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Hypoxia enhances CXCR4 expression favoring microglia migration via HIF-1α activation

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ABSTRACT

Migration toward pathological area is the first critical step in microglia engagement during the central nervous system (CNS) injury, although the molecular mechanisms underlying microglia mobilization have not been fully understood. Here, we report that hypoxia promotes stromal cell-derived factor-1α (SDF-1α) induced microglia migration by inducing the CXC chemokine receptor 4 (CXCR4) expression. Exposure to hypoxia significantly enhanced CXCR4 expression levels in N9 microglia cell. Then, cell migration induced by SDF-1, a CXCR4-specific ligand, was observed accelerated. Blockade of hypoxia inducible factor-1α (HIF-1α) activation by inhibitors of phosphoinositide-3-kinase (PI3K)/Akt signaling pathway abrogated both of hypoxia-induced CXCR4 up-regulation and cell-migration acceleration. These results point to a crucial role of Hypoxia-HIF-1α-CXCR4 pathway during microglia migration.

Hypoxia is one of the important physiological stimuli that are often associated with a variety of pathological states such as ischemia, respiratory disease, and tumorigenesis. In central nervous system (CNS), hypoxia not only causes neuronal cell injury, but also induces pathological microglial activation [1,2]. Microglia is believed to play a crucial role in the development and regeneration of the CNS. During CNS injury, microglia participate in inflammation and wound healing by migrating into damaged tissue, where they proliferate and act as scavengers [3,4]. Thus, the migration of microglia in response to pathological conditions including hypoxia is a critical step to carry out microglia functions, but the mechanisms are not fully understood.

The chemokines are a large family of small, structurally related cytokines which possess chemotactic activities. Chemokine receptors are classified according to which group of chemokines they bind and are designated as CXCR1-CXCR6, CCR1-CCR6, CX_3CR1, and XCR1 [5]. There is growing evidence for the role of chemokines in the regulation of CNS disease. Elevated levels of chemokines have been observed in several brain diseases, suggesting that these molecules function as regulators of brain inflammation. [6,7]. Only recently, the chemokine stromal cell-derived factor-1α (SDF-1α) and its receptor CXC chemokine receptor-4 (CXCR4) have been recognized to control the migration of microglia [8,9].

After exposed to hypoxia, many kinds of cells increase their synthesis of a protein named HIF, which in turn binds to and activates many genes [10]. Hypoxia inducible factor-1 (HIF-1) is a heterodimer composed of a HIF-1β subunit that is constitutively expressed and a HIF-1α subunit that is rapidly degraded by ubiquitination via the proteasomal pathway, a process that is inhibited under hypoxic conditions [11]. A role of HIF-1α in the regulation of CXCR4 mRNA expression is suggested by several previous findings [12,13]. CXCR4 promoter includes four potential hypoxia-response elements (HREs) located within 2.6 kb upstream of the transcriptional start site and one at position −1.3 kb within the intron [14]. This implies that CXCR4 is a hypoxia response gene. Therefore, hypoxia may affect migroglia migration process by altering the expression of CXCR4 via activation of HIF-1α.

To test this hypothesis, we employed N9 microglia cell line to evaluate the effect of hypoxia on microglia. Here, we report that hypoxia increased mRNA and protein expression levels of CXCR4 in N9 cells and enhanced microglia migration. In addition, we also demonstrated that the induction of CXCR4 expression by hypoxia was possibly through the activation of HIF-1α. We believe this knowledge may lead to a potentially important therapy for regulating microglia function targeting CXCR4.

Materials and methods

Cell culture and hypoxia. The N9 murine microglia cell line (kindly donated by Dr. Yun BAI, Department of genetics, the Third Military Medical University, China) was maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT)

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supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 0.05 mM 2-mercaptoethanol, and 2 mM L-glutamine. For the generation of hypoxic condition, cells were washed three times with deoxygenated DMEM, and then immediately placed into an anaerobic chamber (1.0% O_2, 5.0% CO_2, nitrogen-balanced) at 37 °C for varying durations [15].

