Response of nitric oxide pathway to L-arginine infusion at the altitude of 4,350 m

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Response of nitric oxide pathway to L-arginine infusion at the altitude of 4,350 m. J-C. Schneider, I. Blazy, M. Déchaux, D. Rabier, N.P. Mason, J-P. Richalet. ©ERS Journals Ltd 2001.

ABSTRACT: It was hypothesized that hypoxia may inhibit nitric oxide (NO) production by reducing the availability of endothelial NO synthase (NOS III) substrate.

To evaluate the effect of L-arginine on the NO release in high altitude, 11 subjects were infused with L-arginine (0.5 g·kg^{-1}) during 30 min in normoxia and after 36 h at 4,350 m (hypoxia). The L-citrulline and cyclic guanosine monophosphate (cGMP) concentrations were measured to investigate NO synthesis and guanylyl cyclase activity respectively. L-citrulline concentration, arterial oxygen saturation (Sa,O$_2$), systemic blood pressure, heart rate and acute mountain sickness (AMS) score were measured at rest and 15, 30 and 45 min after starting infusion.

The results showed that baseline L-citrulline was lower in hypoxia ($p<0.05$). L-arginine infusion increased L-citrulline concentration in both conditions. However, in hypoxia L-citrulline concentration remained lower than in normoxia ($p<0.05$). The concentration of cGMP was lower in hypoxia ($p<0.05$). In hypoxia, Sa,O$_2$ increased from 15 min after the start of the infusion to 45 min ($p<0.05$). Blood pressure and heart rate were not affected by L-arginine infusion.

Subjects who experienced symptoms of AMS showed a slight decrease in AMS score with L-arginine.

The decreased L-citrulline suggests a hypoxia-induced impairment of nitric oxide synthase III or a decrease in L-arginine availability. The improvement of arterial oxygen saturation by pretreatment with L-arginine could be ascribed to an enhancement of the ventilation/perfusion ratio. Collectively, these results are consistent with a decrease in nitric oxide production in hypoxia that could be antagonized by supplying nitric oxide synthase cosubstrate.
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Exposure to high altitude causes hypoxaemia, which is often accompanied by the clinical manifestations of acute mountain sickness (AMS) and may lead to the development of pulmonary hypertension and high altitude pulmonary oedema (HAPE). Several factors are incriminated: hypoxic pulmonary vasoconstriction (HPV), water retention, and permeability alterations [1]. Increased release of vasoconstrictors, such as endothelin or catecholamines, at high altitude may be partially responsible for HPV [2]. However, impairment of nitric oxide (NO) synthesis by pulmonary endothelial cells occurs in the pulmonary hypertensive process suggesting that the increase in pulmonary vascular tone could have its origin in a decline of endothelium-dependent relaxation [3]. NO is synthesized in pulmonary artery endothelial cells by the action of the endothelial constitutive NO synthase isoform (NOS III) on the cationic amino acid L-arginine (Arg). NO is produced from the terminal guanidino-nitrogen of Arg upon conversion to L-citrulline by NOS III in a reaction that requires molecular oxygen and cofactors. Direct measurement of NO release from vascular endothelial cells needs an NO-selective electrode. However, L-citrulline that is formed stochiometrically with NO could be more easily measured than the short-lived NO. LAGERWERF *et al.* [4] have previously shown that determination of L-citrulline enrichments in plasma was a useful tool for assessment of *in vivo* NO production. Therefore, the release of NO can be measured directly by the L-citrulline concentration as an index of NO synthesis. Synthesis of NO by NOS depends on the availability of its substrate [5, 6]. In normal vessels, the amount of substrate is sufficient for the conversion to NO at a maximal rate. However, addition of Arg causes an endothelium-dependent relaxation in pulmonary arteries depleted from Arg [7]. Chronic hypoxia may impair the uptake of Arg into endothelium or alter its availability or metabolism. However, Arg deficiency does not appear to account for the decrease in NO production reported in endothelial cells exposed to short-term hypoxia [8, 9]. By contrast, Arg restores endothelium-dependent relaxation in pulmonary circulation of chronically hypoxic rats [10], suggesting that the availability of intracellular Arg is a critical factor in the regulation of the rate of NO synthesis in

