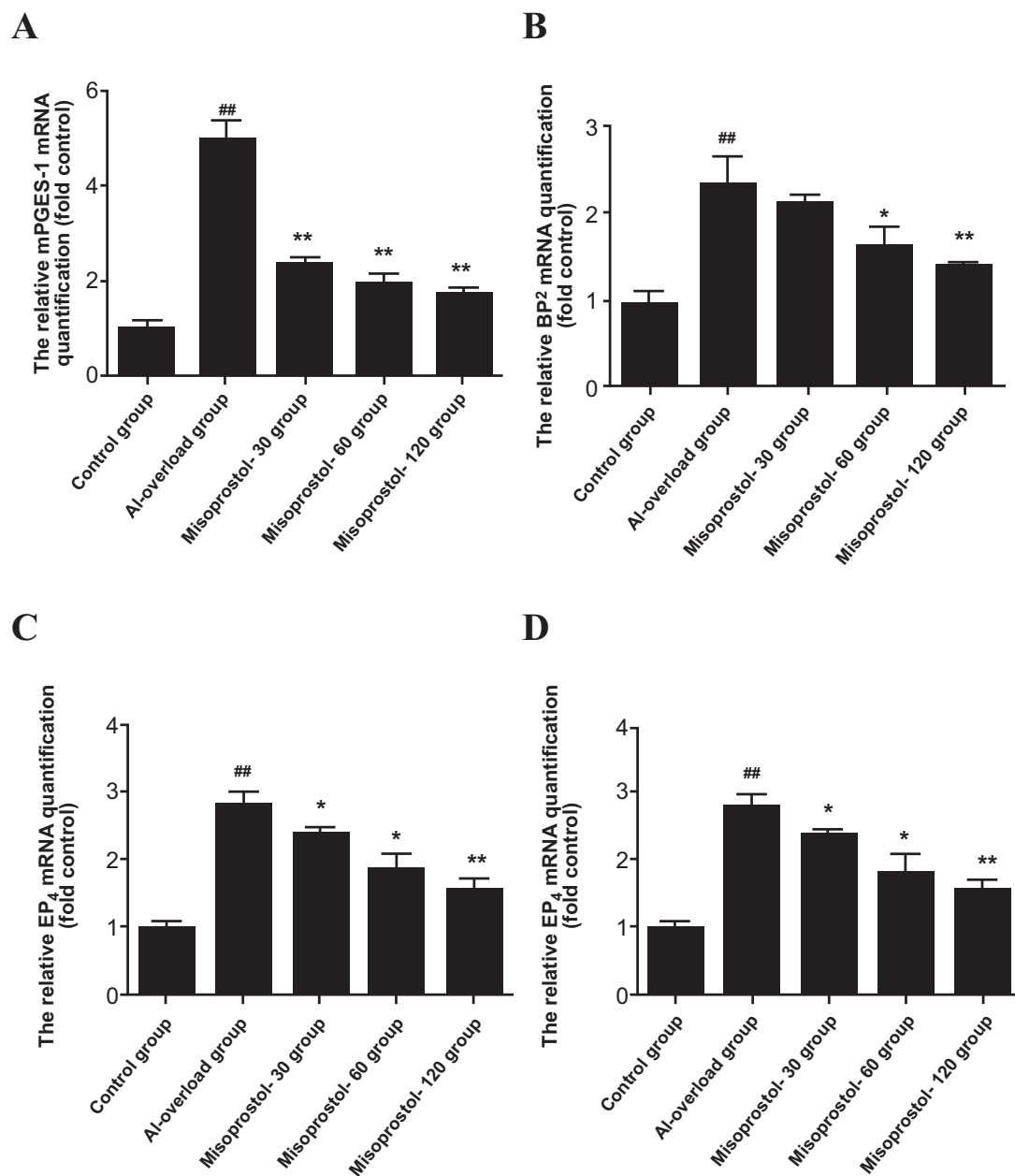
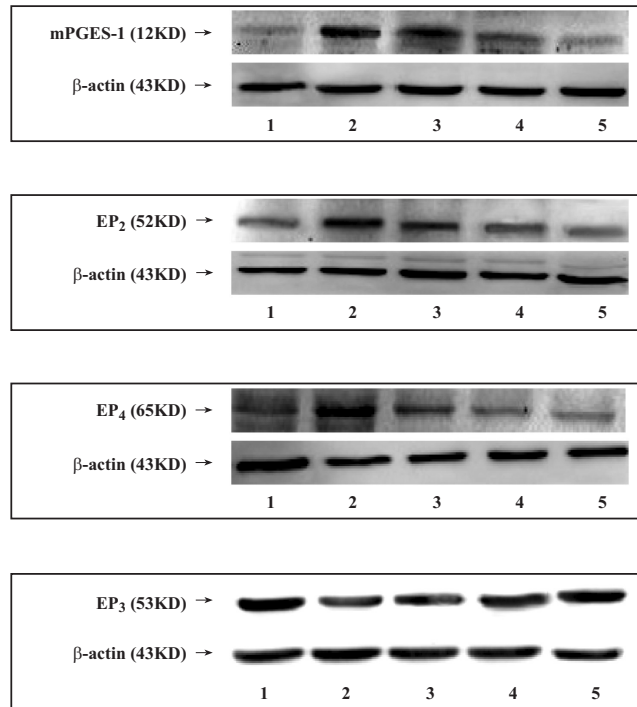


