Systemic hypoxia enhances bactericidal activities of human polymorphonuclear leucocytes

Jong-Shyan WANG and Huang-Chun LIU
Graduate Institute of Rehabilitation Science and Center for Healthy Aging Research, Chang Gung University, Tao-Yuan, Taiwan

ABSTRACT

ROS (reactive oxygen species) generated by hypoxia facilitate the vascular inflammatory response, but whether systemic hypoxia influences leucocyte bactericidal activity by modulating circulatory redox status remains unclear. The present study elucidates how various hypoxic interventions influence the bactericidal activity of PMNs (polymorphonuclear leucocytes) following treatment with an antioxidant, vitamin E (d-α-tocopheryl acetate). Forty healthy sedentary men were randomly assigned to vitamin E (n = 20) and placebo (n = 20) groups. At 1 h following oral administration of 1000 i.u. of vitamin E or placebo, each subject in the two groups was randomly exposed to 12%, 15%, 18% and 21% O2 for 2 h in a normobaric hypoxia chamber. The results demonstrated that exposure to 12% O2 in the placebo group increased urinary 8-isoprostane and plasma malondialdehyde levels and decreased plasma total antioxidant content and superoxide dismutase activity, but did not alter plasma complement-C3a desArg/C4a desArg/C5a concentrations. Moreover, this hypoxic exposure also increased the chemotaxis of PMNs by exposure to N-formyl-Met-Leu-Phe, the phagocytosis of PMNs to Escherichia coli and the release of PMN oxidant products by E. coli, processes which were accompanied by increased expressions of L-selectin, LFA-1 (lymphocyte function-associated antigen 1), Mac-1, FcγIIIBR, C1qRp and C5aR on PMNs. However, exposure to 12% O2 in the vitamin E group did not influence expression of the opsonic/complement receptors on PMNs, and the chemotactic, phagocytic or oxidative burst activities of PMN, whereas the circulatory redox status and complement fragment levels were unaltered following this hypoxic exposure and pretreatment with vitamin E. Additionally, the circulatory redox status, complement systems, PMN-mediated bactericidal processes and the opsonic/complement receptors on PMNs were constant following exposure to 15%, 18% or 21% O2 in the two groups. We conclude that exposure to 12% O2 promotes the chemotactic, phagocytic and oxidative burst activities of PMNs, possibly by increasing lipid peroxidation and decreasing antioxidative capacity. However, this hypoxic effect on PMN bactericidal activity is ameliorated by pretreatment with vitamin E.

INTRODUCTION

Phagocytosis is a critical host defence mechanism by which PMNs (polymorphonuclear leucocytes) and macrophages clear invading pathogens [1]. Abnormal phagocytosis can be associated with numerous clinical disorders that involve the PMN itself or with an immunoglobulin or complement defect, increasing susceptibility...
to bacterial infection [2,3]. As is well known, blood undergoes oxidative stress during severe hypoxia [4,5], and ROS (reactive oxygen species) that are produced by hypoxia facilitate a rapid microvascular inflammatory response that is characterized by enhanced leucocyte-endothelial adherence and emigration, which increases vascular permeability [6,7]. Despite such a change in the vascular inflammatory response, most studies of the effects of systemic hypoxia on the eliminating microbial pathogens by leucocytes have been either controversial or incomplete [8–11]. These discrepancies may be caused by variations in the concentrations of O2 in air to which the subjects are exposed or in other features of the approach. Moreover, to our knowledge, no study employing human subjects has elucidated how systemic hypoxia affects bactericidal activity of PMNs by modulating the circulatory redox status.

The successful arrest of blood PMNs to the vasculature at inflamed tissues depends on the multiple adhesion cascades between PMNs and vascular ECs (endothelial cells), i.e. the selectins initially mediate PMN tethering and slow rolling on vascular ECs; then, the integrins strengthen PMN–EC adhesion and facilitate transendothelial migration of PMNs [12]. Thereafter, the emigrated PMNs recognize the invading pathogens by the Fc receptor for IgG and the complement receptors for complement fragments [1]. By internalizing the pathogens, activated PMNs increase O2 consumption, a process known as oxidative burst, yielding ROS to destroy the invading microbes [13,14]. Herein, we hypothesize that systemic hypoxia impacts the cascade of eliminating microbial pathogens by PMNs, with reactions determined by intervening air O2 concentration and changing circulatory redox status.

To address the discrepancies discussed above, the present study clarifies how interventions with various O2 concentrations in human subjects affect: (i) circulatory redox status, (ii) blood complement systems, (iii) chemotactic, phagocytic and oxidative burst activities of PMNs, and (iv) expression of opsonic and complement receptors on PMNs. Furthermore, the effect of the antioxidant vitamin E (α-tocopheryl acetate) on the bactericidal activity of PMNs, modulated by hypoxia, was also evaluated, revealing the role of oxidative stress in the defence of a host against bacterial infection under various hypoxic conditions.

