HIGH ALTITUDE MEDICINE & BIOLOGY
Volume 12, Number 3, 2011

DOI: 10.1089/ham.2010.1086

Intermittent Hypoxia Mobilizes Hematopoietic Progenitors and Augments Cellular and Humoral Elements of Innate Immunity in Adult Men

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Abstract

Serebrovskaya, Titiana V., Igor S. Nikolsky, Valentyna V. Nikolska, Robert T. Mallet, and Vadim A. Ishchuk. Intermittent hypoxia mobilizes hematopoietic progenitors and augments cellular and humoral elements of innate immunity in adult men. *High Alt. Med. Biol.* 12:243–252.—This study tested the hypothesis that intermittent hypoxia treatment (IHT) modulates circulating hematopoietic stem and progenitor cells (HSPC) and augments humoral and cellular components of innate immunity in young, healthy men. Ten subjects (group 1: age 31 ± 4 yr) were studied before and at 1 and 7 days after a 14-day IHT program consisting of four 5-min bouts/day of breathing 10% O_2, lowering arterial O_2 saturation to 84% to 85%, with intervening 5-min room-air exposures. Five more subjects (group 2: age 29 ± 5 yr) were studied during 1 IHT session. Immunofluorescence detected HSPCs as $CD45^+CD34^+$ cells in peripheral blood. Phagocytic and bactericidal activities of neutrophils, circulating immunoglobulins (IgM, IgG, IgA), immune complexes, complement, and cytokines (erythropoietin, TNF-α, IL-4, IFN-γ) were measured. In group 1, the HSPC count fell 27% below pre-IHT baseline 1 week after completing IHT, without altering erythrocyte and reticulocyte counts. The IHT program also activated complement, increased circulating platelets, augmented phagocytic and bactericidal activities of neutrophils, sharply lowered circulating TNF-α and IL-4 by $>90\%$ and $\sim 75\%$, respectively, and increased IFN-γ, particularly 1 week after IHT. During acute IHT (group 2), HSPC increased by 51% after the second hypoxia bout and by 19% after the fourth bout, and total leukocyte, neutrophil, monocyte, and lymphocyte counts also increased; but these effects subsided by 30 min post-IHT. Collectively, these results demonstrate that IHT enhances innate immunity by mobilizing HSPC, activating neutrophils, and increasing circulating complement and immunoglobulins. These findings support the potential for eventual application of IHT for immunotherapy.

Key Words: complement; cytokines; hematopoietic stem cells; innate immunity; intermittent hypoxia; neutrophils

Introduction

Stem cells and progenitor cells are the pluripotent precursors of myriad cell and tissue types during embryogenesis and postnatal ontogenesis (Weissman et al., 2001). They are capable of self-renewal and possess in varying degrees the ability to differentiate into various cell types. Hematopoetic stem cells (HSC) are among the earliest elements in a well-orchestrated sequence of cell proliferation, differentiation, and migration, which generates all blood cells. HSC also can undergo transdifferentiation to other cell types, such as hepatic oval cells (Lagasse et al., 2000), neurons (Mezey and Chandross, 2000), skeletal and cardiac myocytes (Bossolasco et al., 2004), and vascular endothelium (Jackson et al., 2001). An important property of HSC is their ability to traverse the vascular endothelium and recirculate in the bloodstream, thereby maintaining stem cell pools of numerous tissues in quantities sufficient to sustain reparative processes. Stress can deplete the recirculating HSC pool (Bezin et al., 1975).

Intermittent hypoxia treatment (IHT) is becoming an increasingly popular modality for clinical medicine and athletic training owing to its capacity to protect cells, tissues, organs,

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and the whole organism from more intense and/or sustained hypoxia, ischemia, and other stresses and to enhance physical and mental capacity. The responses of stem cells to hypoxia are pivotal to many of the resultant adaptations. It has been known for almost 40 yr that stem cells can respond rapidly to hypoxia (Murphy and Lord, 1973). Later investigations have shown that hypoxia inhibits the proliferation of $CD34^+$ stem cells and preserves the capacity for colony-forming cell generation in secondary cultures (Desplat et al., 2002), even in the absence of growth factors (Sun et al., 2000). Oxygen status profoundly influences the balance between stem cell pluripotency and differentiation (Ong et al., 2010). Different intensities and durations of hypoxia could have important and diverse effects on stem cell development, but scant information exists on the effects of different IHT regimens on human stem cells.

Hematopoietic stem and progenitor cells are naturally distributed along Po_2 gradients, with HSC occupying the most hypoxic niches (Olive et al., 2002). Transcription factors and cytokines clearly influence HSC development (Lekli et al., 2009). Recent work has revealed an important link between the factors that regulate stem and progenitor cell behavior and the hypoxia-inducible factors, providing a molecular framework for the hypoxic control of differentiation and cell fate (Wang et al., 2007).

Information about hypoxia's effects on human HSC is limited (Ciulla et al., 2005; Mancuso et al., 2008). Exposure of cultured human HSC to 1% to 3% O_2 activated hematopoiesis (Cipolleschi et al., 1993; Danet et al., 2003), and certain intermittent hypoxia protocols were more potent activators of stem cell gene expression and proliferation than sustained hypoxia (Zhao et al., 2008; Tang et al., 2009). However, the effects of IHT on HSPC in intact human subjects are unknown. Moreover, adaptations to IHT are the cumulative effects of multiple hypoxia exposures over several days to weeks (Mallet et al., 2006; Serebrovskaya et al., 2008). The effects of a single intermittent hypoxia session on HSPC in human peripheral blood are unknown. Accordingly, this study was conducted to define the effects of acute and chronic intermittent hypoxia exposure on HSPC, various factors of natural resistance, and the chief humoral and cellular components of adaptive immunity in peripheral blood. This investigation is among the first to examine how chronic and acute intermittent hypoxia influences circulating hematopoietic stem and progenitor cells and factors of nonspecific immune resistance in human subjects.

