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Full Length Research Paper

Neuroprotective effects of ligustrazine on oxygen-glucose deprivation (OGD) in PC12 cells

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Ischemic cerebrovascular disease is a global health problem. According to the World Health Organization, ischemic stroke is actually the most common cause of death in the world. Ligustrazine (4-methyl-pyrazine [tetramethylpyrazine]) protects neurons against toxicity and plays a central role in regulating the brain's response to injury. In the present study, we investigated the mechanisms involved in the neuroprotective effects of ligustrazine in a model of hypoxic-ischemic brain disease. We found that ligustrazine effectively increases the survival rate of PC12 cells and relieves OGD (oxygen-glucose deprivation) damage. The effects of ligustrazine on the ActA/Smad pathway and on the up-regulation of inducible nitric oxide synthase (NOS) and superoxide dismutase (SOD) were investigated using OGD in PC12 cells for clarifying the neuroprotective mechanisms of ligustrazine. The results show that ligustrazine increases the expression of ActRIIA, Smad3 and Smad4; and that 50 and 100 ng/ml of ligustrazine reduces NO levels and increases SOD activity by 78.9 and 79.9%, respectively. These results suggest that the neuroprotective effects of ligustrazine in ischemia are related to the activation of the ActA/Smad signaling pathway and to its anti-oxidant activities.

Key words: Oxygen-glucose deprivation, ischemia tolerance model, ligustrazine, ActA/Smad pathway.

INTRODUCTION

Ischemic stroke occurs when the blood supply to the brain is obstructed, and it is one of the most common causes of health problems, disability and death in the world. Accumulating evidence suggests that the cell death observed during the first few hours of cerebellar ischemia is a result of apoptosis as opposed to necrosis, which was considered the predominant form of cerebellar damage generated by ischemia. Moreover, the ischemic damage of nerve cells leads to the disruption of a series of complex signaling pathways that produces an effect on corresponding biological functions and affects the function of the brain; therefore, it is important to develop therapies that enhance the neuroprotective effects by inhibiting the mechanisms that lead to apoptosis and excessive cell death, before the process becomes irreversible. Ligustrazine is a traditional Chinese Herbal for angina pectoris (Zhang and Zhao, 2009). Ligustrazine plays a role in expanding blood vessels, increasing coronary and cerebral blood flow, preventing platelet aggregation, inhibiting thrombosis, and improving the microcirculation. In this study, the oxygen-glucose deprivation of PC12 cells was used to establish a cerebral hypoxia-ischemia model to investigate the mechanism of ligustrazine neuroprotection (Mei et al., 2011). In addition, we measured the amount of NO and the induction of superoxide dismutase (SOD) in an attempt to elucidate possible mechanisms that underlie ligustrazine-mediated protection against oxygen-glucose deprivation (OGD) in PC12 cells (Qiu et al, 2006).

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MATERIALS AND METHODS

Cell culture

PC12 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. The cell line was maintained in DMEM medium supplemented with 10% (v/v) fetal bovine serum, 5% horse

serum (FBS, GIBCO), 100 IU/ml streptomycin, 100 IU/ml penicillin, pH 7.0, and the cells were detached using 0.25% trypsin (Sigma, USA). PC12 cells were grown at 37°C in 5% CO_2 (Mei et al., 2011).

OGD for *in vitro* ischemia

Before differentiation, the cells were grown in 5% horse serum-containing media on collagen-coated tissue culture dishes. After the cells were attached, they were treated with 100 ng/ml nerve growth factor (NGF 2.5S; Promega, Madison, WI) and were cultured in serum-free DMEM for 6 days (Michiyoshi et al., 2010; Jaehoon et al., 2010). The cells were washed three times with DMEM and were incubated in DMEM containing 10, 20, 30, 50 and 100 ng/ml ligustrazine for 24 h. The cells were washed three times with DMEM and were incubated for 6 h in DMEM containing 1 mmol/l NaS_2O_4 under hypoxic conditions (37°C, 5% CO_2 and 95% N_2) in the absence of sugar (Mei et al., 2011).