endothelial cells. Furthermore, in pulmonary hypertension, basal plasma level of Arg was below average [11], supporting the hypothesis that a relative deficiency of the Arg pool might contribute to the pathogenesis of pulmonary hypertension. Thus, understanding the metabolism of intracellular Arg might clarify the mechanism by which NO formation decreases in hypoxia. Alternatively, low oxygen tension inhibits NOS activity in biochemical assays of the isolated NOS enzyme [12] and chronic hypoxia could interrupt the NO signalling pathway at the level of guanylyl cyclase (GC) [13]. However, chronic hypoxia induced an upregulation of NOS protein expression and activity [14]. Thus, in long-term hypoxia, the part of the Arg availability seems to be lower. To explore the hypothesis that exposure to acute hypoxia is associated with a decrease in Arg availability and to confirm whether decreased NO activity in high altitude could be restored by supplying the precursor of NO, plasma L-citrulline (L-cit) and cyclic guanosine monophosphate (cGMP) concentrations were measured in humans during infusion of Arg at sea-level (normoxia) and after 36 h of exposure to altitude of 4,350 m (hypoxia). Accordingly, the effect of exogenous Arg on arterial oxygen saturation (Sa,O$_2$) and AMS score were examined.

Methods

Subjects

A total of 11 healthy subjects (eight males) volunteered for the study, after approval by the Ethics Committee of Necker hospital (Paris, France). Each subject underwent a medical examination and was fully informed about the experimental procedure. All volunteers were moderately trained, sea-level natives and without experience of HAPE. They had no history of upper respiratory tract infection for $\geqslant 4$ weeks prior to the study, were not acclimatized to altitude before the experiments and did not consume any long-term medication or caffeine before the Arg load. Their age and body weight were (mean±SE) 27.8±1.5 yrs and 73.0±2.8 kg, respectively.

Study design

All experiences were performed in two conditions: at sea-level during the first week (Bobigny hospital France, altitude: 60 m, barometric pressure 761± 5 mmHg) and 3 weeks later at a field laboratory on Mont Blanc (Observatoire Vallot, altitude: 4,350 m, barometric pressure 457±1 mmHg). Subjects were transported by helicopter from Chamonix (altitude: 1,035 m) to 4,350 m in <15 min. The temperature in the laboratory was kept constant at 20–23°C during the whole study. Response to Arg infusion was determined at sea-level and 36 h after arriving at 4,350 m. After 1-h rest in supine position, each subject had a short plastic catheter introduced in a cubital vein 15 min before beginning the infusion. A single dose of Arg (L-arginine-chloride; Laboratoire Veyron

et Froment, Marseille, France) was infused (0.5 g·kg^{-1}). Arg was diluted in 250 mL of a 5% glucose solution and the duration of the infusion was 30 min. Each dose was administered early in the morning before breakfast. Administration of Arg was designed as time 0 (t0). Arterial oxygen saturation (Sa,O$_2$), heart rate (HR) and supine systolic and diastolic blood pressure (SBP and DBP, respectively) were measured by an ear pulse oximeter (Biox II, Ohmeda, Montreuil, France) and an automatic sphygmomanometer (Dinamap 1846 SX P, Critikon, Blanquefort, France). One venous blood sample was collected for the measurement of L-cit concentration and cGMP concentration prior to medication. Then 15, 30 and 45 min after the beginning of the infusion, in resting supine position (t15, t30 and t45 respectively), Sa,O$_2$, HR and BP were measured and blood was withdrawn. Clinical assessment of AMS was performed by the same observer using the Lake Louise score at the same time as blood sampling. Four symptoms were monitored: headache, gastrointestinal upset, dizziness and asthenia. The response to each of the four items was rated with a three-point scale in which a score of 0 indicated no symptoms, 1: slight symptoms, 2: moderate symptoms and 3: severe symptoms. The AMS score is the sum of the scores for the four items [15].

Chromatographic measurements of L-citrulline

Ten millilitres of blood were sampled without venostasis for L-cit and cGMP concentration measurements in plastic tubes containing ethylenediamine tetraacetic acid (EDTA). Blood was immediately centrifuged for 20 min at 900×g at 4°C. Plasma was carefully removed, frozen and stored in liquid nitrogen within 30 min for subsequent analysis. L-cit concentration was measured by ions-exchange chromatography with ninhydrin detection adapted from the technique of SLOCUM and CUMMINGS [16]. Plasma was deproteinized by precipitation with sulphosalicylic acid and centrifuged at 10,000×g. The supernatant was added Beckman Li-S buffer and injected into the amino acid analyser Beckman 6300 (Beckman Instruments, Roisy, CDG, France) calibrated by analysing calibration standard of known concentration. Retention time was used for amino acid identification and integration was used for quantification.

