Intermittent hypoxic training: implications for lipid peroxidation induced by acute normoxic exercise in active men

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ABSTRACT

Oxidant generation during regular physical exercise training may influence the adaptive responses that have been shown to confer protection against oxidative stress induced by subsequent acute exercise. To examine this, we randomly assigned 32 males to either a normoxic (n = 14) or a hypoxic (n = 18) group. During the acute phase, subjects in the hypoxic group performed two maximal cycling tests in a randomized double-blind fashion: one under conditions of normoxia and the other under hypoxic conditions (inspired fraction of O₂ \( f_{O_2} \) 0.21 and 0.16 respectively). During the intermittent phase, the normoxic and hypoxic groups each trained for 4 weeks at the same relative exercise intensity, under conditions of normoxia and hypoxia respectively. During acute exercise under hypoxic conditions, the venous concentrations of lipid hydroperoxides and malondialdehyde were increased, despite a comparatively lower maximal oxygen uptake (\( V_{O_2}^{\text{max}} \)) (\( P < 0.05 \) compared with normoxia). The increases in lipid hydroperoxides and malondialdehyde correlated with the exercise-induced decrease in arterial haemoglobin oxygen saturation (\( r = -0.61 \) and \( r = -0.50 \) respectively; \( P < 0.05 \)), but not with \( V_{O_2}^{\text{max}} \). Intermittent hypoxic training attenuated the increases in lipid hydroperoxides and malondialdehyde induced by acute normoxic exercise more effectively than did normoxic training, due to a selective mobilization of \( \alpha \)-tocopherol (\( P < 0.05 \)). The latter was related to enhanced exercise-induced mobilization/oxidation of blood lipids due to a selective increase in \( V_{O_2}^{\text{max}} \) (\( P < 0.05 \) compared with normoxic group). We conclude that lipid peroxidation induced by acute exercise (1) increases during hypoxia; (2) is not regulated exclusively by a mass action effect of \( V_{O_2} \); and (3) is selectively attenuated by regular hypoxic training. Oxidative stress may thus be considered as a biological prerequisite for adaptation to physical stress in humans.

INTRODUCTION

Research over the last two decades has focused on the potentially adverse effects of reactive oxygen species (ROS) and reactive nitrogen species, and their potential role, be it causal or consequential, in the pathology of disease. However, accumulating evidence suggests that these biomolecules are physiologically essential in controlled amounts due to their contributions to vascular regulation, cell growth and proliferation, gene transcription, mitochondrial biogenesis and cell signalling [1,2]. This concept is consistent with the General Adaptation

Key words: adaptation, antioxidants, exercise, hypoxia, lipid peroxidation.

Abbreviations: CV, coefficient of variation; \( f_{O_2} \), inspired fraction of oxygen; GAS, General Adaptation Syndrome; HDL-C, high-density lipoprotein cholesterol; HR, heart rate; LDL, low-density lipoprotein; LDL-C, LDL cholesterol; LH, lipid hydroperoxides; MDA, malondialdehyde; NEFA, non-esterified fatty acids; Pre and Post, before and after 4 weeks of incremental normoxic or hypoxic training respectively; \( P_{O_2} \), partial pressure of oxygen; ROS, reactive oxygen species; \( S_aO_2 \), arterial haemoglobin oxygen saturation; \( V_{O_2} \), oxygen uptake.

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Syndrome (GAS) developed by Selye [3], which emphasizes the physiological significance of stress and the basic need to disrupt the biological homoeostasis of a living system in order to invoke adaptation. This model can be applied to a variety of physiological settings, and is especially pertinent when attempting to understand responses in humans to the metabolic stress imposed by free radicals.

Acute physical exercise can be considered as one such stressor, with the potential to increase ROS, primarily through mitochondrial superoxide and hydrogen peroxide generation, but also via other mechanisms, such as ischaemia–reperfusion, catecholamine auto-oxidation and neutrophil activation [4]. Consistent with the GAS, regular exercise training has been shown to increase the endogenous production of antioxidants or improve mitochondrial respiratory control, and to provide subsequent protection against further exercise-induced oxidative damage [5]. Such benefits appear to be enhanced when training volume and intensity increase [6], assuming that the adaptive phase is adequate, which suggests the intriguing possibility that the magnitude of ROS generation may to some extent regulate the magnitude of protective adaptation.

Hypoxia, a potent environmental stressor, has also been shown to increase ROS in animals, due primarily to an accumulation of reducing equivalents that cannot be transferred to $O_2$ at the mitochondrial cytochrome oxidase due to a decrease in cellular respiration, an emerging phenomenon known as reductive stress [7]. Other potential sources of ROS generation due to hypoxia include increased xanthine oxidase and phospholipase A$_2$ activation, enhanced nitric oxide release, increased availability of free iron and the release of oxygen radicals from erythrocytes [8]. Human studies are indirectly supportive of a pro-oxidant role for environmental hypoxia, with marked increases in putative metabolic ‘footprints’ of free radical-mediated lipid peroxidation observed at terrestrial high altitude [9]. However, despite an undoubted decrease in the mitochondrial partial pressure of oxygen ($P_{O_2}$), the confounding and potentially pro-oxidant influences of increases in physical activity, UVA/UVB radiation, dehydration and malnutrition during an altitude sojourn cannot be ignored. Whether hypoxia per se increases ROS generation in humans remains to be established.

