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EXPERIMENTAL TRIALS

The antioxidant effect of preischemic dexmedetomidine in a rat model: increased expression of Nrf2/HO-1 via the PKC pathway

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Protein kinase C

Abstract

Background: The precise underlying mechanism of antioxidant effects of dexmedetomidine-induced neuroprotection against cerebral ischemia has not yet been fully elucidated. Activation of Nuclear factor erythroid 2-related factor (Nrf2) and Heme Oxygenase-1 (HO-1) represents a major antioxidant-defense mechanism. Therefore, we determined whether dexmedetomidine increases Nrf2/HO-1 expression after global transient cerebral ischemia and assessed the involvement of Protein Kinase C (PKC) in the dexmedetomidine-related antioxidant mechanism.
Methods: Thirty-eight rats were randomly assigned to five groups: sham (n = 6), ischemic (n = 8), chelerythrine (a PKC inhibitor; 5 mg.kg^{-1} IV administered 30 min before cerebral ischemia) (n = 8), dexmedetomidine (100 μg.kg^{-1} IP administered 30 min before cerebral ischemia (n = 8), and dexmedetomidine + chelerythrine (n = 8). Global transient cerebral ischemia (10 min) was applied in all groups, except the sham group; histopathologic changes and levels of nuclear Nrf2 and cytoplasmic HO-1 were examined 24 hours after ischemia insult.
Results: We found fewer necrotic and apoptotic cells in the dexmedetomidine group relative to the ischemic group ($p < 0.01$) and significantly higher Nrf2 and HO-1 levels in the dexmedetomidine group than in the ischemic group ($p < 0.01$). Additionally, chelerythrine co-administration with dexmedetomidine attenuated the dexmedetomidine-induced increases in Nrf2 and HO-1 levels ($p < 0.05$ and $p < 0.01$, respectively) and diminished its beneficial neuroprotective effects.

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Conclusion: Preischemic dexmedetomidine administration elicited neuroprotection against global transient cerebral ischemia in rats by increasing Nrf2/HO-1 expression partly via PKC signaling, suggesting that this is the antioxidant mechanism underlying dexmedetomidine-mediated neuroprotection.

Introduction

Dexmedetomidine is a highly selective α_2 adrenergic agonist with analgesic and sedative properties; it has been shown to be a potent neuroprotective agent in various experimental models of cerebral ischemia/reperfusion injury.[1–4] The antioxidant effects of dexmedetomidine might represent a possible mechanism of dexmedetomidine-induced neuroprotection. A recent study conducted in patients undergoing brain-tumor surgery demonstrated that dexmedetomidine pretreatment significantly reduced the level of Malondialdehyde (MDA; an oxidative stress marker) and increased the production of Superoxide Dismutase (SOD; an antioxidant enzyme that catalyzes the breakdown of superoxidants).[5] Additionally, an experimental study reported that dexmedetomidine administration after focal cerebral ischemia decreased MDA levels and increased those of glutathione peroxidase and glutathione (an antioxidant enzyme and its substrate, respectively).[6] However, the exact mechanism by which dexmedetomidine activates antioxidant enzymes has not yet been fully elucidated.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the cap'n'collar family of transcription factors and is important in regulating antioxidant-defense genes. Upon phosphorylation, Nrf2 translocates to the nucleus and initiates transcription of cytoprotective genes, thereby promoting the production of antioxidant enzymes, such as Heme Oxygenase-1 (HO-1), SOD, and NAD(P)H-quinone oxidoreductase.[7] Protein Kinase C (PKC) phosphorylates Nrf2, detaches phosphorylated Nrf2 from Kelch-like ECH Associated Protein 1 (KEAP1), and facilitates Nrf2 translocation into the nucleus. Increases in Nrf2 are associated with the neuroprotective effects of Acetyl-11-Keto-beta-Boswellic Acid (AKBA),[8] metformin pretreatment,[9] omega-3 fatty acids,[10] and sevoflurane post-conditioning[11] following cerebral ischemic injury. Additionally, Chen et al.[4] revealed that dexmedetomidine induces the cytoplasm-to-nucleus translocation of Nrf2 and inhibits its acetylation; however, whether dexmedetomidine mediates neuroprotective effects via the upregulation of antioxidant enzyme production remains unknown; moreover, the role of the PKC pathway in the potential antioxidant mechanism of dexmedetomidine is also unclear.

In this study, we tested our hypothesis that dexmedetomidine pretreatment provides neuroprotection against global transient cerebral ischemia/reperfusion injury by increasing Nrf2 and HO-1 expression via the PKC pathway.

