Prazosin blocks apoptosis of endothelial progenitor cells through downregulating the Akt/NF-κB signaling pathway in a rat cerebral infarction model

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Abstract. Endothelial progenitor cells (EPCs) can enhance the recanalization of thrombosis during the progression of cerebral infarction. Prazosin plays a therapeutic role in expanding the peripheral vasculature and regulating infarction cardiosclerosis by inhibiting phosphoinositide signaling. However, the possible mechanisms underlying the therapeutic effects of prazosin have not been fully explored. The purpose of the present study was to analyze the anti-apoptotic effects of prazosin on EPCs in a rat cerebral infarction model. The results showed that prazosin treatment decreased apoptosis of EPCs. Prazosin treatment decreased the serum expression levels of the inflammatory factors, interleukin-1β and tumor necrosis factor-α in rats with cerebral infarctions as well as in EPCs *in vitro*. In addition, prazosin reduced the expression levels of Akt, NF-κB, phosphorylated (p)-Akt and p-NF-κB in EPCs and the middle cerebral artery of rats with cerebral infarction. These findings demonstrated that prazosin inhibited EPC apoptosis in the cerebral infarction rats through targeting the Akt/NF-κB signaling pathway. In conclusion, these results indicated that prazosin has a preventive effect on cerebral infarction by inhibiting EPC apoptosis and by inhibiting the inflammatory response *in vitro* and *in vivo* through regulating the Akt/NF-κB signaling pathway.

Introduction

Cerebrovascular diseases are characterized by systemic vascular disease and neuronal apoptosis (1-3). Cardiovascular diseases are the most common cause of death in the adult population in economically developed countries, accounting for ~33% of deaths in 2017 (4). Cerebral infarction is a type of severe cerebrovascular disease, which often occurs due to brain hypoxic-ischemic necrosis, and leads to embolism and thrombosis (5,6). A previous study found that endothelial cells promote survival, proliferation and neuronal differentiation of transplanted adult ischemia-induced neural stem/progenitor cells after cerebral infarction (7). Maintaining endothelial function and circulating endothelial progenitor cells (EPCs) is associated with a functional recovery for patients with cerebral infarctions (8). Therefore, understanding the molecular mechanisms of action behind cerebral infarction injuries to EPCs is essential for the prevention and treatment of cerebrovascular disease.

Prazosin ($C_{19}H_{21}N_5O_4 \cdot HCl$) is a α-1 adrenergic receptor blocker which prevents the binding of postsynaptic adrenaline. Postsynaptic adrenaline plays an essential role in the progression of coronary artery diseases (9). Prazosin is widely used for treating mild and moderate hypertension (10). The long-term effects of prazosin showed protective effects on blood pressure, heart, carotid artery and acetylcholine in young Wistar rats (11). Furthermore, prazosin treatment causes enduring vulnerability to the transient reinstatement of hemiplegia after traumatic brain injury (12).

A previous study demonstrated that nuclear filament (NF)-κB plays an important role in cerebral ischemia (13). In addition, a previous report indicated that inhibition of apoptosis and inflammation contributes to the recovery folliwng cerebral ischemia/reperfusion injury (14). Expression of Akt is correlated with the expression of apoptosis-related molecules caspase-3 and p38 in acute cerebral ischemia (15). These findings suggest that EPC mobilization following traumatic brain injury may take a different course compared to that associated with body or vascular injuries (16). Activating the Akt signaling pathway can protect rats against cerebral ischemia/reperfusion injury in rat hippocampal neurons (17). This also indicates that the Akt-mediated JNK3/caspase-3 signaling pathway protects rat hippocampal neurons against cerebral ischemia/reperfusion injury in the CA1 region (18). Therefore, the present study hypothesized that prazosin may regulate cerebral ischemia injury through targeting the Akt/NF-κB signaling pathway.

In the present study, the therapeutic efficacy of prazosin in a rat cerebral infarction model was analyzed. The present

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study investigated the association between prazosin and the Akt/NF-κB signal pathway in EPCs in rats with cerebral ischemia injury. The ameliorative effects of prazosin on the cerebral infarct volume, brain water content, cerebral edema, neurological deficits, inflammatory response and EPC apoptosis were also evaluated, which aimed to uncover the potential mechanisms of action of prazosin for the treatment of cerebral infarction *in vitro* and *in vivo*.

Materials and methods

Animal study. The protocols used in the present study were approved by the Animals Committee of Affiliated Hospital of North Sichuan Medical College (Nanchong City, China). A total of 30 male SD rats (8 weeks old, 320-340 g body weight) were purchased from Chongqing Medical University. All rats were maintained at $22\pm2°C$, 50-60% relative humidity and a 12 h light/dark cycle with free access to diet and water. The cerebral ischemia injury model was developed in these rats as described previously (19). Briefly, cerebral ischemia in the area perfused by the middle cerebral artery was induced using an incision in the midline of the neck, where the right carotid bifurcation was observed. Rats were anesthetized using sodium pentobarbital [intraperitoneal (IP), 40 mg/kg]. The internal carotid artery was identified, ligated and then occluded, and the branches of the external carotid artery were dissected and divided. A 4-0 nylon suture with a silicone-coated tip was then advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the middle cerebral artery. Reperfusion was accomplished and rats underwent ischemia for 1 h and reperfusion for 4 h. The sham-operated animals were synchronously intragastrically administrated with equal volume of normal saline. Rat model was established and randomized into three groups: Vehicle, prazosin and sham (n=10 per group). The animals received prazosin (1 mg/kg; Sigma-Aldrich; Merck KGaA) treatment once every day or the same volume of PBS by IP injection. The treatment continued for 30 days.