**MATERIALS AND METHODS**

**Subjects and interventions**

The Ethics Committee of Chang Gung Memorial Hospital reviewed and approved the protocol for this investigation. The procedures corresponded to institutional guidelines and 40 healthy sedentary men were involved after they had given written informed consent. All subjects were recruited from Chang Gung University and were non-smokers, non-medication/vitamin users and were free of cardiopulmonary risk. None of the subjects had engaged in any regular physical activity or mountain climbing for at least 1 year prior to the study. The subjects were randomly divided into placebo (n = 20) and vitamin E (n = 20) groups, and they did not know whether the treated liquid included vitamin E or not. The placebo and vitamin E groups did not differ significantly in their anthropometric data, which were as follows: age, 23.1 ± 0.4 years compared with 24.1 ± 0.7 years; height, 171.8 ± 2.1 compared with 173.20 ± 1.2 cm; and weight, 66.3 ± 1.2 compared with 66.9 ± 1.4 kg respectively. Subjects fasted for at least 8 h and were instructed to refrain from exercise and medicine for at least 48 h prior to the study.

All subjects arrived at the testing centre at 09.00 hours to eliminate any possible diurnal effect. At 1 h following the oral administering of 1000 i.u. vitamin E [5 ml of 200 i.u./ml natural α-tocopheryl acetate in soybean oil without sugar, starch, sodium and artificial colours and flavours (Liquid Natural E, GNC) mixed into 95 ml of 5% (w/v) glucose solution (Sigma)] or placebo [100 ml of 5% (w/v) glucose solution (Sigma)], each subject in each group was randomly exposed to 12%, 15%, 18% and 21% O2, during which period they were asked to remain seated on conventional chairs with single-piece cushion constructions and immobilized upper (elbow flexion 90°) and lower (knee flexion 90°) extremities, in an air-conditioned normobaric hypoxia chamber (Colorado Mountain RoomTM). The chamber was maintained at a temperature of 22 ± 0.5°C with a relative humidity of 60 ± 5%, and had a CO2 scrubber to eliminate CO2 in air (<3500 p.p.m.) [15,16]. The four tests (i.e. interventions with 12%, 15%, 18% and 21% O2) were randomized in a counterbalanced order and performed at 2-week intervals to ensure complete recovery between trials. SaO2 (arterial O2 saturation) was measured by finger pulse-oximetry (model 9500; Nonin Onyx). BP (blood pressure) and HR (heart rate) were monitored using an automatic BP system (model 412; Quinton). For safety reasons, the test was terminated immediately as the level of O2 saturation dropped to less than 70% or the subject complained of obvious discomfort. All subjects were free of acute mountain sickness symptoms during the experimental period.

Before and immediately following acute exposure to various O2 concentrations, 40 ml of blood samples were collected from an antecubital vein using a clean venipuncture (20 gauge needle) under controlled venous stasis at 40 torr (1 torr = 0.133 kPa). The first 2 ml was discarded, and the haematological parameters and PMN functions of the remaining blood sample were measured. The blood cells were counted using a Sysmex SF-3000 cell counter (GMI), and blood pH, PaCO2, HCO3– and lactate concentrations were determined using an i-STAT clinical analyser (+ CG4, i-STAT) [16]. All subjects provided urine
PMN separation
A blood sample (30 ml) was transferred into a polypropylene tube that contained heparin (final concentration 10 i.u./ml) (Sigma) before and immediately after acute exposure to various concentrations of O₂. Peripheral blood PMNs were separated by density-gradient centrifugation in a Polymorphprep™ tube according to the manufacturer’s instructions (Nycomed) [17]. In brief, the heparinized whole blood was carefully layered over 1 vol. of Polymorphprep™ (Nycomed) in a polypropylene tube. After centrifugation at 450 × g for 35 min at 20 °C, the PMN fraction was harvested using a pipette and then the separated cells were washed in RPMI 1640 medium (Sigma) by recentrifugation at 600 × g for 10 min at 20 °C. The purity of PMNs, determined by flow cytometry using an anti-CD16 monoclonal antibody conjugated with FITC (clone CB16; eBioscience) and according to the cell distribution in forward and sideward scatter, was > 95%. Isolated PMNs were re-suspended in RPMI 1640 medium (Sigma) to assay for chemotactic activity, or in heparinized plasma to test the phagocytic and oxidative burst activities; the two PMN suspensions were adjusted to 5 × 10⁶ cells/ml, and then maintained at 4 °C for no more than 1 h prior to use.