Therefore the present investigation was designed to isolate the two stressors and to examine independently the effects of acute hypoxia and physical exercise on metabolic indices of lipid peroxidation in a cohort of physically active males. We also investigated the oxidative stress response to an acute bout of normoxic exercise following a 4-week period of intermittent normoxic and hypoxic training. In accordance with the GAS, we hypothesized that exercise-induced oxidant generation would be compounded further by acute hypoxia which, over time, would be expected to activate counter-regulatory adaptations in an attempt to re-establish homoeostasis. Thus we anticipated that the magnitude of protective adaptation would be more marked after regular hypoxic adaptation compared with normoxic training.

**METHODS**

**Subjects**

A total of 32 Caucasian male University students volunteered for a randomized double-blind placebo-controlled study, which had been ethically approved by Bro Taff Health Authority (South Wales, U.K.). All participants were non-smokers and were free of any overt physical symptoms of poor health. They were 22±3 years old (mean ± S.D.), with a maximal aerobic capacity ($\dot{V}O_{2\text{max}}$) in normoxia of 50±9 ml·min$^{-1}$·kg$^{-1}$. Any antioxidant vitamin or food supplements were excluded 4 weeks prior to experimentation, and participants were subsequently instructed to maintain their normal dietary and lifestyle habits throughout the investigative period.

**Experimental design**

Participants were assigned randomly in a double-blind manner to either a normoxic ($n = 14$) or a hypoxic ($n = 18$) group; the groups were matched for age, body composition and $\dot{V}O_{2\text{max}}$. A pictorial representation of the procedures conducted during the acute and intermittent protocols (described in detail below) is presented in Figure 1.

**Acute protocol**

Only the hypoxic group participated in the acute protocol, which was designed to quantify changes in metabolic indices of lipid peroxidation mediated by acute hypoxia and physical exercise per se. Each participant was assigned randomly in a double-blind fashion to perform both a normobaric normoxic test [$F_iO_2$ (inspired fraction of $O_2$) = 0.21±0.10] and a normobaric hypoxic test ($F_iO_2$ = 0.16±0.30); tests were separated by 48 h to ensure full metabolic recovery, as established previously in pilot studies (D. M. Bailey, unpublished work). Each test involved 30 min of seated rest (rest phase) preceded by a standardized incremental cycling test to volitional exhaustion (exercise phase) for the measurement of absolute oxygen uptake ($\dot{V}O_2$; via a Douglas bag method), heart rate (HR; via a three-lead ECG), arterial haemoglobin oxygen saturation ($SaO_2$; via carilobe pulse oximetry), whole-blood lactate (via a semi-automated analyser) and exercise time to exhaustion according to standard methods that have been described in detail previously [10].

Medical-grade-quality gas (British Oxygen Company Gases, Guildford, Surrey, U.K.) was directed from
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Figure 1 Experimental design

(A) Acute protocol (n = 18); (B) intermittent protocol (n = 32). Arrows indicate the timing of venous samples.

Compressed gas cylinders that contained either a normoxic ($F_{O_2} = \sim 0.21$; balanced $N_2$) or a hypoxic ($F_{O_2} = \sim 0.16$; balanced $N_2$) mixture into a series of 1000-litre meteorological balloons at the prevailing barometric pressure. The respective normobaric inspirates were subsequently presented to each subject in a double-blind manner using a four-way valve and a 2 m length of Falconia tubing (Cranleigh, Birmingham, U.K.) connected to the inspiratory port of a two-way non-rebreathing valve (2400 series; Hans Rudolph, Wyandotte, Kansas City, MO, U.S.A.).

Intermittent protocol

Physiological tests were conducted before (Pre) and after (Post) 4 weeks of incremental normoxic or hypoxic training (see below) to assess whether intermittent exposure to hypoxia affected resting and exercise-induced markers of lipid peroxidation in normoxia, independently of the physical exercise component.

All participants performed three supervised cycling sessions per week on alternate days, while being exposed continuously to the respective inspirate (normoxia for the normoxic group; hypoxia for the hypoxic group). Both groups exercised for the same duration and frequency, and at the same relative exercise intensity (i.e. same percentage of either the normoxic or the hypoxic $HR_{max}$ established previously during the Pre tests). Each session incorporated 20 min of cycling at 70% and 75% of $HR_{max}$ during weeks 1 and 2 respectively, which was increased subsequently to 30 min at 80% and 85% of $HR_{max}$ during weeks 3 and 4 respectively. The inspired $P_{O_2}$ during the hypoxic tests ranged between 111 and 118 mmHg, and that during the normoxic tests was in the range 147–152 mmHg.

Caloric intake and composition

A self-reporting dietary analysis (NutriCheck; Health Options Ltd, Bournemouth, U.K.) was completed in the presence of a qualified nutritionist 7 days before both...
the Pre and Post tests, and caloric intake/composition was analysed subsequently using standard food tables. Dietary characteristics before the Pre tests were identical for the two groups, and thus participants were instructed to maintain their normal dietary behaviour for the duration of the experimental period.