Methods

Human subjects

This work was approved and authorized by the Ethics Committee for Human Experiments of the Bogomoletz Institute of Physiology. All subjects gave their informed consent at the time of enrollment. Two groups of healthy male volunteers participated in the study. Group 1 consisted of 10 men (age 30.9 ± 4.2 yr, mass 76.2 ± 6.2 kg, height 177 ± 12 cm) who completed the 14-day IHT regimen described later. Group 2 consisted of 5 men (age 29.0 ± 4.6 yr, mass 74.8 ± 5.8 kg, height 172 ± 6 cm) who completed 1 day of IHT. All subjects were nonsmoking sea-level residents in good health with no evidence of cardiovascular and pulmonary diseases or immunological disorders. Subjects refrained from all medications, including vitamins and hormones, from at least 2 weeks before the study until its completion. Subjects maintained their usual physical activity and diet.

Intermittent hypoxia protocols

Group 1: 14-day IHT regimen. Intermittent hypoxia sessions were conducted for 14 consecutive mornings, between 10:00 and 12:00, 2 h after a light breakfast. With the subject seated, normobaric isocapnic hypoxia was administered with a Hypoxotron, a modified closed spirometer with CO_2 absorption (Serebrovskaya et al., 2009) for four 5-min periods, with intervening 5-min periods of room-air inspiration, each day for 14 consecutive days. Partial pressures of expired oxygen ($P_{ET}o_2$) and carbon dioxide ($P_{ET}Co_2$) were continuously monitored at the mouth with a medical mass spectrometer (MX-6202, M. Frunze, Sumy, Ukraine). Initial inspired gas contained atmospheric (20.9%) O_2. Inspired O_2 fell to a value of 10% during the first 60 to 90 s rebreathing, and then O_2 was added gradually to the Hypoxotron to maintain inspired O_2 at 10% during the remaining 3.5 to 4 min of hypoxia. The final arterial O_2 saturation was typically 84% to 85% (see Table 1). End-tidal Pco_2 was maintained at the initial pretest value for each subject, typically 38 to 40 mmHg, throughout the intermittent hypoxia session. Subjects easily tolerated the hypoxia periods without any untoward effects. Twelve-lead electrocardiograms were taken before and after each IHT session, and precordial (V_5) electrocardiograms were continuously monitored during IHT sessions. No rhythm disturbances or ischemic ST segment shifts were detected in any subject during IHT.

Venous blood samples for analyses of circulating cells, immunoglobulins, cytokines, and complement were obtained from each subject at 4 times: 2 weeks before IHT (time I); the day before IHT (time II); 24 h after completing the 14-day IHT program (time III); and 1 week after the IHT program (time IV). Blood was withdrawn from the median antecubital vein in the early morning after overnight fast.

Group 2: Acute intermittent hypoxia protocol. Subjects completed a single daily session of 4 cycles of hypoxia as described previously. Blood was withdrawn from the median antecubital vein at 5 times: 5 min before the hypoxia session (time I), during the final 15 to 20 s of the second (time II) and fourth (time III) hypoxia bouts, and at 15 (time IV) and 30 min (time V) after completing the fourth (final) hypoxia exposure. These blood samples and those obtained from group 1 were analyzed by the following methods.

Blood analysis

The content of $CD45^+34^+$-cells in venous blood was measured according to standard direct immunofluorescence techniques with a Becton-Dickinson FACSAria® (Canaan, NY, USA) flow cytometer according to International Society of Hematotherapy and Graft Engineering protocols (Gratama et al., 2001). The analysis consists of four sequential gating steps: (1) leukocytes ($CD45^+$ spanning the range from dim to bright) were selected; (2) $CD34^+$ cells were selected from the leukocytes; (3) $CD45^{+dim}$, SSC^{low}, and HPS^+ cells were selected from among the $CD45^{+dim}$ and $CD34^+$ cells; and (4) cells falling outside the $FSC^{low\text{-}intermediate}$ PS^+ cluster were excluded. The following monoclonal antibodies and isotype controls were obtained from BD Biosciences (San Jose, CA, USA): anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-CD45 (H130), anti-CD34 (581), and isotype mouse IgG1κ (MOPC-21).

Serum contents of immunoglobulins IgM, IgG, and IgA were measured by radial immunodiffusion using

monospecific antisera (Microgen Bioproducts, Moscow, Russian Federation) against the different classes of human immunoglobulins (Manchini et al., 1964). Circulating immune complexes (CIC) were measured by precipitate density photometry (Sunrise, Salzburg, Austria) after precipitation of serum in 3.75% polyethylene glycol 6000 (Hasková et al., 1977). Serum complement content was taken as the reciprocal of the amount of serum necessary to produce hemolysis in a hemolytic system.

The content of hemoglobin in the peripheral blood was measured by the cyanohemoglobin method (van Assendelft et al., 1996). Contents of erythrocytes, platelets, and leukocytes in peripheral blood suspensions were determined by hemocytometry. Contents of reticulocytes and differential leukocyte counts were analyzed by microscopic analysis of blood smears. Phagocytic function of the neutrophils was assessed by their ability to ingest *Staphylococcus aureus* (Harris et al., 1983). Bactericidal activity of neutrophils was determined by the nitroblue tetrazolium test (Delamaire et al., 1997; Hallett et al., 2003) in which active cells are stained by the conversion of tetrazolium to a formazan product. Spontaneous and induced bactericidal activities were assessed from stained neutrophil counts in the absence versus presence of an inducer, *Staphylococcus aureus*. Reserve bactericidal activity equaled induced activity minus spontaneous activity.