Chemotactic, phagocytic and oxidative burst activities of PMNs
The chemotactic, phagocytic and oxidative burst activities of PMNs were evaluated using three commercial kits: Migrastat®, PhagoTest® and BurstTest® kits (Orpegen Pharma) respectively, by the quantitative flow cytometric method, according to the manufacturer’s instructions.

Briefly, for chemotactic activity, 100 μl of PMN suspension (5 × 10⁶ cells/ml) was added to a 3 μm-pore and 10 mm-diameter tissue culture insert, which was then placed into the microchemotaxis well that contained 350 μl of RPMI 1640 medium with or without 10 μmol/l fMLP (N-formyl-Met-Leu-Phe). The well plate with the inserts was incubated at 37 °C for 30 min. After the inserts were removed from the well plate, 200 μl of the transmigrated PMN suspension in the well was collected in a clear polypropylene tube, and then 20 μl of the reagent that contained the FITC-conjugated monoclonal antibody anti-1-selectin and counting beads was added. Then, the mixture was incubated in darkness at 4 °C for 10 min. The percentage of transmigrated PMNs in basal and fMLP-stimulated conditions were determined by FACScan flow cytometry, given by using the following formula: [transmigrated PMNs/total PMNs (5 × 10⁶ cells/ml)] × 100.

To determine phagocytic activity, 100 μl of PMN-rich plasma (5 × 10⁶ cells/ml) was mixed with 20 μl of opsonized FITC-labelled Escherichia coli suspension (1 × 10⁶ cells/mL) (such that the final ratio was 1 PMN to 40 E. coli), and then the PMN/E. coli mixture was incubated at 37 °C for 10 min. At the end of the incubation time, 100 μl of ice-cold quenching solution was immediately added to the cell mixture, which 5 min later was washed twice with 3 ml of the washing solution under centrifugation at 250 g for 10 min at 4 °C. Afterwards, the washed PMNs were re-suspended in HBSS (Hanks balanced salt solution). The percentage of PMNs that had performed phagocytosis was analysed by FACScan flow cytometry (Becton Dickinson) [18], given by using the following formula: [phagocytized PMNs/total PMNs (5 × 10⁶ cells/ml)] × 100.

To measure oxidative burst activity, 100 μl of PMN-rich plasma (5 × 10⁶ cells/ml) was mixed with 20 μl of the opsonized E. coli suspension (1 × 10⁶ cells/ml) (such that the final ratio was 1 PMN to 40 E. coli), and then the PMN/E. coli mixture was incubated at 37 °C for 10 min. Afterwards, 20 μl of fluorogenic substrate solution that contained DHR123 (dihydrorhodamine 123; final concentration 10 μmol/l) was immediately added to the cell mixture, and it was then incubated for 10 min at 37 °C. The non-fluorescent DHR123 following loading was converted into the fluorescent product R123 (rhodamine 123) by interaction with reactive oxygen intermediates [19]. The R123 fluoresences obtained from 5000 events, which represented basal and E. coli-stimulated PMNs, were measured by FACScan flow cytometry [19].

Adhesion molecules and opsonic/complement receptors on PMNs
The PMN suspensions were incubated in the absence or presence of opsonized E. coli (such that the final ratio was 1 PMN to 40 E. coli) for 10 min at 37 °C. Each aliquot was divided into eight tested samples, and then each sample was re-incubated with a saturating concentration of monoclonal anti-human I-Selectin (CD62L; clone DREG56; eBioscience), LFA-1 (lymphocyte function-associated antigen-1; CD11a; clone HI111; eBioscience), Mac-1 (CD11b; clone ICRF44; Serotec), Fcy-IIIBR (CD16b; clone 1D3; eBioscience) or C5aR (CD88; clone PI2/I; Serotec) antibodies all conjugated with FITC, C1qRp antibody conjugated with APC (allophycocyanin, CD93; clone R139, eBioscience), or anti-rabbit IgG control antibody conjugated with FITC or APC (eBioscience) in darkness for 30 min at 4 °C. Following fixation with 2% formaldehyde in HBSS, the fluorescence gained from 5000 events that represented the PMNs was calculated using a FACScan flow cytometer [18].

