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·Original Article·

Protective effects of carbenoxolone are associated with attenuation of oxidative stress in ischemic brain injury

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ABSTRACT

Accumulating evidence has suggested that the gap junction plays an important role in the determination of cerebral ischemia, but the underlying mechanisms remain to be elucidated. In this study, we assessed the effect of a gap-junction blocker, carbenoxolone (CBX), on ischemia/reperfusion-induced brain injury and the possible mechanisms. By using the transient cerebral ischemia model induced by occlusion of the middle cerebral artery for 30 min followed by reperfusion for 24 h, we found that pre-administration of CBX (25 mg/kg, intracerebroventricular injection, 30 min before cerebral ischemic surgery) diminished the infarction size in rats. And this was associated with a decrease of reactive oxygen species generation and inhibition of the activation of astrocytes and microglia. In PC12 cells, H_2O_2 treatment induced more coupling and apoptosis, while CBX partly inhibited the opening of gap junctions and improved the cell viability. These results suggest that cerebral ischemia enhances the opening of gap junctions. Blocking the gap junction with CBX may attenuate the brain injury after cerebral ischemia/reperfusion by partially contributing to amelioration of the oxidative stress and apoptosis.

Keywords: gap junction communication; cerebral ischemia; reactive oxygen species

INTRODUCTION

Gap junctions exist ubiquitously in cell membranes as intercellular channels and are composed of transmembrane proteins called connexins (Cxs), which, when open, permit the intercellular diffusion of ions and small molecules[1,2]. Gap junctions have numerous functions in the brain, such as the synchronization of electrical activity between neurons and the regulation of metabolic action between astrocytes[3,4].

Cx43 hemichannels in astrocytes are susceptible to metabolic inhibition or in solutions free of divalent cations, which suggests that gap-junction communication is determined by the metabolic state[5,6]. Gap junctions remain open after cerebral ischemia[7], suggesting that gap-junction communication among astrocytes is involved in the pathological process after ischemic insult. The open gap junctions accelerate the spreading depression and induce cell death during ischemia or hypoxia[8]. On the other hand, however, communication through gap junctions is important in buffering ionic shifts and the exchange of metabolites generated during hypoxia or ischemia. Recent evidence suggests that this could be a protective strategy against

hypoxic or ischemic insults[9].

Recent studies have suggested that reducing gap-junction coupling with compounds results in decreased neuronal sensitivity to ischemic injury. However, many of the compounds used *in vivo*, such as octanol, heptanol and halothane, have major side-effects[10-12], and intefere with synaptic transmission[13,14]. Most of their neuroprotective effects in cerebral ischemia depend not only on blocking gap junctions, but also other actions. In this study, we attempted to explore whether specific gap-junction blockers affect the cellular sensitivity to ischemic injury in rats and whether such blockade diminishes the diffusion of reactive oxygen species (ROS) *in vitro*. Carbenoxolone (CBX) is effective in blocking gap-junction coupling[15], interferes little with synaptic function *in vivo* and *in vitro*[16-19], and induces minimal changes in intrinsic neuronal characteristics[20,21]. In addition, this agent has been widely used as a mineralocorticoid agonist to inhibit 11-beta hydroxysteroid dehydrogenase and block gap-junction communication in various experimental studies[15,22,23].

Currently, the effect of CBX on ischemic damage remains to be elucidated[24,25]. Perez *et al.* reported that pretreatment with CBX, *via* a cannula implanted into the hippocampus in one hemisphere, results in decreased apoptosis in a global model of transient ischemia in rats[26]. Another study suggested that blocking gap-junctional communication with CBX or downregulating Cx43 increases the survival of pyramidal neurons in the ipsilateral hippocampus and improves behavioral scores in the rodent model of middle cerebral artery occlusion (MCAO)[27]. However, Tamura *et al.* reported that CBX accelerates the initiation and propagation of cortical spreading depression and worsens the outcome of focal cerebral ischemia[28]. Furthermore, some evidence has shown that CBX cannot pass the blood-brain-barrier very efficiently[29,30]. Therefore, the current study was performed to investigate *in vivo* the effects of CBX on transient cerebral ischemia through intracerebral ventricular injection, and further explore whether it can decrease ROS production and glial activation. To investigate whether CBX can block the gap-junction coupling associated with ROS diffusion *in vitro*, we mimicked oxidative injury with hydrogen peroxide and evaluated the coupling by the scrape-loading method in PC12 cells.