The modified neurological severity scores (mNSS) test. Neurological deficits were analyzed using the mNSS test in the prazosin and PBS group on day 30 (n=4) as previously reported (20). The mNSS test was scored on a 5-point scale: 0, no neurological deficits; 1, failure to extend right forepaw fully; 2, circling to the right; 3, falling to the right; 4, inability to walk spontaneously combined with depressed levels of consciousness.

Infarct volume analysis. On day 30, after IP injection of pentobarbital (40 mg/kg) for anesthesia, the brains were dissected and collected. Brain tissues were sliced into 1.0-mm-thick coronal sections and then frozen for 30 min at -80°C. The tissue sections were incubated with 1% 2,3,5-triphenyl tetrazolium chloride (Sigma-Aldrich; KGaA) for 15 min at 25°C and fixed in 4% paraformaldehyde for 12 h at 4°C. The infarct region lacks staining and appears white, whereas the normal non-infarct tissue appears red (14). The stained coronal slices were imaged using a fluorescent microscope (x200 magnification), and the infarction was analyzed using Image J V4.6 (National Institutes of Health). The percentage of infarct

volume was measured as follows: (The total infarct volume of the ipsilateral structure/total volume of the contralateral structure) x100%.

Brain water content. Brains were carefully dissected and cut alongside the sagittal plane and the wet weight was measured. The hemispheres were dried for 24 h at 90°C to determine the dry weight. Based on gravimetrical differences, water content was measured using the following calculation: Brain water content (%)=[(wet weight-dry weight)/wet weight] x100.

Cells cultures. EPCs were isolated from the middle cerebral artery in experimental rats (the sham group) and cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) medium with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. EPCs were treated with 5 mg/ml prazosin (Sigma-Aldrich; Merck KGaA) and/or Akt inhibitor (AktIR; 1 mg/ml; cat. no. ab14088; Abcam) and/or NF-κB inhibitor (NF-κBIR; 1 mg/ml; cat. no. ab141588; Abcam) for 24 h at 37°C to analyze the therapeutic effects of prazosin.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was obtained from EPCs using the RNAeasy Mini kit (QIAGEN, Inc.). RNA was reverse transcribed to cDNA using a reverse transcription kit (cat. no. AB4106C) with β-actin expression as an endogenous control (Invitrogen; Thermo Fisher Scientific, Inc.). The RT temperature protocol was as follows: 37°C for 1 h and then 85°C for 5 min to terminate the reaction. Gene expression levels of Bcl-w, Bcl-2, Bax, Bad, interleukin (IL)-1 and tumor necrosis factor (TNF)-α in cells were measured on the iCycler thermal cycler (Bio-Rad Laboratories, Inc.) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.). The following thermocycling conditions were used for the qPCR: Initial denaturation for 10 min at 94°C; 40 cycles of denaturation for 10 sec at 95°C, annealing for 20 sec at 56-62°C, extension for 10 sec at 72°C; and melt curve analysis at 0.5°C for 6 sec. All the forward and reverse primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Table I). Relative mRNA expression level changes were calculated using the $2^{-\Delta\Delta Cq}$ method (21). The results are expressed as the n-fold expression levels, normalized to β-actin expression.

Overexpression of Akt or NF-κB. Expression plasmids pRK5-Akt and pRK5-NF-κB, with a Flag tag at the C-terminus, were constructed by Invitrogen; Thermo Fisher Scientific, Inc. EPCs ($1x10^5$) were seeded on six-well plates (Corning, Inc.) and transiently transfected with pRK5-Akt, pRK5-NF-κB or pRK5-vector by electrotransfection using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 72 h of transfection, mRNA expression levels of Akt and NF-κB were analyzed. Cells were then treated with 5 mg/ml prazosin for 24 h at 37°C for further analysis.

Activity of AKT and NF-κB. TransFactor AKT kit (cat. no. 6 11437; BD Biosciences) and NF-κB TransFactor NF-κB kit (cat. no. 565446; BD Biosciences) were used to detect relative AKT and NF-κB activity in the treated EPCs, respectively, according to the manufacturer's instructions. Subsequently,

Table I. Primers for reverse transcription-quantitative PCR.

| Gene	Forward (5'-3')	Reverse (5'-3')
TNF-α	CCAGACCCTCACACTCAGATCA	TCCGCTTGGTGGTTTGCTA
IL-1β	GGCTGCTTCCAAACCTTTGA	GAAGACACGGATTCCATGGT
Bcl-w	GCTGGTGGTTGACTTTCTCTCC	GGCTTCAGTCCTGTTCTCTTCG
Bcl-2	GATGAAGTACATCCATTATAAGCTGTCACA	GCGCTCAGCCCTGTGCCACCTGTGGTCCAC
Bad	GGAGCATCGTTCAGCAGCAG	CCATCCCTTCATCTTCCTCAGTC
Bax	CTTCAGGGTTTCATCCAG	CTCCATGTTACTGTCCAG
β-actin	CGGAGTCAACGGATTTGGTC	AGCCTTCTCCATGGTCGTGA