Erythropoietin content in blood serum was measured by two-site ELISA (Biomerica, Irvine, CA, USA). Tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-4 (IL-4) were analyzed using standard ELISA kits (BioSource International, Camarillo, CA, USA), according to the manufacturer's instructions.

Statistical analyses

All values are expressed as means ± SD. Normally distributed data at multiple time points were compared by repeated measures analysis of variance combined with the Student–Newman–Keuls test to identify statistically significant differences. Shapiro–Wilk tests revealed that the circulating cell counts and cytokines data were not normally distributed; accordingly, these results were analyzed by nonparametric statistical procedures. Mean values at different time points (I to V) were compared by a Friedman nonparametric analysis of variance combined with Kendall's coefficient of concordance on ranks. p Values < 0.05 were taken to indicate statistically significant differences.

Results

Cardiorespiratory effects of 5-min hypoxia

Table 1 demonstrates the main cardiorespiratory parameters of healthy men during a single 5-min bout of IHT. During hypoxia, heart rate increased by 18%, but systolic and diastolic blood pressures did not change significantly. The maximal increases in breathing frequency and minute lung ventilation were 21% and 43%, respectively.

Group 1: 14-day IHT program

None of the measured variables changed significantly over the 2 weeks preceding the IHT program (Table 2 and Figs. 1A, 2A, and 3; time points I versus II). Because baseline values were stable, changes in measured variables at 24 h and 7 days after the IHT program were assumed to have resulted from the effects of IHT.

Small numbers of $CD45^+34^+$ cells, that is, hematopoietic stem and progenitor cells, were detected in peripheral blood before the IHT program (time point II: mean 1.6 ± 0.2 cells/μL; range 0.8 to 5.8 cells/μL). $CD45^+34^+$ cell count was unchanged 24 h after IHT (time point III) versus pre-IHT baseline, but by day 7 after IHT (time point IV), the count fell by 24% versus baseline and by 23% versus 24 h post-IHT (Fig. 1A). Neither total leukocyte count nor counts of segmentonuclear leukocytes (i.e., neutrophils and eosinophils), monocytes, or lymphocytes changed 24 h after the IHT program versus the respective baseline values, but segmentonuclear leukocytes fell by 13% and monocytes by 40% at day 7 post-IHT versus the respective pre-IHT counts (Table 2). $CD4^+$- and $CD8^+$- lymphocyte counts did not change.

Neither hemoglobin content, erythrocyte counts, nor reticulocyte counts changed from pre-IHT to 24 h after the IHT program, but erythrocyte count increased slightly and reticulocyte count fell by 15% within 7 days after IHT (Table 2). A modest increase in circulating platelet count was detected at 24 h and day 7 post-IHT versus pre-IHT (Table 2).

The IHT program modulated phagocytic and bactericidal activities of neutrophils. Phagocytic activity increased 7 days after IHT versus the earlier time points (Fig. 2A). Spontaneous

Table 1. Acute Effects of 5-Minute Hypoxia on Cardiorespiratory Function

Parameters	*Rest*	*Hypoxia*		*Recovery*
		3 min	*5 min*	*5 min*
HR, min^{-1}	73.5 ± 5.1	79.7 ± 6.8	86.5 ± 6.3*	72.8 ± 4.7
SBP, mmHg	116 ± 8	127 ± 14	132 ± 12	118 ± 8
DBP, mmHg	75 ± 6	78 ± 7	80 ± 8	76 ± 6
f, min^{-1}	11.1 ± 0.8	13.4 ± 1.3*	11.8 ± 1.1	10.7 ± 0.7
V_E, L/min	7.6 ± 0.7	10.9 ± 1.0*	9.9 ± 0.8*	7.9 ± 0.6
Sao_2, %	98.8 ± 0.7	87.5 ± 3.5**	84.6 ± 4.0**	98.6 ± 0.7
$P_{ET}o_2$, mmHg	104.5 ± 2.1	58.8 ± 7.4**	54.7 ± 6.8**	102.5 ± 1.8
$P_{ET}co_2$, mmHg	38.6 ± 0.6	39.4 ± 1.7	37.8 ± 1.9	38.2 ± 0.8

Values (mean ± SD) were obtained from 10 healthy young men (group I) during the first 5-min hypoxia cycle on day 1 of the intermittent hypoxia treatment (IHT) program. HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; f, breathing frequency; V_E expired minute ventilation; Sao_2, blood arterial oxygen saturation; $P_{ET}o_2$, end-tidal Po_2; $P_{ET}co_2$, end-tidal Pco_2. *$p < 0.05$ versus *r*est; **$p < 0.01$ versus rest.

Table 2. Effects of 14-Day Intermittent Hypoxia Treatment (IHT) on Cellular and Humoral Components of Innate Immunity

