Evidence of a decrease in nitric oxide-storage molecules following acute hypoxia and/or hypobaria, by means of chemiluminescence analysis

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Abstract

Nitrate, nitrite, and other nitroso compounds (NOx) had been proposed as possible nitric oxide (NO) storage molecules. The present work examines, by means of chemiluminescence analysis, changes in NOx serum levels in rats 1 h before and 24, 48, and 72 h after exposure to acute hypobaric hypoxia (HH; barometric pressure [PB] 225 mmHg, oxygen partial pressure [PO2] 48 mmHg), normobaric hypoxia (NH; PB 716 mmHg [Jaén city], PO2 48 mmHg), hypobaric normoxia (HN; PB 225 mmHg, PO2 150 mmHg), and normobaric normoxia (NN; PB 716 mmHg, PO2 150 mmHg) the latter as a control group. Results show a decrease in NOx levels, which reached significance 24 h after exposure in HH animals, 4 h after exposure in the HN and NH groups, and persisted after 48 h of exposure in the HN group. NOx determinations were also performed in brain (cerebral cortex, hippocampus, decorticated brain [basal ganglia–brainstem] and cerebellum), liver, kidney, lung, and heart homogenates, 72 h after the experiment, to detect persistent effects when serum NOx levels had returned to basal values. Only in cerebellum (HN group) and hippocampus (HN and NH groups) were NOx levels significantly lower than in controls. We conclude that not only acute hypobaric hypoxia but also either hypobaria or hypoxia alone induce changes in NOx serum levels. Moreover, all three episodes involve a decrease in NOx, greater and longer-lasting in hypoxia alone than in hypobaria and hypoxia together. The exhaustion of these NO-storage molecules could be critical when, as during a hypoxic episode, the l-arginine/NOS pathway is impaired.

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Most early studies on acute hypobaric hypoxia assumed that physiological responses to high altitude were related only to the low magnitude and duration of the inspired oxygen (PIO2) and to the consequent alveolar hypoxia and arterial hypoxemia [3,10]. Later investigations suggested that the concurrent reduction in barometric pressure (PB) might also affect the physiological responses to high altitude, acting in combination with the decrease in the partial pressure of oxygen (PO2). Thus, Tucker et al. [27] proposed a potential effect of hypobaria on ventilation. Later studies of Loeppky et al. [14] corroborated this hypothesis. Other studies have suggested that body and lung fluid balance may be different during altitude and normobaric hypoxia [12]. More studies on hypobaric normoxia and normobaric hypoxia are thus required as a control condition to determine whether the two variables (reduced PB and reduced PIO2) work in the same or opposite directions to cause the disorders associated with hypobaric hypoxia.

Nitric oxide (NO) is an inorganic free radical involved in many physiological and pathological
processes, such as vasodilatation, inhibition of platelet aggregation, neurotransmission, and host defense mechanisms [20]. NO is formed from L-arginine by NO synthase (NOS), which oxidizes the terminal guanidino nitrogen of L-arginine, releasing NO and citrulline [19]. At least three distinct isoforms of NOS have been located and cloned [18]. Endothelial NOS (eNOS) and neuronal NOS (nNOS) isoenzymes have been described as constitutive proteins whose activities are regulated by calcium increase. Inducible NOS (iNOS) has been detected at low levels under basal conditions, but is induced by endotoxins and cytokines, producing toxic levels of NO; such activity does not depend on calcium increase. Insufficient or excessive production of NO underlies many serious diseases and pathologies [19].

Recent studies correlate hypobaric hypoxia with abnormalities in the synthesis of NO. Thus, Schneider et al. [24] proposed a reduction of NO synthesis during abnormalities in the synthesis of NO. Thus, Schneider underlies many serious diseases and pathologies [19].

Since the half-life of NO in the body is very short, nitrate, nitrite, and other nitroso compounds (NOx), which are stable metabolites of NO, are usually measured either in blood or in urine to determine NO production. Braman and Hendrix [4] have proposed a very sensitive technique for NOx analysis. Those authors indicate that nanogram detection limits are obtained when NOx are reduced at 80–90°C to NO in an acidic medium containing vanadium (III), the NO produced is removed from the reaction solution by scrubbing with helium carrier gas, and is detected by means of a chemiluminescence NOx analyzer.

