Upregulation of PPAR-gamma activity inhibits cyclooxygenase 2 expression in cortical neurons with N-methyl-D-aspartic acid induced excitatory neurotoxicity

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ABSTRACT

This study aimed to investigate the effect of upregulated peroxisome proliferator-activated receptor-gamma (PPAR-γ) activity on cyclooxygenase 2 (COX-2) expression and N-methyl-D-aspartic acid (NMDA)-induced excitatory neurotoxicity in primary cultured cortical neurons. Rat cortical neurons were cultured for 8 days in vitro, and divided into control, NMDA, MK-801 (selective NMDA antagonist), rosiglitazone (ROSI, PPAR-γ agonist), GW9662 (PPAR-γ antagonist), NS398 (selective COX-2 antagonist) and NS398 + ROSI groups. Two hours after treatment in each group, cell viability, intracellular Ca²⁺ concentrations, PPAR-γ and COX-2 protein expression were detected by CCK-8 assay, flow cytometry and western blot assay, respectively. The results showed that compared with the control group, 100 μmol/L of NMDA significantly decreased the neuronal cell viability, increased Ca²⁺ concentrations, which also increased the COX-2 protein expression and decreased PPAR-γ expression in neurons. Compared with the NMDA group, the cell viability was increased, Ca²⁺ concentrations and COX-2 protein expression were significantly decreased, PPAR-γ expression was significantly increased in the MK-801, ROSI, NS398 and ROSI + NS398 groups (both P < 0.01). This finding suggested that upregulation of PPAR-γ activity can inhibit COX-2 expression, decrease Ca²⁺ concentrations in primary cultured cortical neurons, and protect neurons against NMDA-induced excitatory neurotoxicity.

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Introduction

The pathogenesis and prevention of neurodegenerative diseases has become a research hotspot in the field of neuroscience. In the central nervous system, glutamate receptors are classified into ionotropic and metabotropic receptors according to their structural and functional features. The ionotropic glutamate receptors are located in the dense region of the postsynaptic membrane, including N-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors (α-amino-3-hydroxy-5-methylisoxazol-4-propionic acid receptor and kainate receptor). NMDA receptors are ligand- and voltage-gated ion channels, and are involved in the generation and maintenance of synapses between central neurons. Ligand-gated cation channels have high permeability to Ca²⁺. Excessive activation of NMDA receptors can cause excessive influx of Na⁺ and Cl⁻, induce acute osmotic swelling of cells and intracellular Ca²⁺ overload, activate Ca²⁺-dependent enzymes, such as neuronal nitric oxide synthase, and cause the production of large amounts of nitric oxide and oxygen-free radicals, mitochondrial damage, and finally leading to neuronal apoptosis or necrosis and the process called excitatory neurotoxicity [1, 2].

Cyclooxygenase (COX), or prostaglandin-endoperoxide synthase, is a rate-limiting enzyme for prostanoid formation. COX has at least two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in all tissues, and plays an important role in the maintenance of the normal physiological function of the body. COX-2 is an inducible enzyme, which is not expressed or is expressed at extremely low levels in normal tissues, but can be induced by cytokines, growth factors and tumour promoters. COX-2 is involved in the maintenance of synaptic function [3]. Pasinetti and Aisen found that increased neuronal COX-2 expression was associated with the development of chronic neurodegenerative diseases such as Alzheimer’s disease [4].
After brain injury, the COX-2 expression in cortical neurons was increased [5]. These findings suggested that COX-2 expression in the brain is associated with neuropathological processes. A study found that COX-2 inhibitor can protect cerebellar granule neurons from glutamate-mediated neurotoxicity [6]. So, in the present study, we hypothesized that increased expression of COX-2 protein is associated with excitatory neurotoxicity induced by overactivation of NMDA receptors in cortical neurons.