Cell viability assay

The MTT method was used to assess the cytotoxic effects of ligustrazine. The cells were grown to a density of 5×10^4 cells/well and were then treated with 10, 20, 30, 50 and 100 ng/ml ligustrazine in a 96-well plate for 24 h. At the end of the treatment, the ligustrazine-containing medium was carefully removed and the cells were treated with OGD for 6 h. The culture medium was removed and 200 µl medium containing 20 µl MTT (5 mg/ml in PBS) (St. Louis, MO, USA) was added to each well. After 4 h of incubation at 37°C, the medium was removed and 100 µl DMSO was added to each well. The optical absorbance (A) of each well was read at 490 nm. The percentage of viable cells was calculated as follows: (A of experimental group / A of control group) × 100%.

Flow cytometry using Annexin V/PI staining

For the quantitative assessment of apoptosis, Annexin V-FITC and PI double staining, followed by flow cytometry was used. Cells (1×10^5) were harvested and were stained using Annexin V-FITC and a PI double-staining kit (Kaiji Bio Co., Nanjing, China) in accordance with the manufacturer's instructions. Cells were analyzed immediately using flow cytometry. The signals from apoptotic cells are localized in the lower right quadrant of the resulting dot-plot graph.

Western blotting analysis

After treatment with ligustrazine and OGD for 6 h , the cells were washed twice using cold PBS and 1×10^6 cells were lysed using RIPA buffer (50 mmol/l Tris (pH 8.0), 150 mmol/l NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate) containing protease inhibitors (1% cocktail and 1 mmol/l PMSF). Total proteins were separated using 15% SDS-PAGE and were transferred to a PVDF membrane. The membrane was blocked using Tris-buffered saline with 0.1% Tween 20 (pH 7.6, TBST) for 1 h at room temperature and was incubated with the primary antibody solution (1:1000) at 4°C overnight. After two washes in TBST, the membrane was incubated with the HRP-labeled secondary antibody (Santa SC-2073) for 1 h at room temperature and was washed three times with TBST. The final detection was performed using enhanced chemiluminescence (ECL) western blotting reagents (Amersham Biosciences, Piscataway, NJ) and the membrane was exposed to Lumi-Film Chemiluminescent Detection Film (Roche). Loading differences were normalized using a monoclonal ß-actin antibody. The antibodies used in the study included, Smad4 (Santa SC-73040), Smad3 (Santa SC-101154), caspase-3 (Santa SC-7272), SOD (Santa SC-18503), NOS (Santa SC-49055) and ß-actin (Santa SC-2021).

Measurement of NO

Nitrite production was measured by the Griess reaction and used as a measure of NO production. Briefly, 100 µl of culture supernatant was incubated with an equal volume of Griess reagent (1 part 0.1% naphthylethylene-diamine, 1 part 1% sulfanilamide in 5% H_3PO_4) in 96-well tissue culture plates for 10 min at room temperature in the dark. The absorbance at 540 nm was determined using a microplate reader (SpectraMAX 340, Molecular Devices, Sunnyvale, CA).

Measurement of SOD

Cells (1×10^5) were harvested and after treatment with OGD for 6 h and ligustrazine plus OGD for 6 h, the cells were washed twice with cold PBS and were lysed in ice-cold 0.1M Tris/HCl (pH 7.4 containing 0.5% Triton X-100, 5 mM β -ME, 0.1 mg/ml PMSF). The cell lysates were centrifuged at 14,000 rpm for 5 min at 4°C and the cell debris was discarded. The total SOD activities in the supernatants (containing the cytosolic and mitochondrial fractions) were measured using the Superoxide Dismutase (SOD) Activity Assay Kit (Biovision k335-100, San Franciso, USA) in accordance with the manufacturer's instructions.

Statistical analysis

SPSS software was used for statistical analyses and the values are presented as mean ± SD. ANOVA was used to compare the mean values. P values of less than 0.05 were taken to indicate statistically significant differences.

RESULTS

Morphological changes of PC12 cells

The results show that treatment with NGF (100 ng/ml) stimulates neuron-like differentiation of PC12 cells as seen under the microscope. PC12 cells changed into neurons after 1 day of NGF treatment and later formed synapses. Synapses extended up the length of the cell after 3 days of treatment. The synaptic length increased 6- to 8-fold after 6 days of treatment. Bars = 20 µm, Invert microscope, Olympus IX71, Japan (×200) (Figure 1) (Kumar et al, 2006; Greenberg et al, 1985).