Parameters	*Time I 2 weeks before IHT*	*Time II 1 day before IHT*	*Time III 1 day after IHT*	*Time IV 1 week after IHT*
		White blood cells		
TLC, 10^9/L	5.7 ± 1.4	5.9 ± 1.2	5.8 ± 1.1	5.5 ± 0.8
SNL, 10^9/mL	2.9 ± 1.1	3.2 ± 0.8	3.2 ± 1.2	2.8 ± 0.6†
Monocytes, 10^9/L	0.48 ± 0.21	0.48 ± 0.29	0.36 ± 0.22	0.29 ± 0.14*
Lymphocytes, 10^9/L	2.1 ± 0.7	1.9 ± 00.7	1.7 ± 0.6	2.0 ± 0.6‡
$CD4^+$, 10^9/L	0.83 ± 0.35	0.72 ± 0.25	0.75 ± 0.36	0.81 ± 0.32
$CD8^+$, 10^9/L	0.50 ± 0.16	0.47 ± 0.23	0.43 ± 0.15	0.50 ± 0.22
		Red blood cells and platelets		
Hemoglobin, g/L	146 ± 4	144 ± 4	150 ± 4	148 ± 3
Erythrocytes, 10^{12}/L	4.6 ± 0.3	4.6 ± 0.3	4.4 ± 0.4	4.8 ± 0.2‡
Reticulocytes, %	1.40 ± 0.48	1.36 ± 0.59	1.30 ± 0.36	1.16 ± 0.44*
Platelets, 10^9/L	151 ± 29	158 ± 28	172 ± 38*	170 ± 30
		CIC, complement and immunoglobulins		
CIC, optical density units	41.6 ± 10.2	39.0 ± 12.2	32.8 ± 7.1*†	38.2 ± 6.6‡
Complement, mL^{-1}	22.6 ± 14.2	22.8 ± 12.1	39.3 ± 11.4*†	39.0 ± 13.4*†
IgG, g/L	12.7 ± 5.1	11.4 ± 3.2	11.9 ± 3.0	13.5 ± 2.1†‡
IgM, g/L	1.36 ± 0.33	1.35 ± 0.76	1.44 ± 0.30	1.53 ± 0.58*
IgA, g/L	2.4 ± 0.72	2.2 ± 0.72	2.3 ± 0.97	2.7 ± 0.59†‡

Mean values ± SD. TLC, total leukocyte count; SNL, segmentonuclear leukocytes; CIC, circulating immune complexes. *$p < 0.05$ versus time I; †$p < 0.05$ versus time II; ‡$p < 0.05$ versus time III.

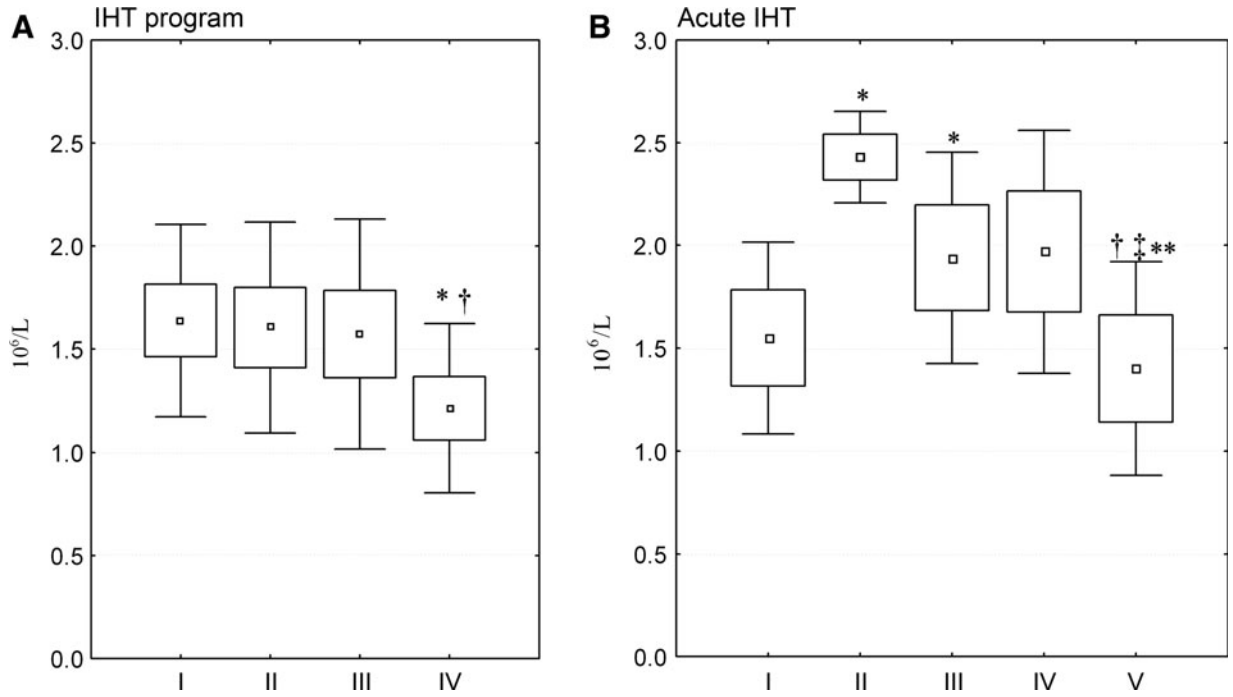

FIG. 1. Effects of intermittent hypoxia treatment (IHT) program (**A**) and acute intermittent hypoxia (**B**) on hematopoietic stem and progenitor cells in human peripheral blood. (**A**) Time I: 2 weeks before IHT; time II: 1 day before IHT; time III: 1 day after the 14-day IHT program; time IV: 7 days after the IHT program. (**B**) Time I: 5 min before the hypoxia session; times II and III: during the final 15 to 20 s of the second and fourth hypoxia bouts, respectively; times IV and V: 15 and 30 min, respectively, after the fourth hypoxia exposure. The boxes define the lower and higher quartiles, and the bars the lowest and highest individual values from 10 (**A**) or 5 (**B**) subjects. *$p < 0.05$ versus I; †$p < 0.05$ versus II; ‡$p < 0.05$ versus III; **$p < 0.05$ versus IV.