Radioimmunoassay measurements of cyclic guanosine monophosphate

NO stimulates guanylyl cyclase to produce cGMP, an intermediate in the endothelium-dependent relaxation of blood vessel. To study the NO-cGMP pathway, cGMP concentration was measured by immunoassay. The nuclear radioimmunoassay kit for the determination of cGMP is adapted from the procedures of STEINER et al. [17]. Increase in sensitivity was achieved by succinylating samples with succinic anhydride, then the tracer was added and incubated for 22 h at 4°C (kit Beckman-Coulter, Immunotech
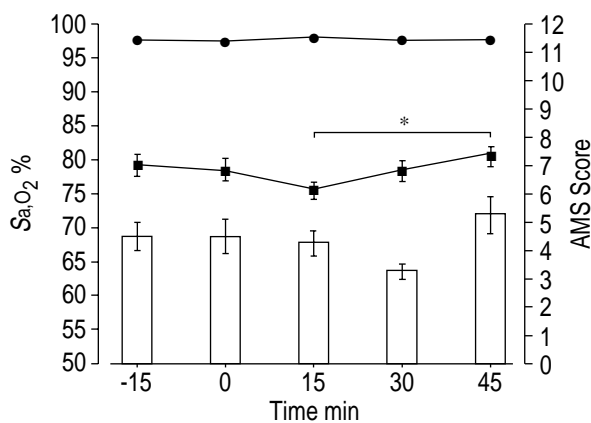
Fig. 1. – Arterial oxygen saturation (Sa,O$_2$) and acute mountain sickness score (AMS score; □) during L-arginine infusion at sea-level (●) and after 36 h of exposure to altitude of 4,350 m (■). Values are mean±SE; n=11. L-arginine infusion (0.5 g·kg^{-1} in 30 min) occurred after 15 min of rest in supine position and was followed by 15 min of recovery. *: p<0.05, significantly different from 15 min after the beginning of the infusion (t15).

SA, Marseille, France). Radioimmunoassays of cyclic nucleotides (*e.g.* cGMP) in biological fluids currently involve the use of specific antibodies and ^{125}I-labelled tracers. Femtomole levels could be measured when ^{125}I-labelled tracers and 2′-O-derivatization procedures are used.

Statistical analysis

All data are expressed as means±SE. A two-way analysis of variance with repeated measures was performed to make comparisons between altitude conditions and across time for clinical data, Sa,O$_2$, cardiovascular data and blood sample. If the F-value indicated significant differences among group means, the overall significance of the variation with time including conditions normoxia and hypoxia were assessed using a paired t-test. Linear regressions were performed by using the least squares method. A p-value <0.05 was considered as significant.

Results

Clinical data

Only four subjects experienced symptoms of AMS at Observatoire Vallot (score >2). Variations of AMS score are shown in figure 1. Subjects who experienced symptoms of AMS showed a slightly decreased AMS score with Arg infusion, mean minimal score was observed at the end of Arg infusion but no significant correlation was found. Fifteen minutes after the end of the infusion, the AMS score retrieved its baseline level, whereas Sa,O$_2$ stayed higher. In the present study, the experiments were performed in the morning, which corresponds to the period of maximal AMS symptoms during the daytime [18]. The most frequent symptom was headache. No subject experienced clinical signs of pulmonary or cerebral oedema.

Physical data

Sa,O$_2$ decreased by 20±2% from sea-level to 4,350 m (fig. 1). In normoxia, Sa,O$_2$ was not modified by Arg infusion. In hypoxia, Sa,O$_2$ tended to decrease before and in the first 15 min of Arg infusion, and then increased from 15–45 min. Sa,O$_2$ was higher by 3.2±0.4% from t15 to t30 and by 5.3±0.3% from t15 to t45 (p<0.05). This increase was parallel to L-cit concentration but no significant correlation was found. SBP and DBP remained unchanged at 4,350 m (table 1). In both conditions, SBP and DBP did not vary during Arg infusion. HR increased by 25±1% (p<0.01) from sea-level to 4,350 m (table 1). HR did not vary during Arg infusion in both conditions.

