ORIGINAL ARTICLE

Bosentan protects the spinal cord from ischemia reperfusion injury in rats through vascular endothelial growth factor receptors

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Study design: Experimental study.

Objectives: To investigate whether Bosentan, an endothelin-A/B dual receptor antagonist, could protect neurons after spinal cord ischemia reperfusion (SCIR) injury in rats and its underlying signaling pathway.

Setting: Department of Neurosurgery, the Second Affiliated Hospital, Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi Province, China.

Methods: Sprague–Dawley rats were randomly divided into two groups, saline group (IRS, $n=48$) and Bosentan group (IRB, 5 mg kg^{-1} , $n=48$). After ischemia for 1 h with occlusion of the infrarenal aorta, spinal cord were reperfused for 6h, 12h, 24h, 3d, 5d, and 7d separately. Enzyme-linked immunosorbent assay was used to detect vascular endothelial growth factor (VEGF) in serum. Immunohistochemistry was performed to detect protein expression of VEGF, VEGF receptor 1 (FLT-1) and VEGF receptor 2 (FLK-1). Gene expressions of VEGF and its receptors were evaluated using the quantitative reverse transcription polymerase chain reaction.

Results: Compared with the IRS group, gene and protein expressions of VEGF, FLT-1 and FLK-1 were significantly increased ($P<0.05$), so was the concentration of VEGF in plasma ($P<0.05$). FLK-1 was expressed on spinal cord neurons.

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INTRODUCTION

Thoracoabdominal aorta surgery causes various complications including neural degeneration and devastating paraplegia owing to spinal cord ischemia reperfusion (SCIR) injury.^{1–3} There are a series of factors that may contribute to it, such as excitotoxicity,⁴ free radical production,⁵ inflammation⁶ and apoptosis.⁷ It was reported that neurovascular remodeling is crucial in the recovery of neural function after central nervous system ischemia reperfusion (IR) injury.⁸ Several studies also demonstrated that vascular endothelial growth factor (VEGF), a major regulator of angiogenesis in development and many pathological conditions, promoted angiogenesis and neuronal remodeling after cerebral ischemia in rats.⁹ A recent study showed that VEGF may reduce neural injury significantly and improve the neural function following SCIR injury.¹⁰ It had been shown that endothelin has an effect on regulating the expression of VEGF and its receptors following cerebral ischemia and hind limbs IR.¹¹ In our previous study, Bosentan played a neuroprotective role in decreasing neuronal apoptosis after SCIR injury.¹² However, the influence of Bosentan on the regulation of VEGF signaling pathway in SCIR injury remains unclear. Therefore, in the current study, we used the established rat model of SIRI to investigate the expression of VEGF and its receptors after Bosentan treatment.

MATERIALS AND METHODS

Experimental animals and grouping; establishment of rat models of SIRI, serum and spinal cord specimen collection

Male Sprague–Dawley rats that weighed between 250 and 300 g were used in this study. All animals were purchased from the Animal Center in Xi'an Jiaotong University, China. The rats were randomly divided into two groups, saline group (IRS, $n=48$) and bosentan group (IRB, $n=48$). The IRB group received Bosentan (Patheon Inc, Canada, 5 mg kg^{-1}) via the caudal vein 20 min prior to ischemia, followed by caudal vein injection with an equal dose of Bosentan at 24-h intervals until they were killed. Group IRS were injected with an equal dose of saline via the caudal vein at each time point. The SIRI model was established according to Zivin's process.¹³ After ischemia for 1 h with occlusion of the infrarenal aorta, spinal cords were reperfused for 6, 12, 24 h, 3, 5 and 7 days separately. After reperfusion, rats in each group were anesthetized. Plasma and L4–L5 spinal cord were collected for future analysis.

Details could be found in our previously published paper.¹²

Enzyme-linked immunosorbent assay for VEGF-A in plasma

Plasma VEGF was detected with ELISA kit for VEGF-A (General Hospital of Chinese PLA, Beijing, China) according to the manufacturer's specifications. Results are expressed as nanogram per milliliter (ng mg^{-1}) of plasma protein.

