Structural alterations in adult rat carotid bodies exposed to hyperbaric oxygenation


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ABSTRACT

Inhibition of carotid body (CB) function is the main mechanism involved in the attenuation of respiratory drive observed during hyperoxia. However, only a few studies at 5.0 atmospheres absolutes (ATA) have analyzed carotid body structure or function in hyperbaric oxygenation (HBO₂) situations. We hypothesized that rats will present CB structural alterations when exposed to different lower hyperbaric oxygen doses enough to alter their chemosensory response to hypoxia.

Methods – Twenty-one adult male Wistar rats, divided into three groups, were maintained in room air or exposed to O₂ at 2.4 or 3.0 ATA for six hours. Histological, ultrastructural and immunohistochemical analyses for neuronal nitric oxide synthase (nNOS) and F2-isoprostane were performed in the excised CBs.

Results – Histological analyses revealed signs of intracellular edema in animals exposed to both conditions, but this was more marked in the 3.0 ATA group, which showed ultrastructural alterations at the mitochondrial level. There was a significant increase in the volume density of intraglomic-congested capillaries in the 3.0 ATA group associated with an arteriolar vasoconstriction. In the 2.4 ATA group, there was a relative increase of glomic light cells and a decrease of glomic progenitor cells. Additionally, there was a stronger immunoreactivity for F2-isoprostane in the 3.0 ATA O₂-exposed carotid bodies. The glomic cells stained positive for nNOS, but no difference was observed between the groups. Our results show that high O₂ exposures may induce structural alterations in glomic cells with signs of lipid peroxidation. We further suggest that deviation of blood flow toward intraglomic capillaries occurs in hyperbaric hyperoxia.

INTRODUCTION

Carotid bodies are the main chemoreceptors that are sensitive to changes in arterial blood oxygen levels (pO₂) in several species, and they respond to hypoxia by driving an increase in ventilation, both in respiratory frequency and in tidal volume [1]. A complex network of vascular tissue forms carotid bodies [2], and this involves the clusters of chemosensitive glomic cells. It is believed that the anatomic distribution of the blood vessels within the carotid bodies has an important role in regulating O₂ chemosensitivity in glomic cells [1, 3, 4].

Oxygen exposures above 0.5 atmospheres absolute (ATA) cause dysfunction of the respiratory system; if exposure is prolonged, respiratory failure and death result [5]. The contribution of regulatory mechanisms associated with the carotid body in the pathogenesis of respiratory failure under such conditions is still unclear.

Inhibition of carotid body function is the main mechanism involved in the attenuation of respiratory drive observed after chronic normobaric or acute high hyperbaric oxygenation (HBO₂). There is a decrease in the specific chemosensitivity to oxygen that can be verified by an attenuated response of the carotid
sinus nerve (impulses per second) and decreased minute ventilation when animals are subsequently exposed to hypoxia at a level with no evident clinical signs of lung intoxication [6,7,8].

Reactive oxygen species (ROS) have been implicated as signaling molecules in several metabolic processes [9,10,11]. Together with nitric oxide (NO), ROS have been implicated in the control of vascular function [12,13,14], and a possible role of NO and ROS in regulating tissue vascular changes during hyperoxia has been suggested [13,15,16,17]. Rats exposed to hyperbaric oxygenation showed a Po2-dependent increase in the concentration of aortic perivascular NO due to an increase in nNOS activity [47]; and nNOS-derived NO has also been implicated in the pathogenesis of hyperbaric oxygen toxicity [13,16,17].

A better understanding of the possible inflammatory and vascular glomic tissue abnormalities that occur in hyperoxic states may thus provide useful information to understand the modifications in respiratory control in these circumstances. There are very few studies that have focused on the histological changes of the carotid bodies exposed to normo- or hyperbaric hyperoxia [18,19,20]. Torbati and co-workers previously described altered physiological responses associated with ultrastructural alterations of glomic cells at the mitochondrial level that were exposed to hyperbaric oxygenation at 5.0 ATA for ~2 hours in cats, which is a dose sufficient to cause overt pulmonary intoxication [18].