IL, interleukin; TNF, tumor necrosis factor.
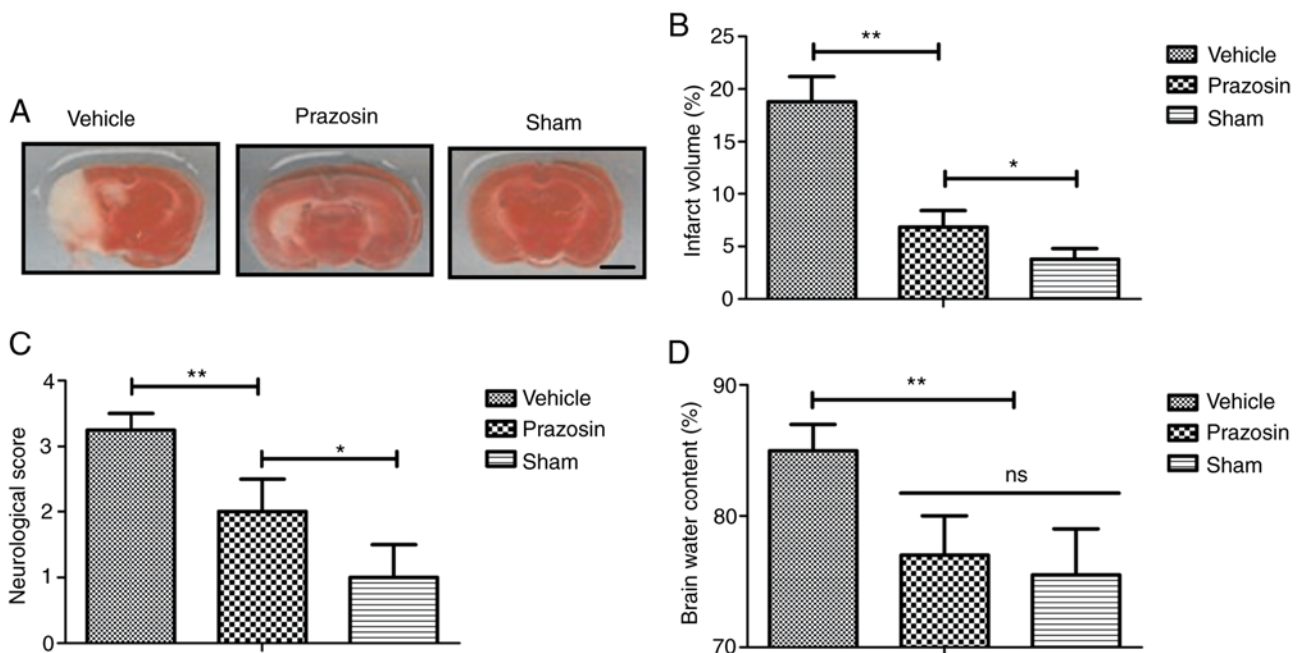
Figure 1. Effects of prazosin on the infarct volume and functional recovery in cerebral ischemia injury rats. (A) Representative images of infarct volumes in the vehicle, prazosin and sham groups. (B) Quantification of the infarct volume in the vehicle, prazosin and sham groups. (C) Neurological score and (D) brain water content in the vehicle, prazosin and sham groups. *P<0.05, **P<0.01. ns, not significant.

the activity of AKT and NF-κB was analyzed as previously described (22,23). Optical density was measured at 405 nm using a microplate reader.

Western blot analysis. EPCs were isolated from experimental rats as previously described (24) and homogenized using RIPA Lysis Buffer (Thermo Fisher Scientific, Inc.). Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Subsequently, protein samples (40 μg) were loaded and separated using 12% SDS-PAGE. Protein were subsequently blotted on a nitrocellulose membrane and labelled using the following rabbit anti-rat primary antibodies at 4˚C overnight: Akt (1:2,000; cat. no. ab185633; Abcam), phosphorylated (p)Akt (1:2,000; cat. no. ab133458; Abcam), NF-κB (1:1,000; cat. no. ab207297; Abcam), pNF-κB (1:1,000; cat. no. ab222494; Abcam) and β-actin (1:1,000; Cell Signaling Technology, Inc.) were added after blocking with 5% BSA for 1 h at 37˚C. The membranes were then incubated with

horse radish peroxidase-conjugated goat anti-rabbit IgG mAb (1:5,000; cat. no. PV-6001; OriGene Technologies, Inc.) at room temperature for 1 h. Protein bands were detected using an ECL detection system and the band intensities were analyzed using ImageJ software version 8.0 (National Institutes of Health).