MATERIAL AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 270–300 g from the Animal Center of Lanzhou University were used. All animals were kept in standard cages at 22–24ºC with 40–60% humidity and lights on from 07:00 to 19:00. The protocols were approved by the Experimental Animal Committee of Lanzhou University. All efforts were made to minimize animal suffering and reduce the number of animals used.

Reagents

Carbenoxolone, cresyl violet and diaminobenzidine were from Sigma (St. Louis, MO); the mouse monoclonal antibodies anti-glial fibrillary acidic protein (GFAP), anti-CD11b, and anti-β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA); and rabbit polyclonal anti-Cx43 antibody was from Sigma. Other reagents were from Biotechnology of Shanghai (Shanghai, China) unless specified otherwise.

Cerebral Ischemia

MCAO was established by the intraluminal filament method as described previously[31]. Briefly, animals were anesthetized by 10% chloral hydrate (360 mg/kg, i. p.) and the right common and external carotid arteries were exposed. A nylon thread (4-0) was carefully inserted into the internal carotid artery and advanced towards the origin of the middle cerebral artery, resulting in MCAO. During the process of ischemia, laser Doppler perfusion monitoring was used to measure the blood flow in the trunk of the middle cerebral artery. A blood-flow drop to 60% of the baseline indicated successful blockage of the middle cerebral artery. After 30 min of MCAO, reperfusion was achieved by withdrawing the filament from the artery. After recovery from anesthesia, animals were kept in single cages for 24 h. Rectal temperature was maintained at 37 ± 0.5°C throughout using an electric blanket. The sham group received only incision of the cervical skin to expose the common carotid artery, without occlusion of blood flow.

Carbenoxolone Administration

The MCAO rats were randomly divided into two groups (12 rats each group). The CBX group received

intracerebroventricular injection of CBX (25 μg/kg, 5 μL) 30 min before MCAO, and the vehicle group received an equal volume of saline. Animals were fixed in a stereotaxic apparatus, and CBX or saline was injected into the right cerebral ventricle at the following coordinates: bregma: AP −0.8 mm, R +1.6 mm (midline) and depth 3.4 mm from dura. Animals were sacrificed after 24 h of reperfusion.

Measurement of ROS

Four rats from each group were randomly selected for ROS measurement. The ipsilateral striatum was dissected out and minced on ice, incubated with 0.25% trypsin at 37°C for 1 h, and washed with Dulbecco's modified Eale's medium (DMEM) containing 10% FBS to harvest the cellular suspension. Free-radical production was measured by the fluorescence method using 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA can cross cell membranes and is hydrolyzed by cellular esterases, then is oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of peroxides. Accumulation of DCF was measured using a spectrofluorometer (Shimadzu Corp., Japan).

Measurement of Infarct Size

Coronal sections were cut serially (25 μm thick, at 250-μm intervals) from 4% paraformaldehyde-fixed brain and stained with cresyl violet. Infarct areas were measured and infarct volume was calculated by integration of the infarct area in each section along the rostro-caudal axis.

Immunohistochemistry

For Cx43, GFAP and CD11b immunostaining, sections (at 0.12 to 2.12 mm from bregma) were incubated with 0.3% H_2O_2 for 30 min and then placed in blocking buffer containing 10% normal goat serum and 0.3% Triton X-100 in 0.01 mol/L PBS (pH 7.2) for 30 min at 37°C and incubated with rabbit polyclonal anti-Cx43 (1:400), mouse monoclonal anti-GFAP (1:1 000) or anti-CD11b (1:200) antibody overnight at 4°C. Sections were then incubated with the corresponding biotinylated secondary antibody (1:400, 2 h at 37°C) followed by streptavidin-peroxidase (1:400, 1 h at 37°C). The same treatment omitting the primary antibodies served as a negative control.

Cell Counts

Total cell numbers were counted in a double-blinded manner. In these experiments, CD11b- and GFAP-positive cells were counted under a 20× objective in the ipsilateral striatum from three sections (at 0.12, 1.12, and 2.12 mm from bregma) from each rat, in four randomly-selected non-overlapping areas (119 × 93 μm^2) in each section.

