The effects of erythropoietin on RhoA/Rho-associated kinase expression in rat retinal explants cultured with glutamate

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Abstract. The aim of this study was to investigate the effects of erythropoietin (EPO) on RhoA/Rho-associated kinase (ROCK) expression in rat retinal explants cultured with glutamate. After the retinal explants were cultured in serum-free R16 nutrient medium for 24 h, the retinal explants were divided into control (R16 nutrient medium), glutamate (R16 nutrient medium containing 5 mM/l glutamate) and glutamate + EPO (R16 nutrient medium containing 5 mM/l glutamate and 6.0 U/ml EPO) groups, and culturing was continued for another 72 h. The mRNA and protein expression of total RhoA, ROCK1 and ROCK2 in the retinal explants was examined by RT-PCR and western blotting, and active RhoA in the retinal explants was detected via GST-RBD binding and immunoblotting with an antibody specific to active RhoA. The total RhoA mRNA and protein expression did not differ substantially between the control, the glutamate and the glutamate + EPO groups. The glutamate increased the active RhoA, ROCK1 and ROCK2 expression in cultured retinal explants ($P<0.05$), whereas the expression of active RhoA, ROCK1 and ROCK2 in the glutamate + EPO group was significantly lower than that in the simple glutamate group ($P<0.05$) and similar to that in the control group. In conclusion, EPO downregulates active RhoA, ROCK1 and ROCK2 expression in retinal explants cultured with glutamate.

Introduction

Rho-associated kinase (ROCK) is a serine/threonine kinase and one of the major downstream effectors of the small GTPase RhoA. The RhoA/ROCK pathway is closely correlated with the pathogenesis of several central nervous system (CNS) disorders and is involved in a number of aspects of neuronal functions, including neurite outgrowth and retraction (1). The effects of these axon growth inhibitors are reversed by blocking the RhoA/ROCK pathway *in vitro* (2-4), and inhibition of the RhoA/ROCK pathway promotes axon regeneration and functional recovery in the injured CNS *in vivo* (2,5,6).

Erythropoietin (EPO) has been found to act on the CNS as a neurotrophic and neuroprotective factor, particularly in conditions of neural damage, such as hypoxia, ischemia or brain hemorrhage (7-10). Previous studies in animal models have indicated that EPO is effective in enhancing neurological recovery following experimental spinal cord injury (11-13). EPO has also been shown to protect retinal ganglion cells (RGCs) in rat models of glaucoma (14,15), axotomy-induced degeneration (16,17) and retinal ischemia (18) and to promote the axonal regeneration of RGCs following optic nerve transection (19). Previous studies also showed that there is crosstalk between hypoxia inducible factor-1 (HIF-1) and the ROCK pathways in neuronal differentiation of mesenchymal stem cells, neurospheres and in PC12 neurite outgrowth (20), and that EPO is one of the major target genes of HIF-1. Therefore, crosstalk may also exist between EPO and the RhoA/ROCK pathway. There are, however, few data available concerning the effects of EPO on the RhoA/ROCK pathway. Therefore, the aim of the present study was to clarify whether EPO regulates the RhoA/ROCK protein expression in rat retinal explants cultured with glutamate.

Materials and methods

Animals. Animals included in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In addition, the Ethics Committee of Ruijin Hospital (Shanghai, China) approved the experiments. Sprague-Dawley (SD) rat pups were used in all experiments, kept under conditions of constant temperature and humidity and fed by their mothers. The day of birth was counted as postnatal day (P)0 and P2-3 rats were used in our experiment. A total of 150 rats was used for this study.

Retinal explant dissection. The SD rats were sacrificed on P2-3 by decapitation. Tissue soaked in 70% ethanol was used

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to wipe clean and wrap the removed heads. The wrapped heads were transported into a culture room to a laminar flow cabinet from which point onwards all handling was performed aseptically. The eyes were enucleated and incubated in serum-free R16 nutrient medium. The anterior segment, vitreous body and sclera were then removed and the retinal explants were collected. Approximately 20 eyes were collected for each experiment.

Retinal explant culture. The complete list of chemicals making up the originally developed R16 nutrient medium for brain tissue (Gibco/BRL, Carlsbad, CA, USA) has been published previously (21,22). The R16 powder is composed of 41 ingredients that can be divided into three groups: group 1 consisted of salts; group 2 included the amino acids with the exception of the potentially neurotoxic amino acids glutamate and aspartate; and group 3 included sugars and vitamins. The retinal explants were cultured in 12-well culture plates with serum-free R16 nutrient medium. Each well contained 4 retinal explants which were cultured in a humidified incubator at 37˚C in an atmosphere of 5% CO_2-95% O_2.

