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Morphological and Neurochemical Plasticity of the Carotid Body after Long-Term Hypoxia: Vascular and Cellular Involvement, Morphometric Study in *Meriones shawi* Rats

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ABSTRACT

Morphological changes in carotid body (CB) were studied in Meriones shawi rats that were made hypoxic by placing them in poor area inspired oxygen (10 % O_2, 28 days) in a normobarique room. The comparisons were carried out with animals kept in normoxia. At the end of the experimental period, hypertrophy CB was observed in all hypoxic animals. It induced an increase in the size of the organ by a factor of 3.8. In an attempt to elucidate the mechanisms involved in this phenomenon, a quantitative study was performed on serial 5-µm thick sections. The morphometric results concern the extravascular compartment (connective tissue islets of glomus cells) and the vascular compartment. ***1)*** *The extravascular surface (by section) has increased by +67.5 % and the extravascular underwent an increase of +88 %. However, when the extravascular volume is expressed relative to the volume of CB no significant change was observed.* ***2)*** *The volume density (Dv) of the islets of glomus cells underwent a slight but non-significant decrease of -7.4%, while the Dv of connective tissue has undergone a significant increase of +50.8%. These results demonstrate that increase observed in the extravascular space of hypoxic CB was not due to cell multiplication, as no mitotic figures were observed. It took place by hypertrophy of type I cells and by an increase in the size of the interstitial space.* ***3)*** *Regarding the vascular compartment, the Dv of microvessels has increased +99.6%, total vascular volume increased by +398% while the volume of the small vessels (<12 microns in diameter) showed only a slight increase of +44.1%. These same parameters when expressed relative to the volume of CB show that the total vascular volume has underwent a slight increase of +32% and that the volume of small vessels has decreased by 619 %. Finally, the variation in the CH of the number density of the small vessels and the change in the surface of the endothelial lining of small and large vessels (> 12 mm in diameter) show that the observed increase in the total vascular volume would largely due to increased volume of large vessels.* ***4)*** *A high number of type I cells that react to the chromaffin reaction was detected in hypoxic animals. Data show that the number of positive type I cells in this reaction was increased by 80.8-times. Their Dv was enhanced by 32-times. In the absence of cell multiplication signs, these results suggest that CH induced the appearance of noradrenaline in glomus cells. All data obtained in Meriones shawi rats suggests that CB hypertrophy is due to an increase in total vascular volume primarily generated by large vessels and an increase in the extravascular volume. Glomus cells react to the CH by hypertrophy and increased, after a delay, of its noradrenaline content. This could be correlated to subsequent increase in the CB sensitivity. We suggest that ventilatory adaptation related to carotid chemoreceptor can induce, at least in part, progressive morphological changes leading to an increase in the extravascular, reducing the density of the small vessels, and enhancing the volume extravascular by small vessels. This should have the effect of increasing the distance between the vessels and the center of the type I and type II islets that would reduce the PO_2 in the tissue and from there an increase in the activity of chemoreceptor. If, as we have proposed, increasing the extracellular volume is the result of the increase in the interstitial space and probably of cell type I and type II it should further increase the blood capillary-tissue distance.*

KEY WORDS: Carotid body; Meriones shawi Rats; chronic hypoxia; microvasculature; Norepinephrine; chromaffin cells; morphometry.

INTRODUCTION

Hypoxia is a stimulus that invades the whole animal body allowing it to react, in a few seconds by increased pulmonary ventilation so that oxygen delivered to the tissues will be maintained at an adequate level. The reaction of

the organism to hypoxia is primarily due to a reflex that originates at the carotid body (CB). This is a peripheral arterial chemoreceptor, located at the bifurcation of the carotid artery, which responds to a lower partial pressure of oxygen (PaO_2), an increase of the partial pressure of carbon dioxide ($PaCO_2$) or to a decrease in blood pH .

Histology of CB is dominated by the presence of islet cells and by blood capillaries that are disseminated in tissue rich in collagen. The islets are formed by glomus cells (also called type I) bordered by supporting cells (type II) and their irrigation is ensured by a dense network of fenestrated capillaries [1, 2, 3]. Chemosensory transduction stimulus occurs from type I cells via their synaptic contacts with the sensory fibers of the carotid sinus nerve (CSN) [4, 5, 6, 7, 8]. The primary sensory neurons in the petrosal ganglion made the connection between the CB, in which are generated chemosensory stimuli and bulbar centers of respiratory control. The mechanism for the increase in sensory discharges into the CSN in response to hypoxia is not yet fully elucidated. Most available models suggests that hypoxia causes a cascade of events in the cell type I, starting with a membrane depolarization, followed by an inflow of Ca^{2+} ions allowing release of catecholamines and neuropeptides by glomus cells and ending with an increase of the discharge frequency in the afferent innervation [4].

Humans and animals are commonly subjected to chronic hypoxia (CH) in their lives. When it occurs, the flow of pulmonary ventilation increase progressively in the same way as hypoxia, in order to ensure blood saturation with oxygen. Later, the hyperventilation leads the body to acclimatize to the CH [9]. The ventilatory adaptation to the CH can be seen in humans living in high altitude [10,11], in patients with chronic respiratory insufficiency, obstructive sleep apnea or asthma attacks [12], or in animals that are subjected experimentally, at sea level, to a poor experimental oxygen environment [9,13].

The underlying mechanisms associated with acclimatization were the subject of numerous studies [for review see [10]. The essential role of CB in the adaptation of the body to the CH is now indisputable since bilateral denervation of the CB in animals is always accompanied by a reduction or abolition of ventilatory acclimatization to CH [13]. Electrophysiological studies have shown that the sensitivity of the CB to a drop in PaO_2 in arterial blood increased after 48 hours of exposure of Cat to hypobaric hypoxia [14] and the activity recorded in the CB in the same animal remained high after 28 days of hypoxia [15]. Finally, a study of sensory activity of CB showed a progressive increase in the frequency of discharges related to the duration of hypoxia [16]. All these results suggest that CH works by increasing the chemosensitivity of the CB and afferent activity in the CSN. The CB becomes progressively more reagent to smaller declines of PaO_2. This important physiological mechanism is the basis of pulmonary function changes that occur during exposure to CH [9, 14, 15].

The efforts that have been made to explain the increased chemosensitivity in the CH effect were mainly focused on mobilizing neurotransmitters contained in the dense-cored vesicles of glomus cells [13, 17]. Significant changes occur in the synthesis, storage and use of dopamine (DA) and noradrenalin (NA) in type I cells [18, 19, 20]. The first CB content in DA begins to increase just few hours after the onset of hypoxia, while the NA begins to rise a week later. However, after long term hypoxia (28 days), the stock of NA jumped to 51 times higher than the control while the DA increased by a factor of 27 [21, 22, 23, 24,25]. In a previous study conducted in rabbits, we demonstrated that the type I cells containing NA differ from those harbouring DA by several cytological and biochemical characteristics. These cells, in addition to their ability to capture specifically the exogenous NA, may be distinguished by their darker cytoplasm contains, large dense-cored vesicles, dopamine-ß-hydroxylase and a large amount of glycogen. They also react to chromaffin reaction and their vesicles resist emptying by reserpine [25, 26, 27, 28]. Despite these differences, the noradrenergic cells cannot be regarded as really different cells in the CB. Indeed, [26, 27] showed that noradrenergic cells of the CB, represent a functional state within a homogeneous population of type I cells. The high amount of NA stored in the CB after a long term hypoxia, causes a variation in the DA/ NA ratio that reflects a change in the dynamic balance between the two functional states of type I cells [28]. However, if the increase in chemosensitivity of CB during the CH is associated, at least in part, to the increased NA content of CB content the histology of the organ should present an increased number of cell type I having the characteristics mentioned above. To the best of our knowledge, no quantitative studies have been yet conducted.