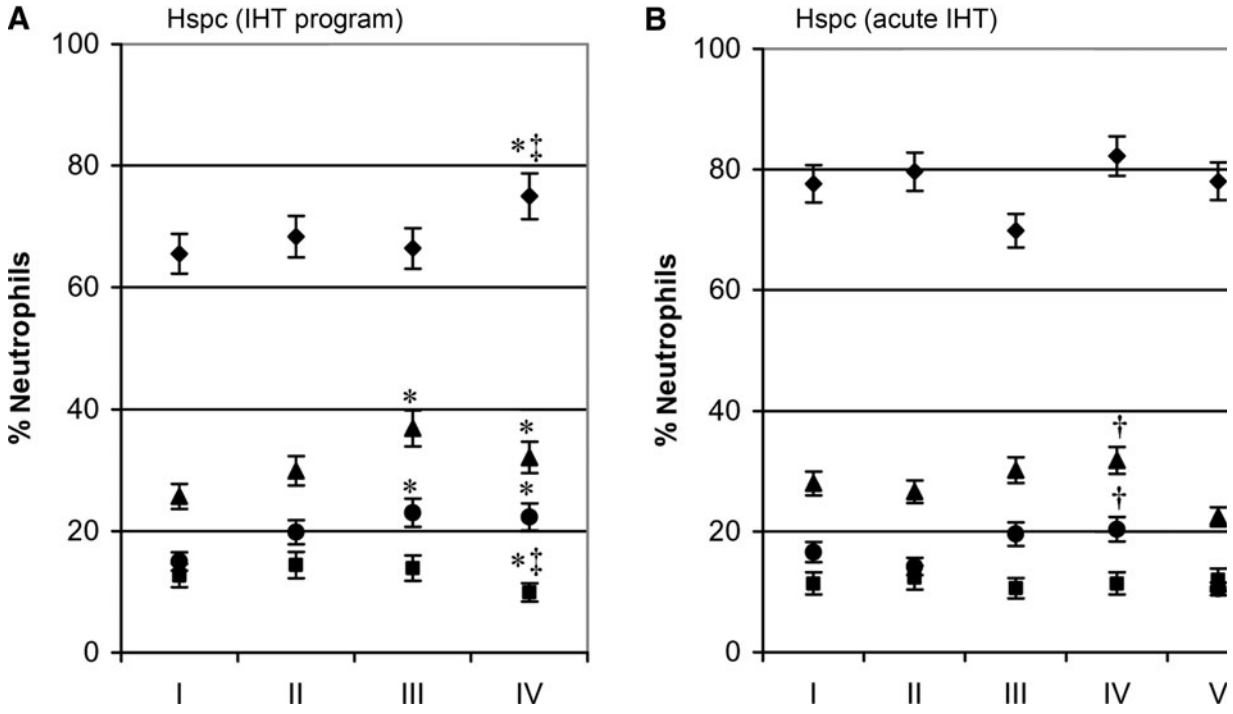

FIG. 2. Effects of intermittent hypoxia treatment (IHT) program (**A**) and acute intermittent hypoxia (**B**) on phagocytic and bactericidal activities of blood neutrophils. Values (means ± SD) are from the same subjects as in Fig. 1. Time points and significance symbols are as defined in Fig. 1. PhA, phagocytic activity; NBT-TS, NBT-test spontaneous; NBT-TI, NBT-test induced; NBT-TR, NBT-test reserve.

bactericidal action fell over the 7 days after IHT, while induced and reserve bactericidity increased at 24 h and day 7 post-IHT (Fig. 2A). Circulating immune complexes fell by 16% at 24 h post-IHT versus pre-IHT and then recovered by day 7 post-IHT (Table 2). Complement activity substantially increased over the same period, by 72% and 71% at 24 h and day7 post-IHT, respectively, versus the pre-IHT value (Table 2). Circulating IgG, IgM, and IgA increased by 18%, 13%, and 22%, at day 7 after IHT versus respective pre-IHT values (Table 2).

The IHT regimen had a marked effect on circulating cytokine concentrations. The proinflammatory cytokines TNF-α (Fig. 3A) and IL-4 (Fig. 3B) fell sharply at 24 h and day 7 post-IHT versus respective pre-IHT baselines. In contrast, although circulating IFN-γ did not change at 24 h, its concentration doubled by day 7 post-IHT (Fig. 3C). The hematopoietic, anti-inflammatory cytokine erythropoietin did not change significantly during IHT course, although a slight upward trend was noted. However, circulating EPO concentration decreased significantly over the first 24 h after completing the IHT program (Fig. 3D).

Group 2: acute effects of intermittent hypoxia

In contrast to 14-day IHT, a single IHT session (Fig. 1B) increased HSC by 55% and 19% during the second (time point II) and fourth (time point IV) hypoxic cycles, respectively, versus pre-IHT (time point I); this effect subsided within 30 min after IHT (time point V). Counts of total leukocytes, segmentonuclear leukocytes, monocytes, and lymphocytes also increased during IHT and then normalized within 15 min after the IHT session (Table 3). Contents of $CD4^+$ and $CD8^+$ lymphocytes were unchanged by acute IHTand recovery.

Neither hemoglobin content nor erythrocyte counts were altered by acute IHT, but the reticulocyte fraction, which did not change during the hypoxia exposures, sharply increased 15 min after the IHT session before subsiding (Table 3). At the same time, counts of leucocytes, neutrophils, lymphocytes, and monocytes also increased. This response was accompanied by increases in inducible and reserve bactericidal activity of circulating neutrophils (Fig. 2B) and a modest, transitory decline in circulating IgM (Table 3). Neither IgA, IgG, nor circulating complement were affected by acute IHT or recovery.

Discussion

This study is the first to investigate the effect of brief cycles of intermittent hypoxia–reoxygenation on the innate immune system in human subjects. A 2-week program of normobaric, cyclic, 5-min exposures to 10% O_2 decreased circulating HSPC, activated complement, increased circulating platelets, and augmented phagocytic and bactericidal activities of neutrophils, while suppressing proinflammatory cytokines. These responses, which persisted at least 7 days after intermittent hypoxia training, may serve to augment the body's immune defenses without exacerbating inflammation.