**Haematology**

Overnight fasted blood samples were obtained following 30 min of supine rest from an antecubital forearm vein using the Vacutainer™ method. Exercise was then begun immediately, and additional samples were obtained within 60 s of the subject achieving volitional exhaustion.

During the sampling periods, tourniquet constriction was standardized due to the potential increase in oxidative stress introduced by an ischaemia–reperfusion manoeuvre (D. M. Bailey, unpublished work). This was achieved by applying the tourniquet to the same anatomical site for precisely 60 s and tightening it so that its circumference was 4 cm less than the relaxed girth measured above the distal region of the biceps brachii. Venous blood was subsequently dispensed into EDTA or serum separation tubes. EDTA tubes were centrifuged immediately at 2500 g for 10 min at 4 °C, whereas the serum separation tubes were allowed to clot at room temperature for exactly 1 h before centrifugation. The serum or plasma supernatant was removed and stored at −70 °C until analysis.

Arterialized capillary samples were also collected for the determination of packed cell volume and haemoglobin, to correct venous metabolites for alterations in plasma volume induced by acute exercise or positional changes [11].

**Assays**

**Biomarkers of lipid peroxidation**

(i) Lipid hydroperoxides (LH). Serum LH were determined using the ferrous oxidation/Xylenol Orange (FOX) assay [12]. Briefly, this assay incorporates the selective oxidation of ferrous to ferric ions by hydroperoxides. This reaction yields a blue/purple-coloured complex due to the selective binding of Xylenol Orange to ferric ions. Catalase was added to prevent spontaneous LH generation during the ferrous oxidation step. Absorbance changes at 560 nm were monitored spectrophotometrically. The intra- and inter-assay coefficients of variation (CVs) for this assay are < 2.0% and < 4.0% respectively.

(ii) Malondialdehyde (MDA). Plasma MDA was measured according to the methods of Young and Trimble [13]. This HPLC method with fluorimetric detection is highly specific and incorporates rigorous steps to separate the thiobarbituric acid–MDA adduct from contaminating compounds, such as biliverdin, bilirubin, urea, creatinine and glucose, which may change as a function of hypoxia and/or physical exercise. This method measures total MDA, including MDA released from protein adducts during the incubation step of the assay. The intra- and inter-assay CVs are 6.2% and 9.1% respectively.

**Lipid-soluble antioxidants**

The plasma concentrations of α-tocopherol, retinol and the carotenoids lycopene, x-carotene and β-carotene were determined using an HPLC method [14,15]. The intra- and inter-assay CVs are both < 5.0%.

**Blood lipids/lipoproteins**

Enzymic assays were used to analyse the plasma concentrations of non-esterified fatty acids (NEFA) (Behring Diagnostics, La Jolla, CA, U.S.A.) and glycerol (Wako Chemicals G.m.b.H., Neuss, Germany). The intra- and inter-assay CVs for NEFA are 1.6% and 5.0% respectively, and those for glycerol are 2.0% and 5.0% respectively. Total cholesterol and triacylglycerol concentrations were determined by routine enzymic techniques using an Olympus AU5200 automated analyser and Olympus reagents (intra- and inter-assay CVs of 3.0% and 5.0% respectively). High-density lipoprotein cholesterol (HDL-C) was assayed enzymically after chemical precipitation of other lipoproteins from the serum with dextran sulphate and magnesium [16] (intra- and inter-assay CVs of ~ 3.0% and 6.0% respectively). Low-density lipoprotein cholesterol (LDL-C) was calculated as [17]:

\[
\text{LDL-C (mmol·l}^{-1}) = \text{total cholesterol} - (\text{triacylglycerols}/2.2) - \text{HDL-C}
\]

Apolipoproteins A1 and B were measured separately by rate immunonephelometry using a Beckmann ARRAY analyser incorporating Beckmann reagents (intra- and inter-assay CVs of 4.0% and 5.0% respectively).

**Statistics**

Parametric statistics were incorporated following mathematical confirmation of a normal distribution using repeated Kolmogorov–Smirnov tests. Physiological responses to maximal exercise during the acute protocol (Table 1) were assessed using an independent-samples t-test. A two-factor [state (rest vs. exercise) × condition (normoxic vs. hypoxic)] repeated-measures ANOVA assessed the oxidative stress response to acute hypoxia (Table 2). Anthropometric, dietary and blood lipid/lipoprotein data were assessed using a two-way mixed ANOVA that incorporated one between-subjects factor (group: normoxic vs. hypoxic) and one within-subjects factor (time: Pre vs. Post) (Tables 3 and 4). Oxidative stress responses to intermittent training (Table 5) were analysed using a three-way mixed ANOVA with one-between subjects factor (group) and two within-subjects factors (state and time). Following a simple main effect and interaction, Bonferroni-corrected paired-sample...
t-tests were employed to make a posteriori comparisons at each level of the between-subjects factor. Between-group comparisons were assessed using a one-way ANOVA with an a posteriori Tukey honestly significant difference (HSD) test at selected levels of within-subjects factors. Separate Pearson product moment correlations analysed the relationships between selected dependent variables (Figures 2 and 3). Significance for all two-tailed tests was established at an $\alpha$ level of $P < 0.05$, and data are expressed as means ± S.D.