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using 1 ml per 25-cm^2 flask of Tripure (Roche, Penzberg, Germany) according to the manufacturer's protocol. The integrity of the RNA sample was confirmed by gel electrophoresis and by reverse transcription-PCR using primers for housekeeping genes. 1ug total RNA was converted to cDNA, using M-MLV Reverse Transcriptase (Toyobo, Tokyo, Japan) at 42 °C. The RT-PCR exponential phase was determined on 20–32 cycles to allow semiquantitative comparisons and β-actin was used for equal loading. The amplification of cDNA was performed with the following primers: mouse CXCR4 (GenBank Accession No. NM00991.3), sense GATGACCCCCAAAAGGATGAAG and antisense GTCATGGGTTACCAGAAGAAGC (642 bp product); mouse β-actin (GenBank Accession No. NM007393.2), sense TAAAGACCTCTATGCCAACACAGT and antisense CACGATGGAGGGGCCGGACTCATC (241 bp product). The amplification reaction was carried out in a Perkin-Elmer GeneAmp. Each cycle consisted of denaturation for 30 s at 94 °C, annealing for 30 s at 56 °C (CXCR4) or 60 °C (β-actin), and an extension for 60 s at 72 °C. A final extension step at 72 °C for 5 min terminated the amplification. The resulting PCR products were separated in 1.5% agarose gels and visualized by ethidium bromide staining. For semiquantitative evaluation, densitometric analysis was performed.

Western blotting. Cells were washed with ice-cold PBS and scraped in RIPA lysis buffer including protease inhibitors. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore. Billerica, MA). Non-specific binding was blocked by incubation overnight at 4 °C with 5% non-fat dry milk in PBS. Membranes were then incubated in primary antibodies for 3 h at 25 °C: rabbit polyclonal anti-CXCR4 antibody (1:250, Santa-Cruz Biotechnology, Santa Cruz, CA), rabbit monoclonal anti-phospho-Akt (Ser473) antibody (1:1000,Cell signaling, Danvers, MA), goat polyclonal anti-Akt1/2 antibody (1:1000, Santa-Cruz Biotechnology, Santa Cruz, CA). Membranes were washed three times with the TBST and then incubated with the Fluor-conjugated secondary antibodies (1:5000, LI-COR, Lincoln, NE). After final washes with PBS, The signal was then detected and quantified with Odyssey infrared imaging system (LI-COR, Lincoln, NE). Loading controls were performed with mouse monoclonal anti-β-actin antibody (1:10000, Sigma, St. Louis, MO).

Transmigration assay. The transwell system was purchased from Millipore Inc (Billerica, MA). A total of 300 μl of 3×10^5 cells/ml was placed in the upper chamber and 700 μl of serum-free medium containing the SDF-1α (Millipore, Billerica, MA) at final concentrations of 200 ng/ml was placed in the lower chamber. Serum-free medium alone were used as negative controls. The migration chamber was incubated for 16 h at 37 °C in anaerobic chamber. Then, non-migrating cells were removed from the top, and migrating cells were stained with eosin and counted. Ten fields under a 50-fold magnification were randomly selected and counted. All experiments were performed at least in triplicates.

Electrophoretic mobility shift assay (EMSA). EMSA was performed on nuclear extracts from N9 cells. The extract preparation and binding reaction were performed as previously described [16]. The biotin-labeled double-stranded oligonucleotides were used included commercially available consensus HIF-1 gel shift oligonucleotide 5′-biotin-TCTGTACGTGACCACACTCACCTC-3′ (Beyotime, Shuzhou, China). Specific binding was confirmed by competition experiments with a 100-fold excess of unlabeled, identical oligonucleotides. After binding, the samples are separated on a non-denaturating PAGE gel and bands are detected by enhanced chmiluminescent (ECL) assay Kit (Priece, Rockford, IL).

Statistics. All results are expressed as mean ± SEM. Statistical analysis was performed by ANOVA, followed by the Tukey post-hoc test. Student's paired *t* test was also used to compare the difference in values between 2 groups. A P value of <0.05 was considered statistically significant.

Results

Hypoxia upregulated CXCR4 expression levels in N9 cells

To investigate the effect of hypoxia on CXCR4 expression in vitro, cultured N9 microglia cells were exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 4 or 16 h, respectively. Then, CXCR4 mRNA levels were analyzed by RT-PCR. The results showed that hypoxia significantly upregulated CXCR4 mRNA expression (Fig. 1A). CXCR4 mRNA was increased as early as 4 h and remained elevated over 16 h treatment interval. Moreover, to determine whether the increase of mRNA for CXCR4 was translated into an upregulation of the protein, western blots were performed. N9 cells expressed higher levels of CXCR4 protein under hypoxic conditions (Fig. 1B). The increase of protein levels was consistent with that of mRNA levels. These data provide evidence that CXCR4 mRNA and protein expression levels in N9 cells are upregulated by hypoxia.