Furthermore, the increase in chemosensitivity of CB by the CH is correlated with another important morphological phenomenon characterized by a hypertrophy of the organ. It is a phenomenon that is observed in physiological or pathological CH, in humans [29, 22, 30] or in animals [18, 20, 24]. An expansion of the microvasculature and an increase in the volume of parenchyma cell were described [18, 31, 32]. Rigorous morphometric study remains necessary in order to highlight the mechanisms that govern these phenomena. The introduction by hypoxia of a new balance between the two functional states of type I cells for NA, and dilation of microvessels may constitute the morphological support increasing the chemosensitivity accompanying the CH.

To answer cheek this hypothesis, we investigated the morphological changes induced by CH in the CB. Further information was sought by combining morphometric measurements in the vascular and cellular compartments of hypoxic CB as well as quantification of glomus cells containing the NA. Detecting the NA was carried out using a technique of the chromaffin reaction [33], adapted to the CB tissue in desert rodent *Meriones shawi Rats*.

MATERIAL AND METHODS

2.1. Animals used:

The animals used in this work are desert rodents belonging to the *Gerbillideae* family: *Meriones shawi* rats (*MS*). The parents *MS* rats were trapped in the semi-arid climate of Boulmane region (located in the Middle Atlas of Morocco) qualified as middle altitude. All animals used in this study were born and raised in pet store under standard conditions of light and temperature and having free access to water and dry food.

2.2. Animal exposure to normobaric hypoxia:

Five groups each formed by eight *MS* rats (male, 16 - 20 weeks old, 240 ± 35 g weight) were exposed to CH for 31 days in a normobaric chamber of hypoxia. To allow acclimatization of the animals, the rate of O_2 in the hypoxic chamber was lowered progressively during the first three days at 17%, 15% and 12%, by injecting the N_2 in the chamber. Finally, the O_2 rate was maintained between 9.5% and 10.5% during the remaining 28 days of exposure. CO_2 concentration in the hypoxic chamber was maintained at a basal level (less than 1%) by circulating the gaseous mixture through the quicklime. Humidity due to the breath was trapped using a desiccant. Animals were removed from the hypoxic chamber about 20 minutes every 3 days for the maintenance of the cage. At the end of 28 days, they were anesthetized and prepared for perfuse. On the other hand, five groups each formed by eight *MS* rats, breathing ambient air have been installed in the same room in a cage next to the hypoxic chamber to serve as a control.

2.3. Animals perfusion:

Animals were anesthetized using ethyl urethane diluted in saline solution (1g / Kg, ip), subjected to midline sternoctomy and quickly sacrificed by a large incision of the right atrium. This step was immediately followed by perfusion of the carotid bifurcation via the aorta with a sodium chloride 0.9 % solution at 37 ° C followed by the solution containing the fixative solution. Perfusion pressure (about 100 mmHg) was made by placing the vial containing the fixative to 1.40 m above the animal.

2.4. Chromaffin reaction:

The chromaffin reaction was adapted to the CB tissue from Tranzer and Richards [33]. The carotid bifurcation was perfused with a 1% glutaraldehyde and 0.4% paraformaldehyde solution in chromate-dichromate buffer at pH 7.2. After taking the carotid bifurcation, the CB was quickly isolated and then immersed for 1 hour in the same fixative at 4 °C. The organ was then incubated for 24 hours at 4 °C in another chromate-dichromate buffer at pH 6.0. Blocks of tissues were rinsed in running water before being dehydrated and embedded in paraffin. Serial transverse sections of 5 µm thick were made using a microtome. A cut out on two followed the rest of the treatment of the chromaffin reaction. These sections were fixed on slides then dewaxed and immersed in an ammoniacal silver nitrate solution (Fontana) before being treated with an aqueous solution of 0.5% gold chloride and then fixed in a solution of 5% sodium thiosulphate to 5%. The optical microscope observation was sometimes preceded by a slight hematoxylin-eosin. Quantification of chromaffin cells was performed in a manner similar to the quantitative histological study described below. The other half of serial sections was reserved for the morphometric study. They were deparaffinized and contrasted with toluidine blue before final mounting between slide and cover slip.

2.5. Histological quantifications:

The procedures used are based on morphometric principles summarized by Weibel [34]. Morphometric measurements were performed by counting using a visual piece grid incorporated in the eyepiece light microscope. The surface of each frame contains 50 lines and 100 points. By this method we determined the area of the sections and the volume of the CB. The volume density (Dv) of the islets of glomus cells was studied under x 40 magnification. Through the entire CB, only half of the cuts made were studied. At each histological section 10 fields randomly arranged, were examined. For each field 100 points were counted and the Dv islets was determined by the following relationship: Dv = Pi / Pt, with Pi representing the number of points that coincide with the islet cells and Pt representing the total number of points considered for each field studied under a microscope.

The relative proportions of the major categories of cells in the CB (CII, Clears CI, Dark CI and degenerating CI) were calculated by dividing the number density of each cell type by the total number of cells counted. The categories of cells are counted in each field by respecting the limits of inclusion and exclusion of the grid with the impartial procedure described by Gundersen and al. [35].

Regarding the vascular compartment, a histological analysis was performed on serial sections of the CB at regular intervals of 10 µm. Values were obtained using direct measures ocular microscope and digital photographs. The parameters studied are: 1- area of the sections and the total volume of CB; 2- total vascular area and volume; 3- extravascular area and volume by subtraction; 4- endothelial surface of the small vessels (diameter <12 microns); 5- endothelial area of the large vessels (diameter> 12 microns); 6- the proportion of the endothelial area of the small vessels related to the volume of the CB. The values of extravascular CB area and the area of the small vessels are obtained by adding the respective areas, measured at the individual sections of the whole organ.

2.6. Statistic study:

In all quantitative studies, the t-test was used to compare means hypoxic animals to those of control animals. Values were considered significant when $P \leq 0.05$.

RESULTS

3.1. Morphological observations:

The CB of the *MS* rats, studied here for the first time, takes the form of a small pair organ on the latero-dorsal face of the carotid sinus at 2-3 mm from the internal carotid origin. The pink color of the CB and the presence of some veins on its surface allows to spot from the upper cervical ganglion nearby. CB dimensions are quite variable from one animal to another, but according to our measurements, the CB of a *MS* rat with an average weight of 200 g measured 375 ± 50 µm long and 300 ± 25 µm wide.