Cell Culture

PC12 cells were cultured in DMEM containing 10% fetal bovine serum and antibiotics (penicillin, 100 IU/mL; streptomycin, 100 μg/mL). The cultures were maintained at 37°C in 95% air-5% CO_2 in a humidified incubator. Cell viability was evaluated with the MTT assay using a microplate reader (Bio-Rad Laboratory, Hercules, CA).

H_2O_2 Treatment

The culture medium was removed and PC12 cells were washed with PBS, then incubated for 20 min in the same medium, followed by incubation for a further 4 h in the presence of 100 mmol/L H_2O_2 at 37°C with or without CBX (dissolved in 0.01 mol/L PBS at a final concentration of 100 or 200 μmol/L).

Scrape-Loading

Gap-junction permeability was determined using the Lucifer Yellow (LY) scrape-loading technique[32]. Confluent cells in 35-mm dishes were washed with PBS containing Ca^{2+} and Mg^{2+}. A scrape line was made using a surgical blade in PBS containing 0.1% LY for 1 min. Cells were then washed again with external solution (in mmol/L: 140 NaCl, 5.5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, pH 7.2). LY was allowed to diffuse in the external solution for 5 min. Thereafter, the culture dishes were photographed under a fluorescence microscope. The fluorescent areas were quantified in five consecutive fields using ImageJ (NIH).

Western Blot

Four rats were selected randomly from each group, anesthetized with pentobarbital, and the ipsilateral striatum was collected on ice. Samples were frozen in liquid nitrogen, homogenized, and total protein was extracted using RIPA buffer containing protease inhibitors. Harvested PC12 cells were lysed with RIPA buffer. Proteins (30 μg) were fractionated on 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blotted with anti-Cx43 (1:1 000) or anti-β-actin (1:5 000) antibody, followed by horseradish peroxidase-conjugated secondary

antibody (1:5 000). The immunoreactive protein bands were visualized by enhanced chemiluminescence.

Acridine Orange/Ethidium Bromide (AO/EB) Fluorescence Staining

Cellular morphological changes were observed by AO/EB staining using fluorescence microscopy. PC12 cells were harvested after different treatments, pooled, pelleted and resuspended in 200 μL medium. A mixture of the fluorescent dyes (8 μL) containing 100 μg/mL AO and 100 μg/mL EB was added to the cells and mixed gently. A drop of the mixture on a microscope slide was covered with a coverslip. The cells were visualized under a fluorescence microscope using a blue filter. At least 200 cells were counted in random fields. The percentage of apoptotic cells was calculated. The tests were repeated three times.

Statistical Analysis

All data are expressed as mean ± SEM. Differences between groups were analyzed by one-way ANOVA. For the influence of CBX on the infarct size relative to whole-brain volume (%), the Mann-Whitney U test was used for each evaluation. For other data, unpaired Student's *t* test was used. P <0.05 was considered statistically significant.

RESULTS

CBX Pre-treatment Reduced Infarct Size Following MCAO

To evaluate the effect of CBX on brain damage after cerebral ischemia, we measured the infarct size by cresyl violet staining. CBX treatment significantly reduced the infarct size compared with untreated rats (Fig. 1A, B).

CBX Pre-treatment Reduced Cx43 Expression and ROS Production after Cerebral Ischemia

To examine the changes of gap junctions after cerebral ischemia, we determined the Cx43 expression in the ipsilateral striatum. Immunohistochemistry showed that Cx43 expression was higher in the ipsilateral striatum of ischemic rats than that in control rats. CBX treatment markedly decreased Cx43 expression in ischemic rats (Fig. 2A). We also quantified the Cx43 level in the ipsilateral striatum using Western blot and found similar results (Fig. 2B, C). Because cerebral ischemia can cause the accumulation of ROS, we measured the DCF fluorescence in the ipsilateral striatum and found that it was higher after cerebral ischemia, and the production of ROS was attenuated by CBX pre-treatment (Fig. 2D).