**RESULTS**

**Effects of acute hypoxia**

**Physiological responses**
The data presented in Table 1 retrospectively confirm that subjects performed to their physiological maximum during both normoxic and hypoxic acute exercise tests. When compared with normoxia, hypoxic exercise decreased $\dot{V}O_2$, $\dot{V}CO_2$, power output and performance time to exhaustion during the maximal exercise test ($P < 0.05$).

**Table 2** Effects of acute hypoxia on selected markers of lipid peroxidation and lipid-soluble antioxidants

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Main effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td>1.09 ± 0.32</td>
<td>1.18 ± 0.26</td>
<td>1.43 ± 0.58</td>
<td>1.73 ± 0.48</td>
<td>Condition and state</td>
</tr>
<tr>
<td>MDA</td>
<td>0.49 ± 0.21</td>
<td>0.57 ± 0.25</td>
<td>0.74 ± 0.28</td>
<td>0.91 ± 0.40</td>
<td>Condition and state</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>21.7 ± 6.7</td>
<td>22.6 ± 7.2</td>
<td>21.2 ± 5.7</td>
<td>24.9 ± 7.2</td>
<td>Condition</td>
</tr>
<tr>
<td>Retinol</td>
<td>1.37 ± 0.41</td>
<td>1.40 ± 0.41</td>
<td>1.28 ± 0.31</td>
<td>1.41 ± 0.31</td>
<td>—</td>
</tr>
<tr>
<td>Lycopene</td>
<td>0.73 ± 0.40</td>
<td>0.75 ± 0.38</td>
<td>0.97 ± 0.55</td>
<td>0.88 ± 0.42</td>
<td>State</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>0.107 ± 0.079</td>
<td>0.115 ± 0.085</td>
<td>0.101 ± 0.072</td>
<td>0.127 ± 0.089</td>
<td>—</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.26 ± 0.16</td>
<td>0.29 ± 0.17</td>
<td>0.52 ± 0.54</td>
<td>0.53 ± 0.30</td>
<td>State</td>
</tr>
</tbody>
</table>

*Metabolites are indicated by a, which signifies a difference ($P < 0.05$) between normoxia compared with hypoxia (pooling rest + exercise values); main effect for state signifies a difference ($P < 0.05$) between rest compared with exercise (pooling normoxia + hypoxia values).

**Figure 2** Lipid peroxidation normalized for $\dot{V}O_{2max}$ and cumulative $\dot{V}O_2$ during acute hypoxic exercise ($n = 18$ for subjects in the hypoxic group)
The peroxidation ratio was calculated by dividing maximal values of LH and MDA by either the absolute $\dot{V}O_{2max}$ at volitional exhaustion or the cumulative $\dot{V}O_2$ required to complete the entire normoxic or hypoxic test. A difference between conditions (normoxia compared with hypoxia; $P < 0.05$) as a function of metabolite and method is indicated by *.

Further evidence of an apparent lack of association between the increased LH ($r = 0.70, P < 0.05$). No associations were observed between the increases in LH/MDA and absolute normoxic or hypoxic $\dot{V}O_{2max}$. Further evidence of an apparent lack of association between $\dot{V}O_2$ and lipid peroxidation is illustrated in Figure 2, which demonstrates clear differences in the exercise-induced increases in LH and MDA when the data were normalized for absolute $\dot{V}O_{2max}$ and cumulative metabolic responses.

Table 2 demonstrates a clear increase in lipid peroxidation during the acute hypoxic trial. The increases in LH and MDA were apparent despite an exercise-induced mobilization of α-tocopherol ($P < 0.05$ compared with normoxia). The increase in α-tocopherol was correlated with the increase in LH ($r = 0.70, P < 0.05$).
Physiological responses to normoxic exercise after intermittent hypoxic training
Values are means ± S.D. Abbreviations: RER, respiratory exchange ratio; \([\text{La}^{-}]_{\text{B}}\), corrected whole-blood lactate. Significance of differences: *P < 0.05 for within-group difference; †P < 0.05 for between-group difference as a function of time.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normoxic group (n = 14)</th>
<th>Hypoxic group (n = 18)</th>
<th>Main effect</th>
<th>Interaction effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{\text{O}2}) (litres · min(^{-1}))</td>
<td>3.97 ± 0.73</td>
<td>3.49 ± 0.47</td>
<td>Time</td>
<td>Time × group</td>
</tr>
<tr>
<td>HR (beats · min(^{-1}))</td>
<td>191 ± 13</td>
<td>190 ± 7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>RER (arbitrary units)</td>
<td>1.23 ± 0.08</td>
<td>1.24 ± 0.22</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>([\text{La}^{-}]_{\text{B}}) (mmol · l(^{-1}))</td>
<td>9.8 ± 2.0</td>
<td>8.3 ± 1.5†</td>
<td>–</td>
<td>Time × group</td>
</tr>
<tr>
<td>(\text{SaO}_2) (%)</td>
<td>96 ± 3</td>
<td>95 ± 2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Time to exhaustion (s)</td>
<td>1188 ± 139</td>
<td>1175 ± 101</td>
<td>Time</td>
<td>–</td>
</tr>
<tr>
<td>Power output (W)</td>
<td>316 ± 55</td>
<td>324 ± 42</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\(V_{\text{O}2}\). In contrast, correlations were observed between the increases in LH and MDA and the decrease in \(\text{SaO}_2\) during hypoxic exercise (Figure 3).