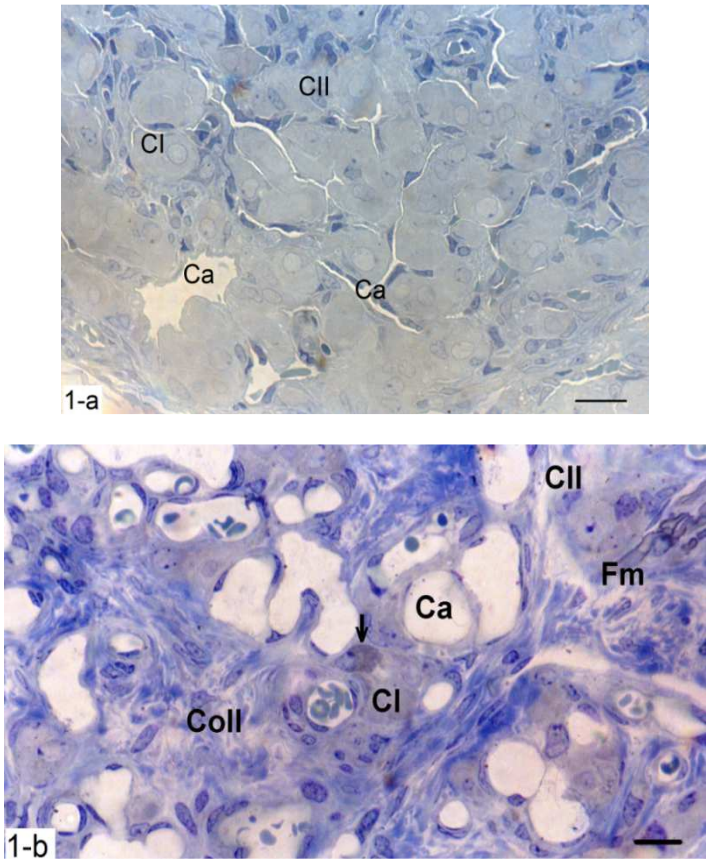


Figure 1: Histological sections of CB from normoxic Meriones Shawi rats. The serial sections are from Meriones Shawi rats that were exposed for 28 days in a normoxic environment (normoxia, ambient air). At this magnification (bar = 10 µm) the parenchyma is essentially characterized by vascularization regularly distributed across the cutting surface. Tissue containing islets of type I cells (glomus cells, CI) are surrounded by cytoplasmic processes of type II cells (CII). Their parenchyma is rich in capillaries (Ca), collagen (Coll) and nerve fibers some of which are myelinated (Fm). Some type I cells that exhibit a dark cytoplasm (arrow) from adjacent cells (CI) which are characterized by a clear cytoplasm are clearly visible (Figure 1-b) .

In control rats (*MS*c) the CB, which forms a small bulge barely marked on the wall bifurcated carotid artery becomes more prominent and visible after 28 days of CH. The thickness of the CB is increased in hypoxic rats (*MS*h). Its more reddish color is due to the peripheral vasculature. These changes were not observed in either the superior cervical ganglion nor in the petrosal ganglion, which are related to the CB by their location and their function.

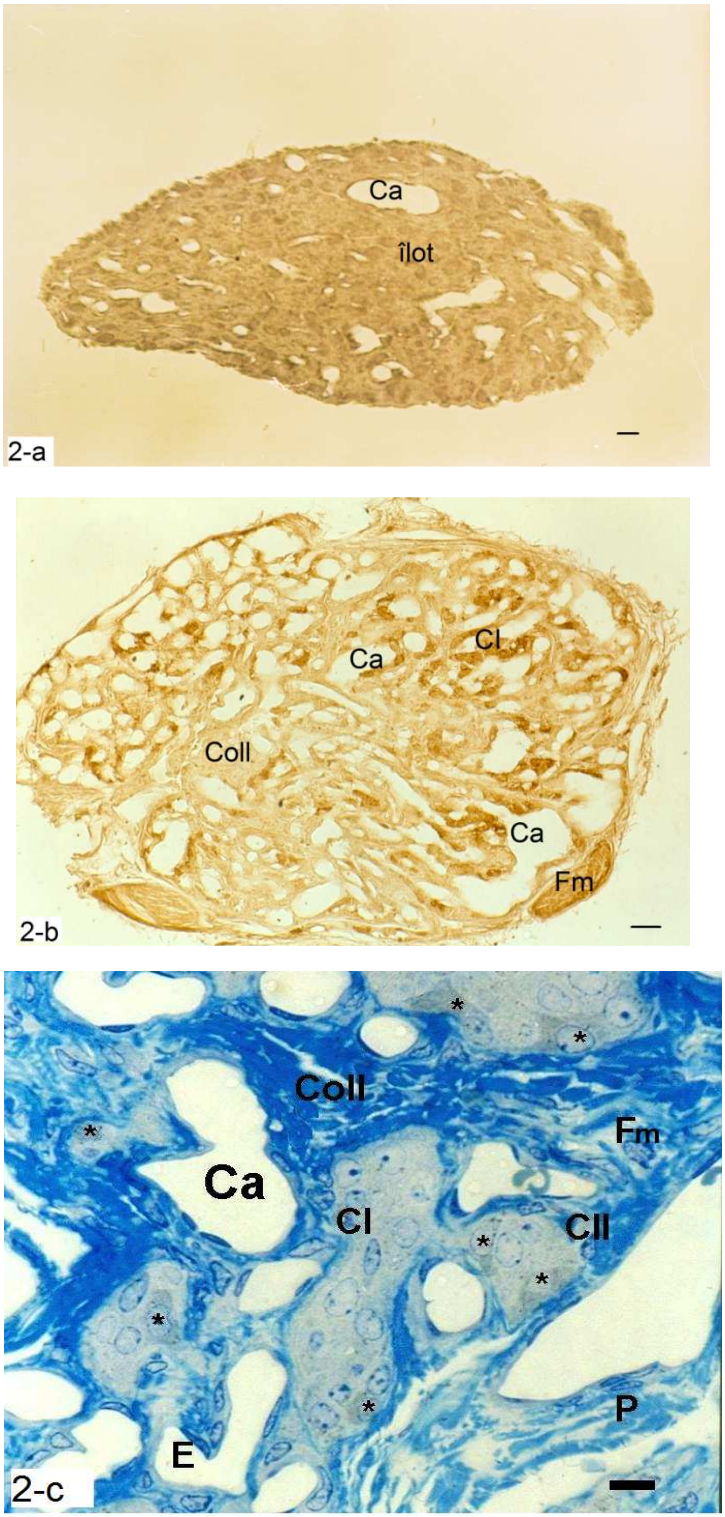


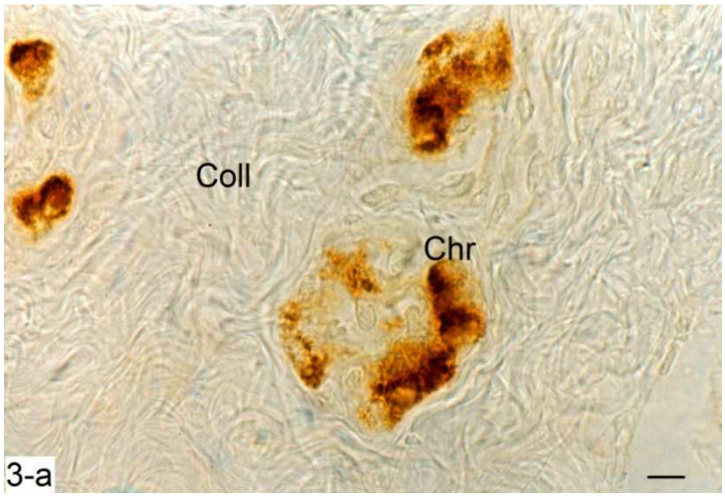
Figure 2: 2b and 2-c are two histological sections of CB from Meriones Shawi rats who undergoes normobaric hypoxia for 28 days (10% oxygen). Compared with normoxic CB in Figure 2-a, the hypoxic CB is essentially characterized by greater size and dilation of these microvessels. It is particularly visible at the periphery of the organ. Type I cells (CI) are stretched and distorted by the dilation of capillaries (Ca). Endothelial cells (E) have more contrast and collagen (Coll) always abundant and occupies large spaces between the capillaries and some myelinated nerve fibers (Fm). Bar: 25 µm.

