Store-operated Ca^{2+} entry mediated regulation of polarization in differentiated human neutrophil-like HL-60 cells under hypoxia

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Abstract. The regulation of neutrophil polarization by calcium entry is critical for maintaining an effective host response. Hypoxia has a major effect on the apoptosis of neutrophils, however the role of store-operated Ca^{2+} entry (SOCE) in neutrophil polarization under hypoxia remains to be elucidated. In the present study, we examined the polarization of differentiated human neutrophil-like HL-60 (dHL-60) cells exposed to hypoxia (3% O_2) and the results demonstrated that the percentage of polarized cells following exposure to an N-formyl-Met-Leu-Phe (fMLP) gradient in the Zigmond chamber was increased. We examined stromal interaction molecule 1 (STIM1) and Orai1 expression in dHL-60 cells during hypoxia, and it was observed that the expression of STIM1 and Orai1 was significantly reduced at day 2. However, no apparent change was observed on the first day, indicating that this effect is dependent on stimulation time. Fluo-4/acetoxymethyl (AM) ester imaging also demonstrated that SOCE was decreased in dHL-60 cells. The plasmid overexpression assay demonstrated that the response of polarization was returned to the control level. We demonstrated the inhibitory role of SOCE on the polarization of dHL-60 cells under hypoxic conditions, which may be the mechanism for the adaptation of neutrophils to hypoxia. SOCE is also suggested to be a key modulator of immune deficiency under hypoxic conditions and is potentially a therapeutic target.

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Introduction

Hypoxia is known to have a detrimental effect on human and animal health. A low O_2 concentration in the human body can cause a variety of physiological changes, including an increased heart rate, pulmonary ventilation and cerebral blood flow. Different cell subpopulations exhibit different responses to hypoxia, for instance, hemoglobin content increases with altitude. However, the functional and physiological changes of other blood components, inlcuding leukocytes, are not completely understood. Neutrophils are suggested to be important in the host defense and acute inflammatory response. Hypoxia is a well documented inflammatory stimulus that contributes to tissue polymorphonuclear leukocyte (PMN) accumulation (1). It also represents an important regulator of inflammatory responses since it can cause inhibition of neutrophil apoptosis mediated by hypoxia-inducible factor (HIF)-1α-dependent NF-κB activity (2,3). Similarly, neutrophil polarization is an initiation signal for cell migration and a constituent of the innate immune response to bacterial infection (4,5). Despite these findings and other parallel clinical and animal studies demonstrating bacterial killing, phagocytic activity and wound healing under hypoxia (6-8), few studies have examined the effect of hypoxia on neutrophil function *in vitro*. It was suggested that extreme hypoxia (<30 mmHg) caused a small (15-25%) but significant reduction in chemotactic migration (9) and that hypoxia impaired the capacity of human peripheral blood neutrophils to generate reactive oxygen species (ROS) and kill *Staphylococcus aureus*. However, the hypoxic challenge did not compromise their motility, migration, receptor regulation or degranulation responses (10).

Ca^{2+} signals have been implicated in numerous cellular functions, including cell movements and are a critical regulator of cell migration and chemotaxis (11-13). Ca^{2+} influx through the plasma membrane is regulated in one of at least two ways; i) depletion of the intracellular Ca^{2+} stores, termed

store-operated Ca^{2+} entry (SOCE) (14,15) and ii) receptor occupation, termed receptor-operated Ca^{2+} entry (ROCE) (16). Previous studies have identified two molecules, stromal interaction molecule 1 (STIM1) and Orai1 (also named CRACM1), that are responsible for SOCE (17-19). STIM1 acts as a Ca^{2+} sensor and Orai1 is an essential pore-forming component of the SOCE channel (20,21). Co-expression of Orai1 and STIM1 is sufficient to reconstitute the store-operated Ca^{2+} channel function (22-24). Store-operated Ca^{2+} influx controls a variety of physiological and pathological processes (25-27), including the migration and polarization of various cell types, including neutrophils. In nonexcitable cells, store-operated Ca^{2+} influx is the predominant Ca^{2+} entry mechanism (15,28). Previous studies have indicated that SOCE is involved in cell polarization, migration and metastasis by regulating a variety of cytosolic Ca^{2+} signals (15,29,30), and it may also be important in the N-formyl-Met-Leu-Phe (fMLP)-induced cell polarization of the neutrophil-like HL-60 cells (31). However, the role of SOCE in neutrophil polarization under hypoxia is unclear and thus needs to be elucidated. This question is addressed in the present study using differentiated HL-60 (dHL-60) cells that have been demonstrated to be a valid model system for the analysis of human neutrophil polarization (32) and easy for genetic manipulation.

