Functional Neuroprotective Effect of CGS 26303, a Dual ECE Inhibitor, on Ischemic-Reperfusion Spinal Cord Injury in Rats

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Endothelin-1 (ET-1) has been implicated in many neurological diseases, including subarachnoid hemorrhage (SAH) and cerebral ischemia. ET-1 is also proved to deteriorate the ischemia-reperfusion injury in many organs. Our previous studies demonstrated that the endothelin-converting enzyme (ECE) inhibitor, CGS 26303, possessed beneficial effects for the treatment of SAH and transient middle cerebral artery occlusion. In this study, we investigated the neuroprotective effect of CGS 26303 on the locomotor function and mRNA expression of heme-oxygenase-1 (HO-1) in rats subjected to a 15-min spinal cord ischemia. The results showed that pretreatment with CGS 26303 significantly preserved the locomotor function and decreased the paraplegia rate at Days 1 and 3 as compared with a saline-treated group. Furthermore, rats pretreated with CGS 26303 had a significant increase in the levels of HO-1 mRNA expression at Day 3 when compared with animals pretreated with saline after spinal cord ischemia and the sham operation group. These results suggest that CGS 26303 may have a promising neuroprotective effect in the spinal cord after ischemia-reperfusion injury, and beneficial result may be due to an adaptive mechanism involved by HO-1 overexpression. Exp Biol Med 232:214–218, 2007

Key words: CGS 26303; dual inhibitor; ischemia-reperfusion injury; heme-oxygenase

Introduction

In thoracoabdominal aortic surgery, aortic cross-clamping may induce transient spinal ischemia and lead to various degrees of spinal cord injury (SCI). Paraplegia is the most serious and catastrophic complication of this kind of SCI. In the previous largest institutional experience reported, the overall incidence of SCI was 16% (1), in which approximately half of these cases were devastating paraplegia. Various approaches, such as monitoring of somatosensory evoked potentials, partial aorta bypass, and hypothermia, have been employed for early detection and prevention of spinal cord ischemia perioperatively (2). Unfortunately, none of these methods were proved to effectively eradicate the development of paraplegia. The mechanisms underlying SCI by transient ischemia, although not fully elucidated, may involve tissue ischemia, the loss of neuronal cells, and ischemia-reperfusion (I/R) injury (3).

Increased production of endothelin-1 has recently been shown to contribute to the pathogenesis of I/R injury in the gastrointestinal system, liver, heart, and kidney (4, 5). Moreover, endothelin-1 has been demonstrated to aggravate I/R injury in the neocortex of rats (6). Although the precise

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molecular mechanisms of endothelin-1 in I/R injury remain to be elucidated, both nitric oxide (NO) and carbon monoxide (CO) were implicated in this pathophysiology of endothelin-related I/R injury (7). Previous studies have demonstrated that intravenous bolus injections of an endothelin-converting enzyme (ECE) inhibitor can provide the neuroprotective effect of rat cortex (8). In our prior studies, CGS 26303, a dual inhibitor of ECE-1 and neutral endopeptidase 24.11, has been proven to have beneficial effects for different experimental neurological disease models including the ischemic stroke and subarachnoid hemorrhage (SAH) (8, 9). In this study, we examined the neuroprotective effect of CGS 26303 on the neurological deficit after transient ischemia. Furthermore, using semi-quantitative reverse transcription-polymerase chain reaction, we also investigated the effect of CGS 26303 on mRNA expression of HO-1 in the spinal cord tissue of rats subjected to I/R injury.

Materials and Methods

CGS 26303 was synthesized at Novartis Pharmaceuticals Corp. (East Hanover, NJ). The compound was dissolved in 0.25 *M* sodium bicarbonate at a concentration of 30 mg/ml.

Experimental Animals. Male Sprague-Dawley rats were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and were housed in an animal center in Kaohsiung Medical University. Experiments described in this study were approved by the Animal Committee of the Kaohsiung Medical University.

Rats were randomized into the following three experimental groups of six animals each: (i) SCI rats pretreated with saline; (ii) SCI rats pretreated with CGS 26303 at a dose of 30 mg/kg, intravenously; (iii) healthy control, sham-operated rats. In groups of pretreatment, CGS 26303 or saline was administered intravenously in the tail vein at 30 mins before the induction of I/R spinal cord injury.

