FEBS Letters 580 (2006) 4252–4260

Involvement of Rho/ROCK signalling in small cell lung cancer migration through human brain microvascular endothelial cells

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Received 8 February 2006; revised 2 May 2006; accepted 21 June 2006

Available online 30 June 2006

Edited by Lukas Huber

Abstract **Small cell lung cancer (SCLC) cells migration across human brain microvascular endothelial cells (HBMECs) is an essential step of brain metastases. Here we investigated signalling pathways in HBMECs contributing to the process. Inhibition of endothelial Rho kinase (ROCK) with Y27632 and overexpression of ROCK dominant-negative mutant prevented SCLC cells, NCI-H209, transendothelial migration and the concomitant changes of tight junction. Conversely, inhibition of phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) had no effects. Furthermore, endothelial RhoA protein was activated during NCI-H209 cells transendothelial migration. Rho/ROCK participated in NCI-H209 cells transendothelial migration through regulating actin cytoskeleton reorganization. These results suggested that Rho/ROCK was required for SCLC cells transendothelial migration.**

Keywords: Small cell lung cancer; Rho/ROCK; Brain endothelial cell; Tight junction

1. Introduction

Brain metastases are a source of significant morbidity and mortality in patients with small cell lung cancer (SCLC) [1]. A key event of brain metastases is SCLC cells migration across the blood–brain barrier (BBB), which separates blood from the brain parenchyma [2]. However, the precise molecular mechanism of SCLC cells penetrating the BBB that mainly consists of endothelial cells with tight junction (TJ) is poorly defined.

It has been reported that leukocytes and breast cancer cells transendothelial migration were associated with intercellular junction disruption [3,4]. Rho/ROCK (Rho kinase), phosphatidylinositol 3-kinase (PI3K)/Akt and protein kinase C (PKC) pathways had all been proposed to be involved in the regulation of paracellular permeability and junctional dynamics in endothelial cells [5–7]. Therefore, we attempt to investigate whether these signalling molecules are involved in SCLC cells migration across brain endothelial cells. Using a Transwell culture system of human brain microvascular endothelial cells (HBMECs) monolayer as an in vitro model for BBB [8], we explored the relevant signalling pathway in brain endothelial cells during SCLC, NCI-H209 cells, migrating across the HBMEC monolayer. We found that Rho/ROCK signalling in HBMECs was required for NCI-H209 cells transendothelial migration.

2. Materials and methods

2.1. Cell culture and transfection

Human brain microvascular endothelial cells were a generous gift from Dr. K.S. Kim (Johns Hopkins University, USA). They were cultured in RPMI 1640 medium, supplemented with 10% FBS, 10% Nu-serum (BD Biosciences, Franklin Lakes, NJ). The human small cell lung cancer cell line, NCI-H209 cells (HTB-172), a suspension cell line, were obtained from American Type Culture Collection (ATCC, Rockville, MD), and maintained in RPMI 1640 medium containing 10% FBS. Stable transfections of HBMECs were carried out with the different expression vectors: pCAG-myc, pCAG-myc-ROCK-WT and pCAG-myc-ROCK-KDIA [9] (provided kindly by Dr. S. Narumiya, Kyoto University Faculty of Medicine, Kyoto, Japan). Single clone cells were selected by G418 and confirmed by Western blot.

2.2. Transendothelial migration assay of NCI-H209 cells

2×10^5 HBMECs were seeded on the upper chamber of Transwell insert with 3 μm pore size (Corning Costar Corp., Cambridge, MA) in 24-well plates. Experiments were conducted when transendothelial electrical resistance (TEER) was >200 ohm · cm^2. 2×10^5 NCI-H209 cells were loaded into the upper chamber of the Transwell and cells that had transmigrated into the lower chamber were counted in a hemocytometer. Inhibition experiments were performed as described in detail elsewhere [10]. Briefly, HBMECs cultured in Transwell insert were pretreated with the inhibitors Y27632 (10 μM), LY294002 (25 μM), wortmannin (100 nM), Gö 6976 (100 nM) and Gö 6983 (100 nM) (Calbiochem, San Diego, CA), respectively, for 1 h. And then culture medium was removed, NCI-H209 cells were loaded into the upper chamber of Tranwell insert and incubated for 8 h. To specifically inhibit the activity of Rho proteins, HBMECs were pretreated with 5 μg/ml *Clostridium botulinum* C_3 exoenzyme for 18 h.

2.3. Transendothelial electrical resistance (TEER)

TEER was measured using an EVOM voltohmmeter (World Precision Instruments, Sarasota, FL). The final TEER values were calculated as ohm · cm^2 by multiplying it with the surface area of the monolayer. The results were presented as a percentage compared to the normal HBMECs without NCI-H209 cells treatments.

2.4. HRP flux measurement

The HBMEC monolayer in Transwell inserts were cocultured with NCI-H209 cells and transferred to a new 24-well plate. 0.5 μM horseradish peroxidase (HRP) (Sigma–Aldrich, St. Louis, MO) in serum-free RPMI 1640 medium was added to the upper compartment of the Transwell system. After 1 h, the media from the lower chamber

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0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.056

were collected and the HRP content of the samples were assayed colorimetrically. The HRP flux was expressed as nanomol passed per cm^2 surface area.