Given the important role of SOCE in cell polarization, we hypothesized that the effect of hypoxia on cell polarization was mediated by SOCE. In the present study, STIM1 and Orai1, essential components in SOCE, were used to study the effect of hypoxia, and we also used plasmids to overexpress STIM1 and Orai1 to further confirm the role of SOCE in the polarization of dHL-60 cells under hypoxia.

Materials and methods

Materials. fMLP, dimethyl sulfoxide (DMSO), thapsigargin (TG) and $CaCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA); Fluo-4 acetoxymethyl (AM) ester was obtained from Invitrogen Life Technologies (Grand Island, NY, USA); monoclonal rabbit anti-STIM1, -Orai1 and -HIF-1α antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA) and cell lysis buffer for western blotting was obtained from KeyGen (Nanjing, Jiangsu, China), respectively.

Cell culture. HL-60 cells, a promyelocytic leukemia cell line provided by the China Center for Type Culture Collection (CCTCC; Shanghai, China), were maintained in RPMI-1640 medium (Gibco-BRL, Karlsruhe, Germany) supplemented with 10% fetal calf serum and 2 mM of L-glutamine (Gibco-BRL) at 37˚C in a humidified atmosphere of 20% O_2 and 5% CO_2. The day prior to the differentiation of HL-60 cells was designated as day 0. HL-60 cells with a cell density of 10^6 cells/ml were induced to differentiate into a neutrophil-like phenotype (dHL-60 cells) with 1.3% DMSO for 4-6 days (31), which was used for subsequent experiments. Non-viable cells were removed by centrifugation at 180 x g for 5 min at room temperature and the cells were washed three times with 5 ml of phosphate-buffered saline (PBS; 0.2 M of Na_2HPO_4, 0.2 M of NaH_2PO_4, pH 7.2±0.1). Then, the dHL-60 cells incubated with DMSO for 4 days were transferred and cultured in a hypoxic incubator (Forma Series-II; Thermo Fisher Scientific, Rockford, IL, USA), which was flushed with a gas mixture consisting of 3% O_2, 5% CO_2 and 92% nitrogen at 37˚C for 1-2 days.

Cell electroporation. dHL-60 cells differentiated with DMSO for 4 days were collected and washed twice with RPMI-1640 medium. Cells were resuspended in ice-cold OPTI-MEMI. Plasmid DNA (2-5 μg/ml; STIM1-mOrange, Orai1-mKO and pcDNA3.1) was added to a 400 μl aliquot of dHL-60 cells at a cell density of $8x10^6$ cells/ml (33,34). The mixture was then transferred to an electroporation cuvette with a 4 mm electrode gap (Bio-Rad, Hercules, CA, USA). Following 10 min incubation on ice, the mixture was electroporated (295 V, 1180 μF, 500 Ω) in a Gene Pulser Xcell Electroporation System (Bio-Rad) (31). Following electroporation, cells were allowed to recover for 30 min on ice and then incubated in RPMI-1640 medium with the presence of 10% fetal calf serum for 2 days. The transfection efficiency of STIM1 was ~80%. The cells were processed for subsequent assays ~48 h following transfection.

Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). dHL-60 cells grown in normoxia for 4-6 days or hypoxia for 1-2 days were suspended at 10^6 cells/ml in Hanks' balanced salt solution (HBSS; pH 7.4) and labeled with 2 μM of Fluo-4 AM at 37˚C for 30 min in the dark. Cells were then washed three times with HBSS on ice and then resuspended in Ca^{2+}-free buffer solution containing 0.3 mM of EGTA. The green fluorescence of Fluo-4 was excited by a 10 mW multitune argon laser at 488 nm and recorded through a 525 nm channel under an inverted laser scanning confocal microscope (FV1000-IX71; Olympus, Tokyo, Japan). For imaging with Fluo-4, $(Ca^{2+})_i$ changes were defined as the ratio of F to F_0 (F/F_0) following background subtraction, where F was the change in fluorescence signal intensity and F_0 was the baseline calculated by averaging three independent experiments prior to the application of the stimulus.

Zigmond assay. HL-60 cells were induced to differentiate into dHL-60 cells with DMSO for 4-6 days and then cultured in a hypoxic environment for another 1-2 days. Cells were allowed to attach to the cover slip (22x40 mm) at room temperature for 5 min before the cover slip was inverted over the chamber (Neuro Probe, Gaithersburg, MD, USA), as previously described (35). One channel of the chamber was filled with HBSS (vehicle) and the other with 100 nM of fMLP. Digital images of the cells were captured every 10 or 15 sec, depending on the experiment, for a total of 30 min using an inverted microscope with a x20 objective (Olympus; IX-71). An average of 100 cells were examined for each experiment and analysis was performed for at least three independent experiments. The percentage of cells that were polarized, i.e. with a directionally oriented leading edge and trailing tail, was calculated as described previously (36).

Western blotting. dHL-60 cells were washed with PBS at 37˚C and the pellets obtained from centrifugation at 100 x g for 1 min were suspended in lysis buffer at a cell density of $1.0x10^7$ cells/ml and incubated on ice for 30 min. Following centrifugation at 15,000 x g for 15 min at 4˚C, the supernatants

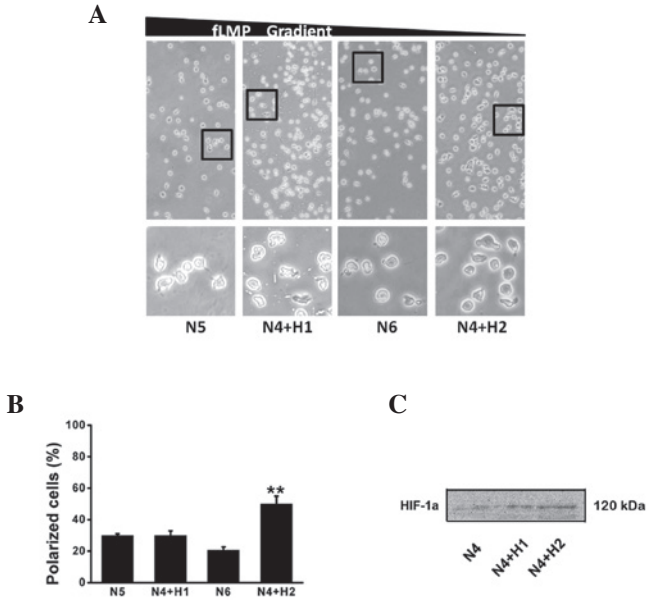

Figure 1. Effect of hypoxia on fMLP-induced cell polarization toward an fMLP gradient. HL-60 cells were induced to differentiate into dHL-60 cells with 1.3% DMSO for 4 days under normoxic conditions and then exposed to hypoxia (3% O_2) for another 1-2 days (N4+H1 and N4+H2), or with 1.3% DMSO all the time for 5 or 6 days under normoxic conditions (N5 and N6), respectively. (A) dHL-60 cells were induced to polarize towards an fMLP gradient in the Zigmond chamber for 15 min at 37˚C. Time-lapse microscopy was used to record cell morphology on the bridge at a 30 sec interval and the direction of the fMLP gradient (0-100 nmol/l) was indicated by the wedge. (B) The proportion of dHL-60 cells polarized along the gradient direction was obtained from three independent experiments. Bars represent the mean ± SD. **P<0.01, as compared with N6. (C) HIF-1α levels exposed to hypoxia for 1-2 days. fMLP, N-formyl-Met-Leu-Phe; dHL-60, differentiated human neutrophil-like HL-60 cells; DMSO, dimethyl sulfoxide; HIF-1α, hypoxia-inducible factor-1α; SD, standard deviation; N5, 5 day incubation under normoxic conditions; N4+H1, 4 day incubation under normoxic conditions and 1 day under hypoxic conditions; N6, 6 day incubation under normoxic conditions; N4+H2, 4 day incubation under normoxic conditions and 2 day under hypoxic conditions.