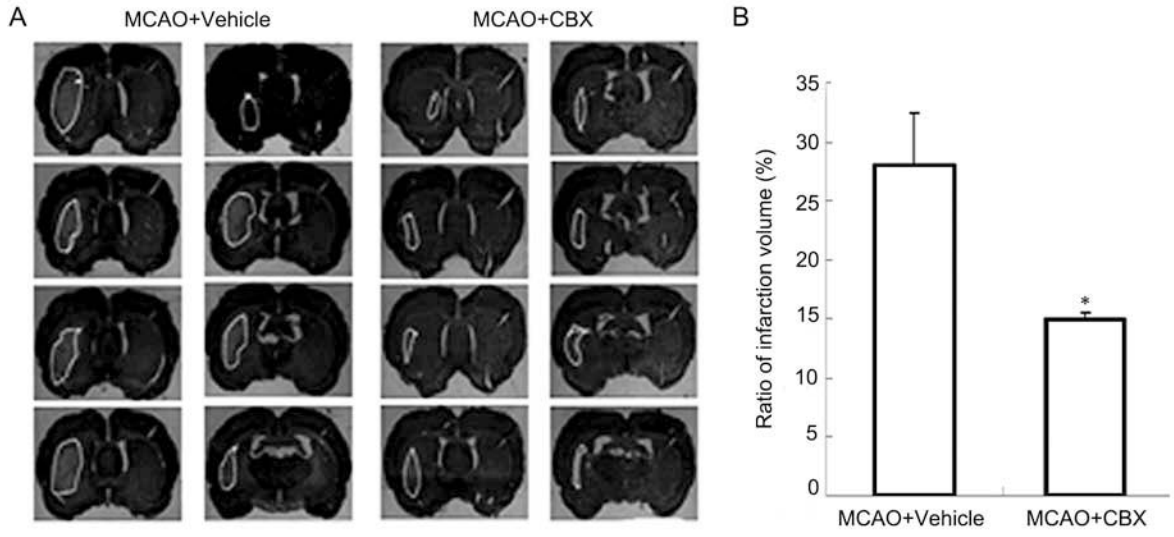

Fig. 1. CBX administration reduced the infarct size following MCAO. A: Representative images of infarcts stained with cresyl violet. Closed outlines denote the infarct foci. B: Infarct area (A_1), contralateral hemisphere area (A_2) and total brain area (A_3) on each coronal slice were directly measured by volume-analysis software, and the volumes were quantified by the formula: $V_{1,2,3} = \Sigma A_{1,2,3} \times 2$. Percentage of infarct volume was calculated as follows: Percentage of infarct volume = $[V_2-(V_3-V_2-V_1)]/V_2 \times 100\%$. *$P$ <0.05 (n = 6).