2.5. Cell fractionation and Western blot

Confluent HBMECs were washed with Dulbecco's phosphate-buffered saline (D-PBS) containing 0.1 mM EDTA without calcium and magnesium for three times, extracted in TritonX-100 lysis buffer (25 mM HEPES/NaOH, 150 mM NaCl, 4 mM EDTA, 1% TritonX-100, protease inhibitors), centrifuged at $14000 \times g$ for 10 min. The supernatant was collected as the soluble fraction (S). The pellets were dissolved in SDS lysis buffer (1% SDS, 25 mM HEPES/NaOH, 4 mM EDTA, protease inhibitors) to eliminate insoluble material (IS). The total cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate, protease inhibitors). Equal amounts of proteins were separated by SDS–PAGE and processed for immunoblotting with antibodies for phospho-myosin light chain (MCL), phospho-cofilin (Cell Signalling Technology Inc., Danvers, MA), occludin (Zymed Laboratories, Inc. South San Francisco, CA) and phospho-Akt (Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were visualized using SuperSignal Chemiluminescence substrates (Pierce, Indianapolis, IN). Quantification of band density was done using Alpha Ease Fc software (Alpha Innotech, CA).

2.6. Immunofluorescence

The HBMEC monolayers grown on glass coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking with 5% BSA in PBS, the cells were incubated with mouse anti-ZO-1, rabbit anti-occludin (Zymed Laboratories)

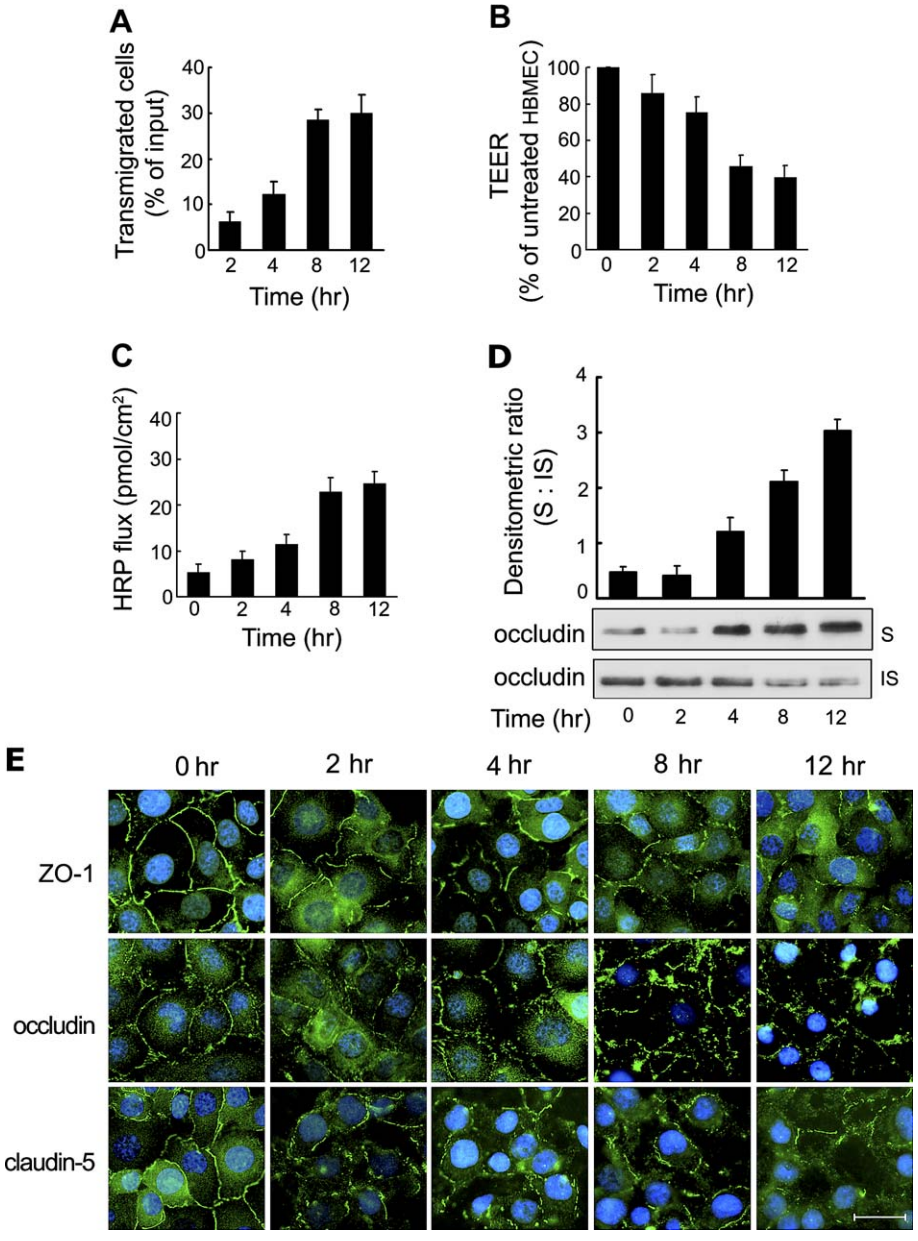

Fig. 1. NCI-H209 cells transendothelial migration and its effect on endothelial permeability. 2×10^5 NCI-H209 cells were loaded on the HBMEC monolayer cultured on the Transwell insert and incubated for the indicated times, then the cells that had transmigrated into the lower chamber (A) and the time-dependent changes of TEER (B) and HRP flux (C) were measured. A shift of endothelial occludin protein from insoluble to soluble phase in response to NCI-H209 cells was detected by Western blot (D). The changes of ZO-1, occludin and claudin-5 distribution in HBMECs cocultured with NCI-H209 cells were visualized by immunofluorescence. Nuclear staining using DAPI was used to visualize all the cells (E). Data were means ± S.D. of three independent experiments. Scale bar: 40 μm.

and goat anti-claudin-5 (Santa Cruz Biotechnology) to visualize the distribution of ZO-1,occludin and claudin-5. The cells were incubated with rhodamine-labeled phalloidin (Sigma–Aldrich) for the actin filaments. The glass slides were analyzed using immunofluorescence microscopy (Olympus, Japan).