Peroxisome proliferator-activated receptors (PPARs) were first discovered by Issmann and Green [7] in 1990. PPARs belong to the steroid hormone receptor superfamily, and have three isoforms (α, δ and γ), which can be activated by peroxisome proliferators and fatty acids. PPARs can regulate the transcription and expression of many genes by binding to specific DNA response elements, which are involved in the regulation of lipid and glucose metabolism, cell differentiation and apoptosis, inflammation and immune responses [8]. PPAR-γ exhibits complex and diverse biological functions. The protective effect of PPAR-γ against cerebral ischemic injury is mainly associated with its inhibitory effect on the secretion of tumour necrosis factor-α (TNF-α), COX-2 and inducible nitric oxide synthase (iNOS) [9]. The aim of the study was to investigate the role of upregulation of PPAR-γ activity on COX-2 protein expression and NMDA-induced excitatory neurotoxicity in rat cortical neurons.

Materials and methods

Experimental animals

Sixty neonatal Wistar rats aged 1 day were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China, license number: SCXK[Hu]2012-0002).

Ethics statement

All experimental Ethics procedures were approved by the Animal Ethics Committee of Hainan Medical University.

Drugs and reagents

Neurobasal-A medium, fetal bovine serum (Grand Island Biological Co. GIBCO, USA); poly-lysine, NS398, rosiglitazone (ROS1), NMDA, GW9662, MK-801 (Sigma, USA); microtubule-associated protein 2 antibody (MAP2, Abcam, USA); B27 (Life Technologies Ltd., USA); Cell Counting Kit-8 (CCK-8) (Dojindo, Japan); polyvinylidene fluoride membrane (Millipore Corp., USA); COX-2 (H-62) polyclonal antibody, PPAR-γ (H-100) polyclonal antibody (Santa Cruz Biotechnology, USA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Bioworld Technology Inc., USA).

Primary culture of rat cortical neurons

Neonatal rats were decapitated under aseptic conditions. Cerebral cortex was obtained, meninges, blood vessels and hippocampus were then removed. Cerebral cortex was cut into 1 mm³ blocks in 4°C D-Hank’s solution (pH 7.0), and digested in an equal volume of 0.25% trypsin (with ethylenediaminetetraacetic acid) in a 37°C water bath for 30 minutes with gentle shaking every 2 minutes. DMEM containing 10% fetal bovine serum was added to terminate trypsin digestion. After the samples were gently blown and beaten, the tube was allowed to stand at low temperature. The supernatant was collected and filtered through a 100-200-μm nylon mesh into 50-mL centrifuge tubes. Then the cell suspension was centrifuged at 1000 g for 6 minutes. After the supernatant was removed, precipitated cells were resuspended in Neurobasal-A medium containing 10% fetal bovine serum. Cells at the density of 5 x 10⁵ cells/mL were seeded on the poly-L-lysine-coated flasks or plates, and incubated in a 5% CO₂ incubator at 37°C for 24 hours. The medium was replaced by serum-free Neurobasal-A medium containing 2% B27. Cytarabine (10 μmol/L) was added to suppress the proliferation of glial cells and non-neuronal cells. One-half of the medium was replaced every 2 days. Primary cortical neurons were cultured for 8 days in vitro.

Immunofluorescence detection of the primary cultured rat cortical neurons

After removal of the medium, primary cultured neurons were washed with phosphate buffered saline (PBS) three times for 5 minutes each, and fixed in 4% paraformaldehyde for 30 minutes at room temperature, followed by three washes in PBS for 5 minutes each. Then the neurons were permeabilized with 0.5% Triton X-100 solution at room temperature for 10 minutes, washed in PBS three times for 5 minutes each. After being blocked with 5% bovine serum albumin at 37°C for 2 hours, the neurons were incubated with primary antibody (1:200) at 4°C overnight. The wet box was then taken out and equilibrated at room temperature for 30 minutes, followed by three washes in PBS for 5 minutes each. Then the neurons were incubated with FITC-conjugated Dnk anti-rabbit
secondary antibody (1:400) at 37 °C for 1 hour in the dark, and washed with PBS three times with 5 minutes each. Cell nuclei were stained with DAPI at room temperature for 15 minutes, and washed in PBS three times for 5 minutes each. After mounting, the cells were then observed and photographed using fluorescence microscopy.