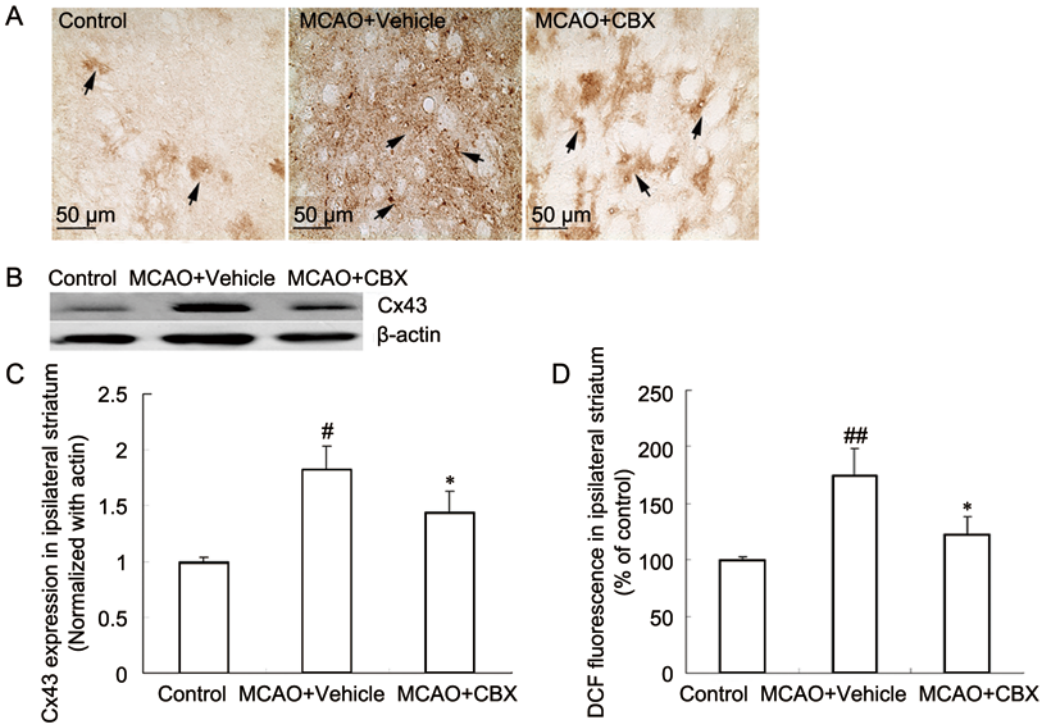

Fig. 2. CBX treatment reduced Cx43 expression and ROS production after cerebral ischemia. A: Immunohistochemical staining for Cx43 from bregma level 1.12 mm in the ipsilateral striatum. Arrows indicate positive immunoreactive staining. B and C: Western blots of Cx43 levels in the ipsilateral striatum. D: Hydrogen peroxide generation determined by DCF fluorescence. $^{\#\#}P$ <0.01 compared with control, $^{*}P$ <0.05 compared with MCAO + vehicle group (n = 4).

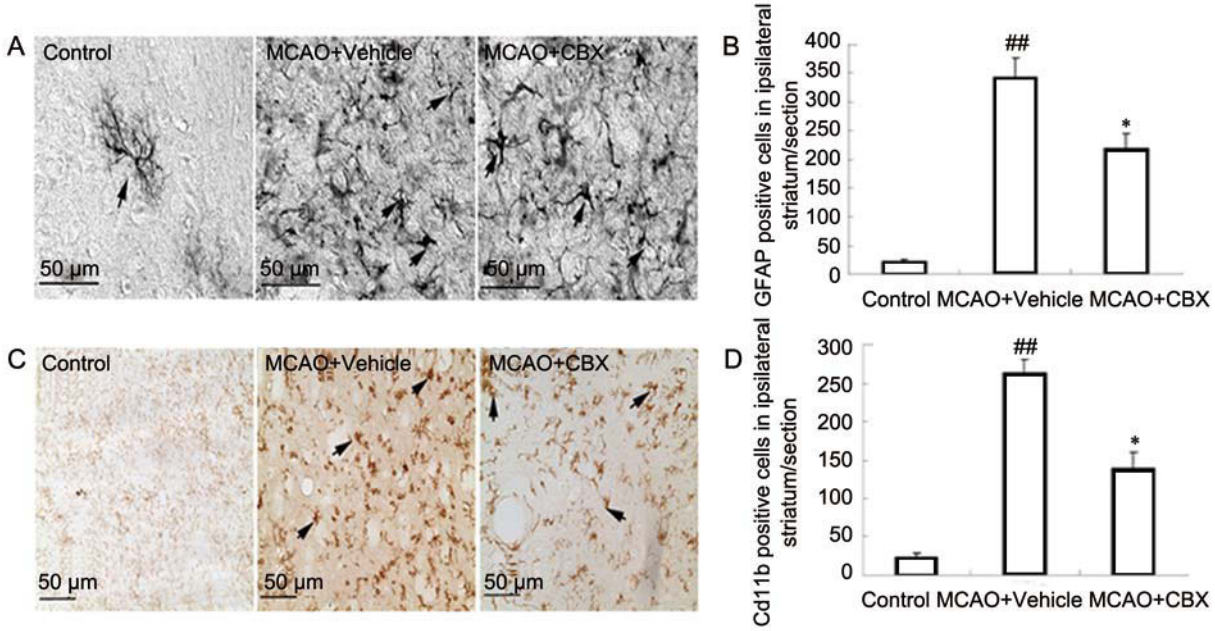

Fig. 3. CBX treatment inhibited the activation of glial cells after cerebral ischemia. A: Representative images of GFAP-positive cells in the ipsilateral striatum. B: Positive cell-counts in three sections at 0.12, 1.12 and 2.12 mm from bregma. C: Representative images of CD11b-positive cells in thse ipsilateral striatum. D: Positive cell-counts in three sections at 0.12, 1.12 and 2.12 mm from bregma. $^{\#\#}P$ <0.01 compared with control, $^{*}P$ <0.05 compared with MCAO + vehicle group (n = 6).