2.7. Rho activation assay

Rho activation assay kit (Upstate Biotechnology, Lake Placid, NY) was used to determine the activation of Rho proteins in HBMECs cocultured with NCI-H209 cells. Briefly, cell lysates were incubated with rhotekin Rho-binding peptide immobilized on

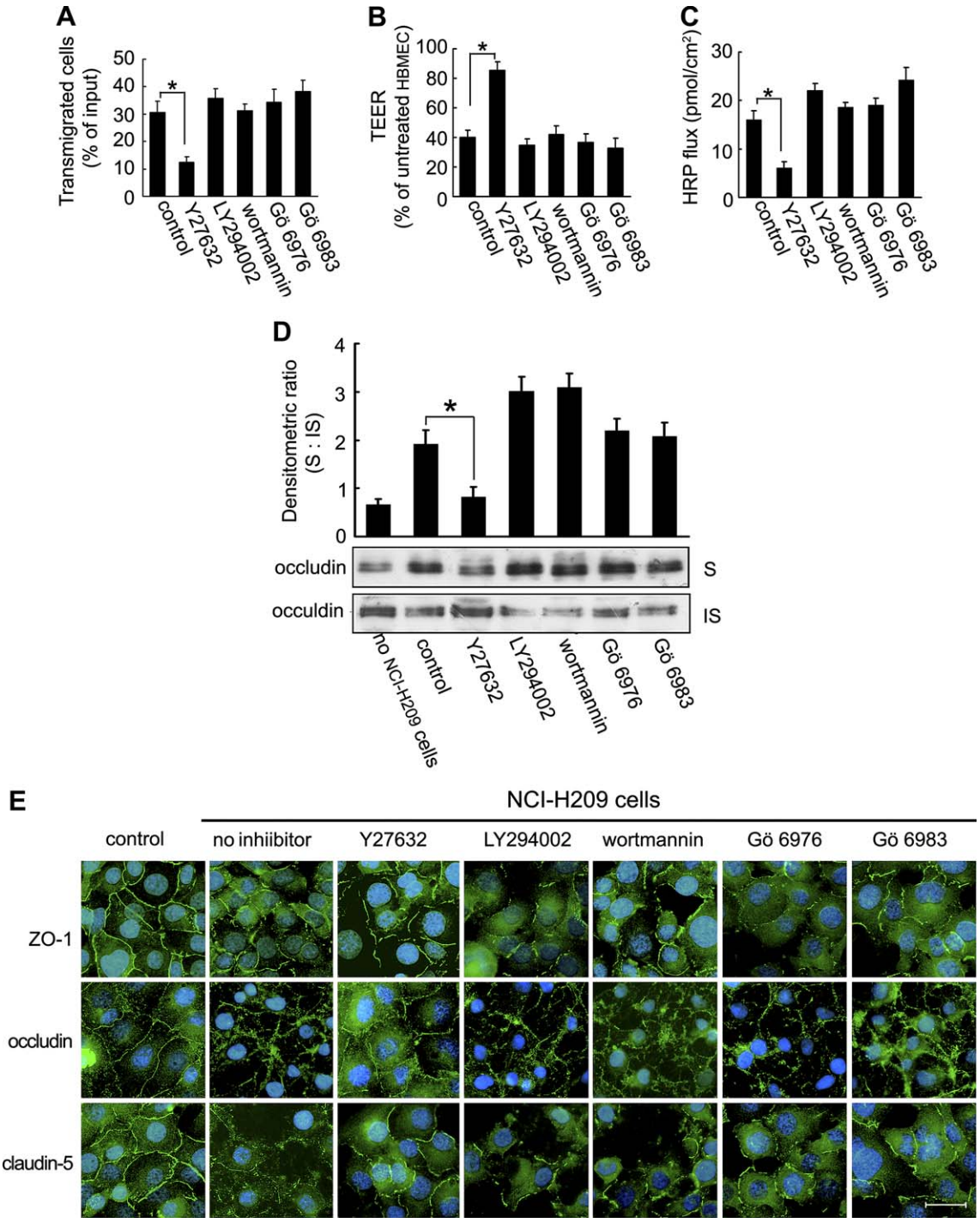

Fig. 2. Effects of inhibitors of ROCK, PI3K and PKC on NCI-H209 cells transendothelial migration. HBMECs were pretreated with specific inhibitors as described in Section 2 and then cocultured with NCI-H209 cells for 8 h. Transmigrated NCI-H209 cells (A), TEER (B) and HRP flux (C) were measured, respectively. The different detergent solubility of endothelial occludin were analyzed by Western blot (D). The changes of ZO-1, occludin and claudin-5 distribution were visualized by immunofluorescence. Nuclear staining using DAPI was used to visualize all the cells (E). The normal HBMECs were served as the control. Data were means ± S.D. of three independent experiments. $^{*}P < 0.05$, Scale bar: 40 μm.

agarose beads for 45 min, and activated GTP-Rho bound to rhotekin-agarose was detected by Western blot using rabbit anti-RhoA antibody (Santa Cruz Biotechnology).

2.8. PepTag assay for nonradioactive detection of PKC activity

PKC activity was evaluated with PepTag Assay for non-radioactive detection of PKC Kit (Promega, Madison, WI) as described in Manufacturer's Protocol. Briefly, after cocultured with NCI-H209 cells, HBMECs total lysates were incubated with PKC reaction mixture and separated by agarose gel. The bands were visualized under UV light and photographed.