Altered glomic physiological responses to hypoxia have also been observed in lower hyperbaric O2 doses that are insufficient to cause pulmonary or other manifestations of oxygen toxicity [8]. We hypothesized that structural alterations in the carotid bodies of rats would also occur, not only in high, but also in low hyperbaric O2 doses as well. Therefore, in the present study, we performed ultrastructural and morphometric analyses and an immunohistochemical evaluation of oxidative stress and nNOS expression in the carotid bodies of rats that were acutely exposed to hyperbaric oxygen concentrations at 2.4 and 3.0 ATA.

MATERIALS AND METHODS
This study was approved by the institutional Ethical Commission Committee.

Animals. Twenty-one male adult Wistar rats that weighed ~330 g were used in this study. The rats were kept in the animal facility at 22-23 degrees C, with controlled humidity and a dark-light cycle of 12 to 12 hours. Food and water were available ad libitum.

Hyperbaric oxygen exposure (HBO2). The animals were divided into three groups. Rats from the control group were kept in room air. Preliminary tests with O2 at 2.4 ATA for three hours showed no relevant histological alterations in the glomic parenchyma (data not shown). A second group was then exposed to doses of O2 that corresponded to a sub-maximal suppressor effect of the ventilatory response to hypoxia (RH) with no clinical signs of lung intoxication [8]. The third group was exposed to an O2 dose high enough to cause overt lung toxicity [5]:

- Group 1 (n=8): control group, kept in room air at 1.0 ATA;
- Group 2 (n=5): exposure to O2 at 2.4 ATA for six hours; and
- Group 3 (n = 8): exposure to O2 at 3.0 ATA for six hours.

The animals were placed in a 480-liter steel hyperbaric chamber (AN-700, Atlas, Sao Paulo, SP, Brazil). Before starting the compression, the chamber was closed and flushed with pure oxygen for five minutes to eliminate the remaining chamber air. The chamber was compressed and decompressed at a rate of 0.1 bar/minute. During the entire period of HBO2 exposure, the chamber was continuously ventilated with 5 liters/minute of pure oxygen to prevent CO2 accumulation.

Experimental procedures after HBO2. Soon after HBO2 exposure, the animals were anesthetized with sodium thiopental (50 mg/Kg body weight, i.p.) and were then reintroduced into the chamber, in order to remain breathing in an oxygen-enriched atmosphere until carotid body extraction. For extraction, both of the carotid arteries were surgically exposed. The region of carotid bifurcation was isolated and fixed in situ by dripping 4% paraformaldehyde in PBS buffer for 10 minutes over it. The entire region was removed in bloc and was then rapidly immersed in the fixative solution.

Tissue processing. Right arterial carotid fragments (n=5 in each group) were prepared for light microscopy analysis. The fragments were fixed for 24 hours in 4% paraformaldehyde in PBS buffer. After alcoholic dehydration, the fragments were embedded for 12 hours in a 1:1 mixture of Historesin solution (Leica Historesin Embedding Kit, Leica, Wetzlar, Germany) and absolute alcohol and were then
embedded for another 24 hours in a pure Historesin solution. Three-micrometer-thick slices were cut at 6-µm intervals and stained with hematoxylin, 0.2% fuchsin and 0.1% toluidine blue (HFT).

The left arterial carotid fragments (n=5 in each group) were prepared for electron microscopy analysis. Briefly, the fragments were first fixed for two hours in 2% glutaraldehyde in 0.15 M sodium and phosphate buffer of pH 7.2 at room temperature. The entire fragment was cut into 2-mm slices. All of the arterial slices were post-fixed in a 1% osmium-tetroxide solution for one hour and in 1% uranyl acetate overnight; the slices were then dehydrated in acetone and embedded in araldite epoxy resin (Araldite 502, Electron Microscopy Sciences, Hatfield, Penn. USA). Ultrathin sections were cut using an ultramicrotome (Leica Ultracut UCT, Leica), and they were subsequently mounted on 200-mesh copper grids and stained with lead citrate. Electron micrographs were recorded with a Jeol electron microscope (Jeol 1010, Jeol, Tokyo, Japan).