Methods

Experimental protocols

The study was approved by the Seoul National University Institutional Animal Care and Use Committee (Seoul, Republic of Korea) and performed using a transient global cerebral ischemia model in rats. This study adhered to the ARRIVE guidelines. Male Sprague–Dawley rats (age: 9–11 weeks; weight: 300–320 g) were housed in individual cages under an alternating 12 h light/dark cycle; all rats used in this study were fasted for 12 hours before the procedure but with ad libitum access to water.

Group assignment

Thirty-eight rats were randomly assigned to five groups using computerized randomization (Microsoft© Excel 2016): sham, ischemic, dexmedetomidine, chelerythrine, and dexmedetomidine + chelerythrine (Fig. 1). In the sham group (n = 6), surgical exposure of the major neck vessels was performed, but transient global cerebral ischemia/reperfusion was not. Rats in the ischemic group (n = 8) underwent surgical exposure and transient global cerebral ischemia for 10 minutes, but no drug was administrated. The dexmedetomidine group (n = 8) underwent the same surgical procedure and cerebral ischemia/reperfusion, but preischemic dexmedetomidine (100 $\mu g.kg^{-1}$; Hospira, Lake Forest, IL, USA) was injected intraperitoneally 30 minutes before ischemic insult. In the chelerythrine group (n = 8), 5 $mg.kg^{-1}$ chelerythrine,[12] a nonselective PKC inhibitor, was administered intravenously 30 minutes before transient global cerebral ischemic insult. In the dexmedetomidine + chelerythrine group (n = 8), both dexmedetomidine and chelerythrine were administered.

Surgical preparation

General anesthesia was used for the surgical procedure. Tiletamine HCl and zolazepam HCl (Zoletil 20 $mg.kg^{-1}$) and xylazine 5 $mg.kg^{-1}$ were injected intraperitoneally before starting the procedure. All rats were intubated: ventilation was maintained with 40% oxygen and 60% nitrogen. Mechanical ventilation was adjusted to maintain the arterial carbon dioxide partial pressure between 35 mmHg and 45 mmHg. Anesthesia depth was monitored by checking the corneal or pedal reflexes and responses to tail pinch.[13] When the reflexes were positive or arterial systolic blood pressure increased by >20% of the baseline systolic blood pressure,

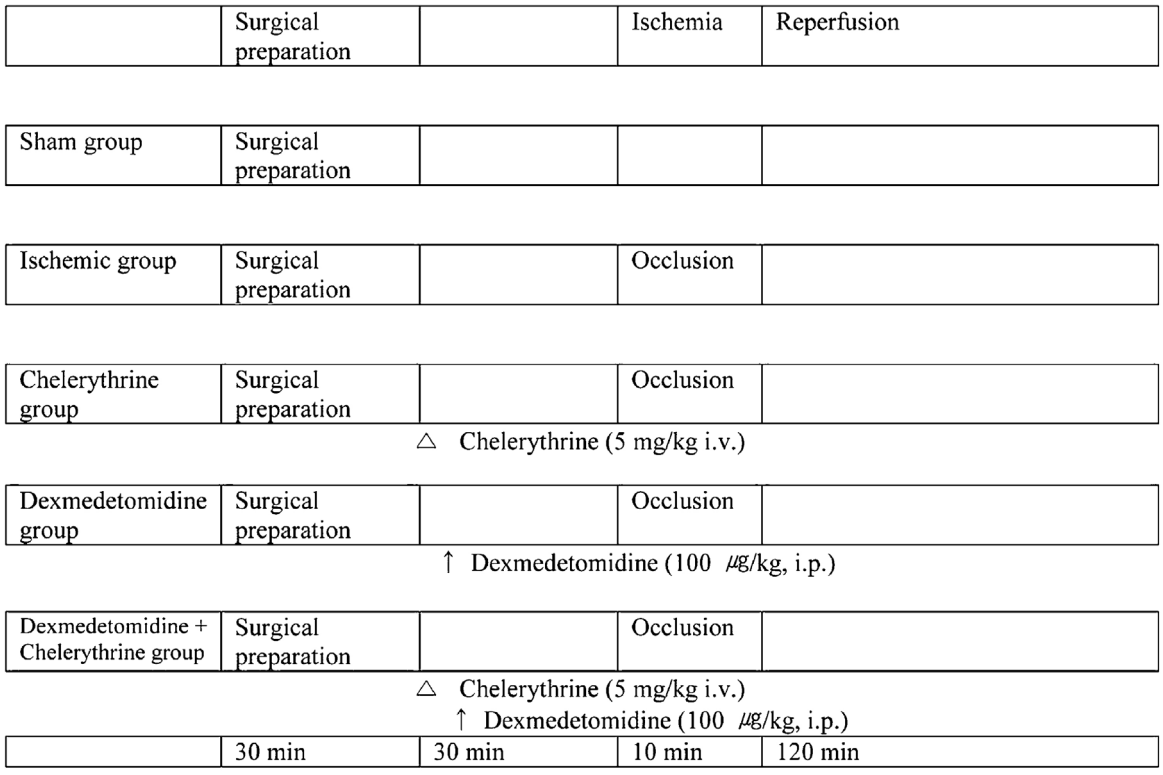

Figure 1 Experimental protocol.