Complement activation, redox status and α-tocopherol
A 10 ml sample of venous blood was collected in a polypropylene tube that contained EDTA at a final
Concentration of 4 mmol/l. The plasma was obtained by centrifugation at 10 000 g for 30 min at 4 °C. Concentrations of plasma complement fragments C3a desArg, C4a desArg and C5a were assayed by three commercial ELISA kits. Additionally, plasma MDA (malondialdehyde) was determined by fluorimetric-liquid chromatography [20]. In the antioxidative capacity, the total amount of antioxidant was assessed by automatically determining the ferric-reducing ability of the plasma assay [21], whereas SOD (superoxide dismutase) activity in plasma was calculated with a commercial SOD assay kit (Cayman). The plasma α-tocopherol concentration was measured by HPLC with fluorescence detection [22]. The quantification of urinary 8-isoprostane has been proposed as being superior to the measurement of circulating 8-isoprostane, since concentrations in urine provide a more precise index of non-enzymatic lipid peroxidation [23]. Moreover, the 8-isoprostane is rapidly cleared in circulation [24] and artefactual generation of 8-isoprostane can occur with improper blood sampling handling and/or prolonged storage, leading to spurious results [25]. Therefore the present study measured the concentration of 8-isoprostane in urine as an index of systemic non-enzymatic lipid peroxidation. The analysis of urinary 8-isoprostane was performed using an Agilent 6890 gas chromatograph linked to a 5975B mass spectrometer using negative-ion chemical ionization [26]. The concentration of the substance was normalized using urinary creatinine levels, which were determined by a modified Jaffe alkaline picate method [27].

Statistical analysis
Values are expressed as means ± S.E.M. The statistical software package StatView IV was applied to analyse the data. Comparisons of the redox status, complement activation, the PMN-mediated phagocytic processes and the expressions of adhesion molecules and opsonic/complement receptors on the PMNs between placebo and vitamin E groups upon exposure to various O2 concentrations were analysed by repeated ANOVA, followed by Tukey’s multiple range test. The criterion for significance was P < 0.05.

RESULTS
Cardiovascular and haematological parameters
Although the decrease in the degree of SaO2 by hypoxia was related to the decline in the concentration of O2 in the air, the levels of both RBCs (red blood cells) and Hb were constant following 12 %, 15 %, 18 % or 21 % O2 exposure (Table 1). Exposure to 12 % O2, but not 15 %, 18 % or 21 % O2, increased the HR (P < 0.01), whereas no intervention changed the SBP (systolic BP) and DBP (diastolic BP). No significant difference was observed between the placebo and vitamin E groups in the parameters that were associated with blood O2 transport (SaO2, RBC and Hb) and cardiovascular function (HR and BP) under various hypoxic conditions. In both placebo and vitamin E groups, exposure to 12 % O2, but not 15 %, 18 % or 21 % O2, increased venous pH and reduced Pco2 and blood lactate levels (Table 1; P < 0.05). Although exposure to 12 % O2 reduced blood lymphocyte and monocyte counts (P < 0.05), total blood leucocyte (including neutrophil, eosinophil, basophil, lymphocyte and monocyte) counts were unchanged following 15 %, 18 % or 21 % O2 exposure in the two groups (Table 2).

Redox status and complement systems
In the placebo group, although exposure to 12 % O2 significantly increased urinary 8-isoprostane and plasma MDA levels, and decreased plasma total antioxidant content, SOD activity and α-tocopherol concentration (P < 0.05), the levels of circulatory redox-related products were independent of 15 %, 18 % and 21 % O2 exposures (Table 3). Conversely, no significant change in the urinary 8-isoprostane level as well as plasma MDA level, total antioxidant content or SOD activity of the vitamin E group was observed following 12 % O2 exposure (Table 3). Following various hypoxic exposures, the vitamin E group had higher plasma levels of α-tocopherol than the placebo group (P < 0.05) (Table 3). Additionally, plasma C3a desArg, C4a desArg and C5a concentrations were unaltered following all O2 exposures in both placebo and vitamin E groups (Table 3).

Chemotaxis, phagocytosis and oxidative burst of PMNs
The results revealed that exposure to 12 % O2 in the placebo group increased the percentages of chemotaxis of PMN by exposure to fMLP (Figure 1A; P < 0.05) and phagocytosis of PMN to E. coli (Figure 1B; P < 0.01), and promoted the production of PMN-derived ROS by E. coli (Figure 1C; P < 0.01). In contrast, exposure to 12 % O2 in the vitamin E group was not associated with any change in the chemotactic (Figure 1D), phagocytic (Figure 1E) or oxidative burst (Figure 1F) activity of PMNs. Moreover, no significant change in the chemotactic, phagocytic or oxidative burst activity of PMNs occurred following 15 %, 18 % and 21 % O2 exposures in the two groups (Figures 1A–1E).