CBX Treatment Inhibited the Activation of Glial Cells after Cerebral Ischemia Previous studies indicated that Cx43 is mainly localized in glia, and ischemia usually causes the activation of astrocytes and microglia, so whether blocking gap junctions affects glial activity after cerebral ischemia is of interest. Immunohistochemistry showed that the number of GFAP-positive astrocytes induced by ischemia in the ipsilateral striatum was decreased by CBX treatment (Fig. 3A, B). Likewise, CBX-treated rats had fewer CD-11b-positive microglia than the untreated rats after ischemia (Fig. 3C, D).

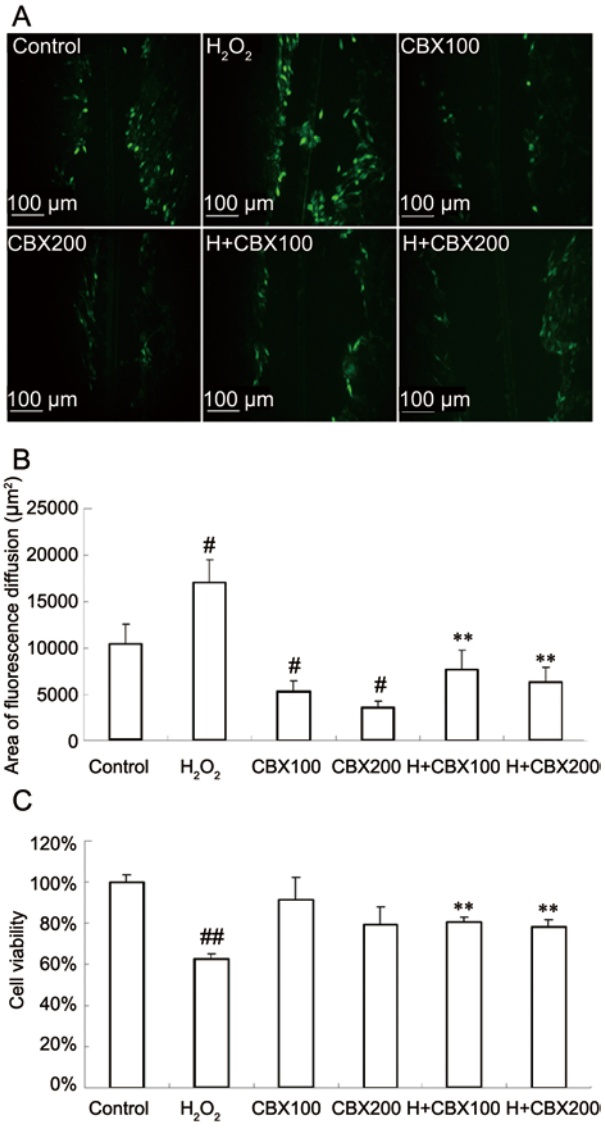

Fig. 4. CBX inhibited gap-junction coupling and improved the viability of PC12 cells after H_2O_2 treatment. A: Representative images of diffusion of LY into PC12 cells. CBX100, 100 μmol/L CBX; CBX200, 200 μmol/L CBX. Diffusion areas are shown in B. C: Cell viability was assessed using the MTT method. H_2O_2 treatment decreased viability, but this was reversed by CBX administration. $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 compared with control; $^{**}P$ <0.01 compared with H_2O_2 treatment (n = 3).

CBX Inhibited the Gap Junction Coupling and Improved the Viability of PC12 Cells after H_2O_2 Treatment

The above results *in vivo* showed that CBX protected against transient cerebral ischemia partly due to a decrease of ROS and inhibition of Cx43 expression, but whether ROS result in the coupling of gap junctions and CBX reverses it were unknown. Therefore, PC12 cells were treated with H_2O_2 to mimic ROS injury. The scrape loading test showed that diffusion of LY into PC12 cells was increased after H_2O_2 treatment, while 100 μmol/L and 200 μmol/L CBX both clearly inhibited the diffusion of LY (Fig. 4A, B). The cell survival was also improved by treatment with CBX under H_2O_2 stress (Fig. 4C).