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Immunohistochemistry for VEGF-A, FLT-1 protein expression

Transverse sections of the lumbar spinal cord were paraffin-embedded, at a thickness of 4 μm , mounted on slides, deparaffinized and hydrated for immunohistochemistry. After incubation with normal goat serum at room temperature for 30 min, the sections were incubated with primary polyclonal rabbit anti-VEGF-A antibody (1:200 dilution, Abcam, Cambridge, MA, USA) and rabbit anti-FLT-1 antibody (1:50 dilution, Abcam) at 4 °C overnight, followed by washing in PBS for 5 min 3 times and incubating with biotinylated goat secondary antibody (1:100 dilution, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. Thereafter, the tissue sections were washed in PBS once again and incubated with horseradish peroxidase complex solution provided in the immunohistochemical staining kit (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions. After a final wash in PBS, the tissue sections were covered with 3,3'-diaminobenzidine (Beijing Biosynthesis Biotechnology Co., Ltd) for 2 min and then rinsed twice in distilled water for 5 min and mounted onto slides. This was performed blind and was repeated in three rats from each group with an image analysis collection system (Q553Cw; Leica, Wetzlar, Germany). We examined the average optical densities (gray values) to quantify the positive area stained in the pictures. In this analysis collection system, small gray values were present, indicating stronger protein expression. It was evaluated at least in five different vision fields per section.

Immunofluorescence for FLK-1 protein expression

Fluorescence double-labelling was performed to localize the expression of FLK-1 with specific cell-type markers for neurons. After incubation with normal goat serum at room temperature for 30 min, the sections were incubated with primary polyclonal rabbit anti-FLK-1 antibody (1:50 dilution,

Abcam), with mouse anti-MAP-2 (1:500 dilution, Abcam) serving as a specific neuron marker, at 4 °C overnight. These sections were then washed 3 $\times$ 10 min with PBS and incubated with an appropriate Alexa-conjugated secondary cocktail: Alexa 488 goat anti-rabbit, Alexa 555 goat anti-mouse (1:100 dilution, Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Following 3 $\times$ 5 min washes in PBS, the sections were incubated for 5 min in 4,6-diamidino-2-phenylindole (1:15000 dilution, Santa Cruz Biotechnology, Inc.). IgG-negative controls were also included to determine nonspecific background staining. All sections were mounted with Fluorescence Mounting Medium (DAKO, Carpinteria, CA, USA). Images were captured with a Nikon fluorescence microscope (i90, Nikon instruments (Shanghai), Shanghai, China). This experiment was repeated in three rats from each group.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) for VEGF, FLK-1 and FLT-1

Total RNA was extracted from L₄–L₅ spinal cord tissue according to an acid-phenol method as described previously.¹⁴ Quantitative reverse transcription polymerase chain reaction was then conducted according to the instructions available in our previously published paper.¹² The following primers were used: β -actin: forward: 5'-TACCCACACTgTgCCCATCTATgA-3', reverse: 5'-CATCggAACCGCTCATTgCCgATAg-3'; VEGF-A: forward: 5'-TgCAgATC ATgCggATCAAAAC-3', reverse: 5'-TTTCTCCgCTCTgAACAAggC-3'; FLT-1: forward: 5'-TACCCgCAACggAgAA-3', reverse: 5'-ggCTTggAAgggACgA-3'; FLK-1: forward: 5'-AACgCTTgCCTTATgAT-3', reverse: 5'-AAgTCgCTGT CTTTCg-3'. The outcome of the PCR was determined using the 2- $\Delta\Delta\text{Ct}$ method.¹⁵ We used mRNA levels to predict the levels of VEGF, FLK-1 and FLT-1 gene expression.

Figure 1 The expression of VEGF following the treatment of Bosentan after IR. (a) Immunohistochemistry showing VEGF expression prior to and following Bosentan intervention ($\times 400$). IR, ischemia reperfusion; IRS, IR + saline group; IRB, IR + Bosentan group; 24 h or 7 days, reperfusion 24 h or 7 days. (b) Histogram revealing the changes in gray values of VEGF in neural cells of the anterior horn of the spinal cord following Bosentan intervention. Strong protein expression exhibited a lower gray value. * $P < 0.05$ vs IRS group. (c) Changes in VEGF plasma content following Bosentan intervention ($\pm$ S, ng ml^{-1}). * $P < 0.05$ vs IRS group. (d) Histogram demonstrating the changes in VEGF mRNA levels in spinal cord tissue following IR and Bosentan intervention. Representative images from four rats in each group are presented here. Scale bar: 50 μm .