Inflammation-related adhesion molecules on PMNs
Treating PMNs with E. coli increased l-selectin shedding (Figures 2A and 2D; P < 0.01) and Mac-1 expression (Figures 2C and 2F; P < 0.01) on the cell surface. In the placebo group, exposure to 12 % O2, but not 15 %, 18 % and 21 % O2, was associated with increased basal l-selectin (Figure 2A; P < 0.01), LFA-1 (Figure 2B,
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<td><strong>Sao₂ (%)</strong></td>
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<td>98 ± 1</td>
<td>98 ± 1</td>
<td>95 ± 1*</td>
<td>98 ± 1</td>
<td>89 ± 2*</td>
<td>98 ± 0</td>
<td>77 ± 2*</td>
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<td><strong>RBC (× 10⁶/μl)</strong></td>
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<td><strong>Hb (g/dl)</strong></td>
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<td><strong>HR (beats/min)</strong></td>
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<td><strong>PCO₂ (mmHg)</strong></td>
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<td>42.6 ± 0.6*</td>
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<td><strong>HCO₃⁻ (mmol/l)</strong></td>
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<td><strong>Lactate (mmol/l)</strong></td>
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<td>1.11 ± 0.09</td>
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* Values are mean ± S.E.M. MBP, mean BP; Post, post-intervention; Pre, pre-intervention. *P < 0.05, pre-intervention compared with post-intervention.
Comparisons of blood leucocyte counts between placebo and vitamin E groups during the experimental periods

Values are means +− S.E.M. Pre-intervention compared with post-intervention.

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<th>Leucocyte type</th>
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<td>Neutrophil (×10^3/μl)</td>
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<td>Lymphocyte (×10^3/μl)</td>
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<td>Monocyte (×10^3/μl)</td>
<td>0.86±0.11</td>
<td>0.80±0.16</td>
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*P < 0.05) and Mac-1 (Figure 2C, P < 0.01) expression and promoted E. coli-treated LFA-1 (Figure 2B; P < 0.05) and Mac-1 (Figure 2C; P < 0.01) expression. However, in the vitamin E group, no significant change occurred in the L-selectin (Figure 2D), LFA-1 (Figure 2E) and Mac-1 (Figure 2F) contents on the PMN membrane following 15%, 18% and 21% O2 exposures.

**Opsonic and complement receptors on PMNs**

With regard to opsonic and complement receptors on PMNs, treating PMNs with E. coli increased the C1qRp level (Figures 3B and 3E; P < 0.01) and decreased the C5αR level (Figures 3C and 3F; P < 0.01) on the cell surface. Exposure to 12% O2 increased the basal content of FCγIIIbR (Figure 3A; P < 0.01) and C5αR (Figure 3C; P < 0.01) on PMNs, as well as enhancing the E. coli-mediated expression of the FCγIIIbR (Figure 3A; P < 0.01) and C1qRp (Figure 3B; P < 0.05) on PMNs. However, basal or E. coli-mediated expression of FCγIIIbR (Figure 3D), C1qRp (Figure 3E) and C5αR (Figure 3F) on PMNs was constant following 12% O2 exposure and pretreatment with vitamin E. Additionally, neither the expression of FCγIIIbR, C1qRp nor C5αR on PMN under basal and E. coli-treated conditions changed significantly following 15%, 18% and 21% O2 exposures in the two groups (Figures 3A–3E).

**DISCUSSION**

The present study employing human subjects shows clearly that oxidative stress that is caused by systemic hypoxia is an important factor in PMN bactericidal cascades in sedentary males. Acute exposure to 12% O2 promotes the chemotactic, phagocytic and oxidative burst activities of PMNs and enhances expression of L-selectin, LFA-1, Mac-1, FCγIIbR, C1qRp and C5αR on PMNs, processes which are accompanied by increased lipid peroxidation and reduced antioxidative capacity. However, such hypoxic effects are eliminated by pretreatment with vitamin E (d-α-tocopheryl acetate). In contrast, acute exposure to 15%, 18% or 21% O2 does not change the PMN-mediated bactericidal activity or the circulatory redox status.

**The role of hypoxia in chemotaxis**

Abnormal chemotaxis was observed and described with a variety of clinical immune disorders [28]. The integrin β2, in a similar manner to LFA-1 and Mac-1 on PMNs, is crucial to the motion of PMNs through an inflamed vascular endothelial layer [12]. Circulating PMNs that lack integrin β2 receptors, such as are present in patients with leucocyte adhesion deficiency-1, are unable to complete a multiple adhesion process that is required for the PMNs to be recruited to the sites of inflammation [29]. Animal studies
Table 3  Comparisons of α-tocopherol, complement fragments and redox status between placebo and vitamin E groups during the experimental periods
Values are means ± S.E.M. Post, post-intervention; Pre, pre-intervention. *P < 0.05, pre-intervention compared with post-intervention; + P < 0.05, placebo compared with vitamin E.