Venous plasma

L-cit and cGMP concentration are shown in figures 2 and 3 respectively. Baseline L-cit concentration decreased from 33.1±1.7 μM·L^{-1} at sea-level to 28.5±2.1 μM·L^{-1} at 4,350 m (p<0.05). In both conditions, L-cit concentration increases during Arg

Table 1. – Systolic (SBP) and diastolic (DBP) blood pressure and heart rate (HR) during L-arginine infusion at sea-level and after 36 h of exposure to altitude of 4,350 m

Variables	Rest	L-arginine infusion			Recovery
		Baseline	15 min	30 min	45 min
SBP mmHg					
Normoxia	116.8±4.3	117.4±3.4	119.0±5.1	116.8±3.4	117.9±3.7
Hypoxia	117.6±3.8	119.6±2.6	116.7±3.6	115.1±3	116.7±3.5
DBP mmHg					
Normoxia	70.0±2.6	69.9±2.6	71.4±4.3	68.1±3.9	70.2±4
Hypoxia	73.6±3.3	72.3±3.2	71.9±3.1	70.2±2.3	68.6±3.9
HR bpm					
Normoxia	62±2.9	61.9±2.9	62.2±3	59.8±2.3	59.8±2.6
Hypoxia	75.2±3.5**	75.1±3.5**	76.0±3.8**	77.5±3.8**	75.7±3.8**

Values are mean±SE; n=11. L-arginine infusion (0.5 g·kg^{-1} in 30 min) occurred after 15 min of rest in supine position and was followed by 15 min of recovery. bpm: beats per minute. **: p<0.01 compared to normoxia.
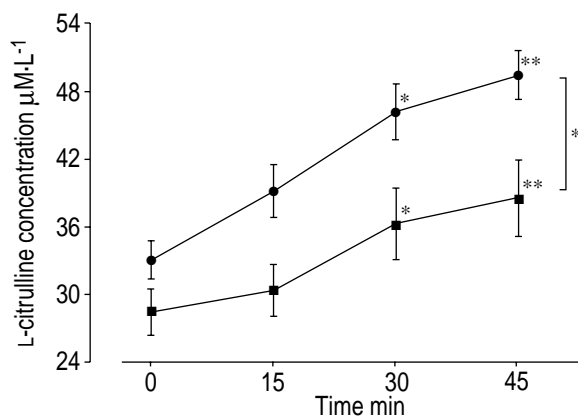
Fig. 2. – L-citrulline plasma concentration during L-arginine infusion at sea-level (●) and after 36 h of exposure to altitude of 4,350 m (■). Values are means±SE; n=11. L-arginine infusion (0.5 g·kg^{-1} in 30 min) occurred after 15 min of rest in supine position and was followed by 15 min of recovery. *: p<0.05; **: p<0.01, significantly different from baseline in normoxia or hypoxia.

Fig. 4. – Relationship between L-citrulline (L-cit) and cyclic guanosine monophosphate (cGMP) concentration in 10 subjects during L-arginine infusion at sea-level (●) and after 36 h of exposure to altitude of 4,350 m (□). The regression equation is [cGMP]= 0.09 [L-cit] -0.38, r=0.96 (p<0.05) at sea-level, where [cGMP] is cGMP concentration and [L-cit] is L-cit concentration, no correlation was found at 4,350 m.

infusion from respective baseline to 46.2±2.5 μM·L^{-1} at sea-level and 36.3±3.2 μM·L^{-1} at 4,350 m (p<0.05). Moreover, concentration remained higher after 15 min recovery (t45). Therefore, L-cit concentration increased by 49±1% at sea-level and 35±2% at 4,350 m from t0 to t45 (p<0.01). However, L-cit concentration in hypoxia remained below the normoxia value by 22±1% throughout Arg infusion and recovery (p<0.05). cGMP concentration was lower at 4,350 m than at sea level (p<0.05). In normoxia, cGMP concentration increased from 2.8±0.5 nM.L^{-1} at t0 to 4.2±0.6 nM·L^{-1} at t45 (53±6%, p<0.05). Moreover, cGMP concentration increase was positively correlated to L-cit concentration increase (r=0.96, p<0.05) (fig. 4). In hypoxia the concentration of cGMP remained unchanged during Arg infusion and the correlation was not significant.
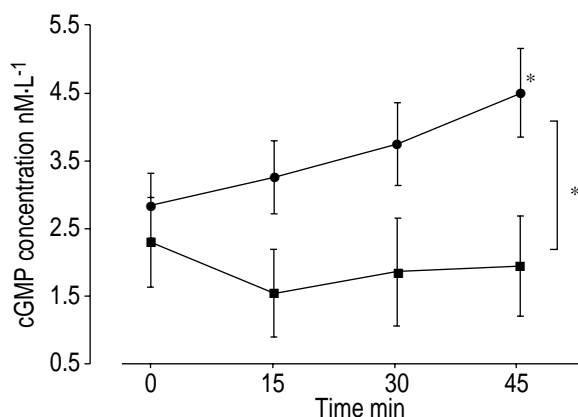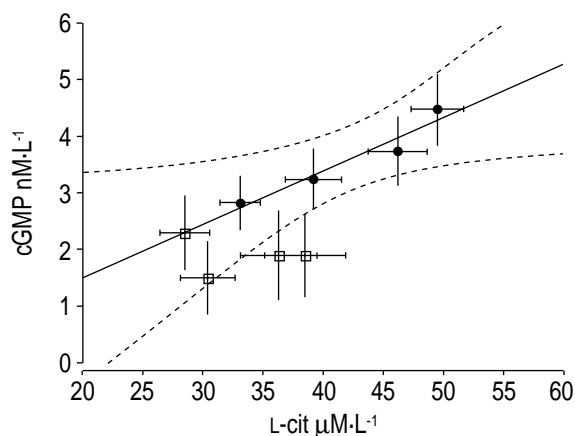