CBX Suppressed Cx43 Expression in PC12 Cells after H_2O_2 Treatment

Recent studies have shown that Cx43 is distributed in PC12 cells after induction[33]. To address whether CBX

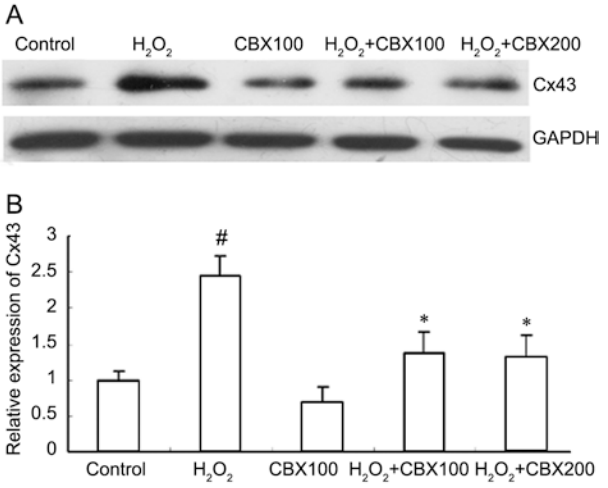

Fig. 5. CBX suppressed Cx43 expression in PC12 cells after H_2O_2 treatment. A: Representative images of Western blots. B: Cx43 expression levels. CBX100, 100 μmol/L CBX; CBX200, 200 μmol/L CBX. $^{\#}P$ <0.05 compared with control, $^{*}P$ <0.05 compared with H_2O_2 treatment (n = 3).

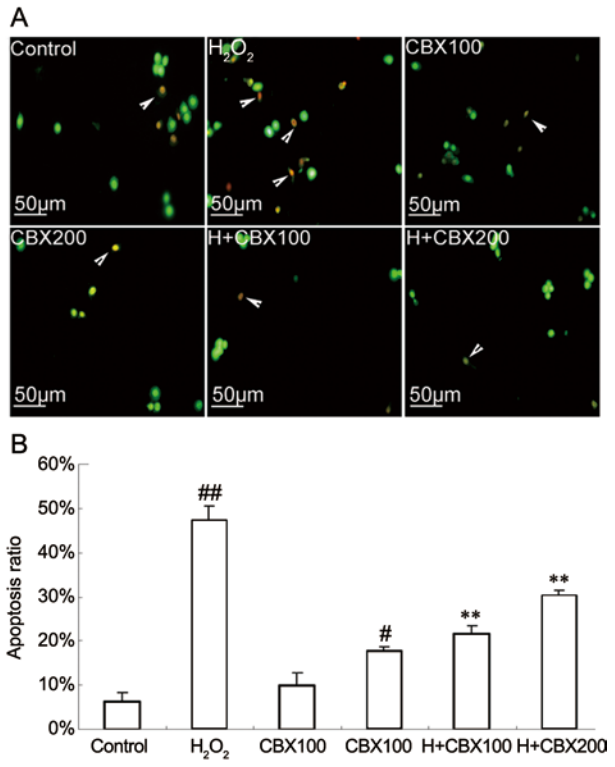

Fig. 6. CBX administration reduced the apoptotic ratio in H_2O_2-treated PC12 cells. A: Representative images of AO/EB staining. Arrows indicate apoptotic cells. B: The apoptotic ratio was calculated according to the following formula: Apoptotic ratio (%) = (number of early apoptotic cells + number of late apoptotic cells) / (number of all cells). $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 compared with control; $^{**}P$ <0.01 compared with H_2O_2 treatment (n = 3).

inhibited Cx43 expression in H_2O_2-treated PC12 cells, we determined the Cx43 level using Western blot and showed that it was overexpressed in H_2O_2-treated cells compared with control. Moreover, CBX treatment significantly decreased the overexpression of Cx43 in PC12 cells with H_2O_2 stress (Fig. 5A, B).

CBX Administration Reduced the Apoptosis Ratio of PC12 Cells with H_2O_2 Treatment

In vitro, we found the CBX inhibited the extensive opening of gap junctions induced by H_2O_2 stress. To address whether CBX can protect PC12 cells against ROS injury, the apoptosis induced by H_2O_2 stress was measured by AO/EB staining. We found that the apoptosis ratio of PC12 cells was increased by 100 μmol/L H_2O_2 treatment for 4 h. In addition, after PC12 cells were incubated with H_2O_2 and CBX together, the apoptosis ratio was clearly decreased (Fig. 6A, B).