<table>
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<tr>
<th>Measurement</th>
<th>Experimental group</th>
<th>21 % O₂</th>
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<th>18 % O₂</th>
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<th>15 % O₂</th>
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| α-Tocopherol (µg/dl)         | Placebo            | 525 ± 29 | 528 ± 26+ | 540 ± 16 | 532 ± 22+ | 536 ± 21 | 520 ± 19+ | 536 ± 29 | 484 ± 14+*+
|                              | Vitamin E          | 529 ± 29 | 633 ± 35* | 529 ± 39 | 643 ± 32* | 537 ± 29 | 649 ± 35* | 533 ± 30 | 571 ± 32|
| Complement fragments         |                    |        |      |        |      |        |      |        |      |
| C₃a desArg (ng/ml)           | Placebo            | 440 ± 22 | 421 ± 32 | 444 ± 29 | 426 ± 30 | 436 ± 15 | 433 ± 14 | 413 ± 16 | 434 ± 12|
|                              | Vitamin E          | 440 ± 22 | 421 ± 32 | 444 ± 29 | 426 ± 30 | 436 ± 15 | 433 ± 14 | 413 ± 16 | 422 ± 12|
| C₄a desArg (ng/ml)           | Placebo            | 862 ± 32 | 868 ± 42 | 872 ± 38 | 874 ± 46 | 839 ± 23 | 827 ± 22 | 814 ± 29 | 835 ± 24|
|                              | Vitamin E          | 845 ± 55 | 863 ± 80 | 858 ± 53 | 863 ± 86 | 792 ± 50 | 829 ± 77 | 805 ± 28 | 813 ± 35|
| C₅a (ng/ml)                  | Placebo            | 330 ± 19 | 350 ± 18 | 332 ± 10 | 352 ± 14 | 334 ± 10 | 336 ± 18 | 326 ± 16 | 343 ± 21|
|                              | Vitamin E          | 352 ± 12 | 326 ± 21 | 356 ± 18 | 327 ± 27 | 323 ± 19 | 302 ± 12 | 305 ± 19 | 300 ± 20|
| Redox status                 |                    |        |      |        |      |        |      |        |      |
| B-isoprostan (ng/mg of creatinine) | Placebo            | 2.21 ± 0.12 | 2.30 ± 0.24 | 2.30 ± 0.34 | 2.52 ± 0.34 | 2.40 ± 0.23 | 3.12 ± 0.40 | 2.35 ± 0.33 | 4.78 ± 0.23+*+
|                              | Vitamin E          | 2.41 ± 0.21 | 2.28 ± 0.32 | 2.53 ± 0.35 | 2.39 ± 0.42 | 2.51 ± 0.20 | 2.74 ± 0.39 | 2.24 ± 0.40 | 2.68 ± 0.38|
| MDA (µmol/l)                 | Placebo            | 0.916 ± 0.091 | 0.962 ± 0.108 | 0.917 ± 0.102 | 1.062 ± 0.089 | 0.928 ± 0.108 | 1.087 ± 0.119 | 0.902 ± 0.086 | 1.340 ± 0.140+*+
|                              | Vitamin E          | 0.951 ± 0.048 | 0.965 ± 0.089 | 0.914 ± 0.079 | 0.957 ± 0.121 | 0.915 ± 0.107 | 0.968 ± 0.074 | 0.961 ± 0.089 | 0.953 ± 0.153|
| SOD activity (units/ml)      | Placebo            | 5.24 ± 0.21 | 5.14 ± 0.45 | 5.31 ± 0.52 | 5.22 ± 0.54 | 5.44 ± 0.52 | 5.12 ± 0.34 | 5.42 ± 0.44 | 4.24 ± 0.35+*+
|                              | Vitamin E          | 5.34 ± 0.31 | 5.42 ± 0.62 | 5.55 ± 0.45 | 5.32 ± 0.35 | 5.52 ± 0.35 | 5.62 ± 0.39 | 5.38 ± 0.33 | 5.42 ± 0.32|
| Total antioxidant (mmol/l)   | Placebo            | 0.651 ± 0.022 | 0.632 ± 0.041 | 0.602 ± 0.042 | 0.581 ± 0.064 | 0.623 ± 0.043 | 0.570 ± 0.042 | 0.654 ± 0.04 | 0.513 ± 0.031+*+
|                              | Vitamin E          | 0.590 ± 0.031 | 0.623 ± 0.053 | 0.624 ± 0.034 | 0.630 ± 0.042 | 0.644 ± 0.062 | 0.634 ± 0.033 | 0.633 ± 0.043 | 0.584 ± 0.052|
have demonstrated that exposure to environmental hypoxia can slow leucocyte rolling velocity and promote the adherence of leucocytes and their subsequent emigration into the extravascular space of microcirculation [6,7,30]. Moreover, the number of leucocytes that adhered to normoxic microcirculation was also increased when animals breathed a hypoxic gas mixture [30]. The findings of these studies suggest that a mediator released from a central site during systemic hypoxia triggers the inflammatory response. Additionally, the administration of an antibody to integrin \( \beta 2 \) or Mac-1 eliminated any effect of systemic hypoxia on leucocyte adhesion or emigration [31]. The results of the present study using human subjects further indicate that exposure to 12 % \( \text{O}_2 \), but not 15 %, 18 % or 21 % \( \text{O}_2 \), enhanced the transmigration of PMNs induced by fMLP, as revealed by increased chemotactic activity, along with increased expression of LFA-1 and Mac-1 on PMNs; however, no such hypoxic effects on the integrin \( \beta 2 \) expression or chemotaxis of PMNs occurred when the subjects were pretreated with vitamin E. Vitamin E, a lipophilic antioxidant, prevents tissue injury caused by free oxygen radicals through a chain-breaking effect on lipid peroxidation [32]. Upon treatment with this antioxidant to scavenge circulating free radicals [32], the blood \( \text{O}_2 \) transport parameters of 12 % \( \text{O}_2 \) exposure, such as blood \( \text{SaO}_2 \), RBCs and Hb, did not change. Hence, the elevated oxidative stress, rather than hypoxaemia, during systemic hypoxia is likely to activate the PMN integrin \( \beta 2 \), triggering the emigration of PMNs to inflammatory sites along a gradient of chemotactic factors.