Fig. 3. – Cyclic guanosine 3′,5′-monophosphate (cGMP) plasma concentration during L-arginine infusion at sea level (●) and after 36 h of exposure to altitude of 4,350 m (■). Values are means± SE; n=10. L-arginine infusion (0.5 g·kg^{-1} in 30 min) occurred after 15 min of rest in supine position and was followed by 15 min of recovery. *: p<0.05.

Discussion

The presented results showed that 36 h of exposure to altitude of 4,350 m, decreased plasma L-cit and cGMP concentrations. Arg infusion increased L-cit concentrations in normoxia and hypoxia, whereas Arg infusion only increased cGMP in normoxia. In addition, Arg infusion tended to increase Sa,O$_2$ without systemic effects at high altitude.

L-arginine availability

The availability of intracellular Arg is a critical factor in the regulation of the rate of NO synthesis in endothelial cells. The Arg content of endothelial cells is derived primarily from plasma membrane-dependent transport of extracellular Arg, but can also be synthesized from L-cit [19]. BLOCK et al. [20] showed that exposure to hypoxia causes significant reduction in the transport of Arg, only accompanied by a decrease in intracellular Arg content after long-term exposure to hypoxia. SU and BLOCK [21] demonstrated that hypoxia time dependently inhibits the synthesis of Arg from L-cit in pulmonary artery endothelial cells. These results suggest that hypoxia-induced L-cit concentration decrease might not be due to the capacity of endothelial cell to recycle L-cit and since the Arg-NO pathway represents the major metabolic pathway, it can be assumed that decreased L-cit was caused by an effect of hypoxia on Arg metabolism. However, NO production is linked to the rate of transformation of Arg to L-cit, which depends on cardiac blood flow and arteriovenous difference. In acute hypoxia, cardiac blood flow increases and L-cit concentration decreases suggesting that NO production might not be correlated to L-cit concentration. Although, hypoxia resulted in a decrease in the conversion of L-cit, intracellular Arg content was increased in the hypoxic pulmonary artery endothelial cells [22, 23]. Thus, lack of availability of intracellular

Arg does not appear to account for the decrease in NO production unless the intracellular pools of Arg are inaccessible to NOS III in hypoxia. This is of particular relevance to endothelium because NOS III is membrane-associated and thus, a membrane-related pool of Arg may be critical to the activity of the enzyme. In normal vessels, the amount of the substrate is sufficient for conversion to NO at a maximal rate. However, addition of Arg causes an endothelium-dependent relaxation in pulmonary artery depleted of Arg [7]. Administration of substrate for NOS restores endothelium-dependent vasodilatory response in hypoxia-induced pulmonary hypertension [10, 24]. In patients with pulmonary hypertension, Arg reduced the pulmonary vascular resistance and the magnitude of the pulmonary vasodilatory response was correlated to the level of L-cit [11]. Furthermore, although NOS expression was upregulated in the early course of development of HPV [25], NOS activity was not increased if the substrate for the NOS enzyme was limiting [26, 27]. Thus, the increase in L-cit and the short-term oxygenation effect of exogenous Arg at high altitude may be due to enhanced NO production in the pulmonary vasculature, and increase in Arg availability can promote L-cit/NO synthesis in high altitude. The vascular effect of Arg may be mediated through NO-independent mechanisms such as the effect of pH. Although alkaline solutions of Arg can induce vasodilatation, Arg hydrochloride solution was slightly acidic, weakening an effect of the pH. As Arg was infused with glucose, the direct effect of carbohydrates on ventilation/perfusion ratio should not be ruled out. However, this effect could be NO-independent since hyperglycaemia did not affect endothelium-dependent vasoreactivity in humans [28]. Alternatively, a nutritional deficiency might not have contributed to the concentration decrease of L-cit during 36 h of hypoxia and Arg availability is low owing to extensive protein binding.