Physical exercise was also shown to increase LH and MDA, and additional increases in lycopene and \(\beta\)-carotene were also observed (P < 0.05 compared with rest). The increases in \(\beta\)-carotene during both normoxic and hypoxic tests were associated with the absolute normoxic/hypoxic \(V_{\text{O}2\text{max}}\) values (r = 0.54, P < 0.05 and r = 0.41, P < 0.05 respectively).

Effects of intermittent hypoxia
Training programme
There were no differences between the normoxic and hypoxically trained groups during weeks 1–4 with regard to submaximal measures of power output, HR, systolic blood pressure or Borg rating of perceived exertion (results not shown). In contrast, \(\text{SaO}_2\) was lower and the whole-blood lactate response was comparatively higher during the hypoxic training sessions (P < 0.05 compared with normoxic group).

Caloric intake and dietary composition
Total caloric intake and dietary composition were not affected by training, and were identical between groups at all stages of the experimental period. Similarly, there were no within- or between-group differences for daily intakes of ascorbic acid, \(\alpha\)-tocopherol, retinol or total carotene (results not shown).
Hypoxic training increased $\dot{V}O_{2\max}$ (+0.47 ± 0.077 litres min⁻¹; $P < 0.05$ compared with pre-training value), whereas no changes were observed after normoxic training (Table 3). Cycling time to exhaustion and maximal power output were also increased after training, but these changes were not significantly different between the groups.

**Physiological responses**

Hypoxic training increased $\dot{V}O_{2\max}$ (+0.47 ± 0.077 litres min⁻¹; $P < 0.05$ compared with pre-training value), whereas no changes were observed after normoxic training (Table 3). Cycling time to exhaustion and maximal power output were also increased after training, but these changes were not significantly different between the groups.

**Metabolic responses**

Resting plasma concentrations of total cholesterol, HDL-C and derived LDL-C were decreased after physical training, whereas no changes were observed for triacylglycerol (Table 4). These responses were not different between groups. In contrast, apolipoproteins A1 and B decreased after normoxic training only.

Lipid peroxidation was markedly lower in the hypoxic group/time (hypoxic group). Significance: *$P < 0.05$ for within-group difference (exercise compared with rest); †$P < 0.05$ for within-group difference (Pre compared with Post) as a function of state (rest/exercise); ‡$P < 0.05$ for between-group difference as a function of time and state.

**Table 4** Effects of intermittent hypoxic training on resting plasma concentrations of blood lipid/lipoprotein cholesterol

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Normoxic group (n = 14)</th>
<th>Hypoxic group (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Total cholesterol (mmol l⁻¹)</td>
<td>4.47 ± 0.90</td>
<td>3.86 ± 0.61</td>
</tr>
<tr>
<td>Triacylglycerols (mmol l⁻¹)</td>
<td>0.93 ± 0.32</td>
<td>0.92 ± 0.38</td>
</tr>
<tr>
<td>HDL-C (mmol l⁻¹)</td>
<td>1.6 ± 0.4</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Derived LDL-C (mmol l⁻¹)</td>
<td>2.4 ± 0.9</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>Apolipoprotein A1 (g l⁻¹)</td>
<td>1.46 ± 0.14</td>
<td>1.24 ± 0.12*</td>
</tr>
<tr>
<td>Apolipoprotein B (g l⁻¹)</td>
<td>0.85 ± 0.25</td>
<td>0.71 ± 0.14*</td>
</tr>
</tbody>
</table>