**The role of hypoxia in phagocytosis**

Adequate phagocytosis of PMNs depends on the expression of the opsonic/complement receptors on the cell...
The deficiencies of the opsonic/complement receptors can result in increased susceptibility to infection due to defective bactericidal capacity of PMNs [2,3,28]. Intervention of various immunomodulators, such as cytokines, offers an attractive therapeutic approach to treating phagocytosis-associated with immune disorders [33]. However, previous studies of the effect of hypoxia, as a non-pharmacological intervention, on PMN phagocytosis and expression of opsonic and complement receptors on PMNs, yielded contradictory results [8–11]. Early studies established that acute hypoxia caused an opsonic mismatch by reducing the number of CD16 [FcγRIII, low-affinity Fc receptor for phagocytosis and ADCC (antibody-dependent cell-mediated cytotoxicity)] and CD32w (FcγRII, low-affinity Fc receptor for aggregated Ig and immune complexes) without affecting complement receptors [CR1 and CR3 (Mac-1)] [10,34], whereas other investigators have observed that hypoxaemia increased cytokine-induced PMN phagocytosis and both CD32w and CD64 (FcγRI, high-affinity Fc for IgG, phagocytosis and ADCC) mRNA levels in PMNs [9,35]. These discrepancies may be associated with differences among the designed hypoxic conditions in terms of type/intensity/duration, or among other aspects of the methodology. In the present study, although various hypoxic conditions did not significantly change classical/alternative pathways of complement systems in plasma, acute exposure to 12% O₂, but not 15%, 18%, or 21% O₂, for 2 h enhanced phagocytosis of PMNs to E. coli and up-regulated basal or E. coli-induced FcγRIIBR (CD16b) and complement (C1qR, CR3 and C5aR) receptors on PMNs. These results imply that systemic hypoxia affects PMN phagocytic activity and the expression of opsonic/complement receptors on PMNs, with reactions determined by intervening air O₂ concentration.
Figure 3  Effects of various hypoxic interventions on the expression of opsonic/complement receptors on PMNs (A and D, FcγRIIBR; B and E, C1qRp; C and F, C5aR) in placebo (A–C) and vitamin E (D–F) groups
MFI, mean fluorescence intensity; Post-basal, basal level at post-intervention; Post-stimulated, stimulated level at post-intervention; Pre-basal, basal level at pre-intervention; Pre-stimulated, stimulated level at pre-intervention. Values are means ± S.E.M. + P < 0.05, pre-intervention compared with post-intervention; * P < 0.05, basal level compared with stimulated level.

The role of hypoxia in oxidative burst
As invading pathogens are opsonized or induce complement activation, the ligation(s) of IgG and/or complement fragments to Fcγ and/or complement receptors respectively, also cause(s) the activation of the NADPH oxidase of PMNs, leading to oxidative burst, thereby effectively killing the pathogens [13,14]. Reduced or absence of oxidative burst activity may result in repeated and life-threatening infections caused by bacterial and fungal organisms. The results reveal that exposure to 12% O2, but not 15%, 18% or 21% O2, promoted the oxidative burst of PMNs to the opsonized E. coli. Just as PMNs respond chemotactically to systemic hypoxia, treatment with d-α-tocopheryl acetate suppressed the enhancement of phagocytosis and oxidative burst of PMNs and the activation of opsonic/complement receptors on PMNs following exposure to 12% O2. Accordingly, elevated oxidative stress may mediate this effect of extreme hypoxic intervention on the phagocytic and oxidative burst activities of PMNs.