**Immunohistochemistry.** An immunohistochemical analysis was performed in three controls and in three 3.0 ATA six-hour-exposed animals. Left arterial carotid fragments were fixed in 4% paraformaldehyde in PBS buffer for 24 hours and embedded in paraffin. Three-micron-thick histological sections were incubated overnight at 4 degrees C with the primary antibodies. Primary antibodies against nNOS (BD Transduction Laboratories, San Diego, Calif. USA) and F2-isoprostane (anti-8-epi-PGF2α, Oxford Biomedical Research, Oxford, Ind. USA) were used at a dilution of 1:600 and 1:500, respectively. The sections were then washed in PBS buffer, and the secondary biotinylated antibodies, anti-mouse IgG (Vector Elite PK6102, Vector Laboratories, Burlingame, Calif. USA) for nNOS and anti-goat IgG (Vector Elite PK-6105, Vector Laboratories) for F2-isoprostane, were utilized and incubated for 60 minutes at 37 degrees C. The slides were further incubated in avidin-biotin-peroxidase complex (ABCKit Vectastain, Vector Laboratories) for 30 minutes at 37 degrees C. The color was developed by a 3.3 diaminobenzidine tetrahydrochloride (DAB) kit (Sigma Chemical Co, St. Louis, Mo. USA), and the slides were counterstained with Harris’ hematoxylin (Merck, Darmstadt, Germany). For the negative controls, the primary antibody was replaced by a phosphate buffer solution (PBS) during the staining process. For nNOS staining, we used the nodose ganglion of the control animals as a positive control.

**Histological and morphometric procedures.** A descriptive analysis was performed on histological and ultrastructural sections of the carotid bodies. The HFT staining used in this study identified light glomic cells as having large, faintly stained nuclei with poorly condensed chromatin and an abundant cytoplasm. Dark glomic cells had a strongly stained nucleus with more condensed chromatin and an abundant cytoplasm. Progenitor glomic cells had small, deeply stained basophilic nuclei. The ultrastructural analysis was performed only in the controls and in animals exposed to 3.0 ATA for six hours.

The quantitative analysis of the histological samples was based on the stereological principles summarized by Weibel [21] and Gundersen and co-workers [22]. We performed measurements of the total carotid body volume, the volume densities of capillaries (with or without red blood cells – RBCs) and the interstitial stroma. We also quantified the relative proportion of sustentacular cells, total glomic cells and their variants as well as light, dark and progenitor cells. Finally, we performed measurements of the arteriolar luminal-wall ratios (L/W ratio, as an index of vasoconstriction). Morphometry was performed using a standard point-counting procedure with the aid of a coherent test system of 25 points and 30 lines in a grid of known area [22].

The total carotid body volume was calculated using Cavalieri’s method [22]. To ensure a random selection of sampling for histological analysis, an initial section was chosen at random, and one of 10 sections was then systematically selected for total volume calculations. In each section, a field was selected at random at 40x. Then, in this field, measurements of the numerical and volume densities were obtained at 400x. A total of ten 40x fields, corresponding to 10 distinct sections per carotid body, were analyzed. The relative proportions of each cell type were calculated by dividing their respective estimated number by the total number of cells. The volume densities were computed by dividing the number of points that hit the structure by the total number of points [22].

Quantification of the L/W ratio was performed on transversally cut arteriolar sections from intraglomic regions, according to Rivero and co-workers [23]. Transversally cut vessels were defined as the
vessels exhibiting a variation between their maximum and minimum diameter of $\leq 10\%$. Briefly, after the same sampling procedure, a total of five sections per carotid body were analyzed. The measurements were performed at 1,000x using a grid of 900 $\mu m^2$. The L/W ratio was determined by placing the entire arteriole within the limits of the grid; the number of points that hit the lumen or wall of the vessel was then determined. The areas of the arteriolar lumen and wall were then calculated according to equations (1) and (2):

1. **luminal area** = number of points hitting the lumen $\times$ 36;
2. **wall area** = number of points hitting the arteriolar wall $\times$ 36.

where 36 $\mu m^2$ is the area associated with one point in this magnification.

To verify the existence of a linear correlation between the doses of oxygen and the degree of arteriolar vasoconstriction, the doses of oxygen respective to the HBO$_2$-exposed groups were converted to UPTD units in order to facilitate future comparisons with different oxygen doses. The concept of unit pulmonary toxic dose (UPTD) encompasses the number of UPTD units that are equivalent to the duration, in minutes, of a similar oxygen exposure at 1 ATA [24]. The control, 2.4 ATA six-hour and 3.0 ATA six-hour groups, have associated UPTD values of 0, 1094.4 and 1375.2, respectively (for calculations see Clark [24]).