TUNEL assay. EPCs were prepared and fixed with 10% paraformaldehyde for 10 min at room temperature. Apoptosis of EPCs as analyzed using TUNEL assays (DeadEnd™ Colorimetric Tunel System; Promega Corporation) according to the manufacturer's instructions. EPCs (1x10^5) were incubated TUNEL solution at 37˚C for 1 h. Cells were washed with PBS three times for 5 min at 37˚C followed by incubated with 5% DAPI (Sigma-Aldrich; Merck KGaA) for 15 min at 37˚C in a dark wet box. Finally, images in three fields were captured using a ZEISS LSM 510 confocal microscope at 488 nm. The apoptosis rate was calculated using Developer XD 1.2 (Definiens AG).
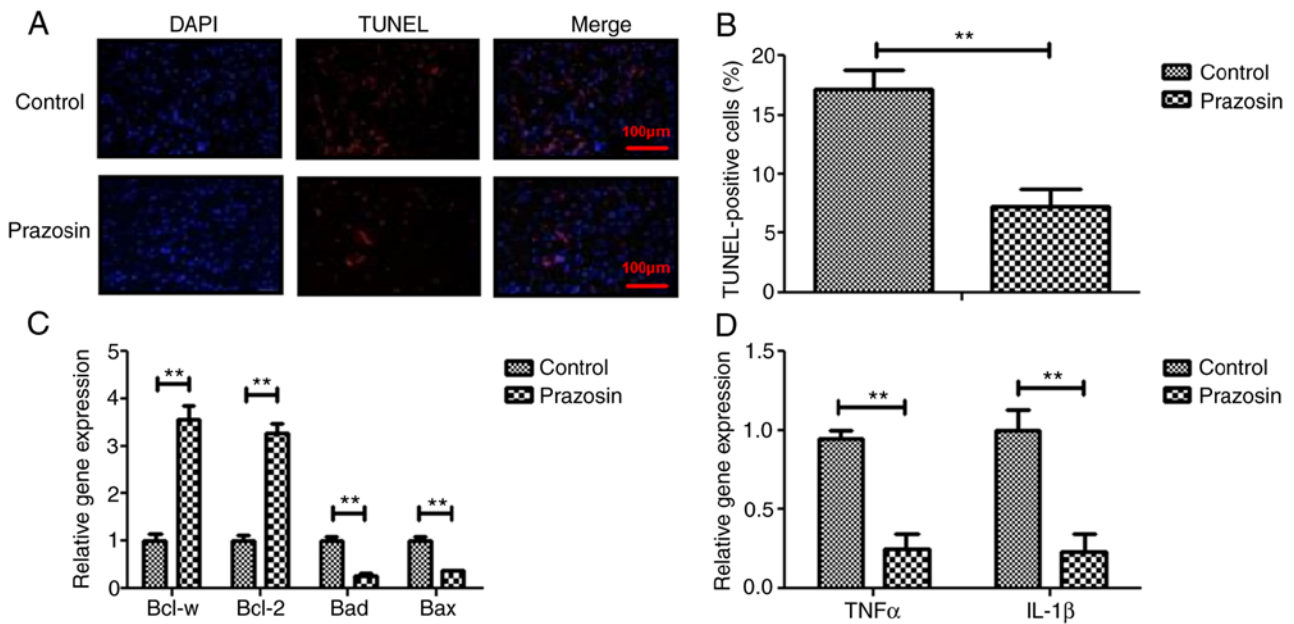
Figure 2. Effects of prazosin treatment on apoptosis inflammatory factors in EPCs *in vitro*. (A) Anti-apoptotic effects of prazosin treatment on EPCs induced by TNF-α. (B) Quantification of apoptotic EPCs induced by TNF-α. (C) Gene expression levels of Bcl-w, Bcl-2, Bad and Bax in EPCs after treatment with prazosin. (D) TNF-α and IL-1β gene expression levels in EPCs after treatment with prazosin. **P<0.01. EPC, endothelial progenitor cell; IL, interleukin; TNF, tumor necrosis factor.

Statistical analysis. All data are reported as the mean and the SD. The mNSS test was analyzed using a Kruskal-Wallis test with post hoc Dunn's tests for multiple comparisons. Statistical significances between two groups were analyzed using Student's t-tests. Other multiple groups were analyzed using one-way ANOVAs followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Prazosin alleviates infarct volume and improves neurological deficits in cerebral ischemia injury rat. Infarct volume and functional recovery were analyzed in experimental rats with myocardial ischemia after treatment with prazosin. The results showed that the infarct volume was markedly decreased following prazosin treatment compared to the vehicle determined by the infarct volume analysis (Fig. 1A-B). Prazosin was found to improve the neurological deficits, as determined by the mNSS test (Fig. 1C). Treatment of prazosin also decreased brain water content compared to the vehicle group, as determined by the brain water content assay (Fig. 1D). These results suggested that prazosin may attenuate ischemia-induced brain injury.

Prazosin treatment inhibits apoptosis of EPCs in vitro. The anti-apoptotic effects of prazosin were investigated in EPCs. The results demonstrated that prazosin treatment decreased the numbers of apoptotic EPCs induced by TNF-α (Fig. 2A). The number of TUNEL-positive EPCs was higher in the control group compared with the prazosin group (Fig. 2B). RT-qPCR showed that the gene expression levels of the anti-apoptotic genes, Bcl-w and Bcl-2, were upregulated in EPCs compared to the control (Fig. 2C). The expression levels of pro-apoptotic genes, Bad and Bax, were downregulated in myocardial cells compared to that of the controls (Fig. 2C). Prazosin was also

found to downregulate TNF-α and IL-1β gene expression levels in EPCs (Fig. 2D). These results suggested that prazosin treatment may inhibit apoptosis and inflammation in EPCs *in vitro*.