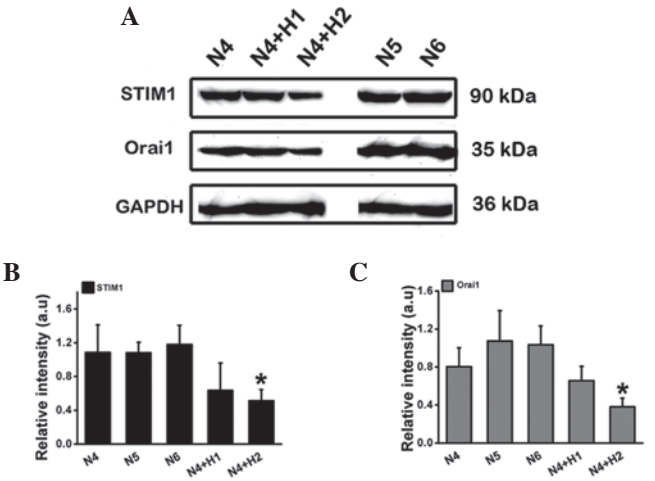

Figure 2. Effect of hypoxia on STIM1 and Orai1 expression level. Cell lysates were analyzed by SDS-PAGE and then western blot analysis using antibodies against STIM1 and Orai1. (A) STIM1 and Orai1 expression of dHL-60 cells stimulated by hypoxia for 1-2 days. (B and C) The relative intensity of the expression of STIM1 and Orai1. Data are expressed as the mean ± SD from three independent experiments. *P<0.05, as compared with N6. STIM1, stromal interaction molecule 1; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SD, standard deviation; dHL-60, differentiated human neutrophil-like HL-60 cells; N4, 4 day incubation under normoxic conditions; N5, 5 day incubation under normoxic conditions; N4+H1, 4 day incubation under normoxic conditions and 1 day under hypoxic conditions; N6, 6 day incubation under normoxic conditions; N4+H2, 4 day incubation under normoxic conditions and 2 day under hypoxic conditions.

were collected and the protein preparations were subjected to a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride membrane for western blot analysis using antibodies against STIM1, Orai1 and HIF-1α.

Statistical analysis. Data are presented as the mean ± standard deviation from three independent experiments and the Student's t-test was used for the comparison between two means and one-way analysis of variance (ANOVA) for the comparison of more than two means using SPSS software, version 13.0. P<0.05 was considered to indicate a statistically significant difference.

Results

Polarization in dHL-60 cells under normoxia and hypoxia. HL-60 cells differentiated for 4-6 days with 1.3% DMSO can be used for polarization assays. In the present study, HL-60 cells differentiated for 4 days and then exposed to hypoxia (3% O_2) for another 1-2 days were selected as the polarization capacity was improved and the DMSO induced differentiation of HL-60 cells to the neutrophil-like phenotype (dHL-60 cells) was largely completed at day 4 (37). We compared the polarization between N5 (5 day incubation under normoxic conditions) and N4+H1 (4 day incubation under normoxic conditions and 1 day under hypoxic conditions) and between N6 (6 day incubation under normoxic conditions) and N4+H2 (4 day incubation under normoxic conditions and 2 day under hypoxic conditions). No significant differences between the N5 and the N4+H1 groups were identified. However, a higher polarization was observed in the N4+H2 group than that in the N6 group, suggesting that hypoxia can induce cell polarization and the effect is associated with the stimulation time. It was also observed that the percentage of cells polarized in the direction of fMLP declined from N5 to N6, however increased from N4+H1 to N4+H2. Western blot analysis results also demonstrated that HIF-1α expression increased with hypoxia stimulation time, as shown in Fig. 1.