Effects of ligustrazine on cell proliferation

The cytotoxicity of OGD for 6 h and ligustrazine plus OGD for 6 h treatments were determined by examining their effects on the proliferation of PC12 cells. PC12 cells were treated with 10, 20, 30, 50 and 100 ng/ml ligustrazine for 24 h before OGD for 6 h (Figure 2). MTT

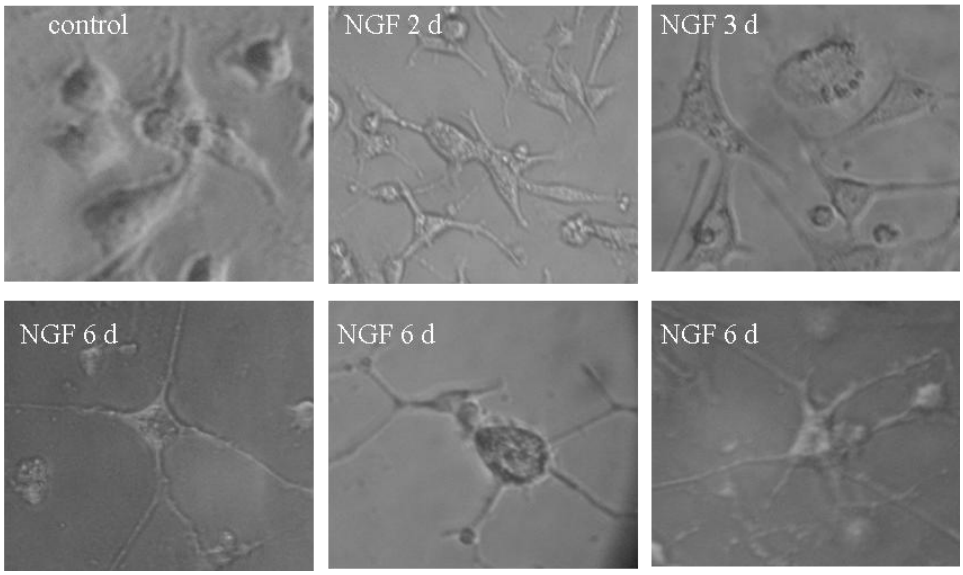

Figure 1. The morphological changes of PC12 cells (×200).