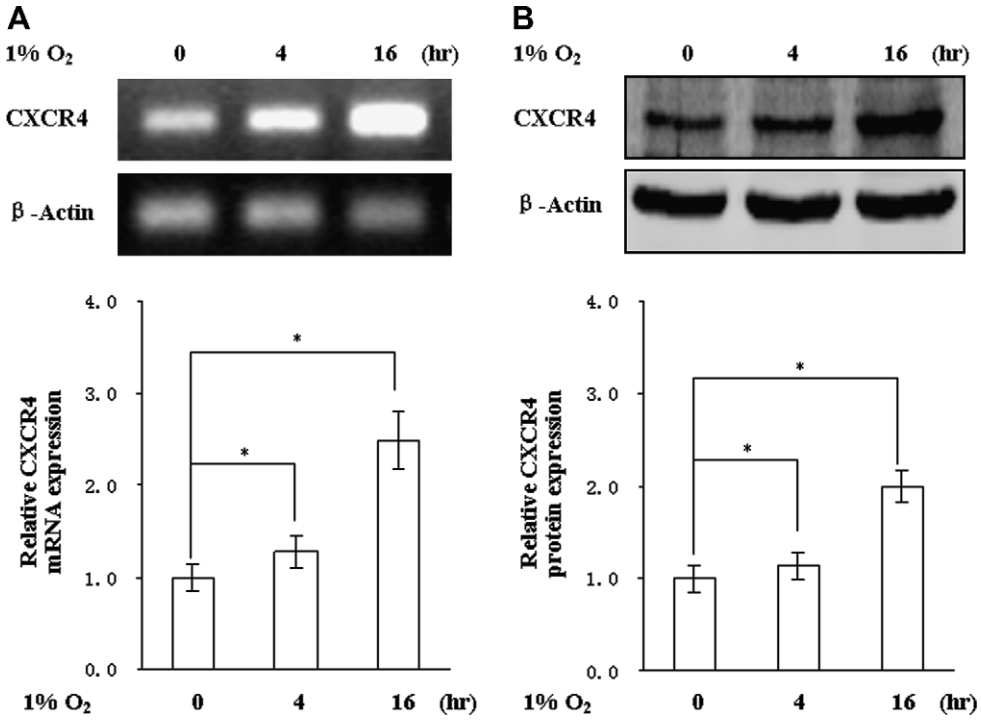

Fig. 1. Effects of hypoxia on CXCR4 expression in N9 cell line. Cultured N9 cells were grown under normoxia or hypoxia for 4 h and 16 h, respectively. (A) CXCR4 mRNA expression were upregulated under hypoxia determined by RT-PCR. (B) CXCR4 protein levels were increased under hypoxia determined by Western blot. β-Actin was used as an internal control. Representative RT-PCR or Western blot results are shown from three independent experiments. Bar graphs represent data pooled from three independent experiments for statistical analysis comparing levels of CXCR4 expression in different groups. * $P < 0.01$.

PI3K signaling pathway blockade inhibits HIF-1α activation and also abrogates hypoxia-induced CXCR4 expression

To investigate whether HIF-1α was involved in the increase of CXCR4 expression induced by hypoxia, we firstly determined the activation status of HIF-1α by EMSA. As shown in Fig. 2B, HIF-1α-DNA binding was significantly increased when N9 cells were exposed to hypoxia for 4 or 16 h, respectively. Previous studies have implicated a role for phosphoinositide-3-kinase (PI3K)/Akt transduction pathway in hypoxia-induced HIF-1α activation [17,18]. To elucidate the signaling pathways involved in the regulation of CXCR4 expression, we pretreated N9 cells with PI3K/Akt inhibitors, Wortmannin (200 nM) or LY294002 (50 μM), for 1 h and then subjected cells to hypoxia conditions for 4 h or 16 h. Exposure of N9 cells to hypoxia led to an increase in the phosphorylation of Akt (Fig. 2A). Subsequently, we observed that in the presence of the PI3K/Akt inhibitors, HIF-1α activation was strongly inhibited (Fig. 2B). Next, we wanted to know whether these PI3K/Akt inhibitors that blocked HIF-1α activation would also block the upregulation of CXCR4 expression. As expected, the PI3K/Akt inhibitors abrogated the upregulation of CXCR4 mRNA and protein expression induced by hypoxia for 16 h, although basel expression of CXCR4