Ventilation/perfusion ratio

PISON [29] demonstrated that NO may improve ventilation and perfusion matching, as well as excretion and retention dispersion indexes, suggesting an improvement in gas exchange because of a redistribution of both ventilation and blood flow. In HAPE, inhalation of NO redistributed blood flow in the lungs away from oedematous regions, which improved the matching of ventilation and perfusion and reducing the alveolar-arterial oxygen difference [30]. Thus, the improvement of the Sa,O$_2$ with Arg suggests that the NO substrate could be a useful adjuvant in the treatment of high altitude pulmonary hypertension, which is implicated in the pathogenesis of HAPE. Alternatively, NO induces vasodilatation of bronchial artery in both normoxia and hypoxia [31] and modulates agonist-induced bronchoconstriction [32]. The improvement of Sa,O$_2$ may be due to the vasodilatory effect of NO on the small vascular pulmonary arteries thereby increasing the perfusion/ventilation ratio. However, Arg-induced hyperventilation might not be ruled out. Furthermore, as subjects prone to HAPE have a less compliant pulmonary circulation due to multifactorial mechanism, including reduced NO synthesis [33], Arg infusion may prevent, at least in part, the complication of HPV. The initial decrease in the Sa,O$_2$ may be due to reclined position during the procedure, since lying flat in bed raises intrathoracic blood volume and consequently, pulmonary arterial pressure [18].

Guanylyl cyclase

In bovine arterial rings, a depletion of tissue Arg is associated with a decrease in basal cGMP levels and impairment of endothelium-dependent cGMP formation [7]. However, basal guanylyl cyclase activity did not differ significantly between normoxic and hypoxic lungs [25]. The decreased cGMP at 4,350 m assumes that Arg content was lower at 4,350 m. However, in hypoxia no correlation was found between L-cit and cGMP concentration variation, suggesting that the cGMP metabolism differs from sea-level to 36 h of exposure to altitude of 4,350 m. Conversely, the nonspecificity of the plasma cGMP level may explain the discrepancy of these results in hypoxia.

Systemic vessel

Administration of Arg results in an increase in the concentration of NO in the exhaled air matched by an increase in the concentration of NO$_3^-$ in the plasma [34]. BODE-BÖGER *et al.* [35] showed that Arg induced vasodilatation in healthy humans in normoxia and BLITZER *et al.* [36] demonstrated that NOS inhibition increased systemic vascular resistance and BP and decreased cardiac output in short-term hypoxia. However, the presented results show that Arg infusion was not associated with any measurable change in systemic haemodynamics, suggesting the absence of a direct effect of NOS substrate on heart and vascular smooth muscle of the systemic vessels. The discrepancy of the findings may be explained by the baseline level of SBP and DBP (150/90 for BODE-BÖGER *et al.* [35] and 120/70 for the present study) and by inactivation of free NO by high-affinity binding to haemoglobin in hypoxia. Furthermore, in the protocol of BLITZER *et al.* [36], decrease in the fraction of inspired oxygen was induced by breathing hypoxic mixture and the duration of hypoxia was less than the present study.

Conclusion

In the present study, L-arginine infusion at doses avoiding systemic haemodynamic effects have allowed for the improvement of arterial oxygen saturation at 4,350 m after intravenous administration of a nitric oxide synthetase substrate used as a surrogate for directional changes in nitric oxide pathway. This effect may be due to the confounding effects of changes in flow through the pulmonary circulation and ventilation from increase in nitric oxide activity. Potential mechanisms to explain the decrease of

L-citrulline at 4,350 m include: 1) impairment of the nitric oxide synthase activity in high altitude; 2) decrease of the nitric oxide substrate bioavailability; and 3) enhancement of the catabolism of L-citrulline. However, the improvement during L-arginine infusion suggests that L-arginine could preserve endothelial function and obviate the pathological consequences of hypoxia.

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