**Table 5** Metabolic responses to acute normoxia following intermittent hypoxic training

Values are means ± S.D. for n = 14 (normoxic group) or n = 18 (hypoxic group). Significance: *$P < 0.05$ for within-group difference (exercise compared with rest) as a function of time (Pre/Post); †$P < 0.05$ for within-group difference (Pre compared with Post) as a function of state (rest/exercise); ‡$P < 0.05$ for between-group difference as a function of time and state.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Group</th>
<th>Rest</th>
<th>Exercise</th>
<th>Rest</th>
<th>Exercise</th>
<th>Main effects</th>
<th>Interaction effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Tocopherol (µmol l⁻¹)</td>
<td>Normoxic</td>
<td>1.59 ± 0.32</td>
<td>1.40 ± 0.26</td>
<td>1.53 ± 0.31</td>
<td>1.30 ± 0.33</td>
<td>State</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>1.37 ± 0.41</td>
<td>1.20 ± 0.31</td>
<td>1.41 ± 0.38</td>
<td>1.30 ± 0.30</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$\alpha$-Carotene (µmol l⁻¹)</td>
<td>Normoxic</td>
<td>0.110 ± 0.085</td>
<td>0.158 ± 0.082</td>
<td>0.102 ± 0.075</td>
<td>0.154 ± 0.047</td>
<td>State</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>0.107 ± 0.079</td>
<td>0.101 ± 0.072</td>
<td>0.094 ± 0.049</td>
<td>0.133 ± 0.062</td>
<td>State × group</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$-Carotene (µmol l⁻¹)</td>
<td>Normoxic</td>
<td>0.28 ± 0.21</td>
<td>0.35 ± 0.22</td>
<td>0.35 ± 0.29</td>
<td>0.36 ± 0.29</td>
<td>State</td>
<td>Time × state</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>0.26 ± 0.16</td>
<td>0.52 ± 0.54</td>
<td>0.30 ± 0.18</td>
<td>0.57 ± 0.43</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NEFA (mmol l⁻¹)</td>
<td>Normoxic</td>
<td>0.26 ± 0.15</td>
<td>0.41 ± 0.08</td>
<td>0.24 ± 0.23</td>
<td>0.45 ± 0.15</td>
<td>State</td>
<td>Time × state</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>0.35 ± 0.13</td>
<td>0.43 ± 0.11</td>
<td>0.18 ± 0.10</td>
<td>0.51 ± 0.15</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glycerol (µmol l⁻¹)</td>
<td>Normoxic</td>
<td>29.5 ± 10.0</td>
<td>47.9 ± 12.6*</td>
<td>31.4 ± 10.2</td>
<td>49.6 ± 13.5*</td>
<td>Time/state</td>
<td>Time × group/time × state × time</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>34.1 ± 14.1</td>
<td>42.2 ± 13.6</td>
<td>33.1 ± 9.3</td>
<td>79.4 ± 24.4*†</td>
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group, 0.78 ± 0.38 μmol·mmol⁻¹; Pre hypoxic group, 0.75 ± 0.33 μmol·mmol⁻¹; Post normoxic group, 0.88 ± 0.30 μmol·mmol⁻¹; Post hypoxic group, 0.76 ± 0.22 μmol·mmol⁻¹ (no main effects or interaction). HDL-C-corrected MDA (MDA/HDL-C): Pre normoxic group, 0.43 ± 0.21 μmol·mmol⁻¹; Pre hypoxic group, 0.34 ± 0.16 μmol·mmol⁻¹; Post normoxic group, 0.50 ± 0.09 μmol·mmol⁻¹; Post hypoxic group, 0.39 ± 0.11 μmol·mmol⁻¹ (main effect for group only, P < 0.05).

Whereas exercise increased lycopene and α-/β-carotene (P < 0.05 compared with rest), a significant increase in plasma x-tocopherol was observed only after hypoxic training (P < 0.05 compared with normoxic group). The increase in x-tocopherol after hypoxic training (Post exercise minus Post rest) was related to the increases (Post minus Pre) in \( V_{\text{O}_2\text{max}} \) (r = 0.71, P < 0.05) and arterial oxygen content (r = 0.42, P < 0.05). No between-group differences were observed when resting plasma x-tocopherol data were lipid-corrected (expressed relative to the summed concentrations of triacylglycerols + total cholesterol) and expressed relative to derived LDL-C: Pre normoxic group, 1.82 ± 0.80 μmol·mmol⁻¹; Pre hypoxic group, 1.70 ± 0.66 μmol·mmol⁻¹; Post normoxic group, 2.04 ± 0.61 μmol·mmol⁻¹; Post hypoxic group, 2.01 ± 0.65 μmol·mmol⁻¹ (main effect for time only; P < 0.05).

Acute exercise increased plasma concentrations of NEFA and glycerol (P < 0.05 compared with rest). The latter was more marked after hypoxic training (P < 0.05 compared with normoxic group) and correlated with the increase (Post minus Pre) in \( V_{\text{O}_2\text{max}} \) (r = 0.59, P < 0.05). A correlation was also observed between the increase in glycerol after exercise (Post exercise minus Post rest) and the corresponding increase in x-tocopherol (r = 0.67, P < 0.05).

The resting haemoglobin concentration was not altered by hypoxic training (15.1 ± 0.7 compared with 15.1 ± 0.8 g·dl⁻¹), but decreased after normoxic training (from 15.2 ± 0.8 to 14.8 ± 0.9 g·dl⁻¹; P < 0.05). The red blood cell count also decreased after training, presumably due to a plasma volume expansion, but this response was not significantly different between groups. The derived arterial oxygen content (e = haemoglobin × \( S_aO_2 \) × 1.34) at maximal exercise decreased after normoxic training (–0.5 ± 0.9 ml of \( O_2 \)·dl⁻¹; P < 0.05 compared with Pre), whereas an increase was observed after hypoxic training (+0.3 ± 1.6 ml of \( O_2 \)·dl⁻¹; P < 0.05 compared with Pre).