Fig. (2). Effects of misoprostol on alterations of mPGES-1, EP₂, EP₄ and EP₃ mRNA expression induced by aluminum overload in rat hippocampus. A-D represents mPGES-1, EP₂, EP₄ and EP₃ mRNA expression, respectively. (Data are expressed as mean ± SD. n=4).

A



B

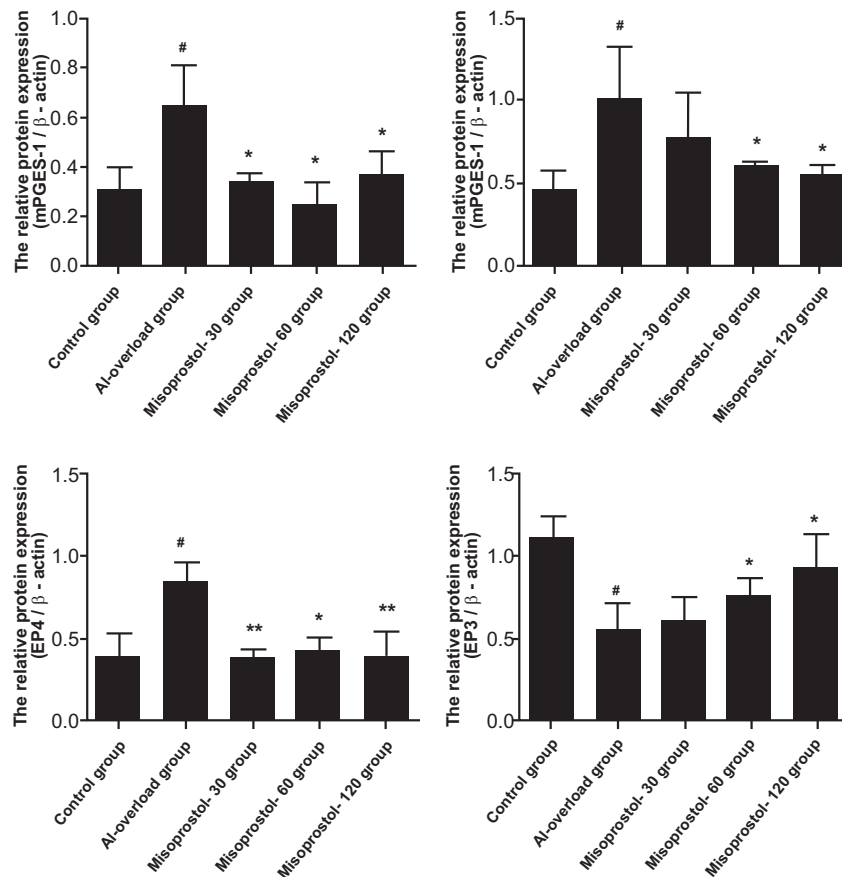


Fig. (3). Effects of misoprostol on alterations of mPGES-1, EP₂, EP₄ and EP₃ protein expression induced by aluminum overload in rat hippocampus. A: Lane 1-5 represents Control, Aluminum-overload, Misoprostol-30, Misoprostol-60 and Misoprostol-120, respectively. B: The relative protein level of mPGES-1, EP₂, EP₄ and EP₃ was normalized to endogenous β-actin protein for each sample. [#]P < 0.05 compared with Control group, ^{*}P < 0.05 compared with Al-overload group (Data are expressed as mean ± SD. n=4).

tients [12, 13, 28, 29]. Epidemiological studies have shown that NSAIDs act distinctly in different stages of AD. Therefore, selected prostaglandin signaling systems downstream of COX-2 exert potent beneficial effects in the setting of AD [3, 12, 30].