3. Results

3.1. NCI-H209 cells transendothelial migration induced TJ disassembly in HBMECs

Using the HBMEC monolayer cultured on the Transwell insert, we examined NCI-H209 cells transendothelial migration. As shown in Fig. 1A, a time-dependent increase in NCI-H209 cells transendothelial migration was observed. Meanwhile, NCI-H209 cells significantly induced a decrease of TEER and an increase of HRP flux in a time-dependent manner (Figs. 1B and C). Because the integrity of TJ was mainly responsible for the brain endothelial permeability [11], we next detected the changes of TJ proteins in the process. The detergent-insoluble occludin usually indicated the TJ integrity and once TJ was weakened, occludin relocated to the detergent-soluble fraction in cells [12]. As shown in Fig. 1D, there was an obvious shift in occludin distribution from insoluble to soluble fractions prepared from HBMECs cocultured with NCI-H209 cells throughout the 12-hour period. Meanwhile, the distribution of TJ proteins was visualized by immunofluorescence. Normal HBMECs showed a characteristic polygonal shape and linear pattern of immunostaining for ZO-1, occludin and claudin-5 at cell-cell borders. In the HBMECs cocultured with NCI-H209 cells, continuous

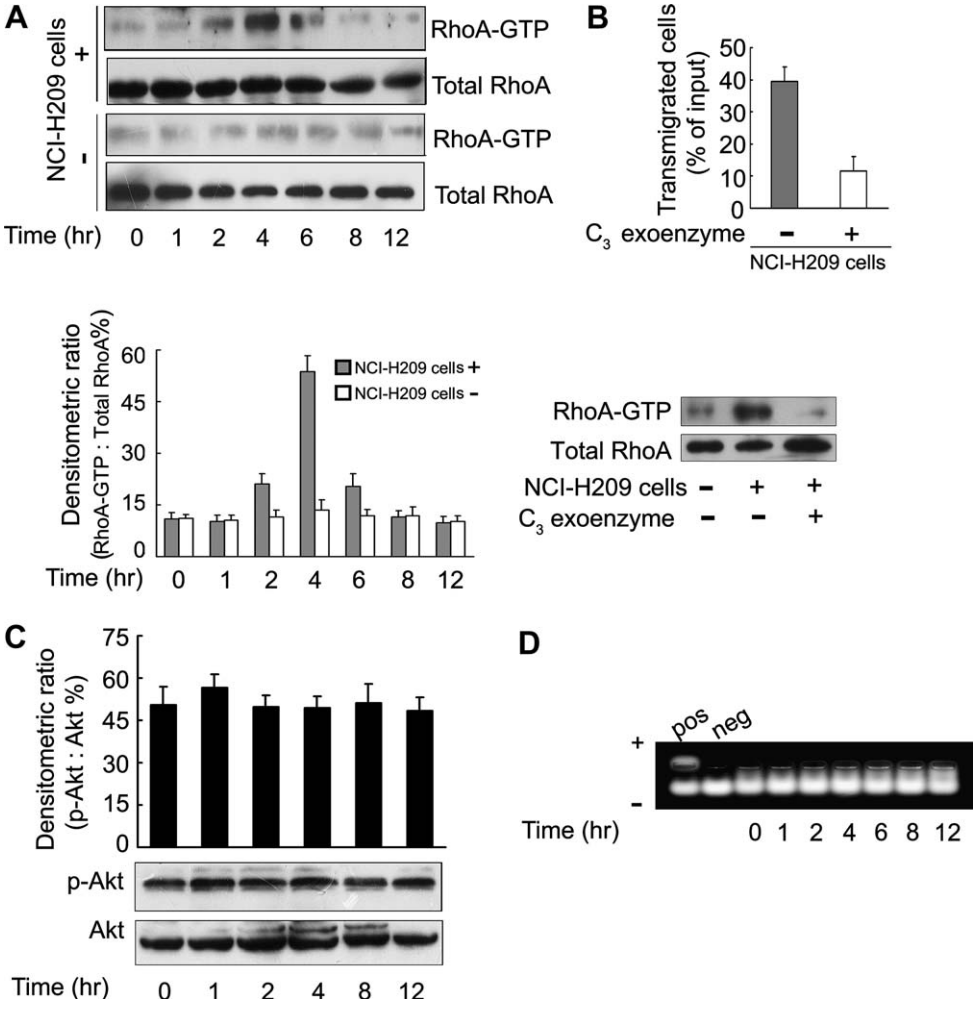

Fig. 3. Changes of Rho, PI3K and PKC activity in HBMECs during NCI-H209 cells transendothelial migration. (A) Confluent HBMECs were cocultured with NCI-H209 cells for the indicated time. The HBMECs alone washed with D-PBS containing 0.1 mM EDTA at the different time points were severed as the control. RhoA activity assay were performed as described in Section 2. Top: Western blot analysis of RhoA-GTP; Bottom: quantification of band density was done using Alpha Ease Fc software. (B) Confluent HBMECs were pretreated with *Clostridium botulinum* C_3 exoenzyme (5 μg/ml) for 18 h, and then cocultured with NCI-H209 cells. NCI-H209 cells transendothelial migration (8 h) and RhoA activity (4 h) were measured. PI3K (C) and PKC (D) activity assay were performed as described in Section 2, respectively. The PKC activity was reflected by the fluorescence of phosphorylated peptides migrated toward the positive electrode (+), whereas the nonphosphorylated peptides migrated toward the negative electrode (−). Data were means ± S.D. of three independent experiments.

lines of ZO-1, occludin and claudin-5 staining gradually became discontinuous, segmented and dotted (Fig. 1E). These data suggested that TJ disassembly in HBMECs were correlated with the progressive appearance of NCI-H209 cells transendothelial migration.