Possible mechanisms of enhanced bactericidal activity by hypoxia
Circulatory redox status changed by acute hypoxic exposure may result from the imbalance between radical-scavenging and radical-generating systems in the circulation [4]. In radical-scavenging systems, results from the present study demonstrated that exposure to 12% O2 suppressed plasma SOD activity along with decreasing total antioxidant content and α-tocopherol concentration in plasma. According to previous studies, the SOD activity depressed by hypoxia may occur for the following reasons: (i) delayed secretion of active extracellular SOD [36], (ii) down-regulated expression of Mn-SOD and Cu,Zn-SOD mRNA by decreasing the stability
of the mRNA transcripts [37], and (iii) depressed SOD activity without changing SOD gene expression [38]. On the other hand, elevated oxidative stress by hypoxia may also be associated with an increase in NADPH oxidase activity and gene/protein expression [39]. NADPH oxidase in PMNs, an O$_2^*$-generating enzyme, comprises a flavocytochrome b558 (p22phox and gp91phox) and cytosolic components (P47phox, p67phox, p40phox, and Rac 1 or 2) [13]. Upon activation of NADPH oxidase, cytosolic components become phosphorylated on specific sites, subsequently translocating the cytosolic proteins to form a complex with the membrane-associated cytochrome and improving the ability to convert O$_2$ into O$_2^*$ [13]. Activation of the PKC (protein kinase C) family in PMNs appears to be important in regulating the assembly and activation of NADPH oxidase [40]. Previous studies have indicated that α-tocopherol supplementation suppressed the release of monocyte oxidant products by reducing the phosphorylation of PKCα and the translocation of the cytosolic p47phox, as well as subsequently inhibiting the assembly and activation of NADPH oxidase in monocytes [41,42]. In the present study, we speculate that exposure to 12% O$_2$ enhances PMN bactericidal activity by ROS-dependent mechanisms, possibly by promoting PKC-mediated activation of NADPH oxidase, and that this hypoxic effect on PMNs is ameliorated by pretreatment with the antioxidant vitamin E.

The molecular mechanisms by which systemic hypoxia influences the PMN-mediated bactericidal cascade by modulating circulatory redox status are unclear, perhaps because stabilized HIF-1α (hypoxia-inducible factor-1α) by elevated oxidative stress under hypoxia induces the gene expression of phagocytic-related adhesion molecules and receptors on PMNs [43–45]. HIF-1α can directly regulate the bactericidal capacity of phagocytes in the inflammatory microenvironment [46,47]. According to in vitro cell culture experiments, hypoxia can promote mitochondrial ROS generation at Complex III, causing the accumulation of HIF-1α protein which is responsible for inducing the expression of a luciferase reporter construct under the control of a hypoxic-response element [48]. Additionally, Kong et al. [31] demonstrated that the HIF-1-dependent induction of β2 integrin gene expression mediates leukocyte adhesion during hypoxia. Whether systemic hypoxia triggers a PMN bactericidal cascade by HIF-1 stabilization in humans, as in the response of local hypoxia in in vitro studies, must be investigated further.

**Clinical applications and limitations of the present study**

A functional chemotaxis defect diminishes the number of granulocytes which migrate from the circulation into inflamed tissues. These deficiencies are usually accompanied by recurrent infections of the skin or respiratory tract [28]. Acquired defects associated with altered PMN phagocytic or oxidative burst activity have been observed in patients with recurrent bacterial skin and sino-pulmonary infections [49], in patients with wound infections from burns [50], or in patients undergoing therapies with glucocorticoids [51]. In the present study, acute hypoxic exposure influences PMN chemotactic, phagocytic and oxidative burst activities in a concentration-dependent manner; it is enhanced by exposure to 12% O$_2$, but not by exposure to 15%, 18% or 21% O$_2$. Furthermore, oxidative-stress-induced expression of opsonic and complement receptors on PMNs probably mediates the hypoxic effects on the PMN bactericidal cascade. By scavenging products of oxidative stress, the antioxidant vitamin E (α-tocopheryl acetate) can attenuate the increases in PMN chemotactic, phagocytic and oxidative burst activities by 12% O$_2$ exposure. These experimental findings help to determine a hypoxic regimen that improves PMN bactericidal capacity, effectively defending individuals against bacterial infection. As for various other investigations, one limitation of the present study is that the subjects were young and healthy. Accordingly, further clinical evidence is required to extrapolate the results herein to patients with bactericidal deficiencies.

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**REFERENCES**

1 J.-S. Wang and H.-C. Liu

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Anand, R. J., Griñar, S. C., Li, J., Kohler, J. W., Branca, M. F., Dubowski, T., Sodhi, C. P. and Hackam, D. J. (2007) Hypoxia causes an increase in phagocytosis by macrophages in a HIF-1α-dependent manner. J. Leukocyte Biol. 82, 1257–1265


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