Experimental I/R Spinal Cord Injury. To induce spinal cord I/R injury, male Sprague-Dawley rats weighting 300–350 g were anesthetized, and a 2F Fogarty catheter (Baxter, Deerfield, IL) was passed through the left femoral artery to the descending thoracic aorta so that the tip reached the level of the left subclavian artery, as described previously (10). This level corresponded to a distance of 10–12 cm from the site of insertion. The intra-aortic balloon catheter was inflated with 0.2 ml of CO_2 for 15 mins. After ischemia, 4 mg of protamine sulfate was administered subcutaneously. Stabilization of the arterial blood pressure was then monitored for an additional 20 mins, after which arterial lines were removed and the wounds were closed.

Behavioral Testing for Neurological Assessment. Behavioral tests were performed and graded in all animals before ischemia and at Days 1 and 3 after ischemia as previously described (11). Motor function was quantified by assessment of ambulation and placing and stepping responses. The scoring for ambulation, walking with lower extremities (LE), was: 0 = normal (symmetric and coordinated ambulation); 1 = toes flat under body when walking, but ataxia present; 2 = knuckle-walking; 3 = movement in LE but unable to knuckle-walk; and 4 = no movement, drags LE. Placing/stepping reflex was assessed by dragging the dorsum of the hind paw over the edge of a surface. This normally evoked a coordinating lifting and placing response (e.g., stepping): 0 = normal; 1 = weak; and 2 = no stepping. A motor deficit index was calculated for each rat at each time interval. The final index was the sum of the scores (walking with LE and placing/stepping reflex). The maximum deficit was indicated by a score of 6. Animals with motor deficit index (MDI) $\geqq 3$ were considered paraplegic, whereas animals with MDI <3 were considered nonparaplegic.

Isolation of RNA in Spinal Cord Tissue and Detection of HO-1 mRNA Using Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR). At the end of the neurological assessment, animals were sacrificed *via* the intracardial perfusion with saline solution. The spinal cord was removed, frozen in liquid nitrogen, and pulverized with a pestle in a mortar immediately. Total RNA was isolated using a SV Total RNA Isolation System (Promega, Madison, WI). The integrity of the isolated RNA was confirmed by agarose gel electrophoresis. The RNA concentration was determined by spectrophotometry. For reverse transcription, samples of 1 μg total RNA were reverse transcribed (RT) into cDNA in 20 μl of reaction mixtures containing 200 units of Superscript II (Gibco BRL, Rockville, MD), 0.5 μg oligo(dT)12–18, 200 pmol dithiothreitol, 10 pmol dNTP, and 40 units of ribonuclease inhibitor (Gibco BRL) in a buffer supplied with the enzymes. The RT procedure was performed according to the manufacturer's protocol (Superscript II; Gibco BRL). The resulting cDNA was frozen at –20°C for mRNA detection.

Target genes were amplified by polymerase chain reaction using a Taq DNA polymerase kit (FastStart Taq DNA polymerase; Roche Diagnostics GmbH, Mannheim, Germany). A volume of 1.5 μl cDNA from RT was replicated in PCR reactions in a total volume of 25 μl containing Taq DNA polymerase (0.63 units), buffer supplied with enzymes, $MgCl_2$ (50 pmol), dNTP (200 μmol), and primers (20 pmol). Duplex PCR was performed using primers for both porphobilinogen deaminase (PBG-D) and heme-oxygenase-1 gene (HO-1). PCR conditions were 94°C for 4 mins followed by 30 cycles of 94°C for 30 secs, 60°C for 30 secs, and 72°C for 45 secs, and finally 72°C for 7 mins. Primer sequences of PBG-D (accession number X06827) and HO-1 (accession number J02722) were designed *via* commercial computer software (Lightcycler probe design; Roche Diagnostics GmbH). All primers were made by Gibco BRL. Five microliters of the reaction products of each transcript were mixed with 1 μl loading

Table 1. Motor Deficit Index (MDI) in Different Groups of Rats on Days 1 and 3 after Ischemia-Reperfusion Spinal Cord Injury

Treatment group	Day 1	Day 3
Sham	0	0
Saline	5.1 ± 1.2[a]	5.5 ± 1.8[a]
CGS 26303	2.3 ± 0.9	2.1 ± 1.5

[a] Statistical difference: $P < 0.05$ between the saline- and CGS 26303-treated groups by one-way Mann-Whitney test.

dye, and the mixtures were then electrophoresed on a 1.5% or 2.0% agarose gel containing ethidium bromide (0.1 μg/ml). After electrophoresis, the gels were visualized by ultraviolet-induced fluorescence. The expected size for the DNA fragments of PBGD and HO-1 were 186 and 335 base pairs, respectively. The PCR products were quantified by a densitometer interfaced with Bioprofil image analysis software (Vilber Lourmat, Marne-la-Vallée Cedex 1, France). The results were expressed as ratios relative to PBG-D.