Hypoxia and hematopoietic stem cells

Tissue O_2 content determines the balance between pluripotency and differentiation of stem cells (Ong et al., 2010). Many stem cell types, including hematopoietic stem cells, reside in hypoxic niches within the host tissues (Eliasson and

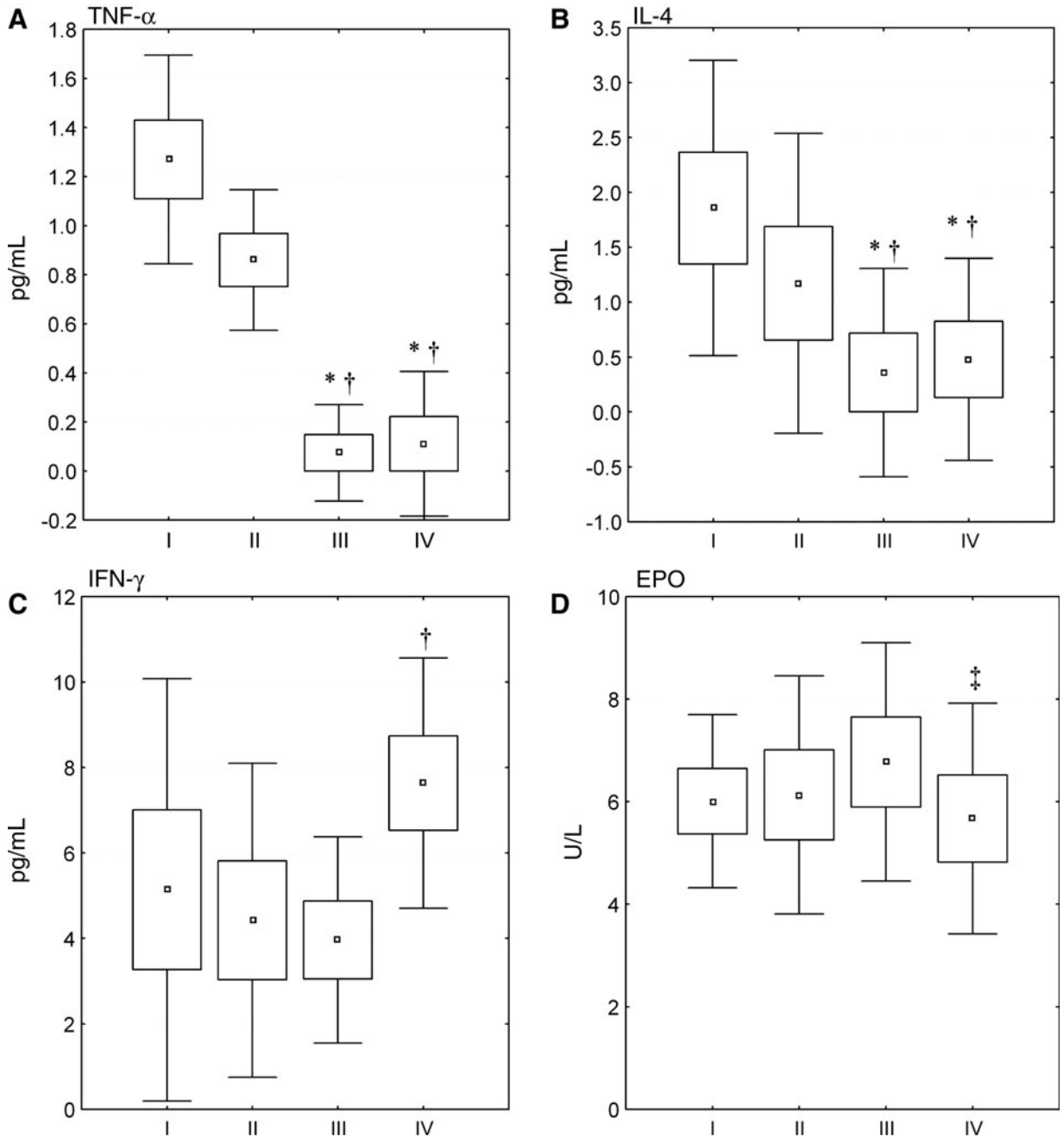

FIG. 3. Effects of intermittent hypoxia treatment (IHT) on cytokine contents in human peripheral blood. (**A**) TNF-α, tumor necrosis factor-α; (**B**) IL-4, interleukin-4; (**C**) IFN-γ, interferon-γ; (**D**) EPO, erythropoietin. Times I to IV are the same as in Fig. 1A. Values are median ± percentiles (boxes) and ± nonoutlier range (bars) from 10 subjects. $^{*}p < 0.05$ versus I; $^{\dagger}p < 0.05$ versus II; $^{\ddagger}p < 0.05$ versus III.

Jönsson 2010; Eliasson et al., 2010; Voog and Jones, 2010). The hypoxic environment helps maintain stem cells in a quiescent but self-renewing state. Hypoxia maintains HSC pluripotency by suppressing NADPH oxidase formation of reactive oxygen species (Fan et al., 2007) and protects the cells against toxic and mutagenic effects of oxyradicals.

Exposure to hypoxia could augment the stem cell system. Theiss and colleagues (2008) demonstrated that 1-week sojourns at moderate altitude (1700 m) with physical activity increased circulating progenitor cell counts in healthy adults. Ciulla and colleagues (2005) reported increased numbers of mature endothelial progenitor cells and endothelial colony-forming unit capacity in a healthy subject after a trek in the Himalayas. But another study in healthy adults (Mancuso et al., 2008) revealed declines in circulating HSC, endothelial cells, and progenitor cells after 12 days at 3000-m altitude. Recently, Viscor and colleagues (2009) reported no appreciable changes in circulating leukocytes or $CD34^{+}$ cells after 3 consecutive days of 3-h exposure to 405-mmHg barometric pressure, equivalent to 5000-m altitude. Thus, evidence is equivocal regarding the effects of sustained or prolonged intermittent hypoxia bouts on circulating HSPC counts.