**Statistical analysis.** Data were expressed as means $\pm$ SEM. Morphometric data were compared using analysis of variance (ANOVA) followed by the Student-Newman-Keuls post-hoc test. The correlation between the L/W ratio and the UPTD values were analyzed using the Pearson’s test, and the control group was set as zero. For all tests, the level of significance was adopted at 5%. The statistical package, STATISTICA v. 5.1 for Windows (StatSoft, Tulsa, Okla. USA), was utilized.

**Figure 1** – Photomicrographs of rat carotid bodies in the (A) control, (B) 2.4 ATA six-hour and (C) 3.0 ATA six-hour O$_2$-exposed groups. The arrows indicate light (black), dark (red) and progenitor (green) glomeric cells and a sustentacular cell (yellow), present in all groups. The exposed groups presented capillary congestion and intracellular edema, more prominent in C (*).

(Hematoxylin, Fuchsin, Toluidine blue – HFT.)
(Scale bar = 25 $\mu m$.)
RESULTS
No animals presented signs of CNS oxygen toxicity during the entire study period. Qualitative analyses revealed normal organ architecture in all of the study groups, with signs of intracellular edema in both of the O₂-exposed groups. Edema and congestion were more prominent in the 3.0 ATA six-hour group than in the other groups (Figure 1, facing page).

Ultrastructural analysis
The ultrastructural analysis of the control carotid bodies revealed mitochondria with intact cristae and typical dense-cored vesicles surrounded by a clear halo (Figures 2A and 3A, above). In this group, sustentacular cells presented few organelles. Conversely, the glomic cells of the 3.0 ATA six-hour-exposed animals showed intracellular edema, deformed...
Mitochondria with loss of cristae and sustentacular cells with a prominent endoplasmic reticulum (Figures 2B and 3B, Page 423). No evident difference in the vesicle distribution was observed between the two groups. In the exposed group, occasional platelets were found in the congested capillaries, a common feature of oxygen toxicity (Figure 4, above).

(Take bar = 2.4µm.)

**Morphometrical analysis**

There were no significant differences in the total carotid body volumes of the animals exposed to O₂ in relation to the controls. The mean total volume of the controls was 81.54±3.76 x10⁶µm³, while those of the 2.4 ATA and 3.0 ATA groups were 78.84±3.76 x10⁶µm³ and 79.38±6.67 x10⁶ µm³, respectively (p>0.05).

**Volume densities of capillaries and interstitial stroma**

There was a significant increase in the volume density of capillaries, including those with RBCs, in the carotid bodies of the 3.0 ATA six-hour group in relation to the other two study groups (p<0.05) (Table 1, below).

The volume density of the interstitial stroma revealed an increase in the two groups exposed to HBO₂ for six hours, but the difference was not significant (p>0.05) (Table 1).

**Luminal-wall ratio (L/W ratio)**

There was a decrease in the L/W ratio of the intra-glomic arterioles. Values of the 3.0 ATA six-hour group (0.296±0.019) were significantly different (p<0.005) from the control and the 2.4 ATA six-hour groups (0.494±0.051 and 0.433±0.032, respectively), which were not significantly different from each other (Figure 5, facing page).

There was a statistically significant negative correlation between the L/W ratios and the UPTD values when all of the groups were considered (r=-0.25, p=0.0005).