Prazosin treatment decreases apoptosis and inflammatory factors in vivo. TUNEL assays showed that the number of apoptotic EPCs significantly increased in the cerebral ischemia injury rats compared to the vehicle controls (Fig. 3A-B). Prazosin treatment decreased the expression levels of pro-apoptotic genes, Bad and Bax, and increased Bcl-w and Bcl-2 gene expression levels in EPCs compared to the vehicle group (Fig. 3C). Gene expression levels of TNF-α and IL-1 in EPCs were also decreased by prazosin treatment compared to the vehicle (Fig. 3D). These results suggested that prazosin treatment can decrease apoptosis and the expression of inflammatory factors *in vivo*.

Prazosin inhibits Akt/NF-κB signal pathway and inflammation in vivo. The effect of prazosin on Akt and NF-κB expression levels was analyzed in the middle cerebral artery *in vivo*. As shown in Fig. 4A, prazosin decreased the expression and phosphorylation levels of Akt and NF-κB in in middle cerebral artery in experimental rats compared to that in the PBS-treated rats. Protein expression levels of TNF-α and IL-1 in the middle cerebral artery were also decreased by prazosin treatment compared to the vehicle *in vivo* (Fig. 4B). These data indicated that prazosin may inhibit the Akt/NF-κB signaling pathway and inflammation in the middle cerebral artery *in vivo*.

Prazosin inhibits EPC apoptosis through targeting the Akt/NF-κB signaling pathways. Cells were transiently transfected with pRK5-Akt, pRK5-NF-κB or pRK5-vector by electrotransfection. Fig. S1 shows that transfection was
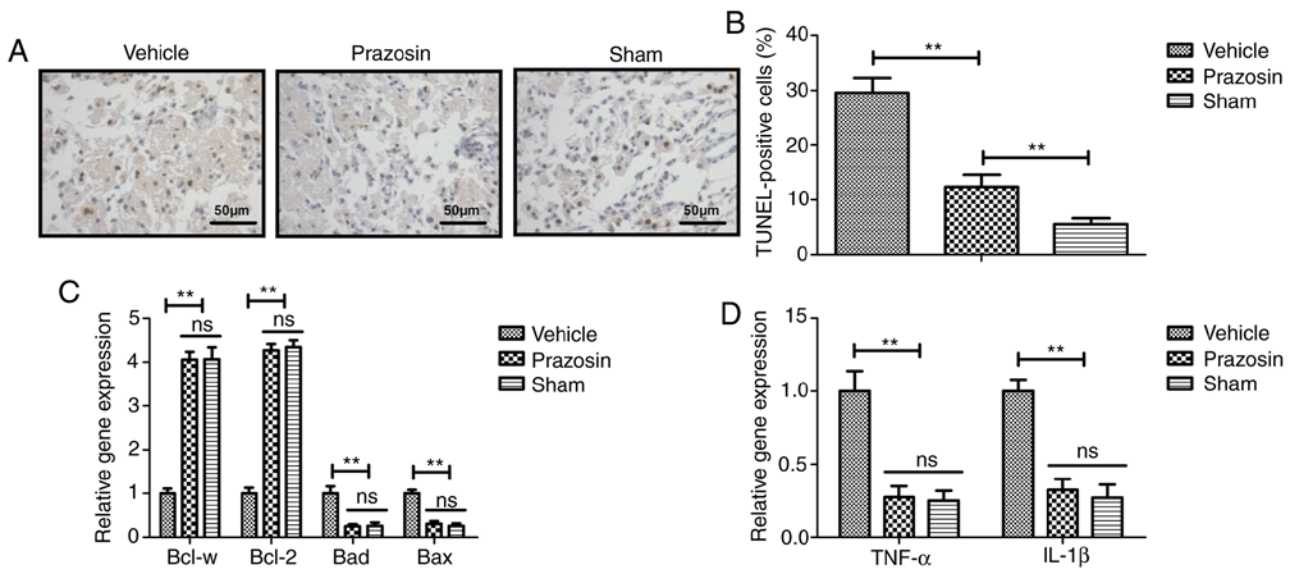
Figure 3. Effects of prazosin treatment on apoptosis and inflammatory factors in EPCs *in vivo*. (A) Representative images of apoptosis of EPCs in the cerebral ischemia injury rats in the vehicle, prazosin and sham groups. (B) Quantification of EPC apoptosis in the vehicle, prazosin and sham groups. (C) Gene expression levels of Bad, Bax, Bcl-w and Bcl-2 gene expression in EPCs in the vehicle, prazosin and sham groups. (D) Gene expression levels of TNF-α and IL-1 in EPCs in vehicle, prazosin and sham groups. **P<0.01. EPC, endothelial progenitor cell; IL, interleukin; ns, not significant; TNF, tumor necrosis factor.
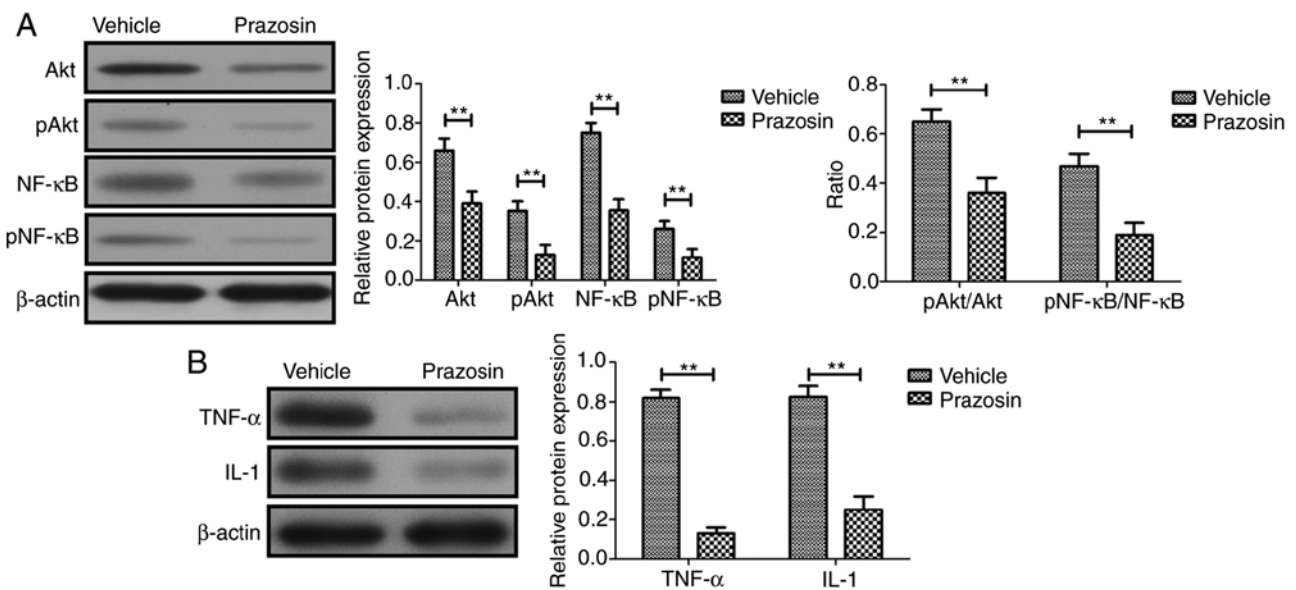