In the present study, to evaluate the effects of reduced Pb and reduced P1O2 on the rate of NO production, we have determined the NOx levels produced during and after acute hypobaric hypoxia, and during and after hypobaric normoxia and normobaric hypoxia episodes, in serum and other rat tissues. We have attempted to determine whether reduced Pb and P1O2 can, independently, cause the same effects as when conjugated during acute hypobaric hypoxia.

Methods

Animals

Studies were performed on 32 male Wistar rats (2 months old), kept under standard conditions of light and temperature, and allowed ad libitum access to commercial rat chow and water until and after exposure to the different conditions in the experimental chamber. All the experiments were carried out according to EU guidelines for the use of animals for biochemical research (86/609/ EU).

Experimental procedure

Animals were separated in four experimental groups: sham control (C), hypobaric hypoxia (HH), normobaric hypoxia (NH), and hypobaric normoxia (HN). To create the acute hypobaric hypoxic conditions, the chamber was decompressed to 225 mmHg, resulting in an O2 partial pressure (PO2) of 48 mmHg (±30,000 feet). Normobaric hypoxia (716 mmHg barometric pressure [Jaén city] and 48 mmHg PO2) was achieved by regulating the oxygen/nitrogen proportion (1/13.7) introduced into the chamber. For hypobaric normoxia (225 mmHg barometric pressure and 150 mmHg PO2), the chamber was decompressed to the desired pressure and the oxygen/nitrogen mixture proportion was changed (1/0.53). Sham control animals were maintained in the experimental chamber under normobaric normoxic conditions. In all cases, values of pressure and O2/N ratio were attained in 20 min and maintained for 30 min. Return to the normobaric normoxic conditions was also attained in 20 min. Ventilation inside the chamber was continuous.

NOx analysis

To estimate NOx level, rats were lightly anesthetized with ether and blood was collected from the subclavian vein 1 h before and 4, 24, 48, and 72 h after the stay in the chamber. Blood collection before 4 h was not advisable due to the exhausted state of the rats after their stay in the acute conditions of the experiment. Specimens were centrifuged for 5 min at 13,500 rpm, and the sera obtained were immediately frozen and stored at −80°C until used. After the last blood extraction (72 h), rats were killed by cervical dislocation and cerebral cortex, hippocampus, cerebellum, decorticated brain (this piece includes basal ganglia, thalamus, hypothalamus, tectum, and tegmentum), heart, lung, kidney, and liver were quickly dissected and frozen until used.

To carry out the analysis, the tissue samples were thawed and homogenized directly, at low temperature, in 1/2/2 (w/v/v) deproteinization buffer (0.5 N NaOH and 10% ZnSO4), let stand at room temperature for 15 min and centrifuged for 5 min at 13,500 rpm. Afterward the supernatants were removed and maintained at 4°C until analysis. Serum samples were also deproteinized following this same protocol.

The total amount of NOx in the deproteinized samples was determined by a modification of the procedure described by Braman and Hendrix [4] using the purge system of Sievers Instruments, model NOA 280i. A saturated solution of vanadium chloride (VCl3) in 1 M HCl
was added to the nitrogen-bubbled purge vessel fitted with a cold water condenser and a water jacket to permit heating of the reagent to 90 °C, using a circulating bath. HCl vapors were removed by a gas bubbler containing 1 M NaOH. The gas flow rate into the detector was controlled by a needle valve adjusted to yield a constant pressure. Once the detector signal was stabilized, samples were injected into the purge vessel to react with the reagent, converting NO₅ to NO, which was then detected by ozone-induced chemiluminescence. NOₓ concentrations were calculated by comparison with standard solutions of sodium nitrate.

**Statistical analysis**

Statistical analyses were conducted using paired Student’s t tests for the measurement of NOₓ serum levels, and unpaired Student’s t tests for the measurements of NOₓ tissues, with P < 0.05 as the significance level.

**Results**

**Serum NOₓ**

As shown in Fig. 1, the serum in HH animals underwent a progressive decrease in NOₓ level, reaching significance at 24 h after the stay in hypobaric hypoxic conditions in the chamber (P < 0.005). In the NH and HN groups, a significantly diminished NOₓ level was observed 4 h after the experiment (P < 0.05 and P < 0.01 respectively). In the NH group, a significant decrease (P < 0.005) 48 h after the experiment was also observed. No significant changes were observed at any time in control normobaric normoxic group (data not included).