3.2. NCI-H209 cells transendothelial migration was associated with Rho/ROCK signalling activation but not PI3K/Akt or PKC

Several different types of intracellular signalling pathways have been implicated to participate in the regulation of endothelial permeability. Among them, Rho/ROCK, PI3K /Akt and PKC appeared to be crucial regulators [5–7]. To investigate which signalling pathway in HBMECs might be associated with NCI-H209 cells transendothelial migration, the effects of specific inhibitors for the signalling pathways above were tested. Fig. 1 showed that the transmigration rate of NCI-H209 cells reached the peak at 8 h, so we decide to work only with an 8-h transmigration time-point thereafter. Fig. 2A showed that only ROCK inhibitor, Y27632, could block NCI-H209 cells transendothelial migration, whereas PI3K (LY294002, wortmannin) and PKC (Gö 6976, Gö 6983) inhibitors had no effects. Similar results were obtained from the TEER assay (Fig. 2B), HRP flux (Fig. 2C), the detergent solubility of occludin (Fig. 2D) and the TJ proteins redistribution (Fig. 2E). These findings suggested that ROCK other than PI3K or PKC was involved in NCI-H209 cells transendothelial migration.

To further confirm the inhibition experiments, we detected the activity status of signalling molecules in HBMECs cocultured with NCI-H209 cells. Because the ROCK activation is mostly regulated by the small GTPase RhoA, the activity of endothelial RhoA protein was investigated by affinity precipitation. As we know, it is very important for the biochemical analysis to ensure that the HBMECs lysates do not have any protein contamination coming from NCI-H209 cells. Therefore, prior to cell lysis, the confluent HBMECs cocultured with NCI-H209 cells were washed three times with Dulbecco's phosphate-buffered saline (D-PBS) containing 0.1 mM EDTA without calcium and magnesium, and about 95% of the adherent NCI-H209 cells were washed out (data not shown). The HBMECs alone washed with D-PBS containing 0.1 mM EDTA at the different time points were severed as the control. Fig. 3A revealed that NCI-H209 cells induced activation of RhoA, reaching the peak at 4 h, whereas the alteration of RhoA activity was not observed in the control group. This time point is not consistent with the NCI-H209 cells transmigration which reached the peak

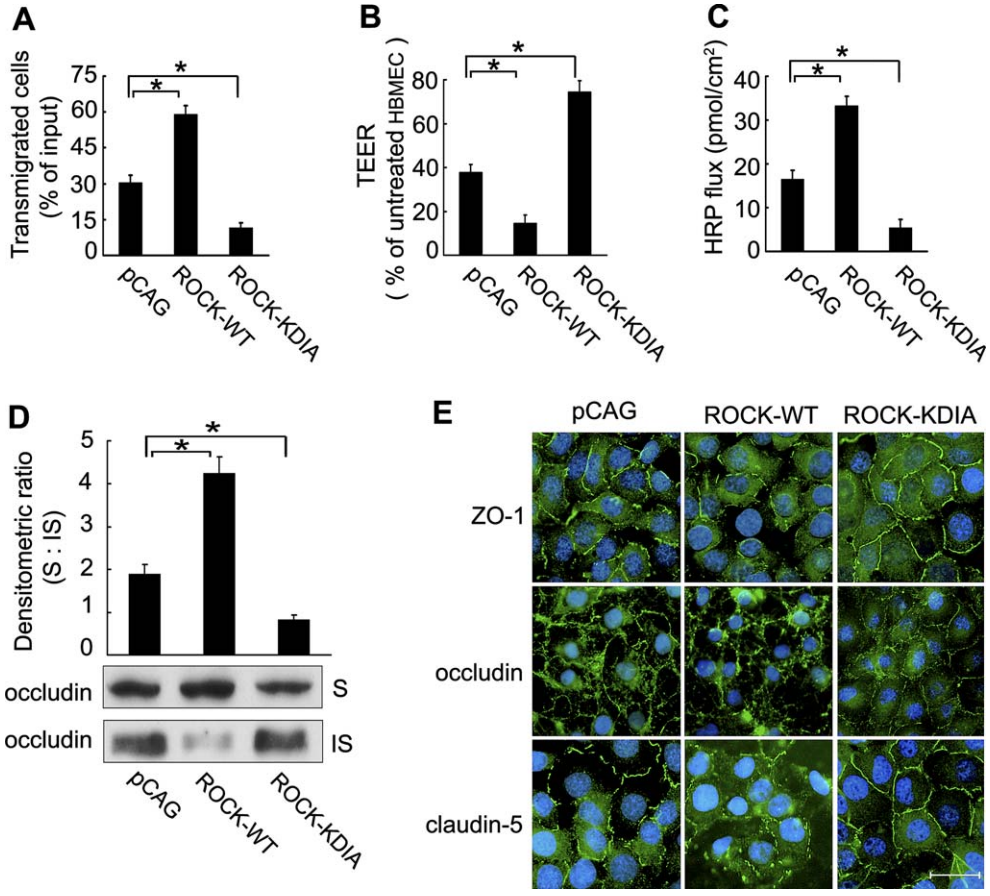

Fig. 4. Effects of overexpressing ROCK dominant negative mutant in HBMECs on NCI-H209 cells transendothelial migration. 2×10^5 NCI-H209 cells were loaded on the HBMEC transfectants cultured on the Transwell insert and incubated for 8 h. Transmigrated cells (A), TEER (B) and HRP flux (C) were measured, respectively. The detergent solubility of occludin proteins of different transfectants in response to NCI-H209 cells were detected by Western blot (D). ZO-1, occludin and claudin-5 distribution in different transfectants cocultured with NCI-H209 cells were visualized by immunofluorescence. Nuclear staining using DAPI was used to visualize all the cells (E). Data were means ± S.D. of three independent experiments. $^*P < 0.05$. Scale bar: 40 μm.