Figure 4. Prazosin inhibits the Akt/NF-κB signaling pathway and inflammation in the middle cerebral artery in cerebral ischemia injury rats. (A) Prazosin decreased the expression and phosphorylation levels of Akt and NF-κB in middle cerebral artery in experimental rats compared to that in vehicle-treated rat. (B) Relative protein expression levels of TNF-α and IL-1 in the middle cerebral artery of prazosin and vehicle-treated cerebral ischemia injury rats. **P<0.01. EPC, endothelial progenitor cell; IL, interleukin; NF, nuclear factor; p, phosphorylated; TNF, tumor necrosis factor.

successfully performed. The mRNA expression level of Akt and NF-κB in pRK5-Akt and pRK5-Akt groups were significantly compared with vector groups, respectively. The potential mechanism of action mediated by prazosin in EPCs was further analyzed. The expression and phosphorylation levels of Akt and NF-κB were downregulated by prazosin treatment in EPCs (Fig. 5A). The results also demonstrated that the ratio of pAkt and pNF-κB to total AKT and NF-κB, respectively, was decreased by prazosin treatment in the EPCs (Fig. 5B). AktIR decreased NF-κB activity, while Akt overexpression (AktOR) increased NF-κB activity (Fig. 5C-D). AktIR

increased prazosin-inhibited apoptosis of EPCs, while AktOR decreased prazosin-inhibited apoptosis of EPCs (Fig. 5E-F). These results suggested that prazosin inhibited EPC apoptosis through targeting the Akt/NF-κB signaling pathway.