A comparison of NOₓ values between the different groups evidences a decrease of serum NOₓ levels in all the experimental groups after their submission to the different conditions in the chamber. This diminution is delayed in the HH group and long-lasting in the NH group.

**Brain NOₓ levels**

Cortex, cerebellum, hippocampus, and decorticated brain of the rats, sacrificed 72 h after their stay in the chamber, were analyzed to quantify NOₓ (Fig. 2). In the cerebellum, significant differences versus control were detected only in the HN group (P < 0.04). In the HN and NH groups, hippocampus NOₓ levels were significantly lower than in controls (P < 0.02 and P < 0.007, respectively). No statistically significant differences were found in either the cortex or the decorticated brain.

The highest values of NOₓ were found in the hippocampus, with significant differences versus cortex, cerebellum, and decorticated brain in the control (P < 0.003, P < 0.05, and P < 0.02, respectively) and HN (P < 0.01, P < 0.03, and P < 0.04, respectively) groups. NOₓ hippocampal levels also differed from those found in the cortex in the HH group (P < 0.03), and in both cortex and decorticated brain in the NH group (P < 0.007 and P < 0.03; data not shown in Fig. 2).

**Liver, heart, kidney, and lung NOₓ**

Table 1 shows the NOₓ levels in liver, heart, kidney, and lung 72 h after the stay in the chamber. The lung showed the highest values, independent of the experimental conditions. No significant differences between experimental and control animals were found in any tissue 72 h after the stay in the chamber.
Discussion

The present study was designed to analyze serum NO$_x$ as an index of NO production during and after hypobaric hypoxia, normobaric hypoxia, and hypobaric normoxia, and to evaluate the NO$_x$ accumulated in different tissues 72 h after these experimental conditions. Our data show that serum NO$_x$ levels underwent time-course changes, with a decline in the first stages after the stay in the chamber (4–24 h), followed by an increase after 24 or 48 h (depending on the experimental group), and a general later increase to return to the baseline values (72 h). Other authors [26] have found similar time-course changes following ischemia induced by middle cerebral artery occlusion in rats. In that case, the decline in NO$_x$ levels began during the ischemia.

It is known that, during a hypoxic injury, the l-arginine/NOS pathway is impaired [17]. Our results show that a hypobaric injury per se can also affect this mechanism of NO production. This phenomenon must be due to the decrease in the partial alveolar oxygen pressure ($P_{AO_2}$) experimented during a sudden acute hypobaric episode, and should be reverted if the organism is capable of adapting to these conditions.

NOS isoforms produce NO in a process that involves the reduction of molecular oxygen. During an episode of acute hypoxia, the activity of these enzymes may fall [20]. In this situation, the enzyme xanthine oxidase can generate NO by reducing nitrite in presence of NADH, compensating the diminished NOS activity [29]. Moreover, this enzyme is also able to produce NO reducing nitrates [2], the main component of plasma NO$_x$ [1]. Thus, the decreased NO$_x$ levels detected in this study could be explained as a consumption of NO$_x$ to produce NO in a hypoxic episode in which the normal NO synthesis is altered.

Table 1
Detection of NO$_x$ and S-nitroso compounds in deproteinized homogenates of liver, heart, kidney, and lung of sham control, normoxic hypobaric (HN), hypoxic hypobaric (HH), and hypoxic normobaric (HN) animals 72 h after the stay in the experimental chamber.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sham control</th>
<th>HN</th>
<th>HH</th>
<th>NH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>16 ± 3.7</td>
<td>11.9 ± 1.9</td>
<td>19.4 ± 2</td>
<td>42.5 ± 13.6</td>
</tr>
<tr>
<td>Heart</td>
<td>11.6 ± 1.4</td>
<td>10.8 ± 1.4</td>
<td>17.8 ± 3.9</td>
<td>65.5 ± 15</td>
</tr>
<tr>
<td>Kidney</td>
<td>14.5 ± 2.6</td>
<td>11.8 ± 1.7</td>
<td>14.1 ± 2.5</td>
<td>43.8 ± 14</td>
</tr>
<tr>
<td>Lung</td>
<td>9 ± 1.4</td>
<td>10 ± 1</td>
<td>24.8 ± 4.1</td>
<td>38.2 ± 11.5</td>
</tr>
</tbody>
</table>

Values are means ± SEM of concentrations (µM); $n = 8$. No significant differences were observed in any case when compared with the corresponding sham control.