additional zoletil 10 mg.kg^{-1} and xylazine 5 mg.kg^{-1} were administered intravenously. Arterial blood-pressure monitoring was performed via the femoral artery. The femoral vein was catheterized for drug administration. A rectal temperature probe was placed, and an additional 22G needle thermistor (model TCAT-2 temperature controller; Harvard Apparatus, Hollison, MA, USA) was placed under the temporalis muscle. Body temperature was maintained at approximately 37 °C.

Transient global cerebral ischemia/reperfusion model

The transient global cerebral ischemia/reperfusion model was performed based on a previously described method.[13] We used a midline incision between the neck and sternum and identified both the common carotid arteries and right internal jugular vein. For direct measurement of cerebral blood flow, a laser Doppler Monitoring System (VMS-LDF2; Moor Instruments, Axminster, UK) was used, with the Doppler probe placed 2 mm posterior and 5 mm lateral to bregma on the skull surface. After intravenous administration of 50U heparin, cerebral ischemia was initiated by withdrawing blood from the internal jugular vein until the regional cerebral blood flow was <50% of the baseline value and the Mean Arterial blood Pressure (MAP) was 26–30 mmHg. Both common carotid arteries were then clamped using vascular clips; the ischemic period was maintained for 10 minutes. The clips on both common carotid arteries were then declamped, and the blood withdrawn from the vein was slowly reinfused for reperfusion. After hemodynamic stabilization, the surgical devices and catheters were removed, and the incision site was completely closed. For postoperative pain control, local injection of 0.5% bupivacaine was performed. After recovery from anesthesia, the rats were placed back in their cages. Hemodynamic data and arterial blood-gas analysis were recorded at three time points: baseline (10 min before ischemia), ischemia (5 min after ischemia), and reperfusion (10 min after reperfusion).

Histopathologic analyses

At 24 hours post-treatment, rats were anesthetized with an intraperitoneal injection of zoletil (20 mg.kg^{-1}) and then decapitated. The brains were removed and divided transversely into two parts using a rat brain slice matrix. The anterior part of each brain was placed in liquid nitrogen and stored at −80 °C until western blot analysis, whereas the posterior part was fixed in buffered 10% formalin for histopathologic examination. Paraffin wax-embedded brain sections were sliced into serial coronal 5 μm thick sections and stained with Hematoxylin and Eosin (H&E). Terminal deoxynucleotidyl Transferase dUTP nick End-Labeling (TUNEL) staining was performed using an Apoptag peroxidase in situ apoptosis detection kit (S7100; Millipore, Billerica, MA, USA) according to the manufacturer's instructions. An investigator blinded to the groups examined the slides using light microscopy. Necrotic cells were defined as those with nuclear swelling and flocculated chromatin and/or the loss of nuclear basophilia. Cells containing apoptotic bodies were stained dark blue in the nucleus and

identified as apoptotic cells. The total number of viable and pathologic cells was counted in six optical fields (medial, middle, and lateral parts of the hippocampal CA1 region in each cerebral hemisphere) under high-power magnification (400×); the percentage of necrotic and TUNEL-positive cells relative to the total cell number was calculated.

Western blot analysis

Cytosolic and nuclear proteins were extracted using a native membrane protein extraction kit (444810 ProteoExtract; Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions. The cells were incubated in extraction buffer and centrifuged; the cytosolic protein fraction was extracted first, followed by the nuclear portion. The protein samples were electrophoresed through a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. After transfer, the membranes were incubated with primary antibodies: monoclonal anti-rabbit Nrf2 (Santa Cruz Biotechnology, Dallas, TX, USA), cleaved caspase-3 (Sigma-Aldrich, St. Louis, MO, USA), and HO-1 (Santa Cruz Biotechnology), respectively. The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (Sigma-Aldrich) for 1 hour at room temperature. Proteins were detected using an enhanced chemiluminescence kit (Amersham, Little Chalfont, UK), with densitometric analysis for quantification. Cytoplasmic cleaved caspase-3 and HO-1 levels were quantified by comparing with β-actin, whereas the nuclear Nrf2 level was compared to that of histone H3.

Statistical analysis

Descriptive values are expressed as the medians and interquartile ranges. Differences between groups according to histopathological changes and western blot results were analyzed using Kruskal-Wallis and Mann-Whitney *U* tests. A $p < 0.01$ was considered statistically significant. SPSS software (version 22.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Physiological variables were measured at three time points: baseline, during ischemia, and 10 minutes after reperfusion. MAP during ischemia was significantly lower and MAP during reperfusion was significantly higher in all groups with ischemia than in the sham group ($p < 0.01$), and cerebral blood flow during ischemia was significantly lower in all groups with ischemia than in the sham group ($p < 0.01$). Glucose levels did not differ significantly among the five groups.