Prazosin inhibits inflammation in EPCs through targeting the NF-κB signaling pathway. Finally, the relationship between the inhibition of inflammatory cytokine production and NF-κB production in EPCs was investigated. As shown in Fig. 6A, NF-κBIR decreased TNF-α and IL-1 in EPCs and NF-κBIR increased prazosin-inhibited TNF-α and IL-1 expression levels
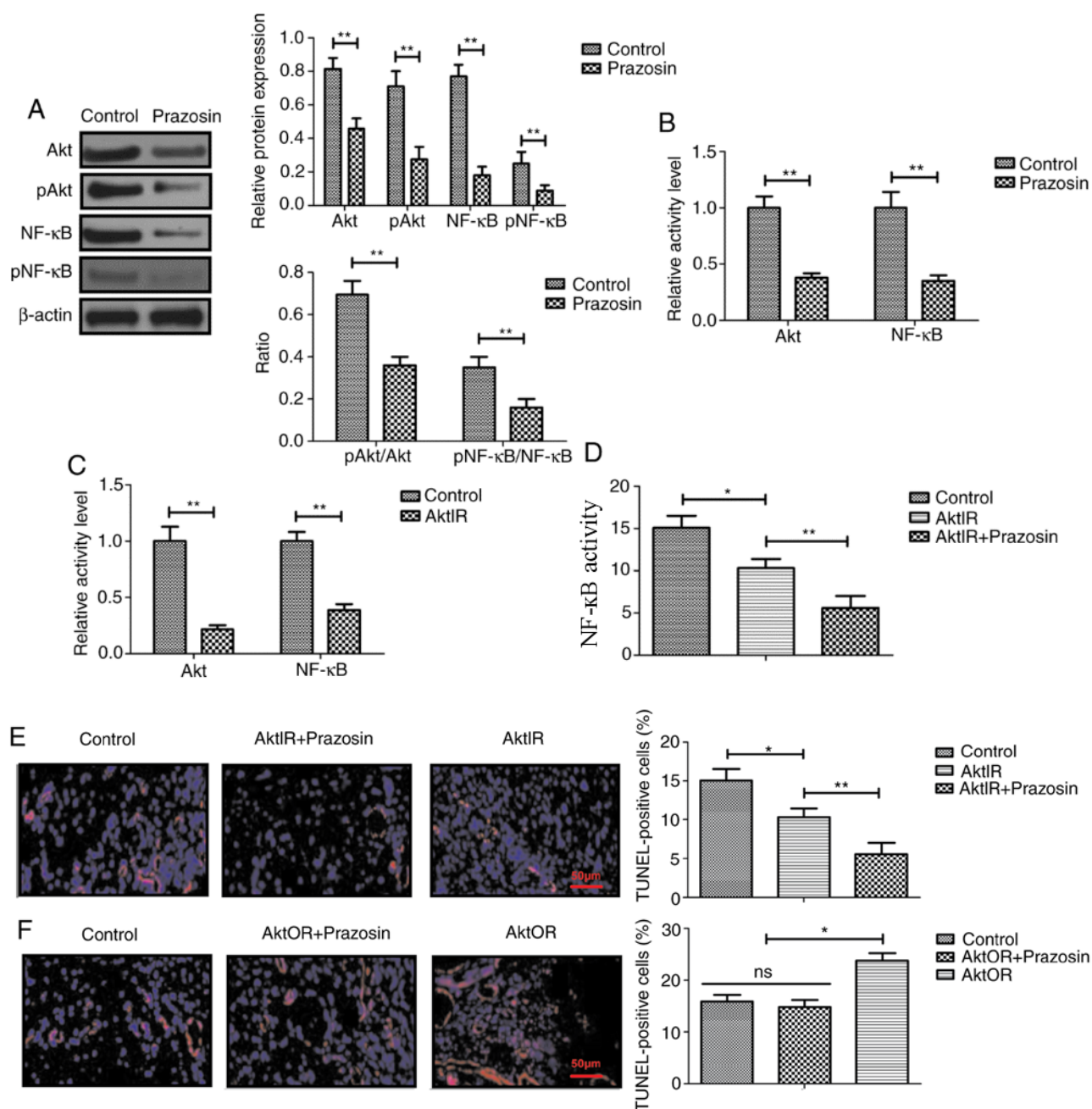
Figure 5. Prazosin inhibits EPCs apoptosis through targeting the Akt/NF-κB signaling pathway. (A) Expression and phosphorylation levels of Akt and NF-κB in prazosin-treated EPCs. (B) Activity of AKT and NF-κB in prazosin-treated EPCs. (C) Effects of AktIR on NF-κB activity in EPCs. (D) NF-κB activity in AktIR + prazosin EPCs. (E) Effects of AktIR on prazosin-inhibited apoptosis of EPCs. (F) Effects of AktOR on prazosin-inhibited apoptosis of EPCs. Control represents PBS-treated cells. *P<0.05, **P<0.01. AktIR, Akt inhibitor; AktOR, Akt overexpression; EPC, endothelial progenitor cell; NF, nuclear filament; ns, not significant; p, phosphorylated.

in EPCs. NF-κB overexpression (NF-κBOP) increased TNF-α and IL-1 expression levels in EPCs, and prazosin was able to prevent the NF-κBOP mediated increase in the expression levels of these proteins (Fig. 6B). These data suggested that prazosin inhibited inflammation in EPCs through targeting the NF-κB signaling pathway.

Discussion

Currently, ischemia-induced brain injury presents an extremely high mortality rate worldwide, that closely associates with metabolic disorders of endogenous substances,