**Grouping**

The cultured cortical neurons were divided into control group (normal control group), NMDA group (neurons were treated with 100 μmol/L NMDA and 10 μmol/L glycine for 2 hours), MK-801 group (neurons were treated with 100 μmol/L NMDA, 10 μmol/L glycine and 10 μmol/L MK-801 for 2 hours), ROSI group (neurons were treated with 1 μmol/L ROSI for 30 minutes, and then treated with 100 μmol/L NMDA for 2 hours), GW9662 group (neurons were treated with 1 μmol/L GW9662 for 30 minutes, and then treated with NMDA 100 μmol/L for another 2 hours), NS398 group (neurons were treated with 30 μmol/L NS398 for 30 minutes, and then treated with 100 μmol/L NMDA for another 2 hours), NS398 + ROSI group (neurons were treated with ROSI and 30 μmol/L NS398 for 30 minutes, and then treated with 100 μmol/L NMDA for another 2 hours). MK-801 is a selective NMDA antagonist. ROSI is a PPAR-γ agonist. GW9662 is a PPAR-γ antagonist. NS398 is a selective COX-2 antagonist.

**Detection of Ca^{2+} concentrations using flow cytometry**

Cells from each group were taken, washed with HBSS, and centrifuged at 251.55 g for 5 minutes. Cells were resuspended with 2.5 μmol/L Fluo 4-AM working solution to a final concentration of 100 μL, incubated at 37 °C in the dark for 30 minutes. After washing three times with HBSS, cells were centrifuged at 1500 rpm for 5 minutes. The mean fluorescence intensity was estimated at the FL-1 channel.

**Statistical analysis**

Data were collected in at least five independent experiments. Results were analyzed with SPSS 13.0 statistical software, and expressed as mean values with standard deviation (± SD). Differences between groups were compared using one-way analysis of variance followed by Dunnett’s test. A P-value of less than 0.05 was considered to indicate a statistically significant difference.

**Results and discussion**

**Identification of primary cultured rat cortical neurons**

Fluorescence microscopy showed that neurons expressed MAP2, which presented green fluorescence. The nuclei displayed blue fluorescence. Neuronal cells showed scattered or massive growth. Neuronal processes exhibited obvious reticulated distribution (Figure 1).

**Upregulation of PPAR-γ activity inhibits NMDA-induced reduction of neuronal cell viability in cultured cortical neurons**

The overactivation of the NMDA receptor can trigger a Ca^{2+} influx, which contributes to neuronal apoptosis or necrosis [10]. NMDA-induced excitatory neurotoxicity is considered to be strongly associated with the development of neurodegenerative diseases. Therefore, inhibition of excitatory neurotoxicity induced by overactive...
NMDA receptors is important for the treatment of neuronal damage and various neurodegenerative diseases. In the present study, we used CCK-8 assay to detect cell viability of each group. The results showed that compared with the control group, the neuronal cell viability was significantly reduced in the NMDA group ($P < 0.01$), indicating that 100 $\mu$mol/L NMDA significantly decreased the neuronal cell viability. MK-801 is a selective NMDA antagonist, which can decrease NMDA-induced neurotoxicity. There was no significant difference between the MK-801 and control groups. Compared with the NMDA group, cell viability was significantly increased in the ROSI, NS398 and ROSI $+$ NS398 groups ($P < 0.01$).

ROSI is an insulin sensitiser, which belongs to the class of thiazolidinediones (TZDs), and acts as a more effective agonist of PPAR-$\gamma$ [11], and GW9662 is a PPAR-$\gamma$ antagonist. The results showed that cell viability was decreased in the GW9662 group compared with the ROSI group, which was increased in the GW9662 group than that in the NMDA group ($P < 0.05$) (Figure 2). The results indicated that ROSI treatment can inhibit NMDA-induced neurotoxicity and reduce neuronal damage through upregulation of PPAR-$\gamma$ activity.