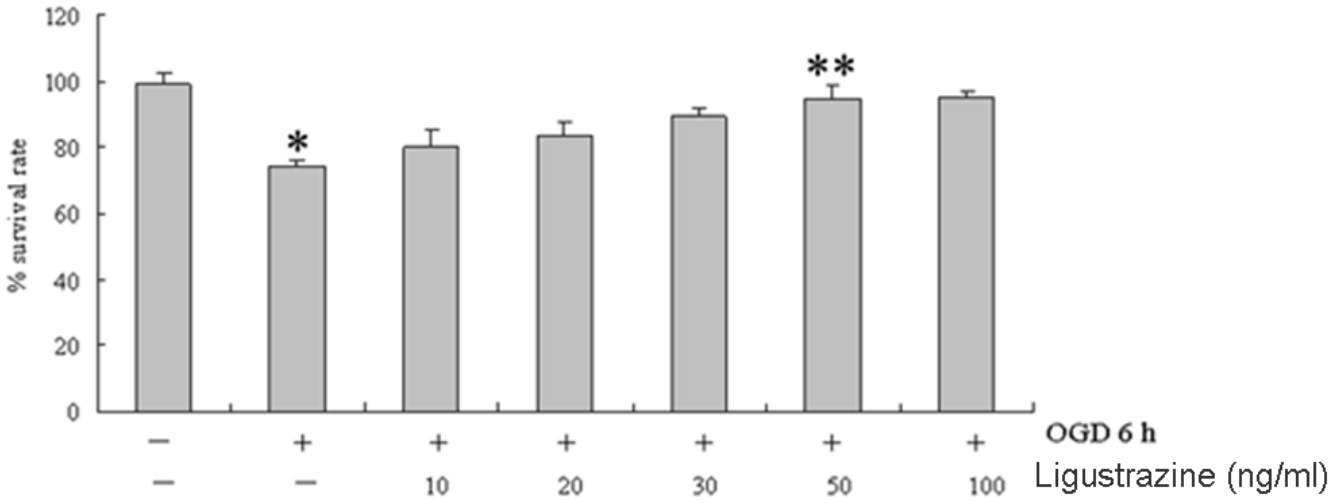

Figure 2. MTT assay showing the growth inhibition of PC12 cells treated with ligustrazine for 24 h plus OGD for 6 h. * denotes a significant difference from control, $P < 0.05$; ** denotes a significant difference from OGD for 6 h, $P < 0.05$ denotes a significant difference from 100 ng/ml ligustrazine plus OGD for 6 h. The data represent the means ± SD obtained from three separate experiments that were performed in triplicate.

assays (Hamid et al, 2004) showed that treatment with OGD for 6 h effectively inhibited the growth of PC12 cells by 25.61%. Compared to the OGD for 6 h-treated group, the Ligustrazine plus OGD for 6 h-treated group inhibited the growth of PC12 cells by 19.82, 16.31, 10.36, 5.41 and 4.92%, in dose-dependent manner, and the survival rates were higher than in the OGD for 6 h-treated group. Because there was no significant difference ($P > 0.05$) between the survival rates of the 50 and 100 ng/ml ligustrazine-treated cells, 50 ng/ml ligustrazine was used in all subsequent experiments.

Ligustrazine plus OGD 6 h/OGD 6 h induced apoptosis in PC12 cells

To assess the apoptosis of differentiated PC12 cells treated with ligustrazine plus OGD and OGD alone, the cells were analyzed using Annexin V-FITC and PI double-staining flow cytometry. The signals from each group of cells are located in the lower right quadrant of the dot-plot graph, and the results are shown in Figure 3. Compared to the control cells, the proportions of apoptotic cells treated with OGD for 6 h was 25.23%. Compared to the

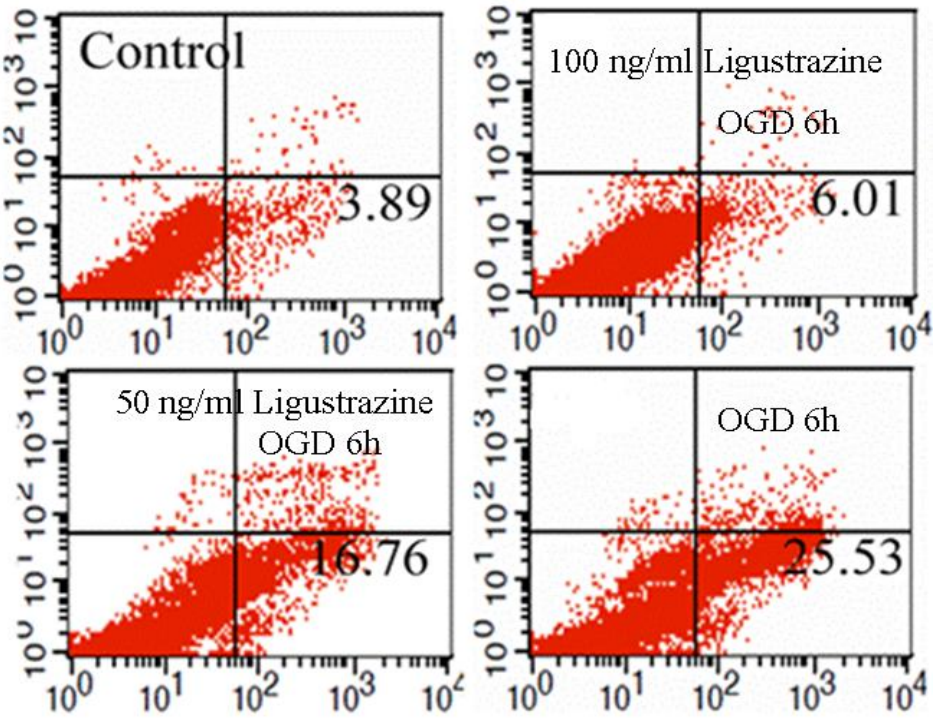

Figure 3. Flow cytometric analysis: assessment of apoptosis. OGD for 6 h treatment induced apoptosis in PC12 cells and ligustrazine reduced cell damage. The cells were treated with OGD for 6 h or with different concentrations of ligustrazine plus OGD for 6 h and were analyzed using flow cytometry following AnnexinV-FITC/PI staining.