STIM1 and Orai1 expression under normoxia and hypoxia. The dependence of cell polarization and chemotaxis on SOCE proteins STIM1 and Orai1 has been described previously (31,38). As shown in Fig. 2A, the expression of STIM1 and Orai1 was significantly reduced at day 2 of exposure to hypoxia, however no apparent change was observed on the first day.

TG triggers SOCE-mediated Ca^{2+} entry in dHL-60 cells under normoxia and hypoxia. dHL-60 cells were treated with TG under Ca^{2+}-free conditions to investigate the mechanisms underlying Ca^{2+} store-depletion and influx in dHL-60 cells under normoxic or hypoxic conditions. SOCE can be initiated by Ca^{2+} store-depletion. No significant difference was observed between N5 and N4+H1 following TG stimulation

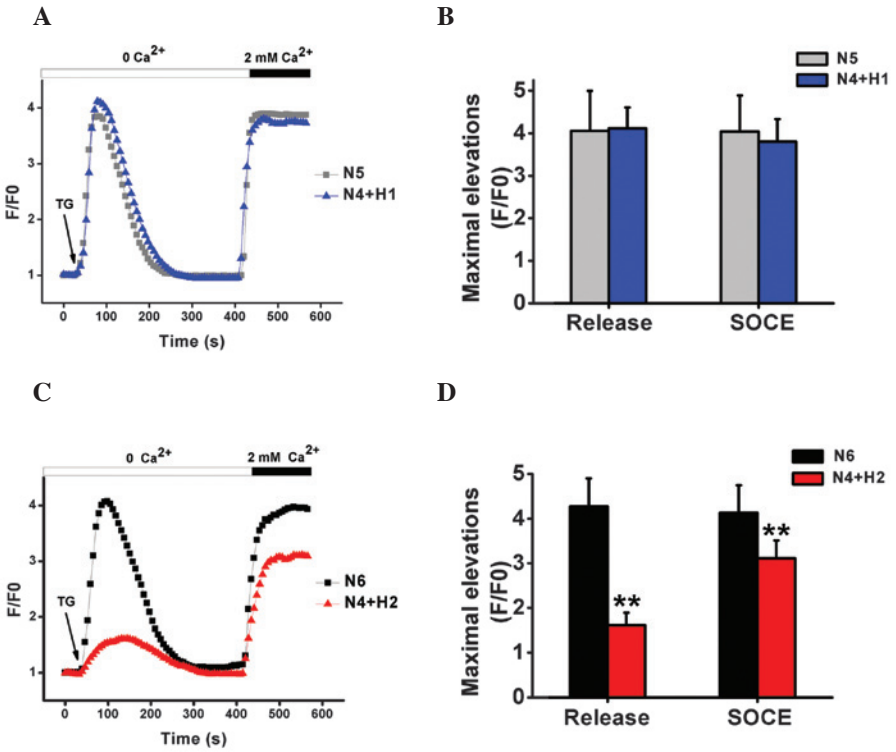

Figure 3. TG triggers SOCE-mediated calcium entry in dHL-60 cells under normoxia and hypoxia. Cytoplasmic Ca^{2+} was assessed in Fluo-4 AM-loaded dHL-60 cells. dHL-60 cells were stimulated with 1 μM of TG, followed by the addition of 2 mM of Ca^{2+} to the extracellular medium. Intracellular Ca^{2+} was monitored using laser scanning confocal microscopy. Each point represents the mean of at least eight observations. (A and C) 2 mM of Ca^{2+} was added to the extracellular medium as indicated following stimulation by TG and Ca^{2+} influx was monitored. (B and D) The bar charts show Ca^{2+} release and SOCE following the addition of TG and Ca^{2+}. Bars represent the mean ± SD from three independent experiments. **P<0.01. TG, thapsigargin; SOCE, store-operated Ca^{2+} entry; dHL-60, differentiated human neutrophil-like HL-60 cells; SD, standard deviation; N5, 5 day incubation under normoxic conditions; N4+H1, 4 day incubation under normoxic conditions and 1 day under hypoxic conditions; N6, 6 day incubation under normoxic conditions; N4+H2, 4 day incubation under normoxic conditions and 2 day under hypoxic conditions.