H&E staining showed a significantly higher percentage of necrotic cells in the ischemic, chelerythrine, dexmedetomidine, and dexmedetomidine + chelerythrine groups relative to that in the sham group ($p < 0.01$) (Fig. 2). Additionally, the number of necrotic cells was lower in the dexmedetomidine group than in the ischemic group ($p < 0.01$). Moreover, both the chelerythrine and dexmedetomidine + chelerythrine groups showed higher numbers of necrotic cells than the dexmedetomidine group ($p < 0.01$).

TUNEL-positive neurons (Fig. 3) revealed that the percentage of apoptotic cells was significantly higher in the ischemic, chelerythrine, dexmedetomidine, and dexmedetomidine + chelerythrine group than in the sham group ($p < 0.01$). Furthermore, the number of apoptotic cells was significantly lower in the dexmedetomidine group than in the ischemic, chelerythrine, and dexmedetomidine + chelerythrine groups ($p < 0.01$).

In the dexmedetomidine and dexmedetomidine + chelerythrine groups, the expression of Nrf2 was higher than that in the sham group ($p < 0.01$) (Fig. 4); the Nrf2 levels were significantly higher in the chelerythrine, dexmedetomidine, and dexmedetomidine + chelerythrine groups than in the ischemic group ($p < 0.01$). Additionally, Nrf2 expression in the dexmedetomidine group was higher than that in the chelerythrine group ($p < 0.01$) and higher than that in the dexmedetomidine + chelerythrine group ($p = 0.015$). In the chelerythrine, dexmedetomidine, and dexmedetomidine + chelerythrine groups, the expression of HO-1 was significantly higher than that in the sham group ($p < 0.01$) (Fig. 5) and significantly higher in the dexmedetomidine group than in the ischemic and dexmedetomidine + chelerythrine groups ($p < 0.01$). Moreover, the level of cleaved caspase-3 was significantly higher in the ischemic, chelerythrine, dexmedetomidine, and dexmedetomidine + chelerythrine groups than in the sham group ($p < 0.01$) (Fig. 6), whereas, in the dexmedetomidine group, the cleaved caspase-3 level was significantly lower than that in the ischemic and chelerythrine groups ($p < 0.01$). However, we observed no difference in the cleaved caspase-3 levels between the dexmedetomidine and dexmedetomidine + chelerythrine groups ($p = 0.195$).

Discussion

In this study, preischemic dexmedetomidine administration showed neuroprotective effects in rats after transient global cerebral ischemia, with dexmedetomidine pretreatment enhancing Nrf2 and HO-1 expression and reducing caspase-3 activity. However, this benefit was reduced by the combined administration of chelerythrine, a nonselective PKC inhibitor, and dexmedetomidine.

Initial hypoxia during ischemic insult induces adenosine triphosphate depletion, resulting in an increased release of glutamate and other excitatory neurotransmitters and an uncontrolled increase in intracellular calcium levels. In response to increased calcium levels and oxidative stress, the permeability of the inner mitochondrial membrane changes abruptly; subsequent reperfusion after ischemia causes further damage via the overproduction of Reactive Oxygen Species (ROS).[14] The accumulation of ROS, which includes both free radicals and oxidants, irreversibly oxidizes macromolecules, such as DNA, lipids, and proteins, thereby causing severe cell injury.[15] Levels of ethidine, an oxidized hydroethidine converted by O_2^-, are elevated in the rat hippocampal CA1 region after global ischemia[16]; therefore, oxidative stress plays an important role in global cerebral ischemic injury; detoxification or prevention of