OGD for 6 h-treated group, the proportions of apoptotic cells treated with 50 and 100 ng/ml ligustrazine plus OGD for 6 h were 16.76 and 6.01%, respectively. In the OGD for 6 h-treated group, the proportions of apoptotic cells was significantly higher than in the control group; the apoptosis rate of the Ligustrazine plus OGD for 6 h-treated groups decreased significantly compared to OGD for 6 h-treated group, suggesting that ligustrazine reduces cell damage. Compared with the 50 ng/ml ligustrazine-treated group, the apoptosis rate of the 100 ng/ml ligustrazine-treated group was slightly lower.

Effects of ligustrazine on ActA/Smad pathway

The ActA/Smad pathway plays a protective role in ischemia following its activation. To investigate the neuroprotective mechanisms of ligustrazine, the expression of ActRIIA, Smad3 and Smad4 were examined using western blots (Figure 3). Compared with the controls, the ActRIIA, Smad3 and Smad4 expression levels in PC12 cells, increased in the OGD for the 6 h-treated group. Compared with this group, the expression levels of ActRIIA, Smad3 and Smad4 increased in the groups treated with ligustrazine for 24 h combined with OGD for 6 h. In this group, as the concentration of ligustrazine increased, the expression levels of ActRIIA, Smad3 and Smad4 increased in a dose-dependent manner. Therefore, ligustrazine may mediate the neuroprotective effect of the ActA/Smad signaling pathway.

Inhibition of NO and iNOS by ligustrazine

To understand further the neuroprotective mechanisms of ligustrazine, we investigated if ligustrazine protects PC12 cells from oxidative injury. The cells were treated with OGD for 6 h, or with 50 ng/ml ActA for 24 h plus OGD for 6 h, and NO production was assayed by measuring the levels of a stable NO metabolite, nitrites, in the conditioned medium. Incubation with ligustrazine (50 ng/ml) for 24 h effectively inhibited NO production in OGD-stimulated PC12 cells (Figure 5).

Increasing the activity of SOD by ligustrazine

The effect of ligustrazine on SOD activity and protein expression were examined (Figure 6). As shown in Figure 5A, compared with the controls, the SOD activity in PC12 cells decreased in the OGD for 6 h-treated