Table 3. Effects of Acute Intermittent Hypoxia (IH) on Cellular and Humoral Components of Innate Immunity

Parameters	*Time I: 15 min before IH*	*Time II: 2nd IH cycle*	*Time III: 4th IH cycle*	*Time IV: 15 min after IH*	*Time V: 30 min after IH*
			White blood cells		
TLC, 10^9/L	5.6 ± 0.9	6.5 ± 1.1*	6.4 ± 1.1	5.4 ± 0.6†	5.6 ± 1.3‡
SNL, 10^9/mL	2.7 ± 0.5	3.1 ± 0.2*	3.0 ± 0.6*	2.6 ± 0.3†‡	2.6 ± 0.3‡
Monocytes, 10^9/L	0.33 ± 0.1	0.38 ± 0.1	0.45 ± 0.3*	0.31 ± 0.1‡	0.30 ± 0.2‡
Lymphocytes, 10^9/L	2.2 ± 0.8	2.6 ± 0.9	2.6 ± 0.7*	2.1 ± 0.6‡	2.2 ± 0.9
$CD4^+$, 10^9/L	0.93 ± 0.44	1.02 ± 0.41	1.01 ± 0.36	0.90 ± 0.32	1.05 ± 0.41
$CD8^+$, 10^9/L	0.59 ± 0.29	0.74 ± 0.36	0.70 ± 026	0.57 ± 0.22	0.55 ± 0.33
			Red blood cells and platelets		
Hemoglobin, g/L	148.6 ± 9.6	145.0 ± 8.3	148.0 ± 8.5	153.8 ± 12.0	149.3 ± 13.0
Erythrocytes, 10^{12}/L	5.0 ± 0.3	4.8 ± 0.4	5.0 ± 0.5	5.1 ± 0.5	4.9 ± 0.3
Reticulocytes, %	0.82 ± 0.26	1.00 ± 0.34	1.04 ± 0.35	1.72 ± 0.72*	0.98 ± 0.25
Platelets, 10^9/L	158 ± 41	156 ± 20	167 ± 32	152 ± 23	153 ± 22
			CIC, complement and immunoglobulins		
CIC, optical density units	27.2 ± 4.8	22.5 ± 5.1*	23.8 ± 5.9	25.1 ± 7.4	25.4 ± 3.0
Complement, mL^{-1}	35.0 ± 13.7	30.0 ± 11.2	30.0 ± 11.2	30.0 ± 11.2	25.0 ± 11.2
IgG, g/L	13.0 ± 1.8	12.2 ± 2.8	11.8 ± 2.4	12.6 ± 1.6	12.1 ± 2.2
IgM, g/L	1.40 ± 0.74	1.27 ± 0.60*	1.32 ± 0.58*	1.20 ± 0.61*†	1.28 ± 0.38*
IgA, g/L	2.86 ± 0.58	2.67 ± 0.22	2.69 ± 0.64	2.69 ± 0.56	2.72 ± 0.67

Mean values ± SD. TLC, total leukocyte count; SNL, segmentonuclear leukocytes; CIC, circulating immune complexes. *$p < 0.1$ versus time I; $^{\dagger}p < 0.1$ versus time II; $^{\ddagger}p < 0.1$ versus time III.

Cyclic, intermittent hypoxia–reoxygenation may be a more potent trigger of transcriptional activation than continuous hypoxia. As reviewed by Nanduri and colleagues (2008), intermittent hypoxia activates hypoxia-inducible factor-1 (HIF-1), the immediate early gene *c-fos*, activator protein-1, nuclear factor kappa-B, and cAMP response element binding protein. It also induces expression of the proteins associated with neuron survival and apoptosis and modulates protein function by posttranslational modifications. Comparison of continuous and intermittent hypoxia showed pronounced differences in both the kinetics of protein kinase activation and the responsive protein kinase subtypes. Panchision (2009) demonstrated in cultured neuronal stem cells that at lower oxygen tensions HIF-1 facilitates signal transduction pathways that promote self-renewal and inhibits pathways that promote cellular differentiation or apoptosis. Indeed, controlled hypoxia can stimulate the proliferation of hematopoietic stem cells in their niche, and HIF-1 appears to play a significant role in their maintenance and homing mechanism (Lekli et al., 2009).

The decrease in HSPC in peripheral circulation after the 14-day IHT regimen is probably associated with changes in HSPC migration capacity. As yet, it remains unclear whether IHT inhibits HSPC migration into circulation and/or activates HSPC efflux from the circulation. In both cases, tissues could accumulate HSPC, which in turn could enhance hematopoiesis and general regenerative potential. In contrast to the 14-day program, a single IHT session provoked appreciable yet transitory increases in circulating HSPC, which quickly subsided after IHT. These results raise the possibility of hypoxia-induced HSPC emigration from niches into the circulation, followed by homing and sequestration in target tissues during post-IHT recovery.

Intermittent hypoxia and circulating formed elements

Like the HSPC, leukocyte and reticulocyte counts also increased, albeit only temporarily, during the single IHT session. It is possible to attribute these changes to hypoxia-mediated release of these cells from the bone-marrow reserve by the sinusoids. Although the present IHT program did not alter circulating erythrocyte counts or blood hemoglobin content, circulating reticulocytes fell 1 week after IHT, presumably owing to their maturation to erythrocytes. Other reports indicate that IHT did not accelerate erythropoiesis despite the increase in serum erythropoietin (Villa et al., 2005; Gore et al., 2006; Abellan et al., 2007). Reticulocytes are considered the most reliable hematology parameters for detecting bone-marrow stimulation (Banfi, 2008), yet data concerning hypoxia effects on reticulocytes are contradictory, possibly owing to the variety of hypoxia regimens employed (Rodríguez et al., 2000; Julian et al., 2004; Abellán et al., 2005; Lundby et al., 2005; Launay et al., 2006).