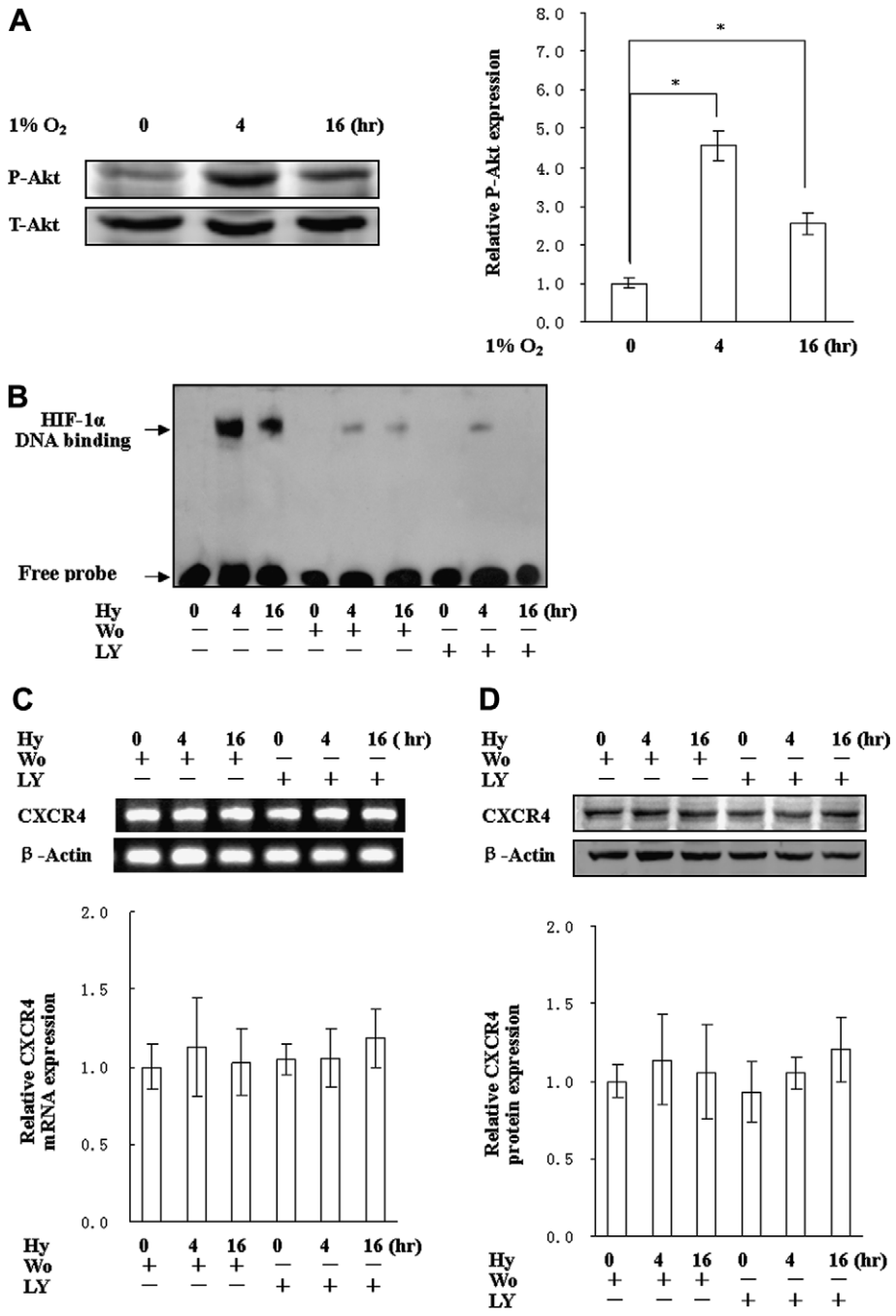

Fig. 2. Inhibition of HIF-1α activation blocked hypoxia-induced increase of CXCR4 expression. N9 cells were pretreated with either vehicle (0.001 DMSO) or inhibitors (Wortmannin, 200 nM or LY29002, 50 μM) for 1 h prior to exposure to hypoxia for 4 h or 16 h. (A) Hypoxia-induced phosphorylation of Akt in N9 cells. (B) EMSA was carried out to determine the effects of PI3K/Akt inhibitors on HIF-1 activation. HIF-1α activation was partly inhibited by Wortmannin or LY294002. (C,D) Increased CXCR4 mRNA and protein expression levels induced by hypoxia were blocked by Wortmannin or LY294002 pretreatment. Representative data from three independent experiments was shown. Hy, hypoxia; Wo, Wortmannin; LY, LY294002; * $P < 0.01$.

remained (Fig. 2C and D). These data further verified that HIF-1α was involved in the upregulation of CXCR4 protein expression.