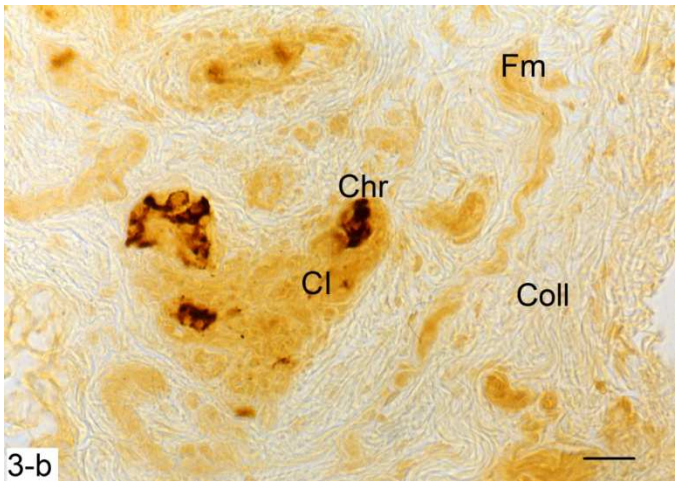
At the histological level, the CB of *MS*c shows a similar architecture to that of the rat, the nearest animal. Thus, we found a significant vascularization the presence of glomus cells islets and abundant nerve fibers [Figure 1: a-b]. According to the cutting plane, each islet seems to be composed by a variable number of type I cells (2-5) recognized by their rounded nucleus, clear and slightly colored by toluidine blue. Their granular cytoplasm is

generally of low contrast. Sometimes, the cytoplasm shows a darker side. The Type II cells can easily recognized by their colorful small nucleus. They envelop type I cells with a thin veil of agranular cytoplasm. According to the cutting plane, the distance between the islets of cells and capillaries is variable. It can be very low. However, the contours of often rounded islets allow contact with the capillaries only in few places.

In hypoxic animals, capillaries are dilated and their endothelial lining may have some thickening [Figures 2 a-b]. The endothelial cells appear more contrasted by toluidine blue but at this stage of hypoxia, their nuclei present no characteristic figures of mitosis. The space between the dilated capillaries is not very different from the normoxic CB. In places, the extravascular space is occupied by the islets which are no longer round but more elongated forms and their contact surface with the capillaries appears to be more important. Although the vascular network is well distributed throughout the hypoxic CB, some areas further inward CB, have large surfaces not or poorly vascularized consisting only of islets of cells surrounded by an abundant collagen [figure 2]. The nuclei of type I and type II cells do not exhibit characteristic figures of cell division. After the period of hypoxia a number of type I cells characterized by a moderately or very dark granular cytoplasm are frequently observed [figure 2-c]. Identical cells are rarely observed in normoxic CB [figure 1-b].

Normoxic CB tissues contain some type I cells that react positively to the chromaffin reaction [Figures 3 a-b-c]. Their pattern is in the form of black granules that are generally limited to the cytoplasm contrasting with the contour of the unlabeled nucleus. The separation limits between cytoplasm of the chromaffin cells and that of adjacent negative cells are generally neat. Although their number is small, these chromaffin cells are found in about two thirds of histological sections. The granular aspect of the chromaffin reaction coincides with the dense-cored vesicles that accumulate NA.





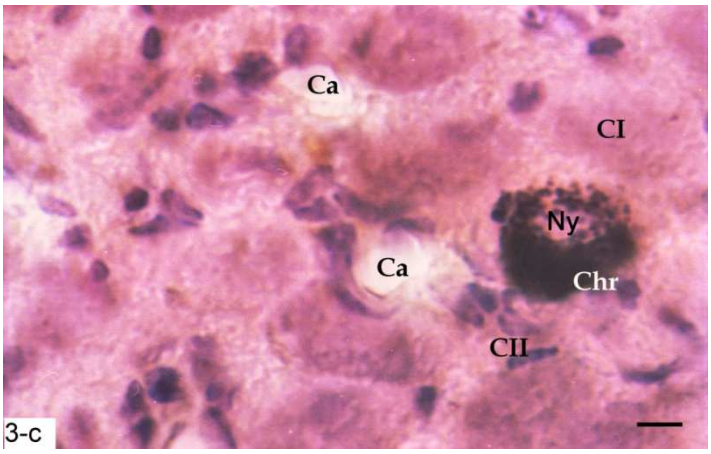


Figure 3: Histochemical revelation of cells containing the NA by reaction chromaffin on sections of 5 µm thick. The serial sections are from CB of *Meriones Shawi* rats that have been exposed during 28 days to normoxic environment (normoxic, ambient air). Only some type I cells that reacted positively to the chromaffin (Chr) while the majority of the CI are negative. The pattern is still visible after a slight staining with hematoxylin-eosin (Figure 3-c). Marking form the black granules restricted to the cytoplasm, contrasting with contour of the unlabelled nucleus (Ny). The boundaries of separation between the cytoplasm of the chromaffin cells and those of negative adjacent cells (CI) are neat. Ca, capillary; CII, type II cells; Coll collagen. Bar: 5 µm.