Fig. 2. Detection of NO$_x$ and S-nitroso compounds in deproteinized homogenates of the cerebral cortex, cerebellum, hippocampus, and decorticated brains of sham control (C), hypobaric normoxic (HN), hypobaric hypoxic (HH), and normobaric hypoxic (NH) animals sacrificed 72 h after the stay in the experimental chamber. Values are means ± SEM of concentrations (µM); $n = 8$. *Significant differences from the sham control, $P < 0.05$; **$P < 0.01$. 
Another possible explanation for the decrease in NOX levels after the onset of hypoxia is a reduction in the oxygen supply and/or in \( \text{L-arginine} \), and/or disturbance of endothelial cells during the hypoxic period [11,16]. Previous studies showed that after a hypobaric hypoxic exposure, there is an increased expression of iNOS and nNOS isoforms in different brain zones [21,15], together with a rise of eNOS protein in the lung [7]. Our results demonstrate a diminution of NOX after the hypoxic injury. Thus, during a hypoxic episode, there is no correlation between NOS expression and NO production. Similar evidence was presented by Sato et al. [23] in hypertensive lungs of chronically hypoxic rats. The hypothesis of NOX consumption, or that of a reduction in the oxygen supply and/or in \( \text{L-arginine} \), and/or disturbance of endothelial cells cited previously, could also explain this event.

It is possible that the decrease in NOX levels reflects a decreased NOX formation due to the lower level of \( \text{NO/} \)oxyHb interaction, but the contrary proposal (i.e., the function of hemoglobin as a nitrite reductase and then the reduction of nitrite to NO) has also been put forward recently [8]. In fact, in highlanders, the most significant increase of NOX concentration in erythrocytes, compared with lowlanders, is detected in nitrites; this increase is explained as an enlargement of the vasodilative reserve [22]. In this sense, Bryan et al. [5] have revealed that tissue nitrite can serve as a significant extracellular pool of NO during periods of hypoxia. Further studies comparing the independent changes in nitrate and nitrite concentrations after a hypoxic/hypobaric injury would shed light on the relative importance of these two compounds as NO-storage molecules.

The quantification of NOX level in the different rat tissues 72 h after the injury, when serum NOX had returned to basal levels, showed significant differences with the sham control group only in cerebellum and hippocampus. These results correlate with other studies proposing that these two cerebral areas are especially sensitive to hypoxia–ischemia episodes [28,6]. Interestingly, these differences were detected only in the cerebellum of the hypobaric normoxic group and in the hippocampus of hypobaric normoxic and normobaric hypoxic groups, but not in the hypobaric hypoxic group. This could be explained as a beneficial effect of hypobaria during a hypoxic episode. A similar conclusion was drawn by Sekhon et al. [25] in a study of pulmonary function alterations in growing rats after exposure to hypobaria and/or hypoxia. In fact, the organism’s capacity of adaptation to hypobaric hypoxia must be higher than the adaptability to the other experimental conditions, due to its similarity with other extreme—although physiological—conditions (altitude).

In summary, we have shown that acute exposure to either hypobaria or hypoxia induces per se effects on NOX levels similar to or even greater than those caused by acute hypobaric hypoxia. In fact, hypoxia alone induces greater and longer-lasting effects on NOX serum levels than those caused by hypobaria and hypoxia together. Consequently, hypobaria could be considered a factor that moderates the effects of hypoxia on NOX level changes. At the same time, this analysis of NOX indicates a decrease of these NO-storage molecules during a hypoxic episode, just when NOS activity is limited due to the absence of molecular \( \text{O}_2 \). This result suggests a nitrate–nitrite reduction to NO in a reaction proposed by Zhang et al. [29], and whose magnitude and kinetics have recently been characterized by Li et al. [13]. We can thus conclude that NO production measurements in hypoxic episodes should take into account not only the \( \text{L-arginine/NOS} \) pathway, but also other mechanisms that can reduce nitrate–nitrite or other NO-storage molecules to NO, and whose exhaustion could mark an inflection point in the evolution of a hypoxic episode.

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