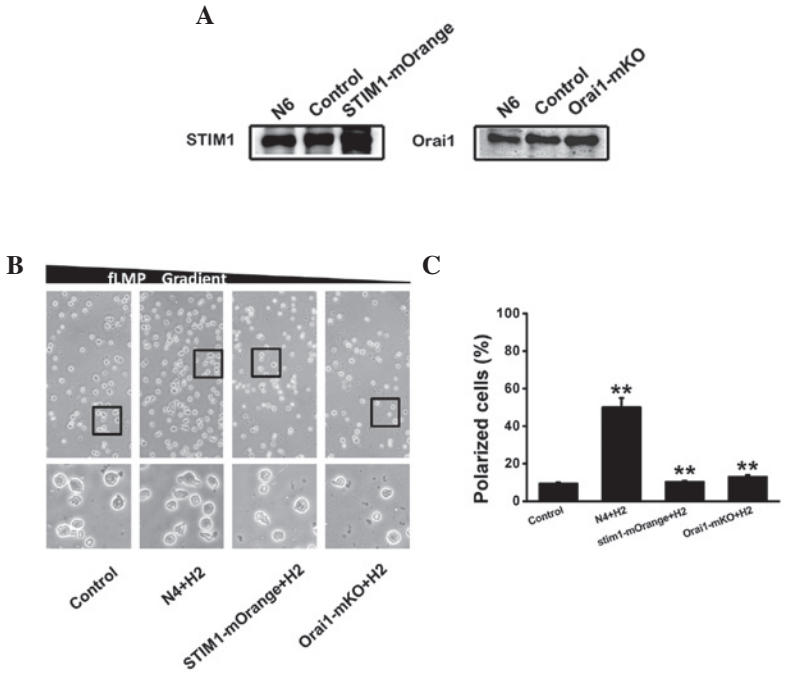

Figure 4. Overexpression of STIM1 and Orai1 reduced fMLP-induced dHL-60 cell polarization under hypoxia. (A) The expression of STIM1 and Orai1 was observed following electroporation with STIM1-mOrange, Orai1-mKO and pcDNA3.1 plasmids. (B) dHL-60 cells were pretreated with cell electroporation during 2 days of hypoxia prior to fMLP stimulation in a Zigmond chamber. (C) The proportion of dHL-60 cells polarized following 15 min exposure to fMLP in a Zigmond chamber was analyzed. Bars represent the mean ± SD; **P<0.01. Cell polarization and reduction were compared between the control and N4+H2, N4+H2 and STIM1-mOrange + H2 and N4+H2 and Orai1-mKO + H2 groups. dHL-60, differentiated human neutrophil-like HL-60 cells; STIM1, stromal interaction molecule 1; fMLP, N-formyl-Met-Leu-Phe; SD, standard deviation; N6, 6 day incubation under normoxic conditions; N4+H2, 4 day incubation under normoxic conditions and 2 day under hypoxic conditions.

and Ca^{2+} addition. However, hypoxia appeared to have a more significant impact on TG-induced Ca^{2+} release in N4+H2 and partially inhibited subsequent Ca^{2+} influx by ~25% following the addition of $CaCl_2$, as shown in Fig. 3.

Polarization in dHL-60 cells following STIM1 or Orail overexpression. In order to evaluate the effect of STIM1 and Orai1 on cell polarization in response to an fMLP gradient (0-100 nM) under hypoxia, plasmid electroporation was used for the overexpression of STIM1 and Orai1, as shown in Fig. 4A. The STIM1-mOrange plasmid appeared to be more effective than Orai1-mKO and the expression was enhanced by 80 and 20% compared with that treated with pcNDA3.1, respectively. We investigated the effect of STIM1 and Orai1 overexpression on cell polarization in response to the fMLP gradient. As shown in Fig. 4B, STIM1 and Orai1 overexpression using plasmids resulted in a substantial suppression of polarization in the direction of the fMLP gradient at day 2 of hypoxia, with the percentage of polarized cells in the presence of STIM1-mOrange, Orai1-mko and N4+H2 being 10, 13 and 50%, respectively.