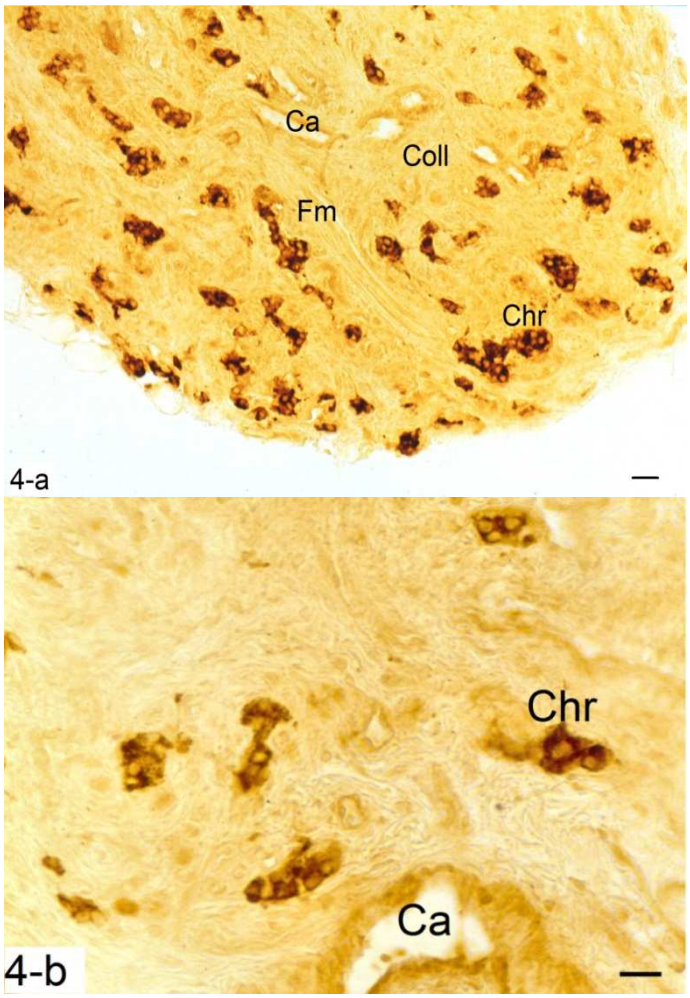


Figure 4: Histological section of hypoxic Meriones Shawi rats CB processed for histochemical detection of cells containing the NA by a chromaffin reaction on 5 microns thick sections. The serial sections of CB are from Meriones Shawi rats that were exposed for 28 days in a hypoxic environment (Hypoxia, 10% O_2). The number of type I cells that have reacted positively to the chromaffin reaction has become very important. According to the cutting plane, chromaffin cells may seem isolated from collagen or part of lobules containing other unlabeled type I cells. The labeling intensity by the chromaffin reaction varies from one cell to another. Bar: 25 µm.

The number of cells that react to chromaffin reaction increases significantly after 28 days of hypoxia [Figures 4: a-b]. According to the cutting plane, chromaffin cells may seem isolated in the collagen or be part of islets formed by other unlabeled type I cells. The labeling intensity by the chromaffin reaction varies from one cell to another. A majority of completely negative cells is always present in CB of *MS*h.

3.2. Morphometric results:

The morphometric study from a few hundred histological sections of CB reveals that the average volume of the organ increased in hypoxic rats [*MS*h +280%; P <0.001] [figure: I]. The main histological parameters (islet cells, connective tissue and microvessels) may be involved in this increase in the CB volume were quantified [figure: II]. Initially, the volume density (Dv) of the glomus cells islets has value higher in the control rat [*MSc*: 0.59]. After CH, Dv showed only a slight but non-significant decrease [*MS*h: -7.4 %]. Dv of the connective tissue showed an increase [*MS*h: +50.8%; P <0.001]. The Dv of microvessels also exhibited a significant increase [*MS*h: +99.6%; P <0.001].

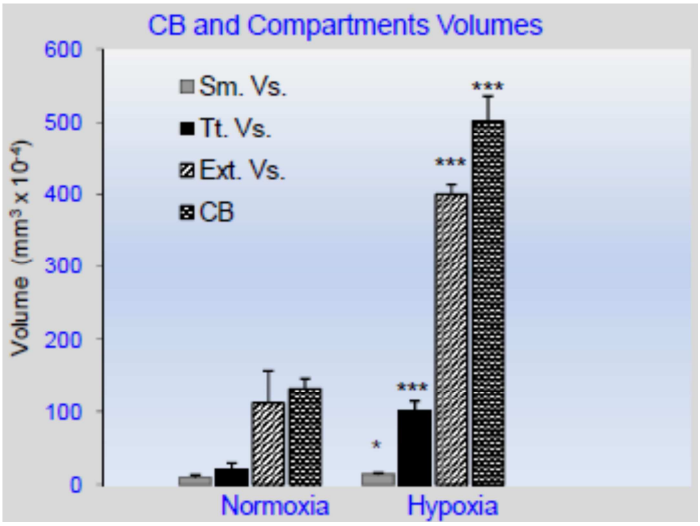


Figure I- Volumes of the carotid body (CB), small vessels (Sm.Vs.), total vascular (Tt.Vs.) and extravascular (Ext.Vs.). Morphometric study was performed on serial sections of CB of Meriones shawi rats that were exposed for 28 days, in a hypoxic environment (Hypoxia, 10% O2) or a normoxic environment (normoxia, ambient air). Each column represents the mean of measurements in 40 pairs of CB (value ± SEM; t test; * P <0.05; ** P <0.01 and *** P <0.001). The CH induced increases in the average volumes of Meriones Shawi rats CB +280%, the total vascular +398%, the small vessels (<12 microns in diameter) +44.1% and the extravascular +257%.

Other changes concern the volume of the microvasculature [figure: I]: The total vascular volume enhanced sharply [*MS*h: +398%; P <0.001]. The volume of the small vessels (<12 mm in diameter) exhibited only a slight increase [*MS*h: +44.1%; P <0.05]. Finally, the extravascular tissue (islet cells and connective tissue) obtained by subtraction, showed a strong increase [*MS*h: +257%; P <0.001].

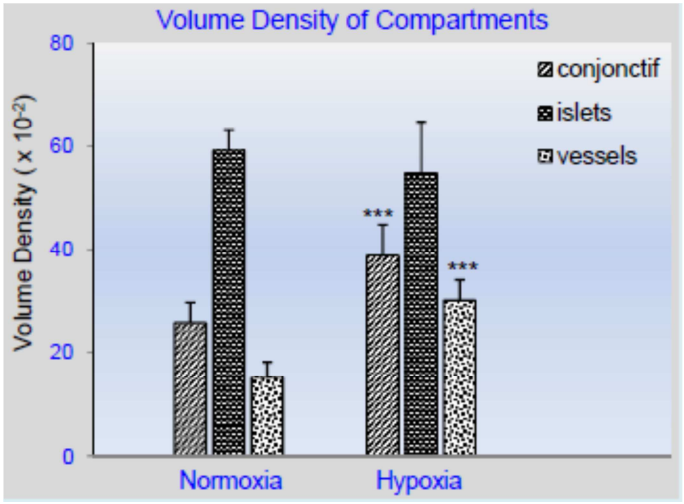


Figure II- Volume density of connective tissue, the islets of glomus cells and blood vessels in the CB. Morphometric study was performed on serial sections of CB of Meriones shawi rats that were exposed for 28 days, in a hypoxic environment (Hypoxia, 10% O_2) or a normoxic environment (Normoxia, ambient air). Each column represents the mean of measurements in 40 pairs of CB (value ± SEM; t test; * P <0.05; ** P <0.01 and *** P <0.001). After CH density of glomus cell islets underwent a slight decrease of -7.4%, while the density of connective tissue shows an increase of +50.8%. Microvascular density underwent a sharp rise of +99.6%.

When they are based on the volume of the CB, the observed changes are not identical [figure: III]. While the total vascular volume increased [*MS*h: +32.0%; P <0.05], the volume of the small vessels undergoes decrease [*MS*h: -61.9%; P <0.01]. The extravascular is not affected since the declines observed in hypoxic rats were not significant [*MS*h: -5.9%].