at 8 h (Fig. 1). It is possible that the activation of signalling molecules is prior to the cellular behavior changes. Further, the treatment of HBMECs with C_3 exoenzyme, a specific inhibitor of Rho, prevented NCI-H209 cells transendothelial migration. It was well known that C_3 exoenzyme can inhibit all three isoforms of Rho, RhoA, RhoB and RhoC. Here, the RhoA activation was observed using anti-RhoA antibody, and the C_3 exoenzyme blocked the NCI-H209 cells-induced activation of endothelial RhoA proteins (Fig. 3B). Whereas, no significant changes of PI3K and PKC activities were observed in the process (Figs. 3C and D). These results strongly supported that NCI-H209 cells transendothelial migration was Rho/ROCK-dependent pathway other than PI3K/Akt or PKC.

3.3. Overexpression of ROCK and its dominant-negative mutant in HBMECs significantly affected NCI-H209 cells transendothelial migration

To gain further insight into the role of ROCK in NCI-H209 cells transendothelial migration, HBMECs were transfected with pCAG vector containing myc-tagged wild type ROCK (ROCK-WT) and a kinase defective, Rho binding-defective dominant negative mutant of ROCK (ROCK-KDIA), respectively. HBMECs transfected with pCAG vector was severed as the control. We found that the transmigration rate of NCI-H209 cells through the HBMECs overexpressing ROCK-WT was significantly raised. In contrast, overexpression of ROCK-KDIA led to a decrease of NCI-H209 cells transendothelial migration (Fig. 4A). These results were further verified by the assessment of endothelial permeability. Overexpression of ROCK-WT caused the decrease of TEER and the increase of HRP flux in response to NCI-H209 cells. However, overexpression of ROCK-KDIA abolished this effect (Figs. 4B and C). In addition, NCI-H209 cells-induced the recruitment of detergent-soluble occludin and the alteration of ZO-1, occludin and claudin-5 staining in HBMECs overexpressing ROCK-WT were not observed in the ROCK-KDIA groups (Figs. 4D and E). These results clearly supported that ROCK activation was exactly required for NCI-H209 cells transendothelial migration.