Discussion

Despite considerable effort directed towards the regulation of neutrophil functions in the past, there remain numerous unknown factors concerning the responses of neutrophils to hypoxia. In an *in vivo* experiment, Klokker *et al* demonstrated that acute hypoxia induced marked alterations in the immune system and natural killer cells were particularly sensitive to the hypoxic stimulus (39). Hypoxic hypoxia was revealed to increase the phagocytic activity of human neutrophils directly with the hypoxia level (8). An *in vitro* study by Rotstein *et al* suggested that extreme hypoxia (less than 30 mmHg) caused a small (15-25%) but significant reduction in chemotactic migration (9). These studies suggest that an altered microenvironment may contribute to the failure of host leukocytes to resolve infection. In humans, hypoxemia (O_2 saturation, 5-20%) significantly increased the percentage of PMN positive cells for phagocytosis via $(Ca^{2+})_i$ mobilization (40). There have been numerous other studies demonstrating that Ca^{2+} influx via SOCE is important in the polarization, migration and metastasis of non-excitable cells following exposure to a variety of stimuli (30,41). Hauser *et al* suggested that prolonged elevations of $(Ca^{2+})_i$ due to enhanced SOCE may alter the stimulus-response coupling to chemotaxins and contribute to PMN dysfunction following injury (42). However, the mechanisms by which SOCE affects cell polarization under hypoxic conditions remain unclear. In the present study, an attempt is made to study the effect of hypoxia on polarization in differentiated human neutrophil-like HL-60 cells and the role of SOCE in this process.

The impact of hypoxia on dHL-60 cells, a phenotype analogous to neutrophils, was examined in the present study. The results demonstrated that there was no significant difference in the percentage of polarized dHL-60 cells between the N5 and N4+H1 groups, however, a higher cell polarization was observed in the N4+H2 group compared with that in the N6 group, indicating that the polarization of dHL-60 cells was increased in hypoxia. STIM1 and Orai1 are important factors of SOCE that can modulate cell polarization. Our experiments also demonstrated that STIM1 and Orai1 were decreased in the N4+H2 group, which may be attributed to the reduction in SOCE. To ascertain the effect of SOCE in this response, $(Ca^{2+})_i$ was measured using Fluo-4/AM imaging. The inhibitory effect of hypoxia on Ca^{2+} influx was observed at day 2 of exposure to hypoxia, suggesting that hypoxia did inhibit SOCE. However, it must be noted that other possibilities which contribute to the inhibition of Ca^{2+} influx cannot be completely ruled out. SOCE is involved in TG-induced Ca^{2+} influx under hypoxia. We also identified that hypoxia not only partially inhibited subsequent Ca^{2+} influx, but also appeared to have a more significant impact on TG-induced Ca^{2+} release in N4+H2. A plausible explanation for the observed difference in Ca^{2+} release appears to be associated with the altered signal response of endoplasmic reticulum Ca^{2+} emptying following hypoxia or a difference in related calmodulin receptor expression. Hypoxia may impact SOCE by the components of SOCE, including STIM1 or Orai1, or it is possible that other factors are involved which change SOCE. Our study supports a complex signaling effect at work in mature dHL-60 cells under hypoxia. Plasmid overexpression resulted in an enhancement of SOCE and an inhibition of cell polarization, which further supports the conclusion that SOCE is involved in the process and inhibits the polarization of dHL-60 cells.

Thus, it can be concluded that hypoxia alters PMN functions, including polarization. This may be explained by the down-regulation of STIM1 and Orai1 expression, and SOCE. SOCE inhibits the polarization of dHL-60 cells under hypoxic conditions, which may be the mechanism by which the neutrophils adapt to hypoxia. SOCE is also a key modulator for immune deficiency under hypoxia, potentially as a therapy target. However, the mechanisms responsible for the differential Ca^{2+} release regulation by TG is not clearly understood. Neutrophils are important in the immune response under hypoxic conditions and this underscores the requirement for further study concerning the regulatory mechanisms and gene expression involved in endoplasmic reticulum calcium emptying.

Acknowledgements

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