Cobalt chloride also up-regulated both CXCR4 transcript level and protein expression through HIF-1α activation

To further prove the involvement of HIF-1α in CXCR4 induction, we utilized cobalt chloride ($CoCl_2$), a well-known chemical inducer of HIF-1α [19], to study the effect of HIF-1α activation on CXCR4 expression in N9 cells. Cells treated with $CoCl_2$ (150 nM) for 16 h induced a significant HIF-1α-DNA binding (Fig. 3A). Furthermore, $CoCl_2$ induced about a significantly increased CXCR4 mRNA and protein expression (Fig. 3B and C). These data confirmed that HIF-1α activation induced by $CoCl_2$ also enhanced CXCR4 expression level in microglia (See Fig. 4).

Exposure to hypoxia enhanced SDF-1α-dependent cell migration in vitro

The migratory response of N9 cells exposed to hypoxia was assessed using SDF-1α at a concentration of 200 ng/ml which had previously been determined as optimum. Serum-free medium alone was used as a negative control. As expected, N9 cells exposed to hypoxia for 16 h acquired increased responsiveness to SDF-1α present in the bottom chamber, and displayed significant migratory properties. Moreover, similar result was observed after $CoCl_2$ (150 nM) was added into culture medium instead of hypoxia for 16 h. However, these effects were blocked obviously by pretreatment of anti-CXCR4 antibody or PI3K/Akt inhibitors. These results demonstrate that hypoxia exposure might induce microglia migration through upregulation of CXCR4 receptor expression.

Discussion

Regulation of cell migration by changes in oxygen availability is a central event during the organization of host response in inflammatory and neoplastic diseases occurred after CNS injury. Here, we report that hypoxia promotes microglia migration through enhancing CXCR4 expression, which was resulted from HIF-1α activation.

Increasing evidences show that expression of CXCR4 on cell surface could be regulated by different microenvironmental factors, ultimately leading to cell migration within tissues during development, angiogenesis, and tissue repair [9,20]. Hypoxia is a primary factor in the pathology of many disease states, specially, in that of CNS [21]. The regulation of CXCR4 expression by hypoxia was suggested by several previous findings [12,13,22]. However, few reports had been studied on microglia. Our finding that CXCR4