**DISCUSSION**

The present investigation examined the independent effects of acute hypoxia and physical exercise on metabolic indices of ROS-mediated lipid peroxidation, and the subsequent implications of regular hypoxic training for oxidant generation induced by acute normoxic exercise. Compared with the normoxic control condition, acute hypoxia markedly increased lipid peroxidation, despite a comparatively lower absolute \( V_{\text{O}_2\text{max}} \) and an exercise-induced increase in x-tocopherol. The increase in lipid peroxidation was related to the exercise-induced decrease in \( S_aO_2 \) rather than to the maximal absolute systemic \( O_2 \) flux per se. In contrast, 4 weeks of intermittent hypoxic training attenuated the acute normoxic-exercise-induced lipid peroxidation more effectively than a comparable programme of normoxic training, despite an increase in absolute \( V_{\text{O}_2\text{max}} \) in the hypoxic group. Exercise-induced lipolysis was subsequently more marked, which may have indirectly increased the transport and corresponding scavenging capacity of the venous circulation by elevating x-tocopherol levels. These data clearly indicate that a mass action effect initiated by an exercise-induced increase in mitochondrial \( O_2 \) flux is not the exclusive source of ROS generation. Our findings also support our original hypotheses and are consistent with the basic tenets of the GAS. ROS may prove an integral component of the signal transduction pathway that initiates adaptive responses to physical and environmental stress in humans.

**Acute hypoxia and oxidative stress**

Whereas animal research indicates clear evidence for increased ROS generation during hypoxia [18], human studies are less convincing, due largely to the lack of appropriate normoxically exposed control groups. However, our controlled findings demonstrate that acute hypoxia and physical exercise are independent sources of oxidative stress. Lipid peroxidation was especially apparent during the combined stress of hypoxic exercise, despite an increase in the plasma concentration of the major lipid-soluble chain-breaking antioxidant x-tocopherol; such a response was observed after acute normoxic exercise due to an increase in the mobilization of blood lipids from hepatic and adipose stores [19]. It was unfortunate that we did not examine potential changes in lipolysis, at least during the acute phase of the present study; a comparatively lower systemic \( P_O_2 \) during hypoxia would have been expected to increase the sympathoadrenergic drive [20] and thus the effective mobilization of NEFA.

We have recently combined the direct spectroscopic technique of EPR with spin trapping to confirm that the peroxidative damage observed during hypoxia is most probably free radical mediated. A marked increase in the intensity of the \( x \)-phenyl-τ-butylnitrore adduct was demonstrated in male subjects during an identical bout of exhaustive normobaric hypoxic exercise (\( F_{O_2} \sim 0.16 \)), a signal we attributed to secondary oxygen-centred peroxyl or alkoxyl radicals derived from initial oxygen-
centred damage to membrane phospholipids [21]. The incorporation of the total radical trapping antioxidant parameter (TRAP) assay may prove a useful measure of the blood redox state in future research, and help to iso- lute further the relative contributions of the peroxyl radical to exercise/hypoxia-induced oxidative stress. However, we must emphasize that our direct/indirect detection of free radical formation/damage is confined to the venous circulation; we suggest that this may be a consequence of the intracellular generation and subsequent diffusion of hydrogen peroxide.

An increase in systemic VO\textsubscript{2} has been traditionally regarded as the major factor responsible for oxidative stress induced by normoxic exercise, due predominantly to enhanced electron ‘leakage’ at the ubiquinone–cytochrome \(b\) level of the mitochondrial electron transport chain [22]. Thus it was intriguing to note a comparatively greater increase in lipid peroxidation after hypoxic exercise, despite a markedly lower cumulative/ maximal VO\textsubscript{2} and hence mitochondrial electron flux. It would therefore appear that a mass action effect of VO\textsubscript{2} was not the exclusive source of exercise-induced oxidative stress, at least during systemic hypoxia. Furthermore, the consistent relationships observed between the increase in lipid peroxidation by-products and arterial desaturation, but not absolute VO\textsubscript{2max}, raise the intriguing possibility that a decrease in the (predicted) mitochondrial \(P_{O_2}\) as opposed to an increase in mitochondrial \(O_2\) flux per se, more precisely regulates ROS generation. The more pronounced arterial desaturation (and hence lower predicted mitochondrial \(P_{O_2}\)) observed during hypoxic exercise would also explain why lipid peroxidation was more marked compared with passive exposure.

Recent \textit{in vitro} research has demonstrated a decrease in the \(V_{\text{max}}\) of cytochrome oxidase during hypoxia, which subsequently increased the reduction state of mitochondrial electron carriers upstream of cytochrome \(a_a\) [23]. An increased mitochondrial redox state for any given VO\textsubscript{2} was considered to increase mitochondrial superoxide generation by increasing the effective lifetime of reduced electron carriers, specifically ubisemiquinone. Emerging evidence now suggests that the increase in ROS is an adaptive response; ROS function as second messengers and are a key element in the signal transduction process initiated by the mitochondria that detect and respond to a fall in intracellular \(P_{O_2}\) [2]. Future studies should consider combining the techniques of EPR and proton magnetic resonance spectroscopy to confirm whether a cause–effect relationship exists between mitochondrial \(P_{O_2}\) and ROS generation in humans.

