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ROCK mediates the inflammatory response in thrombin induced microglia

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HIGHLIGHTS

- Increased expression of ROCK, NO and TNF-α in thrombin induced microglia was found.
- Enhanced phagocytosis in thrombin induced microglia was also found.
- Argatroban or Y-27632 pretreatment decreased ROCK, NO and TNF-α expression.
- Argatroban or Y-27632 pretreatment reduced phagocytosis in thrombin induced microglia.
- ROCK may regulate the inflammatory response in thrombin induced microglia.

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ABSTRACT

To investigate whether the ROCK pathway is involved in thrombin-induced microglial inflammatory response, thrombin-induced microglia were pretreated with the thrombin inhibitor argatroban or a ROCK inhibitor Y-27632. Microglial inflammatory response was evaluated by phagocytosis of fluorescein labeled latex beads analyses and inflammatory mediators' expression such as nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α). Compared to non-induced microglia, thrombin-induced microglia show significantly enhanced phagocytotic capacity and increased ROCK, NO and TNF-α expression. Pretreatment of thrombin-induced microglia with argatroban or Y-27632 significantly decreased phagocytotic capacity and reduced ROCK, NO and TNF-α expression. Therefore, the ROCK pathway may play a vital role in the mechanisms by which thrombin induces microglia in the inflammatory response.

1. Introduction

Intracerebral hemorrhage (ICH) is a devastating disease accounting for 10–15% of all strokes. Clinical advances in ICH have been restricted by a poor understanding of the mechanisms underlying brain injury after hemorrhage [4]. Inflammatory activation plays a crucial role in the pathophysiological mechanisms of ICH, exerting deleterious effects on the progression of ICH-induced secondary brain injury [35]. Therefore, investigation of inflammatory activation under conditions of ICH is warranted.

Thrombin, a blood-derived serine protease, is essential to blood coagulation and is abundantly present in hematoma. Uncontrolled activation of thrombin-induced inflammation after ICH has been reported to be a central mechanism for secondary injury in ICH [15]. Our previous data also indicated that both PKC alpha and PKC delta play important roles in thrombin-induced brain injury after ICH [8]. However, the mechanisms by which thrombin activates microglia in the progression of inflammatory response remain unclear.

Rho-associated protein kinase (ROCK) is a kinase belonging to the AGC family of serine–threonine kinases [23]. The ROCK pathway has been implicated in numerous inflammatory diseases such as rheumatoid arthritis [18], atherosclerosis [10,25], asthma [17], multiple sclerosis [31], and disorders of the central nervous system [24,29]. ROCK appears to also serve as a mediator of thrombin-induced endothelial barrier dysfunction [3,6,16]. However, whether the ROCK pathway is involved in thrombin-induced microglial inflammatory response has not been investigated.

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In the present study, we investigated whether the ROCK pathway is involved in thrombin-induced microglial inflammatory response. To this end, we explored the influence of pretreatment with the specific thrombin inhibitor, argatroban or the ROCK inhibitor Y-27632, in the response of microglia to thrombin. The inflammatory response, induced by thrombin, was evaluated by assessing the phagocytosis of fluorescein labeled latex beads and the expression of the inflammatory mediators, nitric oxide (NO) and tumor necrosis factor-alpha (TNF-a). Molecular mechanisms underlying activated microglia induced by thrombin are described.

2. Materials and methods

All experiments were conducted in accordance with the standards and procedures of the American Council on Animal Care and Institutional Animal Care and Use Committee of Xuzhou Medical College.

2.1. Primary microglial cultures

Primary microglial cultures were isolated from the cortex of neonatal SD rats less than 72 h old, as described previously [36]. Briefly, the cortical brain tissue was isolated from neonatal SD rats and the isolated cells were plated on poly-L-lysine-coated culture flasks at the density of 1.0×10^5 cells/cm^2. After 14 days, the mixed microglia/astrocyte cultures were digested with trypsin at 37 °C. After digestion for 20 min, the remaining adherent cells in the flask are microglia.