Statistical Analysis. All behavior testing data are expressed as the mean ± SEM. Differences between groups in the MDI score and paraplegia rate were carried out by using one-way Mann–Whitney test. Gene expression comparison between groups was performed using the nonparametric Kruskal-Wallis test. Differences were considered significant when $P < 0.05$.

Results

CGS 26303 Alleviated the Locomotor Deficits in Rats after Spinal Ischemia. After 15 mins of transient ischemia in spinal cord, animals in different groups showed variable deficits in locomotor function and paraplegia rate. No paraplegia or adverse effects were found in the sham-operated animals. The neurological outcomes in different groups of animals were summarized in Table 1. The mean MDI for saline-treated rats was 5.1 ± 1.2 and 5.5 ± 1.8 on Days 1 and 3, respectively. In contrast, in CGS 26303-treated group, the mean MDI was 2.3 ± 0.9 and 2.1 ± 1.5 on Days 1 and 3, respectively (Table 1 and Fig. 1a). The paraplegia rate was 80% in saline-treated rats (4 out of 5) and 40% (2 out of 5) in CGS 26303 group, and there was no differences in each group on Days 1 and 3 after ischemia (Fig. 1b). The differences in the mean MDI and paraplegia rate were statistically significant ($P < 0.05$) using one-way Mann-Whitney test carried out between the CGS 26303- and saline-treated groups. These results indicated that CGS 26303 preserved the locomotor function to improve the gait properties and reduced the incidence of paraplegia in rats subjected to transient spinal ischemia.

CGS 26303 Caused HO-1 Overexpression in the Spinal Cord Tissue after Transient Spinal Ischemia. The spinal cord tissues from different groups of rats

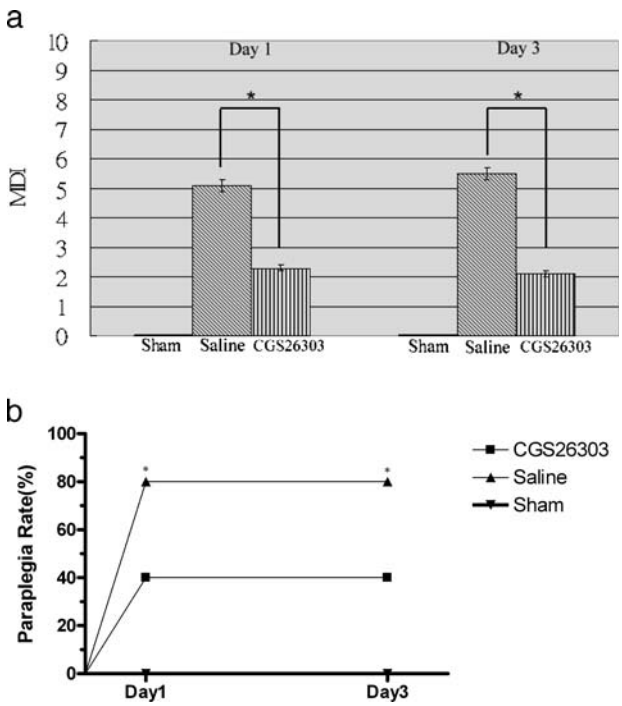

Figure 1. Motor deficit index (MDI) and paraplegia rate among three groups on Days 1 and 3 after spinal cord ischemia. (a) MDI data are presented as mean ± SEM in different groups. (b) Paraplegia rate is present as percentage of rats defined as MDI ≧ 3. *$P < 0.05$ between saline- versus CGS 26303-treated groups by one-way Mann-Whitney test.

were collected for semiquantitative assessment of gene expression for HO-1 and PBG-D using RT-PCR to delineate the underlying mechanism of differential neurological functions. After 30 cycles of PCR amplification, the intensities of HO-1 and PBG-D gene expression were compared between groups using a densitometer interfaced with Bioprofil image analysis software. Furthermore, for internal control, the ratio of HO-1/PBG-D intensity was used to demonstrate the substantial expression by duplex PCR in all groups. In the saline-treated group, the results of HO-1/PBG-D were not significantly different from that of the sham operation group, although there seemed to be a trend toward an increase in HO-1 mRNA expression (Fig. 2a). In contrast, the group pretreated with CGS 26303 showed significant increases in the levels of HO-1/PBG-D ratio as compared with both the saline-treated and sham control rats (Fig. 2b).