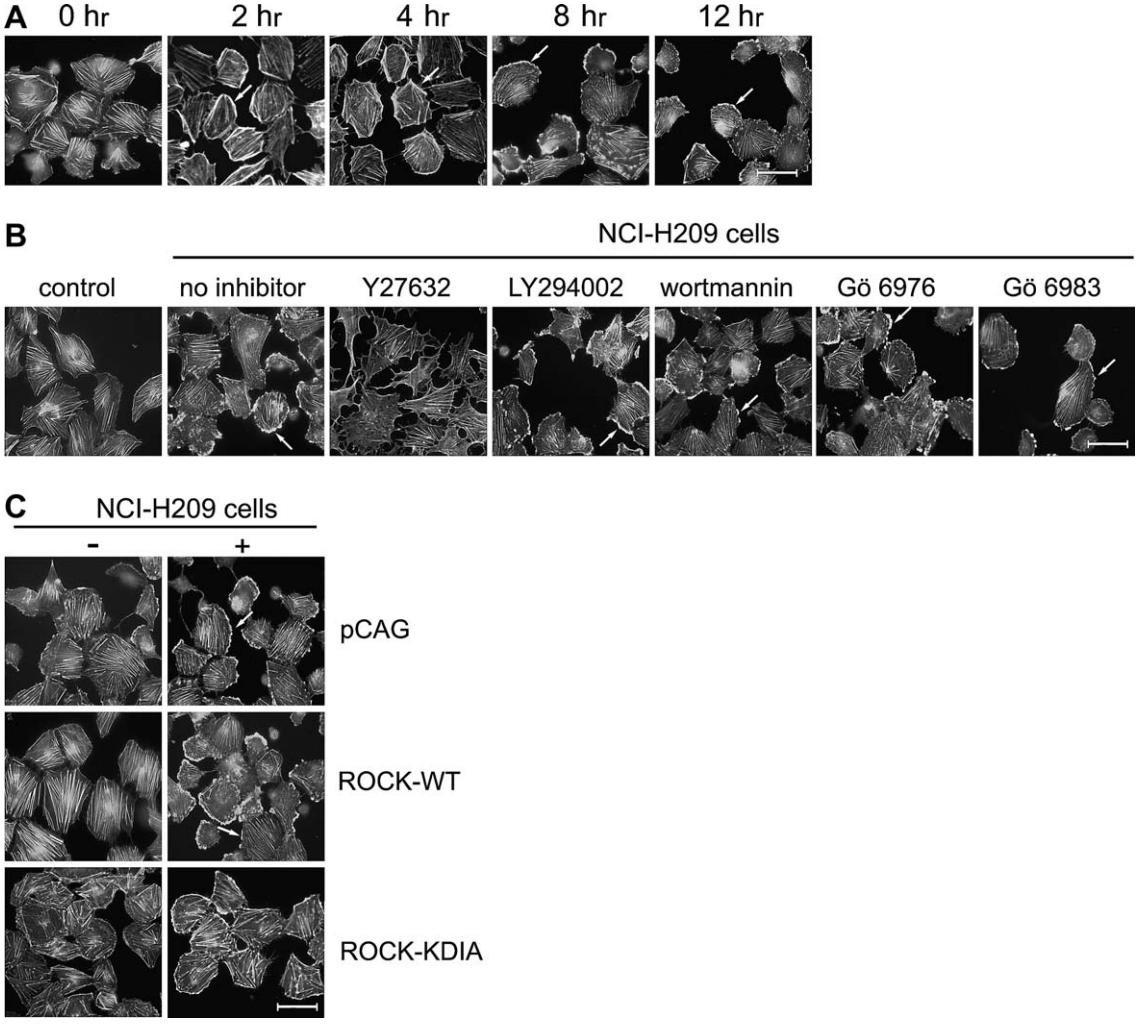

Fig. 5. The alteration of actin cytoskeleton in HBMECs during NCI-H209 cells transendothelial migration. Actin filaments in HBMECs were visualized by immunofluorescence. (A) HBMECs were cocultured with NCI-H209 cells for the indicated times. (B) HBMECs were pretreated with specific inhibitors and then co-cultured with NCI-H209 cells for 8 h. (C) The different HBMEC transfectants were cocultured with NCI-H209 cells for 8 h. Arrows showed the pericellular dense aggregates of actin filaments in HBMECs. Scale bar: 40 μm.

3.4. The regulation of actin cytoskeleton through Rho/ROCK signalling could facilitate NCI-H209 cells transendothelial migration

It had been well documented that Rho/ROCK-mediated actin cytoskeletal organization was an important regulatory mechanism for endothelial barrier function [13]. As shown in Fig. 5A, NCI-H209 cells induced pericellular actin bundles reorganization, stress fibers formation and even some denser actin filaments aggregation in HBMECs. Only ROCK inhibitor, Y27632, could abrogate this effect, whereas PI3K and PKC inhibitors had no effects (Fig. 5B). Furthermore, NCI-H209 cells-induced alteration of actin filaments was not observed in the cells overexpressing ROCK-KDIA (Fig. 5C). These data indicated that the changes of actin cytoskeleton in HBMECs cocultured with NCI-H209 cells were dependent on ROCK activation. Previous studies had shown that the phosphorylation of MLC and cofilin by ROCK appeared to be required for the actin cytoskeleton assembly [14,15]. As shown in Fig. 6A, the levels of MLC and cofilin phosphorylation in HBMECs were increasing after NCI-H209 cells treatment compared with HBMECs alone washed with D-PBS containing 0.1 mM EDTA. Further results showed that the NCI-H209 cells-induced elevation of phosphorylated MLC and cofilin were effectively blocked by Y27632 (Fig. 6B). These results suggested that a signalling cascade downstream of ROCK contributing to the regulation of actin cytoskeleton was involved in NCI-H209 cells transendothelial migration.

4. Discussion

Small cell lung cancer has the propensity to progress aggressively and nearly always to metastasize to extrathoracic organs [16]. The molecule mechanisms of SCLC metastases to bone and bone marrow had been better understood recently [17,18]. However, little was known about how SCLC cells metastasize to brain, especially the mechanism of SCLC cells migrating across brain microvessel endothelial cells which constitute the BBB. In this paper, we reported that SCLC cells transendothelial migration were dependent on Rho/ROCK signalling in HBMECs.

Evidence suggested that breast cancer cells migration across HBMECs require intracellular signals to be generated within the endothelial cells [10,19], which provided a clue to investigate the molecular mechanism of SCLC metastasis to the brain. Our results showed that the TJ disassembly was an important biological event during NCI-H209 cells transendothelial migration. Inhibition of endothelial ROCK with Y27632 and dominant negative ROCK mutant prevented the TJ disruption caused by NCI-H209 cells. Moreover, endothe-