Misoprostol, a synthetic PGE₁ analog (15-deoxy-16-hydroxy-16-methyl PGE₁), is an EP_{2,4} receptor agonist and has the highest affinity for EP₃ (K_d: 7.9 nM, 23 nM and 34 nM for EP₃, EP₄, and EP₂, respectively). It is regarded as a potent agonist of EP₃. Currently, the latest research found that misoprostol promotes axon regeneration and regulates synaptic plasticity in animal brain injury models by reducing systemic oxidative stress and inflammation [22, 31-33]

Our results showed that when compared with hippocampal brain tissue from the control group, MDA levels increased and SOD activity was reduced significantly in hippocampal brain tissue from chronic aluminum overload rats. Because misoprostol decreases MDA levels and increases SOD activity, it also decelerates oxidative stress levels in the hippocampus of rats. When combined with the behavioristics and histopathology findings in rats, the neuroprotective effects of misoprostol enhanced the ability of fragile hippocampal neurons to resist oxygen free radical injury and delayed and (or) reversed the sustainable development of the chronic brain injury state. Similar to our findings, some scholars found that in the LPS-induced brain injury model of rats, misoprostol could dose-dependently down-regulate the expression of caspase-3 and the rate of DNA fragments, decrease MDA content and iNOS activity, increase GSH activity, and play a neuroprotective role [31, 33].

The prostanoids mainly consist of PGE₂, PGI₂, PGD₂, PGF_{2α} and TXA₂. They are lipid autacoids continuously catalyzed by COX and prostaglandin synthetase (PGS) from arachidonic acid. Some studies have shown that PGs play a vital role in the process of occurrence, development and recovery in a variety of CNS diseases [6, 34]. The study of the neurotoxic effects from PGs has found that compared with normal human brains, the PGE₂ content of AD, ALS, and HD patients increased 5, 6, and 1.4 times, respectively [35]. The PGE₂, PGI₂, PGD₂, PGF_{2α} and TXA₂ levels of the rats' cortex were enhanced by copper load in the demyelination model [36]. Other researchers found that PGs might have a neuroprotective effect. Echeverria *et al.* found that exogenous administration of PGE₂ increased hippocampal neuron activity and LDH activity in the primary hippocampal neuronal damage model induced by Aβ₁₋₄₂, and they speculated that the mechanism might be PGE₂ activating PKA, which increases cAMP levels and triggers CREB phosphorylation [37]. Our experiments found that the PGE₂ level in the hippocampal tissue of aluminum-overload rats significantly increased because misoprostol dose-dependently reduces PGE₂ content. These results suggested that PGE₂ has nerve injury/protective effects in chronic brain injury and neurological degenerative diseases, although its net effect presented an injury effect.

Therefore, the mutual conflicting mechanisms of PGs in the CNS are quite complex, and currently, there are no detailed explanations about these mechanisms. However, the generation of PGs plays a physiological role by catalyzing PGS and its corresponding receptor. Furthermore, we con-

nected PGs and its upstream specific terminal synthetase (PGS) with downstream targets (PGs receptor), proposing that the nature of PGs in the CNS depends on the net effect of the PGS-PGs-PGs receptor signal pathways [38].