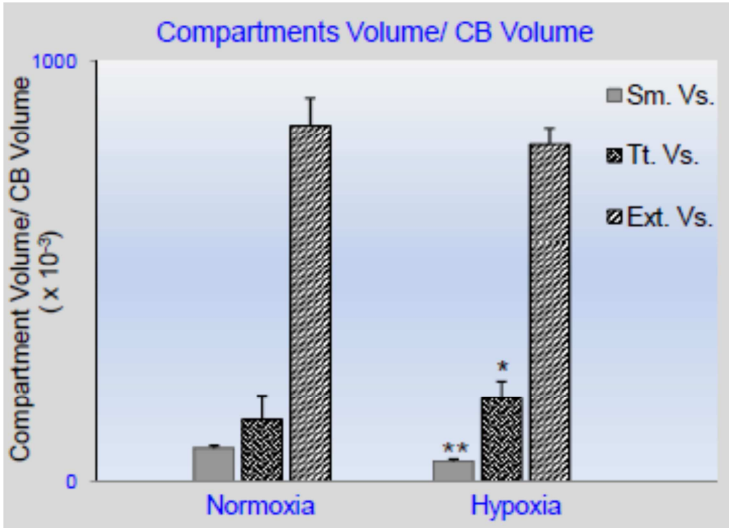


Figure III - Total vascular volume (Tt. Vs.), volume of the small vessels (Sm. Vs.) and extravascular volume (Ext. Vs.) expressed relative to the volume of CB. Morphometric study was performed on serial sections of CB of Meriones shawi rats that were exposed for 28 days, in a hypoxic environment (Hypoxia, 10% O_2) or a normoxic environment (Normoxia, ambient air). Each column represents the mean of measurements in 40 pairs of CB (value ± SEM; t test; * P <0.05; ** P <0.01 and *** P <0.001). After CH total vascular volume shows an increase of +32 %, the volume of small vessels undergoes a decrease of -61.9 %. The extravascular volume is not affected since the decrease of -5.9 % is not significant.

Observation of small vessels was complemented by a quantitative study of their number [figure: IV]: When the number of small vessels is expressed by CB a non-significant increase was observed [*MS*h: +17.4 %]. The same tendency was noticed when the number of small vessels is reported to CB section [*MS*h: +7.5%]. However, when the number of these vessels is expressed per mm^2 sectional area, this decreased [*MS*h: -31.4%; P <0.05].

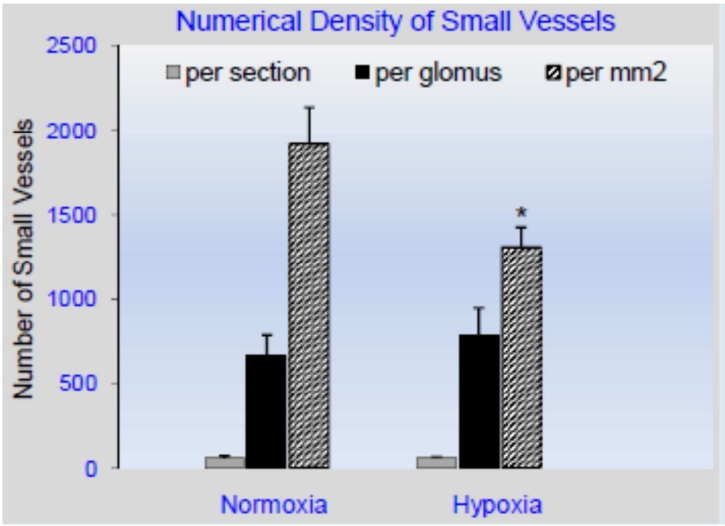


Figure IV- Numerical density of small vessels (number of small vessels per section, mm^2 or carotid body). Morphometric study was performed on serial sections of CB of Meriones shawi rats that were exposed for 28 days, in a hypoxic environment (Hypoxia, 10% O_2) or a normoxic environment (Normoxia, ambient air). Each column represents the mean of measurements in 40 pairs of CB (value ± SEM; t test; * P <0.05; ** P <0.01 and *** P <0.001). After CH the number of small vessels has increased when expressed per section or per CB (+7.5 % and +17.4% respectively). When this number is expressed relative to mm^2, a 31.4% reduction was observed.

The change in the surface of the endothelial envelope (ES) has also been studied for two categories of CB microvessels [figure: V]: ES small vessels exhibits only a slight increase [*MS*h: +65.1 %; P <0.01], whereas the increase of the ES of the large vessels is greater [*MS*h: +189.1%; P <0.01].

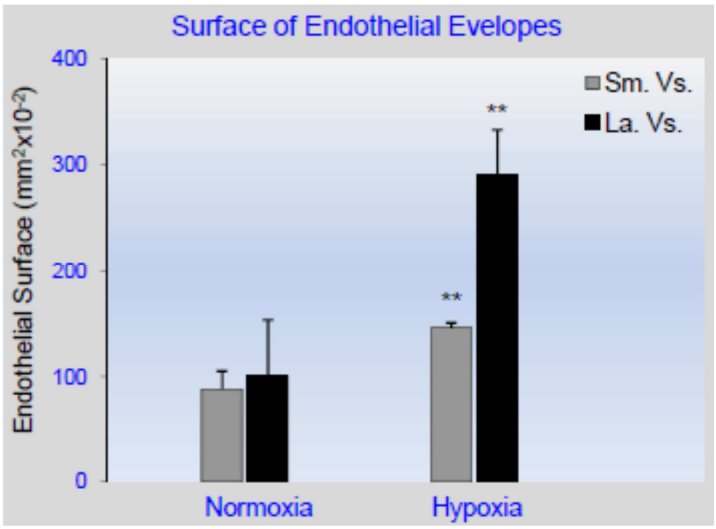


Figure V- Surface of endothelial envelope of small vessels (Sm. Vs.) and large vessels (La. Vs.) in the carotid body (CB). Morphometric study was performed on serial sections of CB of Meriones shawi rats that were exposed for 28 days, in a hypoxic environment (Hypoxia, 10% O_2) or a normoxic environment (Normoxia, ambient air). Each column represents the mean of measurements in 40 pairs of CB (value ± SEM; t test; * P <0.05; ** P <0.01 and *** P <0.001). After CH the surface of endothelial envelope of small vessels has an increase by +65.1%, while the increase of this surface is more important in large vessels +189.1%.

The ES of small vessel expressed relative to the volume of the CB or with respect to the extravascular surface has in both cases a decrease [*MS*h respectively: -21% and -12.2%; P <0.05], [figure: VI]. The large vessels reacted differently since EES large vessels expressed by the volume of the CB reveals an increase in the rat hypoxic [*MS*h: +52.6%; P <0.01], [figure: VI].