**TABLE 1 – Volume densities of capillaries with red blood cells (RBC), total vascular sinus space and interstitial stroma in the control and O₂-exposed animals.**

<table>
<thead>
<tr>
<th></th>
<th>Capillaries with RBC</th>
<th>Total of capillaries</th>
<th>Interstitial stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Air; 1.0 ATA</strong></td>
<td>0.140 ± 0.013</td>
<td>0.166 ± 0.013</td>
<td>0.186 ± 0.013</td>
</tr>
<tr>
<td><strong>6h; 2.4 ATA</strong></td>
<td>0.106 ± 0.011</td>
<td>0.146 ± 0.012</td>
<td>0.201 ± 0.013</td>
</tr>
<tr>
<td><strong>6h; 3.0 ATA</strong></td>
<td>0.192* ± 0.015</td>
<td>0.212† ± 0.015</td>
<td>0.197 ± 0.014</td>
</tr>
</tbody>
</table>

Values are mean ± standard error (SE). * p<0.01 compared to control, p<0.001 compared to 2.4 ATA six-hour group; † p<0.05 compared to control, p<0.01 compared to 2.4 ATA six-hour group.
Figure 5 – Luminal-wall ratios (L/W ratio) in carotid bodies from the control and O2-exposed animals. Values are mean ± SE. * p<0.005 compared to 2.4 ATA six-hour O2-exposed group; p<0.0001 compared to control.

**Figure 5**

**Cellular proportions**

Table 2 (below) shows the relative proportions of total glomic cells (and their variants) and of sustentacular cells in the carotid bodies for the different study groups. There were no significant differences in the proportion of sustentacular cells or total glomic cells between all of the groups (p>0.05). Dark cells were predominant in all of the groups, and there were no differences between the groups (p>0.05). On the other hand, there was a reduction in the proportion of progenitor cells with a statistical significance in the intermediate group (2.4 ATA six-hour) in relation to the control group (p<0.001) and to the highest dose group (p<0.01). Similarly, in the intermediate group, there was a significant increase in the proportion of light cells when compared to the other groups (p<0.05).

**DISCUSSION**

In this study, we describe histological and ultrastructural alterations of the carotid bodies of rats exposed to 2.4 and 3.0 ATA hyperbaric hyperoxia. Overall, the most striking findings were the intense glomic cell edema, mitochondrial disarrangement and vascular abnormalities, such as capillary congestion and arteriolar vasoconstriction. Animals exposed to the highest O2 doses also presented an increased immunoreactivity to F2-isoprostane in the glomic parenchyma. To the best of our knowledge, this is the first study to describe histological and ultrastructural alterations of carotid bodies induced by acute hyperbaric hyperoxia in rats.

**Immunohistochemistry**

Figures 6 and 7 (Pages 426-427) show representative sections of histological slides stained with nNOS and F2-isoprostane, respectively, obtained from the carotid bodies of controls and rats exposed to O2 at 3.0 ATA for six hours. In both groups, there was a positive reaction for nNOS in the arterioles and, interestingly, in the glomic cells. The pattern of staining in the glomic cells was similar to that of the neuron cells in the nodose ganglion (Figure 6C). F2-isoprostane immunostaining occurred at the interstitial stroma involving glomic cell clusters, which were mainly at the periphery of the organ, in both of the groups. The carotid bodies of exposed rats presented a more intense staining than did the controls, with some cytoplasmatic staining in the glomic cells (Figure 7).

**TABLE 2 – Proportion of the different glomic cell variants, total glomic cells and sustentacular cells of carotid body from the control and O2-exposed animals.**

<table>
<thead>
<tr>
<th>Light cells</th>
<th>Dark cells</th>
<th>Progenitor cells</th>
<th>Total glomic cells</th>
<th>Sustentacular cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air; 1.0 ATA</td>
<td>15.2 ± 1.1</td>
<td>38.1 ± 1.8</td>
<td>15.8 ± 1.4</td>
<td>69.1 ± 2.1</td>
</tr>
<tr>
<td>6h; 2.4 ATA</td>
<td>19.1* ± 1.4</td>
<td>39.2 ± 1.8</td>
<td>9.2† ± 1.2</td>
<td>67.5 ± 1.8</td>
</tr>
<tr>
<td>6h; 3.0 ATA</td>
<td>16.2 ± 1.0</td>
<td>39.4 ± 1.3</td>
<td>13.5 ± 1.2</td>
<td>69.2 ± 1.6</td>
</tr>
</tbody>
</table>

Values (% of total of cells) are presented as mean ± SE. * p<0.05 compared to control and 3.0 ATA six-hour O2-exposed groups; † p<0.001 compared to control; p<0.01 compared to 3.0 ATA six-hour group.
Histological and ultrastructural carotid body alterations

In the present study, we found signs of intracellular edema in the oxygen-exposed groups (Figures 1B, C, Page 122), which was more prominent in animals exposed to the 3.0 ATA six-hour group. In this group, electron microscopy confirmed the presence of intracellular edema associated with significant mitochondrial alterations in the glomic cells. Torbati et al. [18] exposed cats to O₂ at 5 ATA for 90-135 minutes and, in line with our findings, also observed mitochondrial alterations. The authors suggested that the generation of reactive oxygen species (ROS) during hyperoxygenation could be implicated in the mitochondrial degeneration. It is well known that a rise in pO₂ elevates the ROS production by mitochondria [25,26].