such as acetylcholine and histamine (25-27). Prazosin can protect myocardial cells against anoxia-reoxygenation injury through the extracellular signal-regulated kinase signaling pathway (28). However, the role of prazosin in EPCs has not reported previously. In the present study, the ameliorative effects of prazosin were investigated in a rat cerebral infarction model. The present study found that prazosin decreased infarct volume, brain water content and attenuated neurological deficits in ischemia-induced brain injury. These findings indicated that prazosin inhibited apoptosis of EPCs through targeting the Akt/NF-κB signal pathway in EPCs.
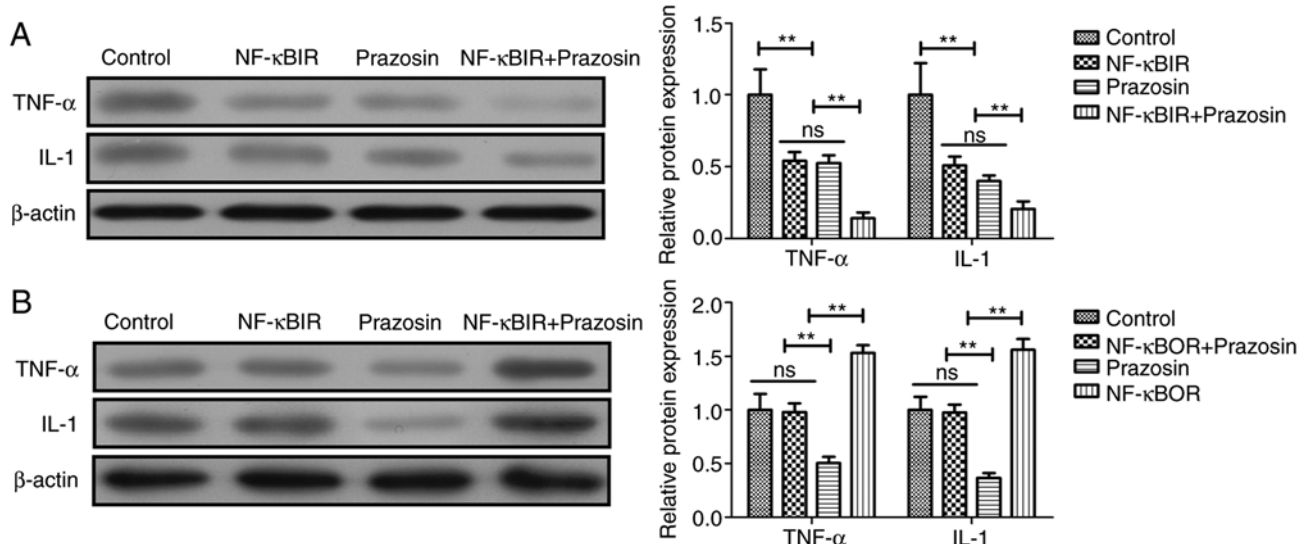
Figure 6. Prazosin inhibits inflammation in EPCs through decreasing activity of the NF-κB signal pathway. (A) NF-κBIR decreased and increased prazosin-inhibited TNF-α and IL-1 expression levels in EPCs. (B) NF-κBOP increased and decreased prazosin-inhibited TNF-α and IL-1 expression levels in EPCs. **P<0.01 vs. control. EPC, endothelial progenitor cell; IL, interleukin; NF, nuclear factor; NF-κBIR, NF-κB inhibitor; NF-κBOR, NF-κB overexpression; ns, not significant; TNF, tumor necrosis factor.

Inflammation plays an essential role in the occurrence and development of cerebral infarction (29). A previous study reported that the levels of TNF-α in the serum is associated with the severity of acute cerebral infarctions (30). It has also been found that IL-1β expression is upregulated in the brain tissue and sera of focal cerebral ischemia/reperfusion injury model rats (31). The present study found that prazosin decreased TNF-α and IL-1β gene expression levels in EPCs, and also reduced serum levels of TNF-α and IL-1β in the rat cerebral infarction model. Administration of prazosin also decreased the brain water content and reduced neurological deficits in the cerebral infarction rat model compared to the control group, which suggested that prazosin may be a potential drug for the treatment of cerebral infarction.

Inhibition of apoptosis and proliferation of EPCs may repair the blood-brain barrier and improve the cognitive function of amyloid precursor protein/presenilin 1 in transgenic Alzheimer's disease mice following the development of cerebral infarction (32). To the best of our knowledge, this is the first study which has analyzed the anti-apoptotic effects of prazosin in EPCs both *in vitro* and *in vivo*. Prazosin treatment was found to decrease the pro-apoptotic gene expression levels of Bad and Bax, and increase Bcl-w and Bcl-2 gene expression levels in EPCs both *in vitro* and *in vivo*. Previously, it has been indicated that EPCs and neural progenitor cells synergistically protect cerebral endothelial cells from hypoxia/reoxygenation-induced injury through activating the Akt pathway (33). Notably, inhibition of NF-κB activation decreases TNF-α-induced inflammation and atherosclerotic activity in EPCs (34). Mechanistically, prazosin inhibited the Akt signaling cascade, which prevented the apoptosis of EPCs. The results demonstrated the protective effects of prazosin through inhibition of apoptosis mediated by the Akt/NF-κB signaling pathway in animals with cerebral infarctions.

In conclusion, the present study indicated that prazosin protects EPCs against apoptosis by downregulating the activity of the Akt/NF-κB signaling pathway in the rat cerebral infarction model. The present findings provided evidence of the anti-apoptotic efficacy of prazosin in the progression of cerebral infarction, which illustrated a possible mechanistic pathway for the treatment of ischemia-induced brain injury.

Acknowledgements

Funding

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL and WL were responsible for guaranteeing integrity of the entire study, study concepts and design, definition of intellectual content, literature research, experimental studies, data acquisition, manuscript preparation and editing and review. WL was responsible for the experimental studies and manuscript editing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethical Committees of Affiliated Hospital of North Sichuan Medical College.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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