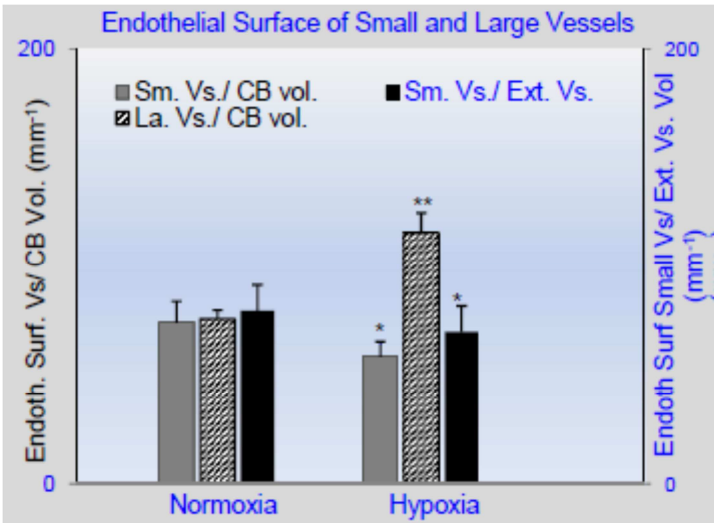


Figure VI- Surface of endothelial envelope of small vessels and large vessels expressed relative to the volume of the carotid body (Sm Vs / CB vol. and La Vs / GC vol.) Or expressed relative to the extravascular surface (Sm. Vs /Ext. Vs.). Morphometric study was performed on serial sections of CB of Meriones shawi rats that were exposed for 28 days, in a hypoxic environment (Hypoxia, 10% O_2) or a normoxic environment (Normoxia, ambient air). Each column represents the mean of measurements in 40 pairs of CB (value ± SEM; t test; * P <0.05; ** P <0.01 and *** P <0.001). After CH the surface of the endothelial envelope of small vessel showed a decrease when expressed relative to the volume of the CB (-21 %) as well as when it is expressed relative to the extravascular surface (-12.2 %). However the surface of endothelial envelope of the large vessels expressed relative to the volume of the CB showed an increase of +52.6%.

Extravascular surface (connective tissue + cells islets by section) is experiencing a significant increase in [*MS*h: +67.5%; P <0.01], [Figure VII]. This increase is still visible when the extravascular surface is expressed relative to the number of small vessels [*MS*h: +48.8%, P <0.01] [Figure VII].

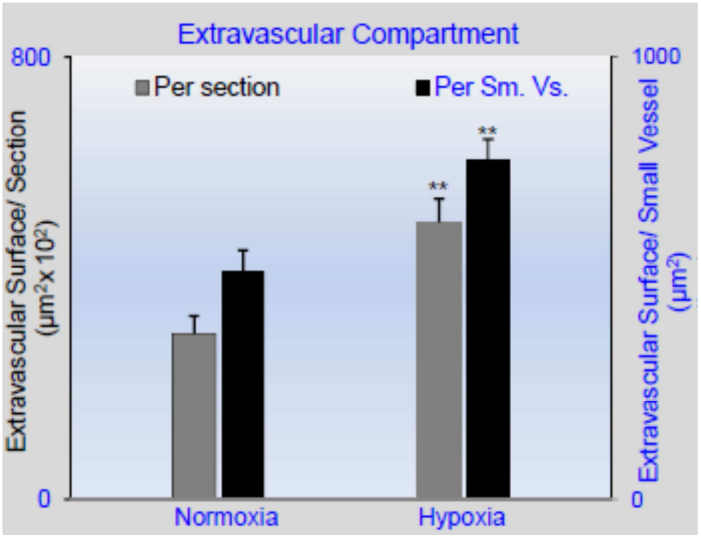


Figure VII: Extravascular compartment: The extravascular surface was expressed per section (Per section) or expressed per small vessel (Per Sm. Vs.). Morphometric study was performed on serial sections of CB of Meriones shawi rats that were exposed for 28 days, in a hypoxic environment (Hypoxia, 10% O_2) or a normoxic environment (Normoxia, ambient air). Each column represents the mean of measurements in 40 pairs of CB (value ± SEM; t test; * P <0.05; ** P <0.01 and *** P <0.001). After CH, the average area of extravascular per section exhibits an increase +67.5 %. When the extravascular surface is expressed by small vessel, the increase is + 48.8 %.

Furthermore, our morphometric study shows that the CH has induced other changes at the glomus cells [figure: VIII]: An increase in the proportion of dark type I cells [*MS*h: +386.1%; P <0.001] and a decrease in the proportion of clear type I cells [*MS*h: -11.3%; P <0.01], were visualized. The proportion of type I cells characterized by a dark cytoplasm and pyknotic nucleus (degenerating) have only a very small non-significant increase [*MS*h: +5.2%]. The proportion of type II cells shows a slight decrease [*MS*h: -31.0%; P <0.05].

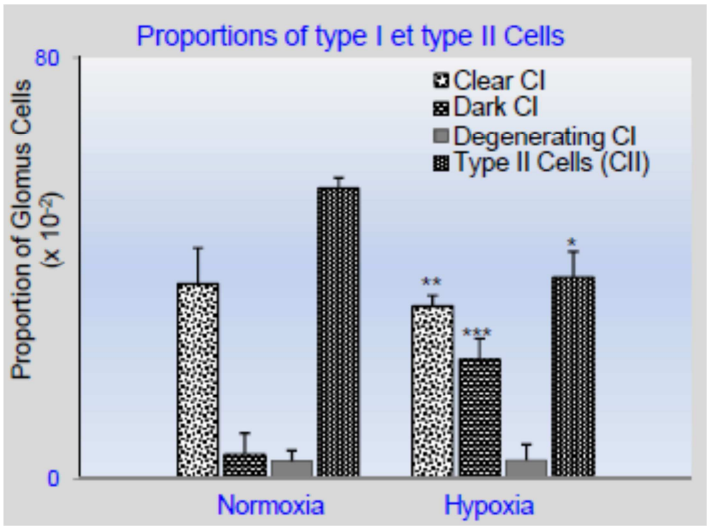


Figure VIII- Proportions of type I cells (CI) and type II (CII) in the carotid body (CB). Type I cells are differentiated by their appearance (clear CI), dark (dark CI) or degenerating (Degenerating CI.). Morphometric study was performed on serial sections of CB of Meriones shawi rats that were exposed for 28 days, in a hypoxic environment (Hypoxia, 10% O_2) or a normoxic environment (Normoxia, ambient air). Each column represents the mean of measurements in 40 pairs of CB (value ± SEM; t test; * P <0.05; ** P <0.01 and *** P <0.001).

After CH, CB shows strong increase in the proportion of dark type I cells (+386.1%), a slight decrease in the proportion of clear type I cells (-11.3 %), and a non significant increase in the proportion of degenerating type I cells (+5.2%). The proportion of type II cells shows a slight decrease (– 31.0%).

Finally, the results of immunohistochemistry [figure: IX] show that after CH the number density of positive type I cells in the chromaffin reaction is increased [*MS*h: factor x80.8; P <0.001]. The volume density of these cells is also increased [*MS*h: factor x32; P <0.001].

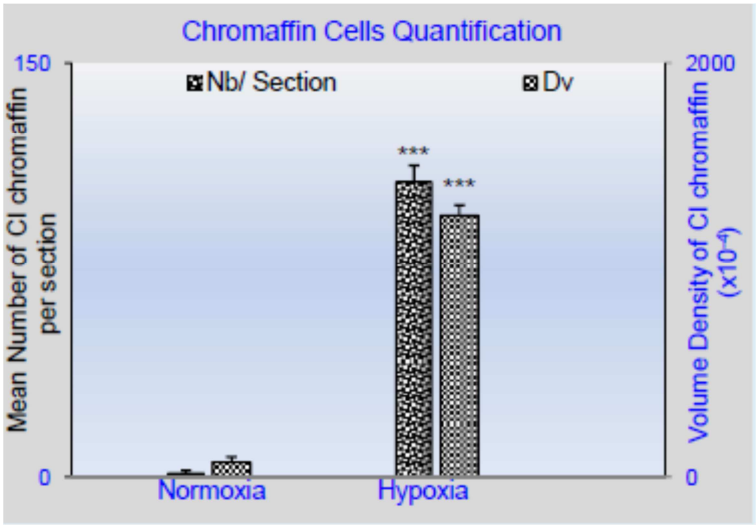


Figure IX-Quantification of chromaffin cells. The study of the average number of chromaffin cells per section of carotid body (Nb / section) and their volume density (Dv) was performed on serial sections of CB of Meriones shawi rats that were exposed for 28 days, in a hypoxic environment (hypoxia, 10% O_2) or a normoxic environment (normoxia, ambient air). Each column represents the mean of measurements in 40 pairs of CB (value ± SEM; t test; * P <0.05; ** P <0.01 and *** P <0.001). It is noted that after the CH number density of positive type I cells after the chromaffin reaction is increased by a factor x 80.8. The volume density of these cells is also increased by a factor x 32.