The presence of intracellular edema in glomic cells with a lower oxygen dose has never been described. This fact indicates that altered plasma membrane permeability due to changes in physical characteristics or functional properties of plasma membrane has occurred in this phase of oxygen intoxication, and this is likely related to the oxidative stress already present with the lower oxygen toxic dose we used in this study. Intracellular edema also suggests that there is impairment in the membrane potential control of the glomic cells, interfering with the physiological response to hypoxia associated with the opening of voltage-gated Ca²⁺ channels [31]. This result gives support to Liberson et al.’s [8] physiological findings. Our data give new insights for the proper use of HBO₂ in a safe manner because one must consider that a putative glomic intoxication could occur with oxygen doses insufficient to cause clinical pulmonary intoxication.

Evidence for lipid peroxidation

In accordance with the results above, the increase in immunoreactivity to F2-isoprostane in the glomic parenchyma of the 3.0 ATA six-hour exposed animals observed in this study suggests that there was an occurrence of lipid peroxidation in the carotid bodies during high levels of oxygen exposure, a signal indicative of an increase in glomic ROS production. Isoprostanes are metabolic products of arachidonic acid peroxidation by free radicals, independent of the cyclooxygenase enzymes [27]. They are structurally stable bioactive prostaglandin (PG) F2-like compounds produced
in vivo in low quantities. However, isoprostane production increases in situations of oxidative stress. They have been used as biomarkers, especially the F2-isoprostane, in several pathological conditions where oxidant injury is expected, such as asthma [28], hepatorenal syndrome, and scleroderma [29], as well as in experimental models of hepatic oxidant lesion [27,29] and in oxidative lung injury models of pulmonary hypertension [30].

**Arteriolar vasoconstriction, microvascular changes and related causes**

Our findings also indicate that vascular changes occur in the carotid body of animals exposed to hyperbaric hyperoxia. Carotid bodies have a very complex vascular structure with at least three post-arteriolar routes, providing an adequate match between chemosensitive cells and blood flow [2]. Here, we describe an intraglomic capillary congestion associated with arteriolar vasoconstriction. These glomic vascular changes in response to hyperbaric hyperoxic exposures have not been described previously. Hyperoxia causes peripheral vasoconstriction that is more prominent in the arteriolar side of the microcirculation and is induced by local intrinsic mechanisms evoked by an excess of circulating O₂ [32]. We hypothesize that in situations of hyperbaric oxygenation, despite local arteriolar vasoconstriction, a flow deviation towards the intraglomic capillaries occurs, contrary to what has been previously described in situations of hypoxia [1,3,33].

Animals exposed to high O₂ doses presented arteriolar vasoconstriction and a negative correlation between the doses of oxygen converted to UPTD units and the L/W ratio. Several factors could be implicated in the vasoconstrictor effect that we observed. An arterial hypocapnia normally occurs during hyperoxia due to an indirect hyperventilatory effect [34]. An inhibition of vasodilator prostaglandin release from the endothelium was previously described in high levels of tissue oxygen, leading to vasoconstriction [35]. Also, there could possibly be participation of endothelin-1 (ENT-1) [36]. Both ENT-1 and NO modulate glomic chemosensory discharges, mainly due to vascular effects [4].

Certainly NO and ROS must also be implicated in the glomic arteriolar vasoconstriction observed in this study. Besides the well-known increase in NO production by hyperoxia [37], a perivascular NO reduction due to its rapid reaction with oxidative stress-derived superoxide (O₂⁻) [9,10,14] could lead to the arteriolar vasoconstriction observed in this study. Zhilyaev and co-workers [15] ascribed to a decrease in NO content, due to NO degradation O₂⁻-mediated and subsequent formation of peroxynitrite, the cerebral hyperbaric oxygen vasoconstriction they observed. Other authors have documented a similar hyperbaric hyperoxic effect on the CNS [13].