A substantial increase of circulating platelets was seen immediately after IHT, and an upward trend was noted after a single IH session. These results are consistent with hypoxia induction of erythropoietin-activated megakaryopoiesis (Beguin, 1999). In mice, the hypoxia-inducible cytokine erythropoietin exerted a biphasic effect on circulating platelets: moderate doses of exogenous erythropoietin augmented, but chronic, higher doses suppressed platelet counts (McDonald et al., 1992; Beguin, 1999). This dose-dependent response to the cytokine may explain the divergent effects of short- versus long-term hypoxia exposures on circulating platelets. In mice, brief, cyclic hypoxic episodes induced thrombocythemia, in parallel with moderate induction of erythropoietin (Beguin, 1999). In contrast, prolonged hypoxia, which provoked

intense erythropoietin formation, caused thrombocytopenia (Cottrell et al., 1991). Accordingly, the present IHT regimen of brief, cyclic exposures to moderate hypoxia augmented circulating platelets in human subjects; however, more sustained or severe hypoxia might not produce the same effect.

Humoral components of innate defense

In addition to cells, humoral components of nonspecific resistance also responded to brief, cyclic hypoxia. We previously demonstrated that hypoxic training increased NADPH oxidase activity and cationic protein content in neutrophils and modulated myeloperoxidase activity (Serebrovskaya et al., 1996). Hitomi and colleagues (2003) later confirmed that intermittent hypobaric hypoxia induces granulocytosis and potentiates the ability of neutrophils to generate O_2^-. Interestingly, a single IHT session elicited changes in cellular and humoral immunity similar to the 14-day IHT program. Considering that the oxidative burst is essential for phagocyte bactericidity, it seems likely that both acute and chronic IHT activates oxygen-dependent components of innate immunity.

Hematopoietic stem cell migration into the circulation and hematopoietic tissues is regulated by chemokines and cytokines (Muench et al., 2000; Krstić et al., 2009). Our study demonstrates that IHT suppresses IL-4 and TNF-α contents and sharply increases IFN-γ without altering erythropoietin content. Wang and colleagues (2007) also reported that severe versus moderate intermittent hypoxia provoked divergent changes in plasma interleukins. Interferons regulate a multitude of cell-mediated immune responses. Among these key immunoregulatory functions of interferons is the ability to augment natural killer cell activity, an essential part of the early defense mechanism against infections or tumor development (Djeu et al., 1982). TNF-α, a cytokine produced during infection, injury, or invasion, has proved pivotal in triggering the lethal effects of septic shock syndrome, cachexia, and other systemic manifestations of disease. The IHT-induced decrease in blood TNF-α content with simultaneous increase in IFN-γ could contribute to the moderation of infectious inflammatory processes.

Although the activities of the T lymphocyte subpopulations Th1 and Th2 were not assessed directly, changes in circulating activities of the cytokines IL-4 and IFN-γ suggest that IHT may have shifted the Th1:Th2 balance in favor of Th1. IL-4 activity fell sharply at 1 and 7 days post-IHT versus pre-IHT baseline, while IFN-γ activity concomitantly increased. Because Th2 lymphocytes are the main sources of IL-4 (Renauld, 2001; Romagnani, 2006; Gilmour and Lavender, 2008) and IL-4 is a growth factor for Th2 (Romagnani, 2006; Gilmour and Lavender, 2008) and suppressor of Th1 (Fujiwara and Kobayashi, 2005), these results suggest that Th2 lymphocytes may be suppressed following IHT. Th1 and Th2 are reciprocally related (Mühl and Pfeilschifter, 2003; Onoé et al., 2007), so Th1 activity likely remains intact or even increases under these conditions, in accordance with the post-IHT augmentation of IFN-γ, which is produced by Th1 (Rocha et al., 2008) and represses Th2 lymphocytes (Bowen et al., 2008). Th2 lymphocytes play central roles in the pathogenesis of bronchial asthma (Larché et al., 2003). Thus, the shift from Th2 to Th1 could be pivotal to the enhancement of ventilation reported in asthmatic patients following IHT (Redzhebova and Chizhov, 1992; Serebrovskaya et al., 2003; Vogtel and Michels, 2010).

Summary

The current IHT regimen decreases the content of hematopoetic stem cells in human peripheral blood 1week after its completion and modulates both cellular and humoral immunity. Cellular adaptations include increased phagocytic activity, decreased spontaneous bactericidity, and increased reserve bactericidity of neutrophils, indicating a heightened capacity to combat infection. Changes in humoral components also bolster innate immunity, especially the increases in complement activity and circulating immunoglobulins. Although this study was conducted in healthy young men, these results provide empirical support for the eventual clinical application of IHT in immunologically compromised patients or those at risk of infection. Of importance in this regard, the safety of this IHT program in healthy senior men, ages 60 to 74, has been documented (Shatilo et al., 2008).

Acknowledgments

This work was supported by grant #4299 from the Science and Technology Center in Ukraine. The authors are indebted to Professor Gennadiy Butenko, Professor Oleg Korkushko, and Dr. Valeriy Shatilo of the Institute of Gerontology, Academy of Medical Sciences of the Ukraine, and Dr. Lisa M. Hodge, Department of Molecular Biology and Immunology, University of North Texas Health Science Center, for their helpful suggestions and assistance. Drs. Lubov Taranukha, Vitaliy Kirik, and Svitlana Halytska and the staff of the Institute of Genetic and Regenerative Medicine, Kiev, provided skilful analytical expertise.

Disclosures

The authors have no conflicts of interest or financial ties to disclose.

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Received November 22, 2010;
accepted in final form March 1, 2011.