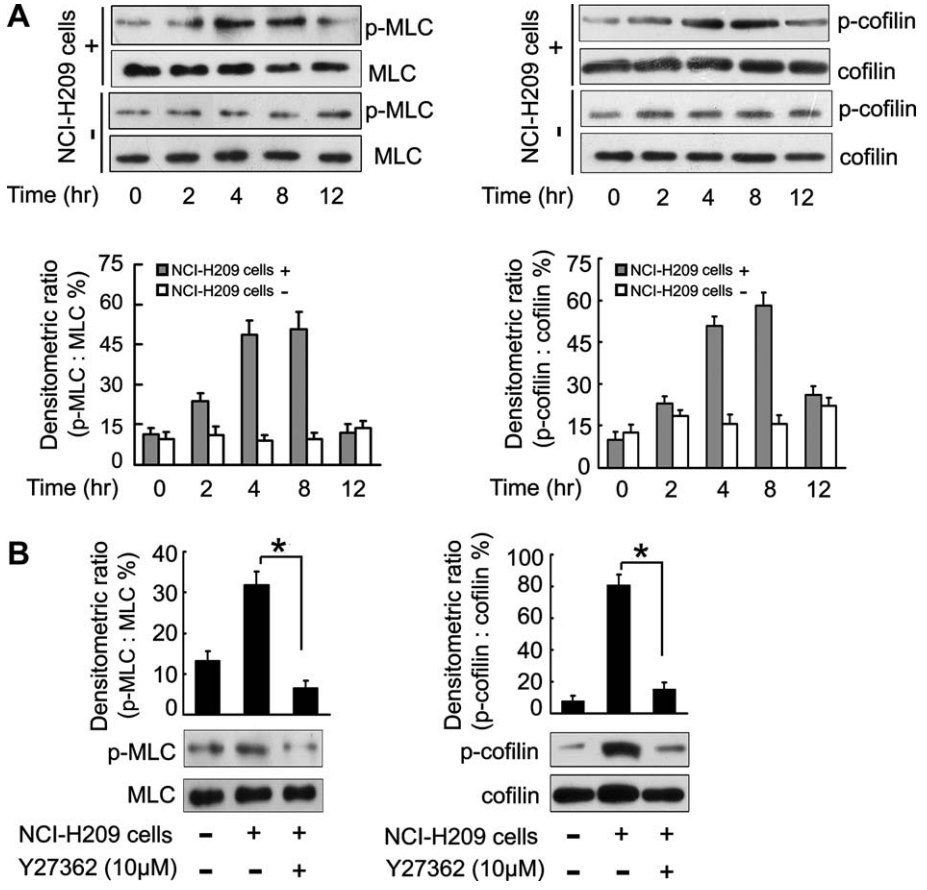

Fig. 6. NCI-H209 cells-induced changes of endothelial actin cytoskeleton were mediated by ROCK downstream molecules. (A) The levels of phospho-MLC (p-MLC) and phospho-cofilin (p-cofilin) in HBMECs cocultured with NCI-H209 cells for different time were detected by Western blot. The HBMECs alone washed with D-PBS containing 0.1 mM EDTA at the different time points were severed as the control. Top: Western blot analysis of MLC, phospho-MLC (p-MLC) and cofilin, phospho-cofilin (p-cofilin). Bottom: quantification of band density was done using Alpha Ease Fc software. (B) Confluent HBMECs were cocultured with NCI-H209 cells for 8 h, in the absence (−) or presence (+) of Y 27632 (10 μM), then subjected to Western blot analysis. Data were means ± S.D. of three independent experiments. $^{*}P < 0.05$.

lial RhoA activity was increased after 2 h incubation of HBMEC with NCI-H209 cells, peak at 4 h. These data strongly supported that endothelial Rho/ROCK signalling was involved in NCI-H209 cells transendothelial migration. As previously reported, Rho/ROCK activation could lead to actomyosin-based contractility and actin cytoskeletal reorganization through indirectly phophorylating cofilin and MLC, which could alter the integrity of TJ [14,15]. In our study, the alterations of endothelial actin filaments and the increases of endothelial MLC and cofilin phosphorylation in response to NCI-H209 cells were also dependent on ROCK activation. The appearance of peak activation of MLC and cofilin was at 4 h, consistent with RhoA. After that, the activation of MLC and cofilin lasted to 8 h, whereas RhoA activity had returned to the basal level. In our presented paper, the peak of NCI-H209 cell transendothelial migration occurred at 8 h and 12 h, which was later than the activation of Rho signalling pathway. This raised the possibility that endothelial trafficking signals for NCI-H209 cells transendothelial migration were transmitted from Rho/ROCK to TJ 'opening' through the rearrangement of actin cytoskeleton. The cross-talk between HBMECs and NCI-H209 cells might be a signal for RhoA activation. It was possible that endothelial Rho/ROCK signalling linked the subsequent events including MLC and cofilin phosphorylation, actin cytoskeletal remodeling, TJ disruption in HBMECs and NCI-H209 cells transendothelial migration. Increasing evidence showed that actomysion-based cell contractility by the MLC phosphorylation caused a subtle change in the physical characteristics of TJ membrane microdomains, such as a shift of detergent-insoluble protein pool [20]. Moreover, ZO-1 was both an actin-binding and cross-linking protein serving as a bridge that allowed regulation of TJ by the myosin-mediated actin contraction [21]. We also observed that the increasing of MLC phosphorylation was correlated with a significant shift of occludin from insoluble to soluble phase and an obvious redistribution of ZO-1 during NCI-H209 cells transendothelial migration. Therefore, it is possible that the myosin-mediated actin contraction through Rho/ROCK activation induced by NCI-H209 cells provide the mechanical forces to drive TJ disassembly, which facilitated NCI-H209 cells transendothelial migration.

It had been reported that PI3K and PKC signalling pathways also regulate the integrity of TJ in endothelial cells [6,7]. However, the activation of PI3K/Akt and PKC were not observed in our study. Therefore, it would be possible that Rho/ROCK signalling directly contributed to NCI-H209 cells transendothelial migration without cross-talking with PI3K or PKC pathways.

How did NCI-H209 cells trigger the transendothelial Rho/ROCK signalling to increase the endothelial permeability for migration across the brain microvascular barrier? Previous studies had clearly indicated that transendothelial Rho signalling was required for leukocytes transendothelial migration [22]. Autocrine and paracrine chemokines, such as monocyte chemoattractant protein-1 (MCP-1), had a role in 'opening' the BBB through a Rho/ROCK pathway and contributed to the transmigration of leukocytes across the BBB [23]. In our study, the interaction between SCLCs and HBMECs obviously resulted in the activation of endothelial Rho/ROCK signalling and the increase of brain endothelial cell permeability. Therefore, it is interesting to investigate whether the chemokines derived from SCLCs could activate endothelial Rho/ROCK signalling, which contribute to SCLCs transendothelial migration.

In summary, our results provided clear evidence that the activation of Rho/ROCK signalling in HBMECs was required for NCI-H209 cells transendothelial migration. Future studies would be necessary to identify the Rho/ROCK upstream molecules which contribute to SCLC cells migration through the brain endothelial TJ.

Acknowledgements: The authors are grateful to Drs. Monique Stins and Kwang Sik Kim for providing HBMECs, and Dr. S. Narumiya for providing expression vectors: pCAG-myc, pCAG-myc-ROCK-WT and pCAG-myc-ROCK-KDIA. This work was supported by the China State Education Ministry, the Trans-Century Training Program Foundation for Talents (JJH2002-48) (Y.H.C.)

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