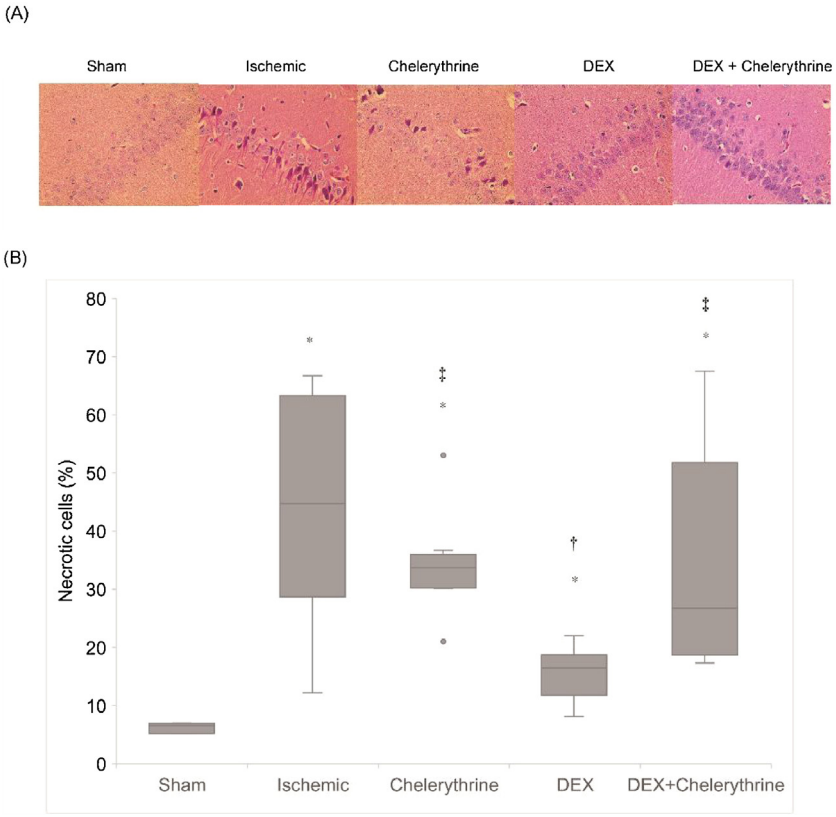

Figure 2 (A) Representative photomicrographs showing necrotic and viable cells in the hippocampal CA1 regions 24 h after transient global cerebral ischemia (H&E staining). (B) Percentages of necrotic cells in the hippocampal CA1 region 24 h after ischemia. Results are presented as the median ± interquartile range. *$p < 0.01$ vs. the sham group; †$p < 0.01$ vs. the ischemic group; ‡$p < 0.01$ vs. the dexmedetomidine group. Che, Chelerythrine; DEX, Dexmedetomidine.

oxidative stress represents a primary concern for organ protection from ischemia/reperfusion injury.

Experimental studies have demonstrated the neuroprotective effects of dexmedetomidine after cerebral ischemia/reperfusion injury. Preischemic subcutaneous injection of dexmedetomidine before ischemia, followed by a continuous infusion for 48 hours after reperfusion, effectively prevented neuronal death in CA3 in a transient global ischemia model in gerbils.[1] Additionally, dexmedetomidine treatment after focal cerebral ischemia/reperfusion decreased cerebral infarct volume and neuron death.[17] Moreover, both sevoflurane postconditioning and preischemic dexmedetomidine administration individually exerted neuroprotective effects, although the combination of sevoflurane and dexmedetomidine showed no additional benefit.[18] In the present study, we observed a greater proportion of viable cells and fewer apoptotic cells in the hippocampal CA1 area, which suggests a neuroprotective effect in rats treated with dexmedetomidine. Furthermore, there were fewer necrotic and apoptotic cells in rats pretreated with dexmedetomidine before ischemic insult. Previous studies reported that dexmedetomidine significantly decreased ischemia/reperfusion injury in other organs, including the kidney, heart, and lung.[19–22]

However, the molecular mechanism underlying dexmedetomidine-induced neuroprotection has not yet been fully explored. A recent study suggested that the protective effect of dexmedetomidine occurs via an intrinsic mitochondria-dependent pathway.[23] Dexmedetomidine is considered to affect both the Phosphoinositide 3-Kinase (PI3K)/Akt and Extracellular Regulated Kinase (ERK) 1/2 pathways during ischemia/reperfusion injury as the neuroprotective effect of dexmedetomidine was reduced following the use of PI3K inhibitors.[17] Additionally, the anti-inflammatory effect via the tumor necrosis factor pathway is a proposed mechanism of dexmedetomidine-induced neuroprotection.[2] Other studies focused on the antioxidant properties of dexmedetomidine to explore its protective effects on other organs, such as kidney, heart, and lung.[19–21] The authors suggested that acute kidney injury following orthotopic liver transplantation was caused by oxidative damage due to an increase in MDA and hydroxyl free radicals (OH); however, pretreatment with dexmedetomidine regulated the ROS levels and enhanced antioxidation via Nrf2 activation.[19] Moreover, a previous experimental study reported a synergistic neuroprotective effect of the combined use of propofol and dexmedetomidine, which caused the increased expression of antioxidant enzymes, such as SOD.[24]

Nrf2 is an essential activator of the transcription of genes encoding antioxidant enzymes, such as HO-1. Previous studies demonstrated a positive relationship between many exogenous materials and neuroprotection via increased Nrf2 expression. A study reported reductions in MDA level and