Another possible contributor to the arteriolar vasoconstriction observed in this study is the increase in F2-isoprostane. Roberts and Morrow described a
direct vasoconstrictor effect from nanomolar concentrations of F2-isoprostane in renal arterioles and an indirect effect from endothelin-1 released from isoprostane-activated endothelial cells of bovine aorta [29]. Finally, it has been proposed by some authors that CNS oxygen toxicity could increase sympathetic activity, even in the absence of convulsions [13,16,17]. If this is the case, although none of our exposed rats presented with convulsions, it is possible that this mechanism may be involved also in the vasoconstriction observed in this study.

On the other hand, we did observe an increase in the volume density of capillaries with red blood cells in the group exposed to the highest dose of oxygen, indicating the presence of capillary congestion. Because there was also a significant increase in the volume density of the total capillary space, it is likely that a greater number of capillaries would have also become functional. These results suggest that a deviation of flow from more calibrated vessels toward the capillaries, effectively perfusing the glomic cells, has occurred, indicative of increased glomic local flow. The results also suggest the existence of local adaptive conditions when presented with oxygen excess, in agreement with the plasma-skimming theory formulated by Acker et al. in hypoxic conditions [1,38].

In this sense, it is possible that a larger amount of dissolved O2 will reach the chemoreceptor cells, attenuating or inhibiting the reflex carotid response in this situation. However, toxic lesions due to the excess of O2 can occur. Therefore, we believe that the local circulation plays an important role in hyperoxia, either having a protective function or, conversely, contributing to the genesis of hyperoxic lesions due to the mechanisms of autoregulation of O2-mediated flow. Hence, as the local flow increases with very high tissue pO2, more erythrocytes come into the glomic parenchyma. Initially, this is beneficial because erythrocytes will act as a sink for some of the generated ROS; however, over time, this mechanism becomes overloaded, and glomic oxidative stress lesions occur. This situation might partially explain why shorter HBO2 exposures causes less carotid body damage than longer normobaric oxygen exposures [6,18,19,39] because with lower tissue pO2, the local flow does not increase sufficiently to cause capillary congestion, and only high amounts of plasma-dissolved O2 will reach glomic cells.

**nNOS expression**

In this study, immunoreactivity for nNOS could be observed in the histological sections of carotid bodies from both the control group and the animals exposed to O2 at 3.0 ATA; however, there were no qualitative differences in immunostaining for nNOS between the control and 3.0 ATA exposed groups. It is well known that hyperoxia increases NO production [37]. nNOS-derived NO has been implicated in the pathogenesis of both CNS and pulmonary hyperbaric oxygen toxicity [16,17]. However, Thom and co-workers did not find any significant difference in the protein concentration of aorta homogenates for both eNOS and nNOS isoforms between the rats exposed to O2 at 2.8 ATA and the controls, which is in line with our findings [40]. Lungs from the adult rats presented an increase in the eNOS content [41] only after prolonged normobaric hyperoxia (85% O2 for 28 days), but not after acute exposure (< 72 hours) [42]. Other studies revealed alterations in eNOS and nNOS expression when the duration of the hypoxic exposure changed [43,44]. Taken together, it is possible that a 3.0 ATA six-hour O2 exposure was not sufficient to cause any detectable change in nNOS content.

An interesting finding of this study was the specific staining for nNOS that occurred in the glomic cells. Although these results are different from those typically found in the literature, which exclude a positive immunoreactivity for any isoform of NOS to glomic cells [45,46], Prabhakar and colleagues [47] used a NADPH diaphorase histochemical method and found positive focal staining for this enzyme in glomic cells, similar to that found in guinea pigs by Tanaka and Chiba [48]. NADPH diaphorase was then verified to be identical to the nNOS molecule [45].