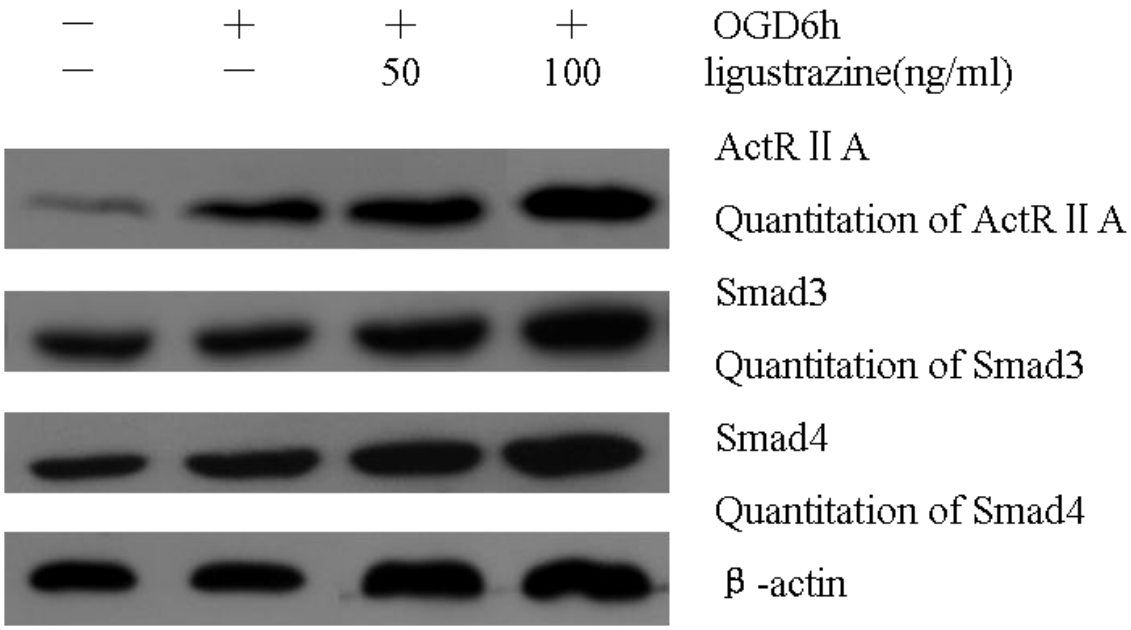

Figure 4. Western blot analysis. PC12 cells were treated with OGD for 6 h or different concentrations of ligustrazine for 24 h combined with OGD for 6 h. ActRIIA, Smad3 and Smad4 expression levels were determined using western blots. The results shown are representative of three independent experiments. NIH imaging indicated that the protein signal densities increased in the groups treated with ligustrazine combined with OGD for 6 h compared with OGD for 6 h-treated groups.

group. Compared with the OGD for 6 h-treated group, the SOD activity increased following treatment with different concentrations of ligustrazine for 24 h combined with OGD for 6 h. Furthermore, the treatment with 50 or 100 ng/ml ligustrazine, led to a significant increase in SOD protein levels (Figure 5B).

DISCUSSION

Ligustrazine can be absorbed rapidly through the gastric wall with a quite short plasma elimination half-life. However, it has a long retention time in blood-rich tissue. It also experiences enterohepatic circulation and is partially reabsorbed into the brain. Repeated circulation increases the contact time between the drug and the intestinal tract as well as the liver. Therefore, first pass effect is more evident when ligustrazine is taken. Several ligustrazine preparations via transdermal administration have been reported to improve its bioavailability and safety (Liu et al, 2011; Yu, 2003; Qiu et al, 2006).

The pathogenesis of ischemic cerebrovascular disease is complex and is related to electrolyte imbalances, the production of oxygen free radicals, lipid peroxidation, NO, monoamine neurotransmitters, phospholipid metabolism and the synthesis of other damaging factors. The generation of oxygen-free radicals and NO plays an important role in the promotion of ischemic cerebrovascular disease. Existing animal models are affected by many factors; therefore, a stable cell model of cerebral ischemia was used in this study because the ability to control the experimental conditions, the small amount of sample required and the short experimental period has clear advantages (Curtis and Walker, 1988).

An OGD-damage model is one of the most commonly used models for the study of cerebral ischemia. The principle of the OGD model is that $Na_2S_2O_4$ quickly clears the oxygen in the culture matrix. In this study, oxygen and glucose deprivation was applied to PC12 cells and the results show that the OGD for 6 h-treated group effectively inhibited the growth of PC12 cells, indicating that the cell model causes cell damage (Mei et al., 2011). When compared with the OGD for 6 h-treated group, the ActA plus OGD for 6 h-treated groups effectively increased the survival rate of PC12 cells (Figures 2 and 3). Therefore, ActA has a protective effect on OGD-induced cell injury. Smad2, Smad3, a total adjustment type Smad4 and inhibitory Smad7 participate in the ActA/Smad pathway. Our results demonstrate that treatment with OGD for 6 h increases the expression of ActRIIA, Smad3 and Smad4. When compared to the OGD for 6 h-treated group, ActRIIA, Smad3 and Smad4 expression levels increased, following treatment with different concentrations of ligustrazine for 24 h combined with treatment with OGD for 6 h (Figure 4). Ligustrazine may mediate the ActA/Smad signaling pathway.