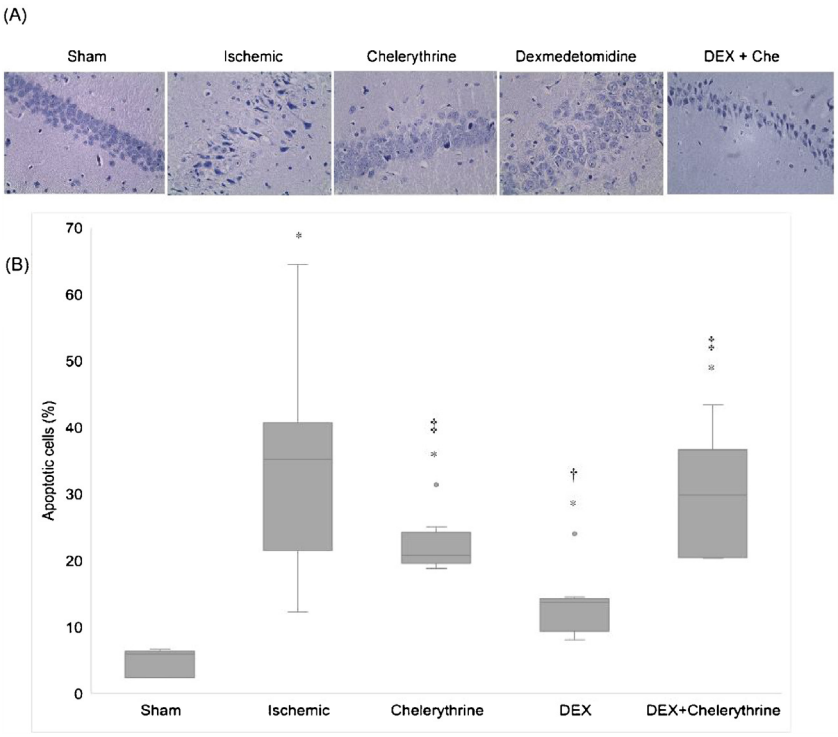

Figure 3 (A) Representative photomicrographs showing apoptotic and viable cells in the hippocampal CA1 region 24 h after transient global cerebral ischemia (TUNEL staining). (B) Percentage of apoptotic cells in the hippocampal CA1 region 24 h after ischemia. Results are presented as the median ± interquartile range. *$p < 0.01$ vs. the sham group; †$p < 0.01$ vs. the ischemic group; ‡$p < 0.01$ vs. the dexmedetomidine group. Che, Chelerythrine; DEX, Dexmedetomidine.

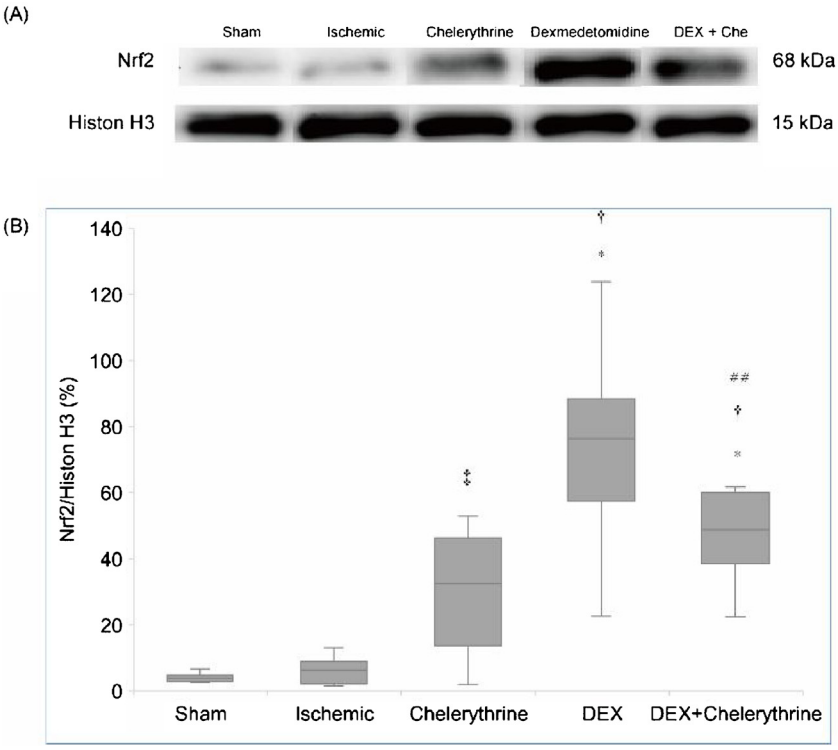

Figure 4 Densitometric evaluation of Nrf2 levels. Results are presented as the median ± interquartile range. Histone H3 was used as a control. *$p < 0.01$ vs. the sham group; †$p < 0.01$ vs. the ischemic group; ‡$p < 0.01$ vs. the dexmedetomidine group; ##$p < 0.05$ vs. the dexmedetomidine group. Che, Chelerythrine; DEX, Dexmedetomidine.