Current research confirmed that PGES was an important terminal rate-limiting synthesis enzyme of PGE₂ followed by COX-2. mPGES-1 expression and activity increased sharply after stimulation by a pathological injury factor, meanwhile mPGES-1 shares the same signal transduction pathway with JNK, PKC, NF-κB, p38 and p42/44 MAPKs for COX-2. Our experimental results showed that in comparison with the normal control group, mPGES-1 levels of hippocampal tissue from the aluminum-overload rat increased significantly and that misoprostol dose-dependently reduces mPGES-1 levels. Similar to our results, Ikeda-Matsuo found that the cerebral ischemic infarct areas and neurological function scores of mPGES-1^{-/-} mice after transient global cerebral ischemic injury were better than wild-type mice [39]. Bastos *et al.* used minocycline against rat microglia cells and found damage induced by LPS; they found that minocycline specifically reduced mPGES-1 expression, inhibited the generation of PGE₂ and 8-iso-PGF_{2α}, relieved inflammatory reactions and increased cell survival rates without affecting the COX-2 expression [40]. In addition to previous studies, we speculated that single inhibition of mPGES-1 could theoretically reduce the adverse effects induced by excessive inhibition of COX-2 activity. Selectively reducing mPGES-1 expression without affecting COX-2 expression might achieve a therapeutic effect superior to a COX-2 inhibitor [39].

PGE₂ triggered a variety of signal transduction pathways, leading to its multiple biological effects by binding to EP₁₋₄. It was found that the G protein receptor coupled with PGE₂ could be divided into four different subtypes according to their coding genes, i.e., EP₁, EP₂, EP₃, and EP₄ [6, 9, 17]. Although many studies have demonstrated that EP₁ coupled with G_q mediated the neurotoxicity effects of PGE₂, our preliminary results demonstrated that there was no significant change for EP₁ in the brain cortical and the hippocampus tissue of the aluminum-overload group compared to the normal control group; this finding suggests that the PGES-PGE₂-EP₁ signaling pathway might not play an obvious role in the CNS injury induced by an aluminum overload.

The EP₂ downstream signal transmission mainly coupled with G_s accelerated AC activation, increased cAMP levels, and promoted CREB activation. However, EP₂ produced distinct neuroprotective and (or) toxic effects under the condition of different nerve damage factor stimulation. For example, in the neuronal injury caused by ischemia, hypoxia and excitotoxic factors, EP₂ activation increased cAMP levels, accelerated CREB phosphorylation, promoted nuclear translocation of NF-κB and played a neuroprotective functional role; in glial cell injury characterized by the persistence of oxidative stress and inflammatory, EP₂ activation up-regulated the expression of iNOS and COX-2. However, this mediated neurotoxicity occurrence by causing secondary nerve inflammation and lipid peroxidation [41]. Our experimental results showed that, in contrast with the normal control group, EP₂ expression of the hippocampal tissue from aluminum-overload rats increased and misoprostol dose-dependently reduced the expression of EP₂ significantly.

Similar to our results, Liang *et al.* demonstrated that the blockage of EP₂ in APP/PS1 aging mice obviously decreased the content of F₂-isoprostanes and F₄-neuroprostanes, reduced the Aβ₄₀ and Aβ₄₂ precipitation and BACE1 activity, and resisted nerve damage of [42]. In the rat model of LPS-induced spinal cord neuron inflammation, Brenneis *et al.* found that EP₂ was a selective agonist (ONO-AE1-259-01) that down-regulated mPGES-1 expression and PGE₂ generation through a negative feedback effect, decreased the generation of TNF-α and IL-1β without affecting COX-2 expression and other PGs generation, and played a neuroprotection role [43]. These results strongly suggested that PGES-PGE₂-EP₂ signaling pathway disorders might have a vital role in the process of CNS damage caused by aluminum overload. However, it is still unknown whether decreased EP₂ expression is beneficial or harmful for the treatment of aluminum-overload chronic cerebral damage.

Similar to the signal transduction pathways when EP₂ acted, EP₄ not only activated the cAMP/PKA pathway dependently by coupling with G_s but also activated the PI3K/AKT pathway. Differing from EP₂, most studies showed that EP₄ had a significant neuroprotective effect for a variety of neurological damage [9, 43]. For example, in the excitotoxicity model induced by unilateral intracerebroventricular injection of NMDA of mice, the EP₄ selective antagonist (ONO-AE1-329) significantly reduced the ipsilateral damage area and decreased cytokines, such as IL-1β, IL-6 and TNF-α, with a neuroprotective effect [44]. Contrary to these studies, our results showed that, in contrast with the normal control group, EP₄ expression of hippocampal tissue in aluminum-overload rats significantly increased, and misoprostol dose-dependently reduced EP₄ expression. The results indicated that PGES-PGE₂-EP₄ signaling pathway disorders might play a vital role in the process of CNS injury caused by aluminum overload; however there is lack of direct evidence to demonstrate that increased expression of EP₄ is deteriorative in this process.