DISCUSSION

So far, many studies have documented the roles of gap junctions in cerebral ischemia. But argument continues, partly due to different experimental models, leading to different and even opposing results and conclusions[34-36]. *In vivo*, most of the studies were designed to down- or up-regulate specific connexins, then investigate the gap-junction effects and underlying mechanisms in cerebral ischemia. But they failed to link the connexin expression to the actual level of opening of gap junctions[37]. *In vitro*, studies cannot duplicate the ischemic condition, although quantitative assays were performed to evaluate the extent of gap-junction coupling[38]. In the present study, we found that ischemia-induced overexpression of Cx43 was accompanied by ROS accumulation and glial activation. Furthermore, the gap-junction blocker CBX inhibited Cx43 expression, decreased ROS accumulation, and ameliorated the activation of glia including microglia and astrocytes. Also, the infarct size was diminished with CBX injection into the lateral cerebral ventricle after transient cerebral ischemia/reperfusion. To further explore the relation between ROS and gap-junction communication, PC12 cells were treated with H_2O_2, which induced the extensive opening of gap junctions and overexpression of Cx43. However, the roles of Cx43 in the ischemic process remain controversial. Cx43 heterozygous-null mice and mice with astrocytic Cx43 removed by *cre* expression are more susceptible to infarct expansion after focal ischemia[37,39,40]. Alternatively, when hypoxic-ischemic brain injury induces an acute inflammatory reaction and reactive astrogliosis associated with peri-lesional upregulation of Cx43, human umbilical cord blood cell transplantation accelerates the regression of inflammatory events, narrows the peri-lesional astrocytic wall, and leads to the downregulation of Cx43 expression[41]. A mimetic peptide that blocks Cx43 hemichannels after perfusion into the lateral ventricle reduces seizure activity and improves the survival of oligodendrocytes and neurons after ischemia in sheep[42].

In vitro, the gap-junction coupling induced by H_2O_2 treatment was partly counteracted by CBX treatment.

Further findings also indicated that the apoptosis induced by H_2O_2 was attenuated by CBX administration. These *in vivo* and *in vitro* results suggested that cerebral ischemia enhances gap-junction coupling associated with the production and accumulation of ROS. And ROS stress enhances the secondary brain injury after cerebral ischemia[43-45]. Inhibition of gap junctions with CBX decreases the diffusion of ROS, limiting the secondary injury factors in the ischemic core, and protecting cells in the penumbra to attenuate the expansion of the infarct[46,47]. Other studies also indicate that CBX acts partially as an antioxidant[26].

Not only do ROS accumulate, but activation of glial cells also occurs after cerebral ischemia. The roles of activated glial cells in brain injury remain controversial; presumably, they have advantages and disadvantages. Glia may play complex roles in the development of neuropathology, for example in triggering secondary injury or promoting self-repair by secreting cytokines[48-50]. Here, we found that CBX reduced the activation of microglia and astroglia localized in the penumbra after transient cerebral ischemia. However, the underlying mechanisms remain to be clarified. Most evidence indicates that Cx43 is localized in the cell membranes of activated astrocytes and microglia[51,52]. Accumulation and diffusion of ROS may activate microglia and astrocytes, and these effects may be amplified by gap-junction communication[53]. In addition, apoptosis signals can diffuse through gap junctions[54]. Previous studies showed that CBX treatment decreases the numbers of TUNEL-positive neurons in global ischemia[26] and its administration to ischemic pups prevents caspase-3 activation[55]. In our experiment, H_2O_2 treatment induced apoptosis in PC12 cells accompanied by gap-junction coupling. And after blocking the gap junctions with CBX, the apoptosis ratio was attenuated under ROS stress. These results confirmed that gap junctions are involved in apoptosis progression in brain injury.

Therapeutic targets have mainly been focused on signals associated with secondary injury, such as inflammation, excitatory amino-acid toxicity, and ROS, after cerebral ischemia. Although gap junctions are only regarded as channels for the diffusion of small molecules, their functional status may affect the efficiency of the above signals and thus participate in the development of neuropathology after cerebral ischemia. The functional pattern and structural changes in gap junctions under neuropathological conditions remain to be elucidated. Our study only investigated the effect of CBX at the beginning of ischemia, so further studies are needed to determine the protective effect of CBX in long-term ischemia.

In brief, the gap-junction blocker CBX reduced the infarct size after cerebral ischemia, partially by inhibiting the accumulation and diffusion of ROS, suppressing the activation of glial cells, and decreasing apoptosis.

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