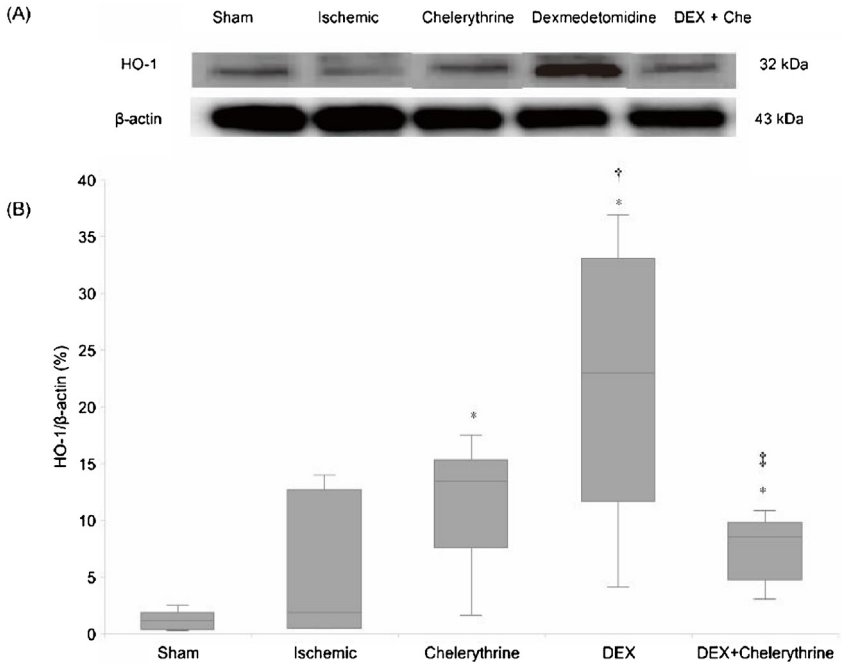

Figure 5 Densitometric evaluation of HO-1 levels. Results are presented as the median ± interquartile range. β-Actin was used as a control. *$p < 0.01$ vs. the sham group; †$p < 0.01$ vs. the ischemic group; ‡$p < 0.01$ vs. the dexmedetomidine group. Che, Chelerythrine; DEX, Dexmedetomidine.

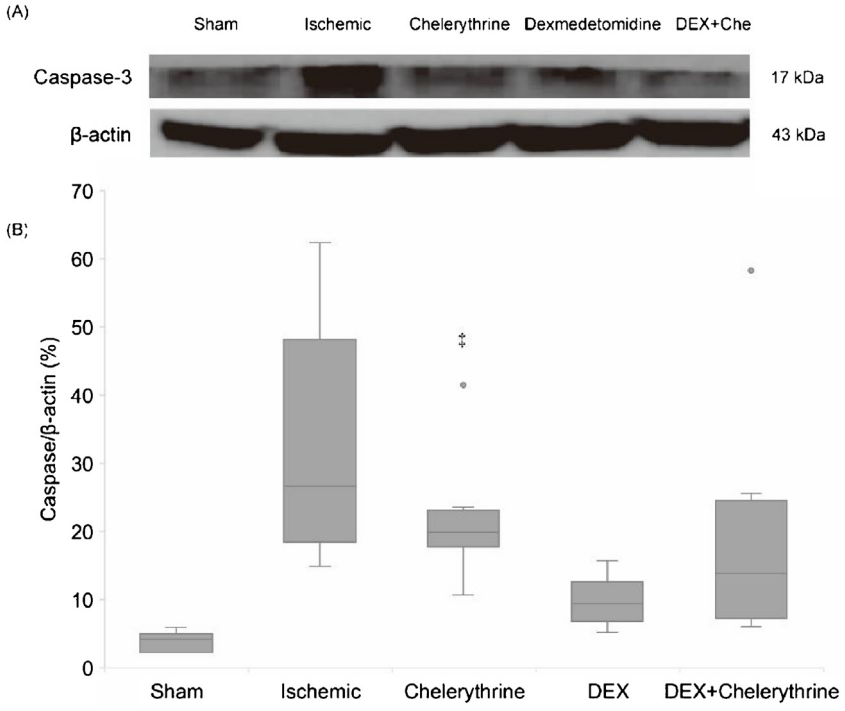

Figure 6 (A) Representative western blot analysis of caspase-3 24 h after transient global cerebral ischemia. (B) Densitometric evaluation of cleaved caspase-3 levels. Results are presented as the median ± interquartile range. β-Actin was used as a control. *$p < 0.01$ vs. the sham group; †$p < 0.01$ vs. the ischemic group; ‡$p < 0.01$ vs. the dexmedetomidine group. Che, Chelerythrine; DEX, Dexmedetomidine.

increases in Nrf2 and HO-1 levels following AKBA treatment after ischemia/reperfusion injury, suggesting that the activation of the Nrf2/HO-1 defense pathway is involved in the AKBA-related neuroprotective effect.[8] Additionally, metformin pretreatment enhanced the levels of Nrf2 and HO-1 in the hippocampus of ischemic rats,[9] with HO-1 level showing

a similar pattern to Nrf2 expression as HO-1 is an antioxidant enzyme activated by Nrf2. In our study, both the Nrf2 and HO-1 levels were higher in the dexmedetomidine group than in the control group. These findings suggest that the antioxidant pathway related to dexmedetomidine might be an important underlying mechanism of dexmedetomidine-induced neuroprotection.