EP₃ has a variety of transcriptional splice variants, such as EP_{3α}, EP_{3β}, and EP_{3γ}, and the EP₃ has the most complex signaling pathway and the most diverse biological effects of EP₁₋₄. EP₃ could not only couple with G_i, inhibiting AC activation, reducing cAMP levels and PKA phosphorylation but also couple with G_q, activating the PLCγ-IP₃ pathway, increasing IP₃ and DAG content, and promoting PI3K / AKT phosphorylation. Furthermore, it also coupled with G_{12 / 13}, regulating the activity of Rho-kinase, which made EP₃ have the potential property of regulating the PGES- PGE₂- EP₁₋₄ signaling pathway [6, 9]. Therefore, it was not entirely clear what role EP₃ played in CNS diseases. Saleem *et al.* found that, compared with the wild-type littermates, EP₃^{-/-} mice could distinctly reduce the area of cerebral infarction caused by MCAO [45]. Ikeda-Matsuo *et al.* found that the ONO-AE-248(selective EP₃ agonist) exacerbated cerebral injury caused by the excitotoxicity and ischemia; otherwise, EP₃ antagonist (ONO-AE3-240) could have neuroprotective effects by reducing the biological activity of G_i and RhoA, which showed that EP₃ had a neurotoxin effect [18]. However, Bilak *et al.* found that in the ALS model mediated by tetrahydroamino-acridine (THA), the selective EP₃ agonist (sulprostone) decreased motor neuronal necrosis by activating the PI3K / AKT pathwa [46]. In the rat CNS ischemia -

reperfusion model, Li *et al.* found that EP₃ agonist (misoprostol) reduced the area of ischemic penumbra, improved neurological function score and survival rate, and had a neuroprotective effect; meanwhile, the neuroprotective effects of misoprostol was not observed in EP₃^{-/-} rats, which showed that EP₃ had a neuroprotective effect [47]. Our experimental results demonstrated that, in contrast with the normal control group, EP₃ expression in the hippocampal tissue of aluminum-overload rats decreased and misoprostol elevated the expression of EP₃. Considering that misoprostol had the strongest affinity for EP₃ and potential regulative ability, we speculated that the PGES-PGE₂-EP₃ signaling pathway might play a core role in the process of the CNS damage caused by aluminum overload and that increasing EP₃ expression might have a protective effect in cerebral damage and the occurrence and development process of neuronal degeneration caused by chronic aluminum overload.

CONCLUSION

In summary, the results of this study confirmed that the imbalance of the mPGES-1- PGE₂- EP₁₋₄ signaling pathway involved in brain injury and neuronal degeneration was caused by chronic aluminum overload in rats. The down-regulation of neurons were protective with EP₃ and the up-regulation of nerve injurious EP₂ and EP₄ in the rat hippocampus involved in the mechanism that brain injury and neuronal degeneration was caused by chronic aluminum overload in rats. Misoprostol had a significant protective effect on brain injury and neuronal degeneration caused by chronic aluminum overload in rats, and the potential mechanism might be that misoprostol acts on EP₃ and reduces the endogenous production of PGE₂ through a negative feedback mechanism to lower EP₂ and EP₄ expression, raise EP₃ expression and reverse the mPGES-1-PGE₂-EP₁₋₄ signaling pathway imbalance in part, and play a counteractive role for inflammation and oxidant stress in the CNS. Regarding the low effector that was in the mPGES-1-PGE₂-PGE₁₋₄ signal transduction pathway of COX-2 downstream as the target, it might be a new strategy for studying brain injury mechanisms and correlative prevention drugs. EP₃ receptor agonists could be candidate drugs for chronic brain injury and neuronal degeneration in clinical treatments.

AUTHOR'S CONTRIBUTION

Yuanxin Guo : carried out the study and wrote the manuscript.

Junqing Yang: designed the study, directed the study and revised the manuscript.

Wenjuan Lei: performed the study and collected data.

Jianfeng Wang: performed the study and collected data.

Xinyue Hu: performed the study and analyzed data.

Yuling Wei: performed the study and analyzed data.

Chaonan Ji: performed the study.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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