Discussion

In the present study, we used a rat model to delineate the relationship between the interruption of the blood flow in the aorta and the development of spinal cord injury. Our study indicated that intravenous administration of CGS 26303, at 30 mins prior to transient spinal ischemia, can improve neurological function assessment significantly and

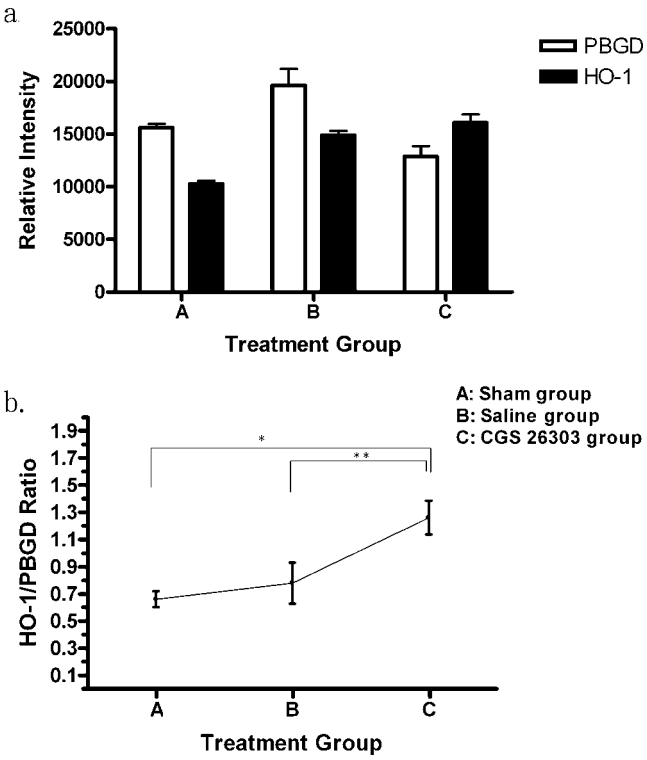

Figure 2. Semiquantitative assessment of gene expression for heme-oxygenase 1 (HO-1) and porphobilinogen deaminase (PBG-D) using RT-PCR. (a) The relative intensity of mRNA in different group and (b) the ratio of mRNA expression of HO-1 relative to PBG-D among different groups are shown. A, sham-operated rats; B, rats subjected to spinal cord ischemia and pretreated with saline; C, rats subjected to spinal cord ischemia and pretreated with CGS 26303. *$P < 0.05$ when CGS 26303 group is compared to sham and saline-treated groups.

decrease the paraplegia rate and locomotor dysfunction after a 15-min spinal cord ischemia.

Previous studies have demonstrated that reduction in the levels of ET by ECE inhibitors can attenuate the macroscopic and microscopic parenchymal damages of various vital organs in I/R injury (12). Patel *et al*. have shown that ECE antagonists can decrease the neuronal damage in experimental stroke (13). However, the potential role of ECE inhibitor in spinal cord I/R injury after transient ischemia has not been evaluated. CGS 26303, a dual combination of ECE inhibitor and NEP inhibitor, has been shown to have beneficial effects in experimental SAH and transient middle cerebral artery occlusion in our prior work (8, 9). In the present study, CGS 26303 resulted in an overexpression of HO-1 in the spinal cord tissue of rats after transient spinal cord ischemia and ameliorated the paraplegia and locomotor dysfunction. It is well proven that HO can catalyze heme degradation to produce CO, resulting in vasodilatation. According to the present finding, HO-1 induced by CGS 26303 pretreatment may play an important role in I/R injury of the spinal cord as an adaptive mechanism in many cell types described previously (14). Several cascades, including NO, TNF-α, and ROS, have been mentioned as possibly being regulated by ET-1 in I/R injury (15), but they were not investigated in this study. The neuroprotective mechanism in the spinal cord ischemia by which CGS 26303 may be involved in NO and prostanoid syntheses remains to be studied.

In conclusion, our present results demonstrate that CGS 26303 possesses neuroprotective effects in an *in vivo* animal model of transient spinal cord ischemia as assessed by neurological score and paraplegia rate. This neuroprotection was correlated to the HO-1 overexpression in spinal cord tissue after CGS 26303 pretreatment. Thus, endothelin-converting enzyme-1 inhibitors such as CGS 26303 may hold promising therapeutic potential in the clinical setting.

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