Statistical analysis

The data were analyzed with SPSS18.0 software (Chicago, IL, USA). One-way analysis of variance was performed to analyze the comparison between groups. The results were represented as mean $\pm$ s.e.. Statistical significance was accepted for $P < 0.05$. In order to detect the protein expression of VEGF, FLK-1 and FLT-1, an image analysis collection system (Q553Cw; Leica, Wetzlar, Germany) was used to analyze the immunohistochemical staining pictures. The average optical densities (gray values) were inversely correlated with the protein expression.

All applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

RESULTS

VEGF expression was enhanced by Bosentan treatment following rat SCIR injury

The protein expression of VEGF in IRB was significantly increased according to the gray value, which is inversely proportional to the protein expression in our analysis system ($P < 0.05$, Figures 1a and b). Compared with the IRS group, treatment with Bosentan resulted in a significant increase in the plasma VEGF concentration ($P < 0.05$, Figure 1c) and VEGF gene expression at each time point ($P < 0.05$, Figure 1d).

Bosentan promoted FLT-1 expression following rat SCIR injury

Protein expression of FLT-1 in the IRB group was significantly higher than that in the IRS group at each time point ($P < 0.05$, Figures 2a and b). Bosentan treatment also significantly increased FLT-1 gene expression compared with the IRS group following SCIR at each time point ($P < 0.05$, Figure 2c).

The expression of FLK-1 was upregulated by Bosentan treatment following rat SCIR injury

The protein expression of FLK-1 in the IRB group was significantly increased compared with the IRS group at each time point (Figure 3A). According to fluorescence double-labelling, FLK-1 was expressed on the spinal cord neurons (Figure 3B). Furthermore, the treatment of Bosentan increased FLK-1 gene expression compared with the IRS group ($P < 0.05$, Figure 3C).

DISCUSSION

We have previously reported that blocking endothelin receptors with Bosentan leads to a significant reduction of neuronal apoptosis following SCIR injury in rats. The purpose of the current study was to investigate the underlying mechanism for the neuroprotective role of Bosentan after SCIR injury. After careful analysis, we found that Bosentan has a very important role in activating the VEGF signaling pathway to improve the recovery of the neural tissue after SCIR injury.

VEGF, also known as vascular permeability factor (VPF) or vascular opsonic, has a potential proangiogenic effect and the ability to promote the permeability of the blood vessel. It binds to the tyrosine kinase receptors FLT-1 and FLK-1 that are expressed by vascular endothelial cells.¹⁶ In the central nervous system, VEGF not only exerts its effect on the vascular system, but also helps to maintain the neural homeostasis and neural development.¹⁷ VEGF is upregulated during various pathological events in the central nervous system, including ischemia,¹⁸ cold lesions, spinal cord injuries, brain contusion and direct wounding.¹⁹ It has also been reported that VEGF had a neuroprotective role in spinal cord injury.

Figure 2 Expression of FLT-1 following the treatment of Bosentan after IR. (a) Immunohistochemistry showing FLT-1 expression in spinal cord tissue from each group ($\times 400$). IR, ischemia reperfusion; IRS, IR + saline group; IRB, IR + Bosentan group; 24 h or 3 days, reperfusion 24 h or 3 days. (b) Histogram showing the changes in gray values of FLT-1 in neural cells of the anterior horn of the spinal cord following Bosentan intervention. Strong protein expression exhibited a low gray value. $*P < 0.05$ vs IRS group. (c) Histogram showing the changes in FLT-1 mRNA levels in spinal cord tissue following IR and Bosentan intervention. $*P < 0.05$ vs IRS group. Representative images from four rats in each group are presented here. Scale bar: 50 μ m.