Drug treatment. EPO was prepared by dissolving 10 μg EPO (R&D Systems, Minneapolis, MN, USA) in 200 μl distilled water (8.3 μg=1,000 units EPO). The retinal explants were cultured for 24 h as described above and then divided into three groups: the control, glutamate and glutamate + EPO groups. The retinal explants in the control group were continually cultured with serum-free R16 nutrient medium; the retinal explants in the glutamate group were continually cultured with serum-free R16 nutrient medium containing 5 mM/l glutamate (Sigma-Aldrich, St. Louis, MO, USA); and the retinal explants in the glutamate + EPO group were continually cultured with serum-free R16 nutrient medium containing 5 mM/l glutamate and 6.0 U/ml EPO. The retinal explants in the three groups were continually cultured for another 72 h. The doses of glutamate and EPO used in the present study were selected according to our previous study in which it was shown that 6.0 U/ml EPO significantly improved the survival of cultured retinal neurocytes incubated with 5 mM/l glutamate (23).

Reverse transcription-polymerase chain reaction (RT-PCR). The mRNA levels of the genes were measured by RT-PCR. Total RNA was isolated from the individual samples using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of the preparations were determined by measuring the absorbance at 260/280 nm using a spectrophotometer (Beckman Coulter, Miami, FL, USA). Total RNA was reverse-transcribed into cDNA in a 20-μl reaction volume containing 2 μg RNA, 4 μl 5X M-MLV buffer, 2 μl dNTP, 1 μl random hexamer primer, 0.5 μl RNase inhibitor and 1 μl M-MLV RTase. The reactions were performed at 25˚C for 10 min, at 42˚C for 60 min and at 70˚C for 10 min.

The nucleotide sequences of the primers were based on previously published sequences (24,25). The primer sequences used for RT-PCR were: RhoA, 5'-GTGATTGTTGGT GATGGAGC-3' and 5'-CTCGTGGCCATCTCAAAAAC-3'; ROCK-1, 5'-TGCGGGAGTTACAAGATCAGCT-3' and 5'-TTTCCGTCAGTCTCATCAGCAC-3'; ROCK-2, 5'-TCTG AAAGGAGGGACCGAACC-3' and 5'-GTTCCTGTTT GTGTCGAGCCATCA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-ATGGGGAAGGTGAAGGTCGG-3' and 5'-CAGGAGGCATTGCTGATGAT-3'. The PCR protocol comprised an initial incubation for 5 min at 94˚C; 30 cycles (for RhoA, ROCK-1 and ROCK-2) or 25 cycles (for GAPDH) of 45 sec at 94˚C, 45 sec at 55˚C and 2 min at 72˚C and a final incubation for 7 min at 72˚C. The PCR products were separated by 2% agarose gel electrophoresis and stained with 0.5 μg/ml ethidium bromide, and the band signals were exposed to ultraviolet radiation before they were scanned and quantified with a gel image analyzer (GelDoc Quantity One; Bio-Rad, Hercules, CA, USA). Band intensities were quantified and normalized against those of GAPDH. Each set of experiments was repeated in triplicate for statistical analysis.

Western blot analysis. Total retinal protein was extracted from pulverized samples using modified radioimmunoprecipitation (modified RIPA) buffer with a Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). The protein concentrations were determined by the Bradford protein assay (Bio-Rad). Each sample contained 4 retinal explants. Equal amounts of protein (20 μg/lane) were separated on polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After blocking for 3 h in Tris-buffered saline with 0.1% Tween-20 (TBST) and 3% bovine serum albumin (BSA), the membranes were incubated overnight at 4˚C with primary antibodies (RhoA, dilution 1:50, sc-418; ROCK-1, dilution 1:50, sc-6056; ROCK-2, dilution 1:50, sc-5561; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in TBST containing 3% BSA. The membranes were then washed and incubated with alkaline phosphatase-conjugated secondary antibodies in TBST for 2 h and developed using nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Promega, Madison, WI, USA). The densities of the bands on the membrane were scanned and analyzed using Image Pro Plus version 6.0 (Media Cybernetics, Silver Spring, MD, USA). Each set of experiments was repeated in triplicate for statistical analysis.

RhoA activity assay. Active RhoA was assayed in tissue lysates using a Rho activation assay kit (Upstate Biotechnology, Milton Keynes, UK), following the manufacturer's instructions as described previously (26,27). Briefly, the retinal protein (200 μg) was mixed with glutathione-S-transferase (GST)-Rho-binding domain (RBD) fusion protein (20 μl) in an ice bath and the mixture was incubated for 45 min at 4˚C with intermittent mixing. After the mixture was centrifuged (13,000 x g) for 10 min at 4˚C, the precipitate was suspended with Mg^{2+} lysis/wash buffer (500 μl) and centrifuged for 10 min. This procedure was repeated three times. The final precipitate was used for active RhoA assay by western blot analysis. Each set of experiments was repeated in triplicate for statistical analysis.