Recent work has clearly shown specific eNOS immunoreactivity to glomic cells, precisely in the mitochondrial membranes [49]. Early antibodies for NOS detection were directed against epitopes of the activated form of the enzymes [50]. In the study by Yamamoto et al. [49], an antibody against amino acids 599-613 of the eNOS molecule was used, enabling detection of the enzyme in an inactivated state. Our antibody was also not directed to an nNOS epitope, but it had an immunogen region directed against nNOS amino acids 1095-1289, which may have also allowed for the detection of the enzyme in the inactive state. Furthermore, the fact that interstitial stromal cells did not present nNOS-positive
immunostaining suggests a specificity of the reaction to glomic cells. This was supported by similar nNOS immunoreactivity at the nodose ganglion (Figure 6C), which is known to have nNOS- and NADPH diaphorase-positive ganglion cells [45]. These results suggest new interpretations to describe the increase in ventilation to hypoxic and cyanide stimulus in nNOS knockout mice [51] because they are results that lend support to the notion that the glomic cells – and not necessarily the nerve endings [52,53] – are the O₂ chemosensing structure in the carotid bodies [54,31].

**Clinical relevance**

A histological analysis of glomic tissue revealed the presence of three types of glomic cells, as previously described for other species [55,56]. There was an increased proportion of light cells associated with a decreased proportion of progenitor cells in the group that received O₂ at 2.4 ATA for six hours, without changes in the total glomic cell proportion. This finding could indicate that there was a dynamic response from this cell population to stressful environmental conditions, similar to that previously described for patients presenting with acute hypoxia in ARDS (acute respiratory distress syndrome) [57], or different oxidative stress susceptibilities in the glomic cell subtypes. It is possible that the more pronounced histological alterations observed in glomic cells at 3.0 ATA may have contributed to non-recognition from the different cell populations and therefore might partially explain the absence of differences in the glomic cell proportions in this group.

The significant lesions of the carotid bodies found at the highest O₂ dose in this study could partially explain the sudden breathing failure that is observed in patients with oxygen toxicity. In animal studies, deaths are usually preceded by a sudden elevation of arterial pCO₂ with high arterial pO₂ levels, pointing to possible misregulation in the ventilatory drive [58,59]. Although the presence of pulmonary mechanoreceptor stimulation could favor an increase in respiratory frequency, the sudden decrease that is observed in the terminal stages of pulmonary O₂ toxicity suggests a failure of peripheral chemoreception mechanisms [59]. With the discrete and progressive increase of arterial pCO₂ due to the progress of intoxication, the central chemoreceptor would tend to adapt to this situation. In this sense, structural lesions of carotid bodies observed at high doses of O₂ exposure could lead to alterations in the chemoreflex to CO₂. Studies of the carotid body chemoreflex function in these situations could supply more information about the role of these organs in terminal breathing failure due to oxygen toxicity.

It is interesting to note that Gelfand et al. [60] have observed no reduction in the hypoxic ventilatory sensitivity in men exposed to toxic hyperbaric oxygen at 1.5, 2.0 or 2.5 ATA for 17.7, 9.0 or 5.7 hours, respectively. Despite the different species susceptibility in oxygen toxicity between rats and men, the oxygen doses used in men had to be less toxic that an exposure to oxygen at 3.0 ATA for six hours due to ethical problems associated with exposing volunteers to such a dose. 3.0 ATA for six hours was also verified to be two-thirds of the LD50 of 9.0 hours at 3.0 ATA for rats [61].

Finally, we used animals maintained in room air as a control group, similar to other reports [18,39]. Other studies using humans or animals exposed to air at 1 ATA have found no significant physiological or histological changes due to presence in the hyperbaric chamber interior [62,63]. In a recent study, rats exposed to hyperbaric normoxia at 3 ATA (a mixture of 7% O₂, 93% N₂) showed no sign of pulmonary oxygen toxicity [16].

In summary, we describe cellular and vascular structural alterations in the glomic parenchyma of rats exposed to elevated oxygen concentrations, and this suggests that lipid peroxidation of glomic tissue occurs at high doses of hyperoxia. Our data advance the current knowledge on glomic microcirculatory function and open new horizons for studies that focus on the characteristics of local carotid body blood flow in hyperoxic or non-hyperoxic situations. Also, our results may help explain further some of the clinical alterations that are seen in hyperoxic conditions like the attenuation of the ventilatory hypoxic drive [6,7,8,39].

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