In this study, the increased Nrf2 expression induced by dexmedetomidine was significantly inhibited upon co-administration of chelerythrine with dexmedetomidine, suggesting that PKC activation is closely associated with the antioxidant pathway induced by dexmedetomidine. PKC activation leads to its binding to diacylglycerol in the cell membrane and its activity as a messenger that phosphorylates other molecules, including Nrf2 as a major transcription factor regulating antioxidant-defense mechanisms.[25] Similarly, another study evaluating the effect of ischemic preconditioning on cardiac protection in an isolated rabbit heart model showed that administration of polymyxin B, a PKC inhibitor, inhibited the nuclear accumulation of Nrf2.[26] These findings and our results suggest that PKC plays a critical role in the nuclear translocation of Nrf2 in response to oxidative stress.

After cerebral ischemia/reperfusion injury, apoptosis is a major and terminal pathway of cell death. The anti-apoptotic effect of B-cell lymphoma-2 (Bcl-2) and pro-apoptotic Bcl-2-associated X protein are related to cell death. Additionally, caspase-3 is a member of the cysteine-aspartic acid protease family; its activation leads to cell death. Nrf2 is involved in the increased expression of anti-apoptotic factors, such as Bcl-2, and the decreased levels of caspase-3.[27] In the present study, we found a significant increase in caspase-3 expression in the control group, whereas, in the dexmedetomidine group, elevated Nrf2 levels were accompanied by decreased caspase-3 levels, indicating the anti-apoptotic effect of dexmedetomidine. However, co-administration of chelerythrine with dexmedetomidine did not significantly reduce the anti-apoptotic effect of dexmedetomidine, possibly because significant apoptosis occurs more than 24 hours after ischemia/reperfusion injury. A previous study demonstrated significant increases in both cell death due to apoptosis as well as caspase-3 levels at 7 days rather than 1 day after cerebral ischemia/reperfusion injury.[11]

This study has several limitations. First, there are various PKC subtypes, including PKC_{α}, PKC_{β}, PKC_{γ}, PKC_{δ}, and PKC_{ε}, with varying activities associated with cerebral ischemia/reperfusion injury.[28] Because a nonspecific PKC inhibitor was used in the present study to evaluate the role of PKC in Nrf2 expression, we could not determine the exact PKC subtype involved in the PKC–Nrf2. Second, we did not completely exclude other pathways involved in Nrf2 activation. In addition to the PKC pathway, Nrf2 is activated by various intracellular signaling pathways, including those associated with PI3K/Akt,[29] protein kinase CK2,[30] c-Jun N-terminal kinase, and ERK.[31] However, although several kinases affect Nrf2 transport, PKC is a representative Nrf2 nuclear transporter. Third, we did not evaluate changes in neurological behavior. Although we found significant histopathological differences among groups, neurological tests might be helpful in strengthening our results. Forth, we did not evaluate the long-term antioxidant effect of dexmedetomidine as the brains were removed for western blot examination 24 hours after ischemia/reperfusion injury. Finally, the biochemical and histological evaluations were done in different part of the brain. As it was technically impossible in our circumstance to use the same small piece of tissue for both tests.

In conclusion, pretreatment with dexmedetomidine not only reduced neuronal necrosis and apoptosis but also enhanced Nrf2 and HO-1 expression via the PKC pathway following global transient cerebral ischemia/reperfusion injury in rats. Our findings suggest that the antioxidant effect of dexmedetomidine contributes to dexmedetomidine-induced neuroprotection and that PKC might be in partly involved in the antioxidant mechanism of dexmedetomidine.

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Availability of data and materials

Data are available within the article or its supplementary materials.

Conflicts of interest

The authors declare no conflicts of interest.

Credit authorship contribution statement

Yong-Hee Park: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. **Hee-Pyoung Park:** Validation, Formal analysis, Visualization. **Eugene Kim:** Methodology, Software. **Hannah Lee:** Methodology, Software, Investigation, Data curation. **Jung-Won Hwang:** Validation, Formal analysis. **Young-Tae Jeon:** Validation, Supervision. **Young-Jin Lim:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

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