**Intermittent hypoxia and oxidative stress**

Lipid peroxidation was comparatively lower in the hypoxic group, which was primarily the result of an attenuation of the normoxic-exercise-induced increase in LH and MDA after physical training. This was an intriguing observation in light of the selective increase in VO\textsubscript{2max}, which appeared to be due to a relative improvement in convective \(O_2\) delivery to skeletal muscle, as suggested by the observed increase in arterial oxygen content. These findings compliment our data under conditions of acute hypoxia and suggest further that a mass action effect of VO\textsubscript{2} is not the primary determinant of exercise-induced oxidative stress. These adaptive responses were induced by hypoxia \textit{per se} and were essentially independent of diet or subtle differences in physical activity, as both relative (\% of HR\textsubscript{max}) and absolute (power output) training intensities were comparable between groups.

Our preliminary findings suggest that the decrease in lipid peroxidation may have been caused by a more efficient peripheral redistribution of \(\alpha\)-tocopherol, due to enhanced exercise-induced lipolysis. It was apparent that, whereas acute normoxic exercise did not influence the venous concentration of \(\alpha\)-tocopherol prior to physical training, a selective increase was observed only after hypoxic training. It was intriguing to note that the protective effects of \(\alpha\)-tocopherol were only apparent after hypoxic training, and not during acute hypoxic exercise. Two explanations could account for this phenomenon. First, the hypoxic-exercise-induced increase in \(\alpha\)-tocopherol appeared to be comparatively lower than the normoxic-exercise-induced increase following intermittent hypoxic training (+2.3 \pm 7.9 and +3.3 \pm 6.7 \mu \text{mol} \cdot \text{L}^{-1} \text{respectively}; approaching statistical significance at \(P = 0.061\)). Although merely a speculation, these findings may provide indirect evidence for a dose–response phenomenon; a threshold exercise-induced venous concentration of \(\alpha\)-tocopherol may need to be attained before its antioxidant properties and subsequent attenuation of lipid peroxides become physiologically apparent. Secondly, we must also consider potential contributions from other sources, specifically the antioxidant enzymes. The initial consequences of a comparatively greater oxidant load in the early, and at present undefined, stages of hypoxic training may have subsequently increased the transcription of genes encoding antioxidant defence enzymes and heat-shock proteins, adaptations that have clearly been associated with not only the intensity (i.e. magnitude of the acute increase) but also the duration of exposure to normoxic-exercise-induced oxidative stress in rats [24,25].

Furthermore, the observed differences in lipid peroxides after hypoxic training may have been influenced by more pronounced changes in the circulating concentration of high-density lipoproteins, which have been implicated previously in the protection of blood lipids against oxidation. While this did not appear to be the case for the resting data at least, we cannot dismiss the possibility that the exercise-induced increase in HDL-C
may have been comparatively greater following hypoxic training.

The increase in α-tocopherol levels appeared to be related to the increases in arterial oxygen content and \( V\text{O}_{2}\text{max} \) and the acute-exercise-induced rise in plasma glycerol, which was also more marked in the hypoxic group, suggestive of enhanced lipolysis. These findings suggest that the improved delivery and utilization of \( O_2 \) by skeletal muscle after hypoxic training may have enabled a given exercise \( V\text{O}_2 \) to be sustained at lower intracellular ADP and \( P_i \) concentrations. This may have increased the hydrolysis of triacylglycerols by lipoprotein lipase and hepatic triacylglycerol lipase following the catabolism of hepatically derived nascent very-low-density lipoproteins, a mechanism that may have increased the secretion of RRR-α-tocopherol, thus raising the corresponding plasma and lipoprotein concentrations [26].

It was unfortunate that we did not examine either resting or exercise-induced changes in oxidized LDL, in particular when one considers their pivotal role in the pathogenesis of coronary artery disease [27] and the observed changes in α-tocopherol. The resting concentration ratio of lipid-corrected α-tocopherol to LDL-C increased as a function of physical training, a response that may have conferred greater protection against circulating LDL.

A previous study has documented a similar increase in the serum concentration of α-tocopherol after acute normoxic exercise (+19%, compared with +15% in the present study), which was also shown to be associated with an increase in serum glycerol [19]. Furthermore, the α-tocopherol was incorporated into the membranes of red blood cells. Thus, while the site of action of α-tocopherol is confined largely to the lipophilic interior of biological membranes [28], its selective mobilization may have increased the effective scavenging capacity of the peripheral circulation, an adaptive acute-phase response to protect against exercise-induced oxidative damage.

There have been some indications to suggest that the magnitude of antioxidant adaptation coincides with the magnitude of ROS generation during an acute bout of physical exercise performed as part of a regular training programme [6]. Our findings indirectly confirm this dose–response relationship and are consistent with the fundamental concepts of the GAS [3]. Acute hypoxia was clearly a more severe oxidative stressor and, when combined with the stress of physical exercise, further compounded the oxidant load. However, normoxic-exercise-induced oxidative stress was comparatively lower after intermittent hypoxic training, which suggests that controlled ROS generation may prove an important component of the signal transduction sequence that can adjust homoeostasis and initiate protective adaptation. Future investigators need to consider that, whereas antioxidant prophylaxis may be an effective strategy for reducing oxidative stress, it could also delay or perhaps even inhibit the ‘normal’ adaptive responses to physical/environmental stress.

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