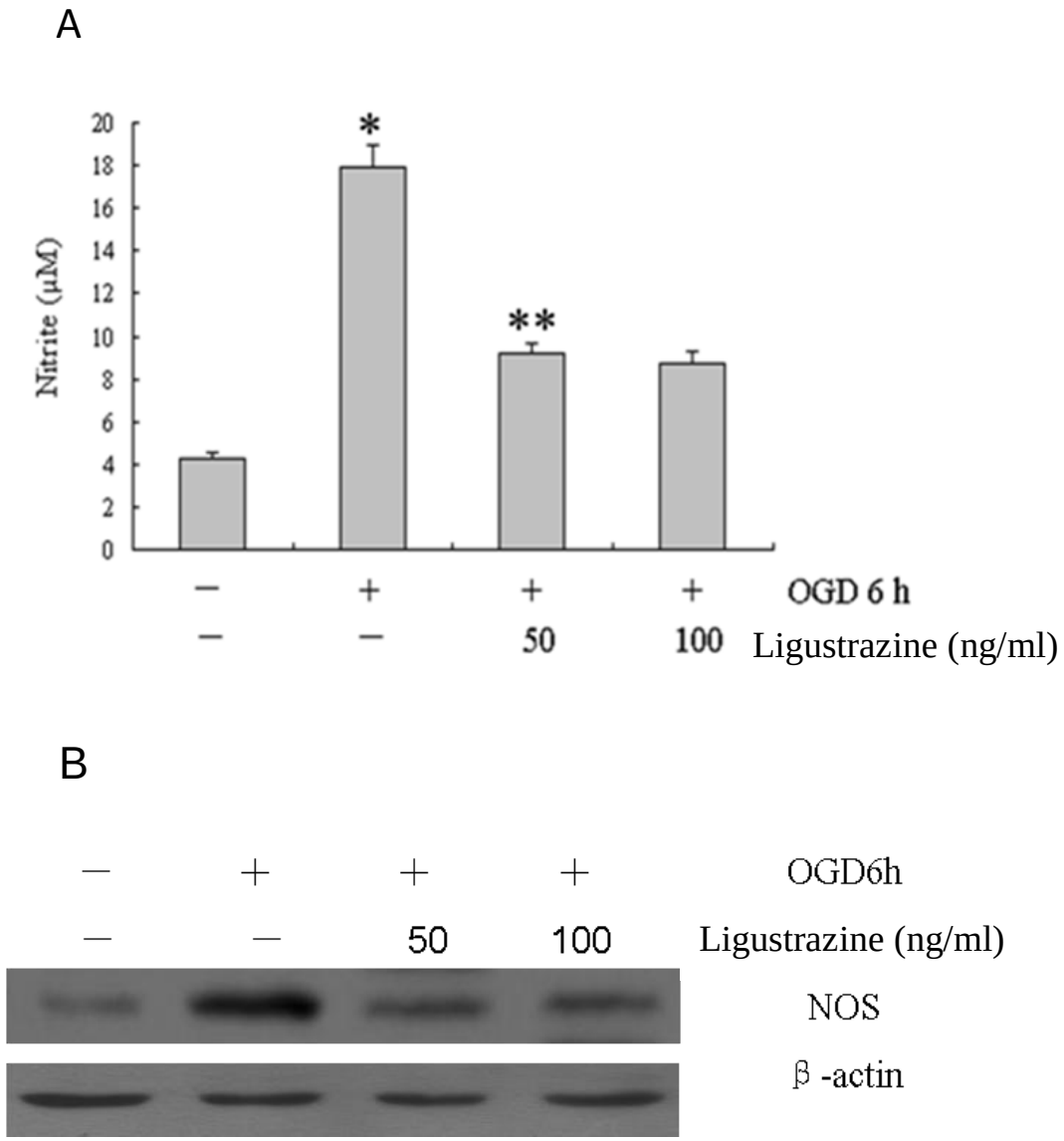

Figure 5. Effect of ligustrazine on oxidative stress in OGD for 6 h-induced PC12 cells. (A) Cells were OGD-induced by incubation with 50 or 100 ng/ml of ligustrazine for 24 h combined with OGD for 6 h. The nitrite content was measured using the Griess reaction. The values indicate the nitrite production in cells that were exposed to the culture supernatants collected from cells that were treated with OGD alone or cells that were exposed to OGD plus Ligustrazine. *denotes a significant difference from the control, $P < 0.05$; **denotes a significant difference from OGD for 6 h-treated cells, $P < 0.05$. The data represent the means ± SD from three independent experiments that were performed in triplicate. (B) Western blot analysis of OGD-induced cells or cells treated with increasing concentrations (50 and 100 ng/ml) of ligustrazine for 24 h combined with OCD for 6h. The western blot shows the levels of NOS protein expression. β-actin was used as an internal control.