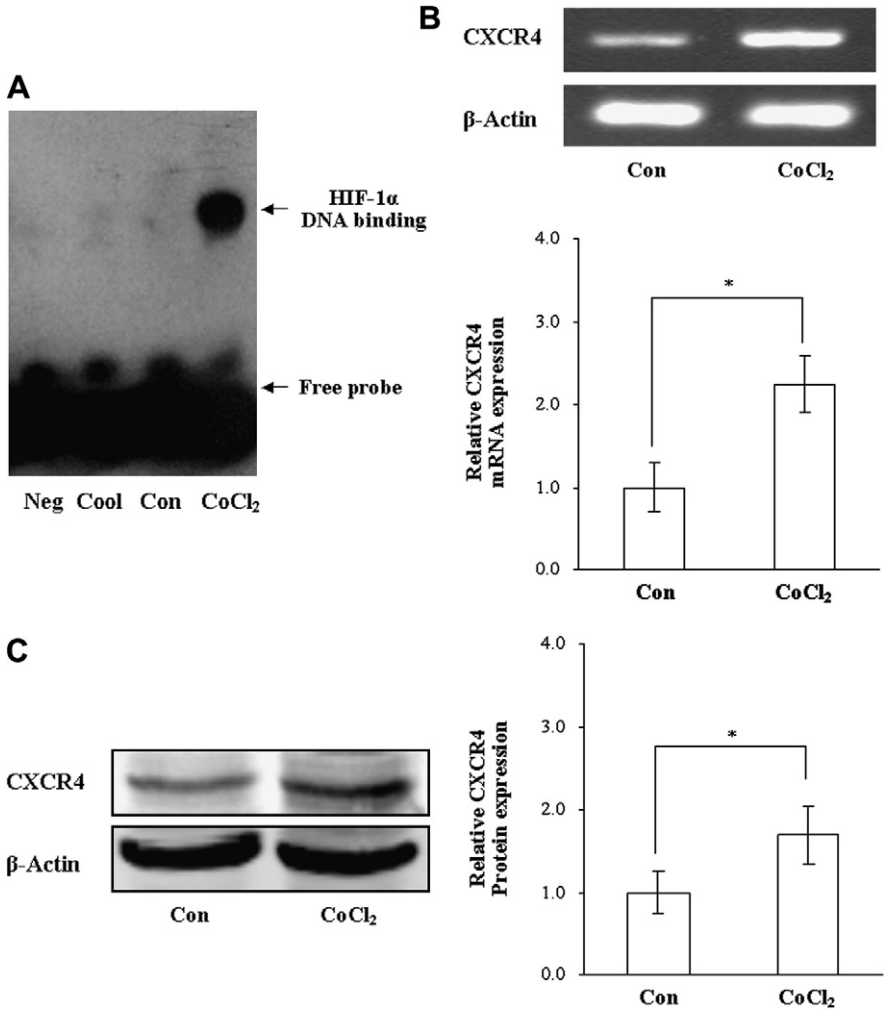

Fig. 3. Elevated activation of HIF-1α by $CoCl_2$ stimulation also enhances CXCR4 expression. Cultured N9 cells were treated with 150 nM $CoCl_2$ for 16 h. (A) $CoCl_2$-induced HIF-1α-DNA binding determined by EMSA. (B,C) $CoCl_2$ enhanced of CXCR4 mRNA and protein expression in N9 cells. Representative data from three independent experiments was shown. Neg, negative control; Cool, cool competition; Con, control; * $P < 0.01$.

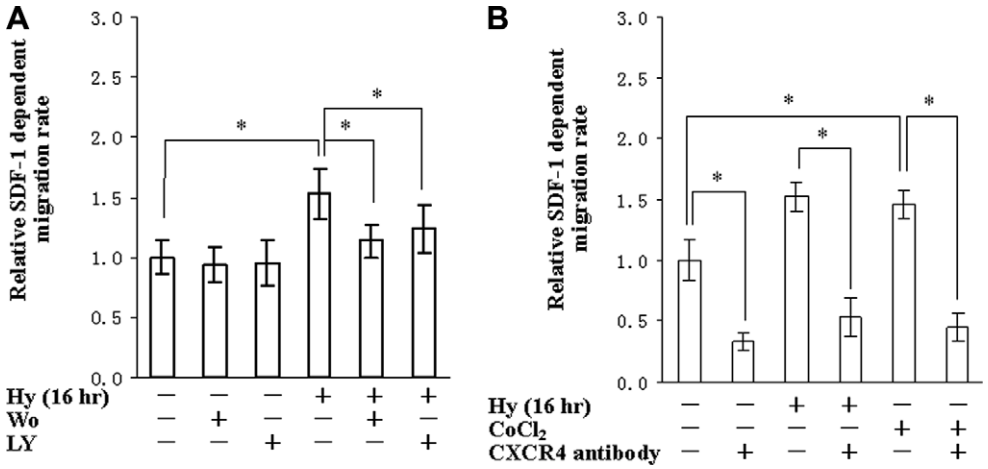

Fig. 4. Effect of hypoxia on N9 cells migration. N9 cells treated by normoxia or hypoxia for 16 h were used for migration assay. SDF-1α (200 ng/ml) was added into the bottom chamber. We estimated migration by counting the number of migrating cells on the lower side of the membrane after eosin staining. Hypoxia and $CoCl_2$ (150 nM) treatment for 16 h enhanced the migration of N9 cells to SDF-1α, respectively. However, hypoxia-induced cell migration was abolished after pretreated by Wortmannin (200 nM) and LY294002 (50 μM) for 1 h prior to hypoxia. Anti-CXCR4 antibody (1:1000) treated for 1 h prior to hypoxia partly blocked the migration of N9 cells. The number of migrated cells in ten fields is counted. The experiment has been repeated three times. Hy, hypoxia; Wo, Wortmannin; LY, LY294002; * $P < 0.01$.