Statistical analysis. Data were presented as the mean ± standard deviation, unless otherwise stated. Statistical analyses were performed using the SPSS software (IBM SPSS Statistics 19.0, SPSS, Inc., Chicago, IL, USA). To compare data among

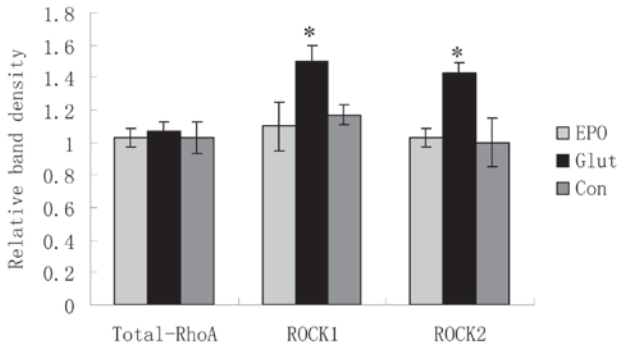

Figure 1. RT-PCR analysis of RhoA, ROCK1 and ROCK2 in retinal explants after 72 h culture with 5 mM/l glutamate and 6.0 U/ml EPO. The RhoA mRNA expression did not differ substantially between the groups. The glutamate increased ROCK1 and ROCK2 mRNA expression in cultured retinal explants. ROCK1 and ROCK2 mRNA expression in the EPO group was significantly lower than that in the simple glutamate group, and similar to that in the control group. EPO: 6 U/ml EPO + 5 mM/l glutamate culture group; Glut: 5 mM/l glutamate culture group; Con: control group. *P<0.05 compared with EPO and control groups. RT-PCR, reverse transcription-polymerase chain reaction; ROCK, Rho-associated kinase; EPO, erythropoietin.

three groups, one-way analysis of variance (ANOVA) was performed followed by post hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of EPO on RhoA, ROCK1 and ROCK2 mRNA expression in retinal explants cultured with glutamate. RT-PCR analysis revealed that the RhoA mRNA expression did not differ substantially between the control, glutamate and glutamate + EPO groups. Compared with the control group, the glutamate increased ROCK1 and ROCK2 mRNA expression in cultured retinal explants (P<0.05; Fig. 1). The ROCK1 and ROCK2 mRNA expression in the glutamate + EPO group was significantly lower than that in the simple glutamate group (P<0.05; Fig. 1) and similar to that in the control group.

Effect of EPO on RhoA, ROCK1 and ROCK2 protein expression in retinal explants cultured with glutamate. Western blot analysis revealed that the protein expression of total-RhoA did not differ substantially between the control, glutamate and glutamate + EPO groups, which was in accordance with the expression of the RhoA mRNA (Fig. 2). However, compared with the control group, the glutamate increased active RhoA expression in cultured retinal explants (P<0.05; Fig. 2). The protein expression of active RhoA in the glutamate + EPO group was significantly lower than that in the simple glutamate group (P<0.05; Fig. 2). Compared with the control group, the glutamate increased ROCK1 and ROCK2 expression in

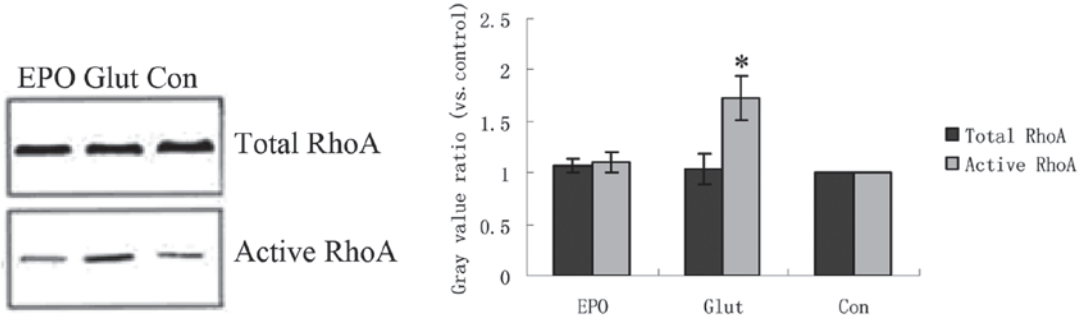

Figure 2. Representative western blots showing the expression regulation of total and active RhoA in retinal explants after 72 h culture with 5 mM/l glutamate and 6.0 U/ml EPO. The protein expression of total-RhoA did not differ substantially between the groups. The glutamate increased active RhoA expression in the cultured retinal explants. The protein expression of active RhoA in the EPO group was significantly lower than that in the simple glutamate group, and similar to that in the control group. EPO: 6 U/ml EPO + 5 mM/l glutamate culture group; Glut: 5 mM/l glutamate culture group; Con: control group. *P<0.05 compared with EPO and control groups. EPO, erythropoietin.