Many studies indicate that oxidative stress plays a key role in ischemic cerebrovascular disease. SOD plays a vital role in the body's oxidant and antioxidant balance by removing superoxide anion radicals and protecting cells from damage. NO is an important messenger and effector molecule *in vivo*, and plays a role in neurotransmitter functions in physiological and pathological events, therefore, SOD and NO are involved in oxidative damage. The results show that OGD significantly increases NO levels and decreases SOD activity, whereas ligustrazine reduces NO levels and increases SOD activity, and this may be the mechanism by which ligustrazine promotes the neuroprotective effect (Figures 5 and 6) (Yu and Gao, 2003).

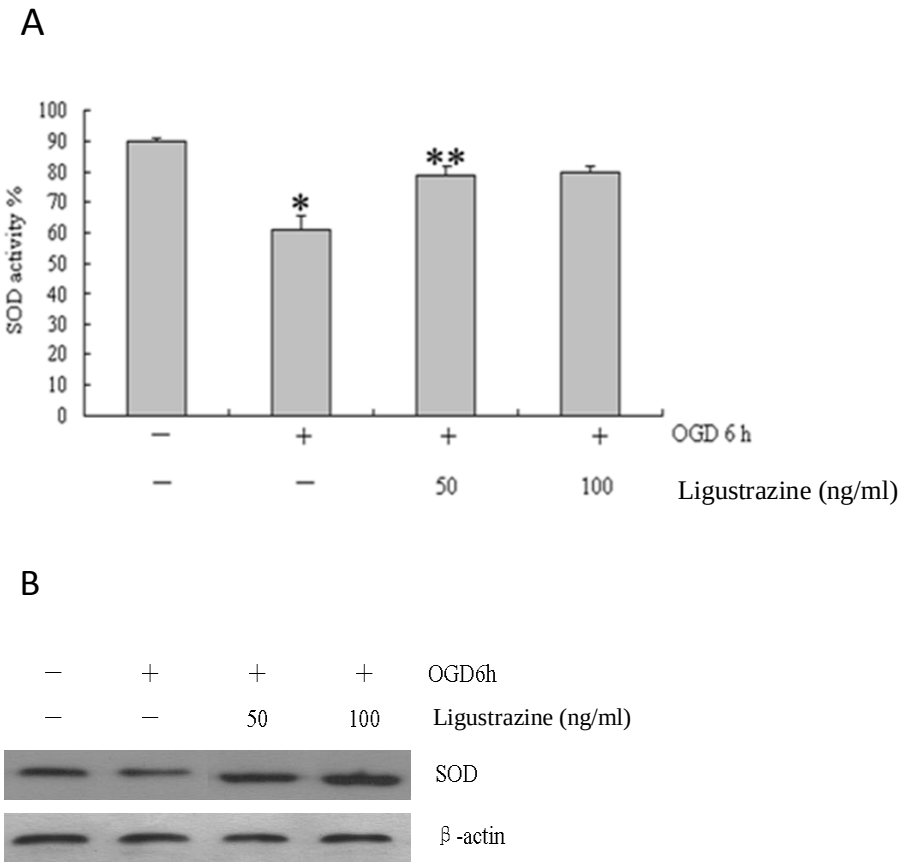

Figure 6. The effect of ligustrazine on SOD activity. (A) Cells were OGD-induced or were treated with 50 or 100 ng/ml of ligustrazine for 24 h plus OGD for 6 h. The effect of ligustrazine on SOD activity was assessed. *denotes a significant difference from control ($P < 0.05$); **denotes a significant difference from the OGD plus 6 h-treated group ($P < 0.05$). The data represent the mean ± SD obtained from three independent experiments that were performed in triplicate. (B) Western blot analysis of cells treated with OGD for 6 h or cells treated with OGD for 6 h in combination with increasing concentrations (50 and 100 ng/ml) of ligustrazine for 24 h. The western blot shows the SOD protein expression. β-actin was used as an internal control.

In conclusion, our results demonstrate that ligustrazine protects PC12 cells in an OGD-deprivation model through the activation of the Smad pathway, the inhibition of NO production and the increased activation of SOD. Further investigation is required to understand fully the beneficial role of ligustrazine in post-ischemic injury and will eventually lead to clinical interventions that will salvage brain cells that are at risk in ischemic cerebrovascular disease.

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