was expressed in N9 microglia cell line is in accordance with previous studies that microglia express various chemokine receptors including CXCR4 [17,23]. The presence of chemokine receptors in microglia suggests a role for chemokine signaling mechanisms in microglia function, particularly in pathological states. In present study, we observed that CXCR4 expressions on N9 microglia cells were significantly enhanced by hypoxia. It is known that SDF-1, the ligand of CXCR4, is induced in brain injuries, including hypoxia [24,25]. In our studies, the SDF-1-induced migration of N9 cells was enhanced by hypoxia but blocked by competitive inhibition by anti-CXCR4 antibody. These data, combined with the result of hypoxia-induced CXCR4 expression in microglia presented in this study, suggest that chemokine peptide and receptor signaling plays an important role in migration of microglia in the neuropathological model of hypoxia.

Several previous findings suggest that HIF-1α is involved in the hypoxia-induced CXCR4 expression in various cells, including hematopoietic cells, immunocytes and tumor cells [15,16,19]. In this study, PI3K/Akt inhibitors and $CoCl_2$ were employed to confirm the involvement of HIF-1α in hypoxia enhanced CXCR4 expression in N9 cells. PI3K/Akt signaling pathway is thought to be involved in the activation of HIF-1α. Both of PI3K and mitogen-activated protein kinase pathways converge on the p70 S6 kinase, which initiates a cascade of events that ultimately leads to an increase in the rate at which HIF-1α mRNA is translated into protein and activation of HIF-1α [26]. $CoCl_2$ is a well-known chemical inducer for HIF-1α. The induction of HIF-1α expression by $CoCl_2$ was probably via inhibiting the prolyl hydroxylase domain containing enzymes (e.g., HIF-1α prolyl-4-hydroxylase, HIF-PH), a family of enzymes that plays a key role in oxygen-dependent degradation of the transcription factors leading to the stabilization of HIF-1α and activation of cellular hypoxia-dependent pathways under the normal oxygen level [27]. In order to investigate whether HIF-1α affects CXCR4 expression in microglia, we examined the changes of CXCR4 expression after down-regulated HIF-1α activation by PI3K/Akt inhibitors or up-regulated HIF-1α activation by $CoCl_2$. We proved that after HIF-1α activation was blocked by PI3K/Akt inhibitors, the increases of the CXCR4 expression induced by hypoxia were abolished. Moreover, $CoCl_2$-induced HIF-1α activation enhanced the expression of CXCR4 in both mRNA and protein levels. In addition, the SDF-1-induced migration of N9 cells was enhanced by hypoxia. PI3K/Akt inhibitors blocked N9 migration to SDF-1 while $CoCl_2$ enhanced this effect. These results prove that HIF-1α plays an important role in induction of CXCR4 expression and hypoxia enhances the migration of microglia through the HIF-1α involved SDF-1-CXCR4 interaction.

Together with the finding that HIF-1α regulates CXCR4, our data suggest that tissue hypoxia may be a fundamental mechanism governing microglia recruitment in brain injuries. As such, hypoxic microenvironments in brain (such as ischemic tissue) may activate HIF-1α and upregulate CXCR4 expression that facilitate microglia recruitment and function exertion in ischemic tissue. Thus, manipulation of HIF-1α activity may be a useful means with which to regulate the microglial functions. A reduction in HIF-1α activity, as occurs in aging, may also alter microglia trafficking and underlie the observed decline in microglial function, which may contribute to neurodegenerative diseases, e.g. Alzheimer's disease (AD), for microglia can clear plaques in AD following Aβ peptide immunization [28].

It has been proposed that the positioning of mobile cells within a tissue is determined by their overall profile of chemokine receptors. We only studied one of the important receptors, CXCR4, in cultured N9 microglia cell line. Studies in vivo are in progress to clarify the effects of hypoxia on microglia migration. In addition, since the changes of CXCR4 expression in microglia induced by hypoxia may affect not only migration, but also proliferation, activation, or other functions of microglia, the interactions of chemokines with receptors in hypoxia condition in microglia are likely to be complex and further studies should be done in this field.

In summary, our results demonstrate that hypoxia induces microglial CXCR4 expression via HIF-1α activation and thus accelerates the migration of microglia. The Hypoxia-HIF-1α-CXCR4 pathway may regulate trafficking and localization of microglia and represents a target for novel therapeutic strategies for hypoxia injury of CNS.

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