2.2. The purity of the primary microglial cultures

To determine the purity of the primary microglial cultures, microglia were plated on 8-chamber poly-D-lysine coated glass slides. After blocking with 10% BSA in PBS for 1 h, the cells were incubated overnight at 4 °C with IBA-1 goat polyclonal antibody (1:100, Abcam, Cambridge, MA), followed by incubation with FITC-conjugated secondary antibody (1:200, ZSGB-Bio, Beijing) for 2 h at 37 °C [34], then we randomly chose 5 visual fields (nearly 20 cells per visual field) to count the percentage of the positive cells by confocal microscopy.

2.3. The optimal dose and time for thrombin to activate microglia

To figure out the optimal dose and time for thrombin to activate microglia, microglia were treated with thrombin with different concentrations (0, 10, 20, 40, and 60 U/ml) for 6 h and with different time (3, 6, 12, and 24 h). ROCK expression was evaluated among them.

2.4. Experiment groups

To investigate whether ROCK mediates the inflammatory response in thrombin induced microglia. Microglia were randomly assigned to different groups and were treated with thrombin with optimal concentration for the optimal time ($n = 3$/group): (1) thrombin group: microglia were incubated with thrombin; (2) argatroban group (25 U/ml): microglial cells were pretreated with argatroban (Mitsubishi Pharma, GuangZhou) for 30 min and then treated with thrombin; (3) Y-27632 group (10 μmol/L): microglial cells were pretreated with Y-27632 (≥95% by HPLC, Merck, USA) for 30 min and then treated with thrombin.

2.5. Western blotting and immunofluorescence

ROCK expression levels in the microglia were analyzed by Western blotting and immunofluorescence. Equal amounts of cell lysate were subjected to Western blot analysis, as described previously [7,40]. The following primary antibodies were used: anti-β-actin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ROCK (1:500; Abcam, Cambridge, MA). For immunofluorescence [41], cells growing on coverslips were fixed and treated with 0.1% Triton-X100, incubated with anti-ROCK (1:500; Abcam, Cambridge, MA) followed by a FITC-conjugated secondary antibody. The absence of primary antibody was the negative control. Samples were examined by a laser scanning confocal microscope. The experiments were done with 3 different cell culture preparations. The data were calculated as a percentage of fluorescence area in each field.

2.6. Quantification of TNF-α release

The release of TNF-α in the culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA). Supernatants (detection limit: 5 pg/ml) were placed on ELISA kit strips (Boster, Wuhan). Sandwich ELISA was then performed according to the manufacturer's instructions.

2.7. Nitrite assay

The level of NO was evaluated by Nitrite assay as described previously [13]. In brief, cell culture supernatant was centrifuged at 1000 rpm for 5 min, aliquots of 100 μl were incubated with 100 μl Griess reagent (0.1% naphthylthylene, 1% sulfanilamide in 2.5% phosphoric acid), and the absorption at 450 nm was measured using a MR5000 ELISA reader.

2.8. Fluorescein labeled latex beads analysis

Microglial phagocytotic activity was measured by the uptake of FITC (fluorescein isothiocyanate) labeled latex beads (Nanozymics, Wuxi) [33]. In brief, culture specimens were exposed to FITC labeled latex beads for 2 h at 37 °C. Surface bound beads were removed by vigorous washing with PBS, then cells were stained with DiI (Molecular probe Inc., USA) for 5 min. The experiments were done with 3 different cell culture preparations. The data were calculated as number of positive cells in each field.

2.9. Statistical analysis

The homogeneity of variances was assumed to hold for all measurements by using Levene test. One-way analysis of variance (ANOVA) was performed for ROCK expression, NO and TNF-α expression, fluorescein labeled latex beads analyses; the analyses started with the testing of overall group effect, followed by a post hoc test of least significant difference (LSD) for multiple comparison if the overall group effect was significant. The significance level was set to 0.05 in the paper and all the data analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago IL). The data are presented as mean ± SD.

3. Results

3.1. Purity of primary microglial cells isolation

To determine the purity of the cultures, a subset of cells was seeded onto multi-chamber glass slides for characterization via immunofluorescence (Fig. 1). Using commonly employed cell-type specific antibodies, the percentage of microglia (Iba-1+) present in the microglial cultures was determined. Quantification showed that primary microglial cell cultures were over 95% Iba-1+ microglia with few astrocytes remaining (Fig. 1).