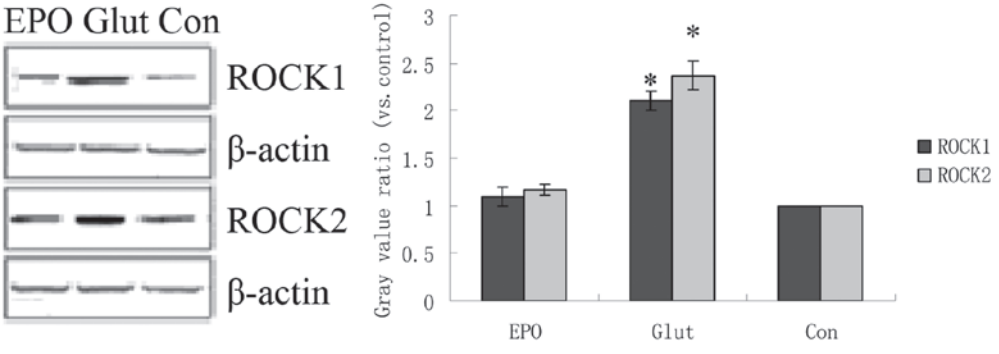

Figure 3. Representative western blots showing the expression regulation of ROCK1 and ROCK2 in retinal explants after 72 h culture with 5 mM/l glutamate and 6.0 U/ml EPO. The glutamate increased ROCK1 and ROCK2 expression in cultured retinal explants. The protein expression of ROCK1 and ROCK2 in the EPO group was significantly lower than that in the simple glutamate group. EPO: 6 U/ml EPO + 5 mM/l glutamate culture group; Glut: 5 mM/l glutamate culture group; Con: control group. *P<0.05 compared with EPO group and control group. ROCK, Rho-associated kinase; EPO, erythropoietin.

cultured retinal explants (P<0.05; Fig. 3). The protein expression of ROCK1 and ROCK2 in the glutamate + EPO group was significantly lower than that in the simple glutamate group (P<0.05; Fig. 3), and similar to that in the control group.

Discussion

A number of studies have confirmed that Rho and its associated signaling molecules are involved in and mediate the biological processes of axon regeneration, extension and fiber projection (28-31). Rho regulates the cell actin cytoskeleton by its downstream effective factor ROCK, which is extensively involved in the biological processes of cell migration, movement, apoptosis, gene transcription and nerve regeneration (32). An important reason that axon regeneration is difficult following CNS damage in adult mammals is the existence of certain growth suppression molecules in the damaged environment. Three types of molecules derived from myelin which can suppress axon growth have been identified thus far: Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (Omgp) (1). Previous studies indicate that Nogo-A, MAG and Omgp may activate Rho by common or different pathways and subsequently cause growth cone collapse (33,34).

Our previous studies demonstrated that EPO had a neuroprotective effect *in vivo* (15) and a neurite outgrowth promotion effect on retinal neurons *in vitro* (23). However, the mechanism of the axonal regeneration effect of EPO on retinal neurons has not been fully clarified. A previous study has shown that EPO promotes the regeneration of adult CNS neurons via activation of the JAK2/STAT3 and PI3K/AKT pathways, and that EPO-facilitated neuritogenesis is paralleled by the upregulation of Bcl-X(L) (35). However, the induced expression of Bcl-X(L) alone cannot completely neutralize the inhibition of axonal growth (36). Thus, the axonal regeneration mechanism of EPO in damaged RGCs in adult rats may also involve other signaling pathways or factors. To gain a deeper understanding of the EPO-dependent axonal regeneration process, we studied the effects of EPO on RhoA/ROCK expression in rat retinal explants cultured with 5 mM/l glutamate. Our results show that glutamate increased the active RhoA, ROCK1 and ROCK2 expression in cultured retinal explants, and that the expression of active RhoA, ROCK1 and ROCK2 in the glutamate + EPO group was significantly lower than that in the simple glutamate group, and similar to that in the control group. This suggests that EPO downregulated the active RhoA, ROCK1 and ROCK2 expression in retinal explants cultured with glutamate.

There were a few limitations in our study. Firstly, the present study did not explore the axonal regeneration of retinal neurons, making it difficult to determine the relationship between the RhoA/ROCK expression and axonal regeneration. Secondly, the study did not determine where the active RhoA, ROCK1 and ROCK2 expression occurred in the cultured retinal explants. Further investigations are required to demonstrate the locations of active RhoA, ROCK1 and ROCK2 expression and the correlation between RhoA/ROCK expression and the axonal regeneration of retinal neurons.

In conclusion, our results suggest that glutamate increases the active RhoA, ROCK1 and ROCK2 expression in cultured retinal explants, and that EPO downregulates the active RhoA, ROCK1 and ROCK2 expression in retinal explants cultured with glutamate.

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