**Upregulation of PPAR-$\gamma$ activity decreases $\text{Ca}^{2+}$ concentration in neurons with NMDA-induced neurotoxicity**

Intracellular $\text{Ca}^{2+}$ concentration of each group was detected by flow cytometry analysis. As shown in Figure 3, compared with the control group, the intracellular $\text{Ca}^{2+}$ concentration was significantly increased in the NMDA group ($P < 0.01$). MK-801 suppressed NMDA-induced neurotoxicity, and $\text{Ca}^{2+}$ concentrations were significantly reduced in the MK-801 group compared to the NMDA group ($P < 0.01$). Intracellular $\text{Ca}^{2+}$ concentrations were significantly decreased in the ROSI, NS398 and ROSI $+$ NS398 groups compared with the NMDA group ($P < 0.01$). GW9662 reduced PPAR-$\gamma$ activity. There were significant differences in the $\text{Ca}^{2+}$ concentrations between the GW9662 and NMDA groups ($P < 0.05$). These results indicated that intracellular $\text{Ca}^{2+}$ concentrations were increased in neurons with NMDA-induced neurotoxicity, and upregulation of PPAR-$\gamma$ activity can decrease intracellular $\text{Ca}^{2+}$ concentrations in neurons.

**Upregulation of PPAR-$\gamma$ inhibits NMDA-induced neurotoxicity through downregulating COX-2 protein expression**

COX-2 can catalyze the conversion of arachidonic acid into thromboxane A2, prostaglandin E2 and prostaglandin...
H2, which is associated with neuroinflammation, and contributes to the progression of neurodegenerative diseases [12]. A recent study had shown that COX-2 expression depends on the activation of calcineurin, which is associated with increased Ca2+ concentration in the cytoplasm of neurons [13]. Selective inhibition of the binding of neuronal nitric oxide synthase to COX-2 can suppress NMDA-induced excitatory neurotoxicity and exert neuroprotective properties [14, 15].

Western blot assay was used to determine the protein expression of PPARγ and COX-2 in neurons of each group. Results showed that compared with the control group, COX-2 protein expression was significantly increased, while PPARγ protein expression was decreased in the NMDA group (P < 0.01). Compared with the NMDA group, COX-2 protein expression was significantly decreased in MK-801 group (P < 0.01); PPARγ protein expression was increased and COX-2 protein expression was decreased in the ROSI, NS398, and ROSI + NS398 groups (P < 0.01). PPARγ protein expression was decreased, and COX-2 protein expression was increased in the GW9662 group compared with the ROsi group (P < 0.01). These results indicated that NMDA-induced neurotoxicity was associated with the increase in COX-2 protein expression, and increased PPARγ activity could downregulate COX-2 protein expression in neurons with NMDA-induced neurotoxicity (Figure 4).

ROSI exerted neuroprotective effect through reducing the COX-2 expression surrounding the infarcted site of the ischemic hemisphere [16–18]. Du et al. [19] found that PPARγ-mediated inhibition of COX-2 expression can protect hippocampal neurons from damage. The protective effect of hyperbaric oxygen preconditioning against hypoxia/hypoglycemia-induced injury of primary cultured cortical neurons is also achieved through activating PPAR-γ expression and increasing antioxidant enzyme activity [20]. Selective COX-2 inhibitor NS-398 and endogenous PPAR-γ ligand can act synergistically and exert neuroprotective effects on rat pheochromocytoma cells [21]. In the present study, we found that combination of ROSI and selective COX-2 inhibitor NS-398 not only decreased Ca2+ concentrations, increased neuronal cell viability, but also increased PPARγ activity and increased COX-2 protein expression are associated with excitotoxic neuronal damage induced by NMDA in cultured cortical neurons. The combination of ROSI and NS-398 can inhibit NMDA-induced excitatory neurotoxicity in primary cultured cortical neurons.

Conclusions
Our results suggested that upregulation of PPARγ activity can increase the neuronal cell viability, and protect cortical neurons against NMDA-induced excitatory neurotoxicity through inhibiting COX-2 expression and reducing Ca2+ concentrations in cultured cortical neurons.

Disclosure Statement
The authors report no conflicts of interest.

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