Figure 3 FLK-1 expression following the treatment of Bosentan after IR. (A) Shown are representative FLK-1⁺-stained images of lumbar spinal cord sections from each group. There was stronger expression of FLK-1 in the spinal cord from the IRB group. By contrast, there was less expression in the spinal cord from IRS. IR, ischemia reperfusion; IRS, IR + saline group; IRB, IR + Bosentan group; 12 h or 24 h or 7 days, reperfusion 12 h or 24 h or 7 days. (B) This was confirmed by double immunolabeling using an Ab against MAP-2, a marker for neurons (FLK-1, g; MAP-2, h; DAPI, i; merge, j). (C) Histogram showing the changes in FLK-1 mRNA levels in spinal cord tissue following IR and Bosentan intervention. Representative images from four rats in each group are presented here. Scale bar: a–f, 100 μ m; g–j, 50 μ m.

For example, VEGF treatment may significantly reduce the oxidative damage following SCIR.¹⁰ Moreover, VEGF promoted angiogenesis, decreased neuronal apoptosis and improved the recovery of the neurological and motor function after spinal cord contusion injury in rats.²⁰ Similarly, local administration of VEGF exerted similar effects after spinal cord injury in rats.²¹

Previously, chronic blocking of the endothelin receptors with Bosentan was shown to improve angiogenesis via the upregulation of VEGF following rat hind limb IR,¹¹ indicating that endothelin receptors may regulate VEGF. We have also reported a significant upregulation of endothelin-1 (ET1) and endothelin receptor type B (ETRB) expression following SCIR in rats treated with Bosentan. However, it remains unclear whether there is any crosstalk between the VEGF signalling pathway and ET1-ETRB.¹² In the current study, we found that treatment with Bosentan results in obvious increased expression level of VEGF protein and gene. In addition, blocking endothelin receptors enhanced the expression of FLK-1 and FLT-1 significantly following SCIR. FLK-1 and FLT-1 have common ligands but have different roles in central nervous system injury. FLK-1 has direct neurotrophic effects and can protect cultured neurons from hypoxia and glucose when activated by VEGF.²² It was also confirmed that VEGF promoted the survival of motor neurons through its binding to FLK-1 in the ischemic brain of mice *in vitro*.²³ These data suggested that FLK-1 may act as a direct neuroprotective receptor in central nervous diseases. VEGF is important for endothelial cell proliferation and survival, which are mediated via FLK-1.²⁴ Enhanced survival of endothelial cells could promote angiogenesis, reduce capillary breakdown and bleeding and preserve adequate microcirculation and perfusion, thereby contributing to increased

neuronal survival.²³ In addition, inhibition of FLT-1 reduced brain edema after cerebral ischemia in rats, which may be associated with the protection of blood–brain barrier permeability and induction of macrophage/monocyte infiltration.²⁵ Interestingly, other groups found that FLK-1 and FLT-1 expression increased from day 1 to day 14 following acute spinal cord contusion injury in rats without any treatment.²⁶ Whereas blocking FLT-1 decreased the infiltration of inflammatory cells, blocking FLK-1 increased neuronal apoptosis and worsened the functional recovery after spinal cord injury, which indicates that FLK-1 plays a crucial neuroprotective role in spinal cord injury.²⁷ The results of the present study suggested that blocking endothelin receptors by Bosentan increased both FLK-1 and FLT-1 expression, and decreased neuronal apoptosis via possible neurovascular remodeling, including increased angiogenesis and neurogenesis. We would like to evaluate the change in vessel density that is associated with angiogenesis following rat SCIR in later research.

The VEGF family consists of six different members; however, only the expression of VEGF-A was evaluated, which was one of the limitations of our study. Therefore, the roles of other members of the VEGF family need to be investigated in the future study. On the other hand, Bosentan treatment may initially result in a dose-dependent reduction of systemic blood pressure. Moreover, other common side effects in clinical administration of Bosentan include abnormal hepatic function, peripheral edema, headache, diarrhea and anemia, and so the safety and tolerability of Bosentan may need to be studied in clinical trials in the near future.^{28–30} Additionally, further study is still needed to evaluate the molecular³¹ mechanisms by which Bosentan upregulates expression of VEGF and its receptors following SCIR injury in rats.

CONCLUSIONS

The results of the present study reveal that the blocking of endothelin receptors with Bosentan increased the expression of VEGF and its receptors in a rat SCIR injury model, which may promote angiogenesis and improve the recovery of spinal cord after IR injury.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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