To summarize the effects of long-term hypoxia on the histology and compartments of the *Meriones shawi* rat CB: 1- we observed an enlarged glomus, which resulted in an increase in the volume of the organ [3.8 times]; 2-the extravascular volume expressed relative to the volume of the CB remain unchanged; 3- the volume density of the islets of glomic cells remained stable; 4- the volume density of connective tissue has suffered a significant increase [+50.8%]; 5- total vascular volume was increased [+398%], and only part of this percentage [+44.1%] may be related to the increased volume of the small vessels. Finally, 6- the number of positive type I cells in the chromaffin reaction was enhanced by 80.8-time and their volume density was multiplied by 32 in the hypoxic CB.

DISCUSSION

Our study on the morphological plasticity of the *Meriones shawi* rats CB following the long-term hypoxia has provided new morphological and morphometric results on the behavior of microvessels, islets of glomus cells and their content of catecholamines.

Regarding the normoxic CB of *MS*, histological study has allowed to recognize the four main components (islets of glomus cells, blood vessels, connective tissue and nerve fibers) well known in normoxic CB of rats and rabbits [1,3]. In this respect, the CB of *MS*c is comparable to that of rat. In addition, the application of a chromaffin reaction on the CB tissue of *MS*c allowed us to reveal the existence of some type I cells that have reacted positively. Their presence proves that part of the NA in the CB of *MS*c is located in the glomus cells. This result confirms previous data of Péquignot and al., [36] obtained by assaying catecholamines after sympathectomy in *rats*. Through this experience, the authors were able to demonstrate that some of the NA is located outside of sympathetic innervation. However, the presence of a minority of positive cells among a majority of cells that do not respond to the chromaffin reaction, suggests that content in CB of *MS*c is dominated by the DA as was described in rats and rabbits [37,38,39,40]. Our results are also consistent with the work of Chen and al. [41] who showed that only a few rat CB type I cells are immunopositive for dopamine-ß-hydroxylase. However, the rat CB, characterized by the abundance of dopamine cells, different from the cat CB in which the NA is more abundant than the DA [42] and the majority of glomus cells show positive immunoreactivity for dopamine-ß-hydroxylase [41]. Finally, we found in the chromaffin cells of the *MS*c cytological features (limited to marking cytoplasmic granules) similar to those we previously obtained by the use of autoradiography, immunohistochemistry and chromaffin reaction in rabbits and rats [26, 27, 25]. It is noteworthy that in this work, the excellent quality of histological preservation offered by the nature of the fixative used, allowed the studies of morphometry and chromaffin reaction on serial sections made in the same CB. Histological sections therefore come from the same group of animals that were placed in the same experimental conditions.

Regarding the effects of Long-term hypoxia, all observations in *MS*h demonstrate that the CH has increased the marking by the chromaffin reaction in the cell type I. This represents a morphological support to the increased

content of NA in CB after the CH as has been shown by other biochemical studies [18, 19, 20]. It is also interesting to note that in our experimental conditions, the number of chromaffin cells and the volume they occupy in the *MS*c-CB were increased by a factor 47 and 29, respectively. These increases are of the same order of magnitude as those obtained by Verna and al. [25] in the rat CB by biochemical methods (x 61) and immunocytochemistry (x 29). However, the method of tissue fixation for immunocytochemistry did not allow performing other morphometric measurements. Our result shows that one of the effects of CH on the CB means is increasing the capacity of glomus cells to store, capture [25] and probably synthesize NA [43]. An effect of the CH on the NA contained in the sympathetic innervation in CB can not be excluded since sympathectomized animals were not used in this study. This suggests that chromaffin cells observed after CH are newly formed cells. However, reports underlining the presence of cell divisions in the CB after the CH were still contradictory. For some authors, the signs of mitosis in type I cells after 1 week and until 4 weeks of hypoxia are absent in the rat CB [32,44], and for others, CH induces cell multiplications in rat CB [21, 45]. However, Dhillon and al. [45] had seen a small increase in the number of type I cells by cellular division (2-4 times), which is not comparable with the increase of chromaffin cells we observed (47 times). Finally, another study using bromodeoxyuridine (similar to uridine) showed that most divisions of type I cells occur before the 3rd day of hypoxia, which would explain the absence of mitosis after the first week [46]. Nevertheless, the content of NA in rat CB starts to rise only after the first week of hypoxia and continued to increase up to 12 times of its original value after 28 days of hypoxia [18]. Recent studies have shown that increases in immunoreactivity DβH and its mRNA appeared only 12 hours after the onset of hypoxia [43]. It is therefore unlikely that the increase in the number of chromaffin cells be assigned to newly formed cells. This rather suggests a chemical and functional plasticity of pre-existing cells. Indeed, glomus cells contain both DA and NA but in varying proportions, depending on the circumstances, and the CH switches the DA / NA ratio in glomus cells to the noradrenergic pole.

Moreover, it seems that the DA / NA balance is controlled by some factors. Indeed, we have shown that the CH and dexamethasone resulted in increased cell storing the NA in rat CB [28]. Denervation may also have an effect on the DA / NA ratio since the section of carotid sinus nerve leads, a week later, to an increase of the NA in rabbit glomus cells [39]. This suggests that the content of NA glomus cells is controlled by humoral, environmental and nervous factors, and it would be more influenced by long-term variations [17].

In this study we also observed an increase in the number of dark type I cells and a decrease in the number of clear type I cells in the *MS*c-CB. An increase in the population of dark type I cells was observed in men with chronic obstructive pulmonary disease [47]. It is also the first morphological alteration observed in human CB died after suffering acute respiratory distress syndrome [48]. However, in animals, the existence of type I cells having dark appearance was correlated with a change in cytoplasmic composition of type I cells in response to hypoxia. Indeed, the CH can lead to various changes in the cytoplasm of type I such an increase in volume of dense cored vesicles [44, 49, 50, 51], an increase in the number of mitochondria [52], or an increase in mRNA of tyrosine hydroxylase in the first few hours after hypoxia [46, 53, 54]. Furthermore, in another ultrastructural study of rabbit CB, we found that the dark appearance of type I cells containing the NA, was linked to the presence in their cytoplasm in large amounts of glycogen grains and large dense cored vesicles with higher contrast compared to clear glomus cells [27]. Dark cells would therefore representative of a higher functional state. The increase in their number could be interpreted as an appropriate response to the decrease in partial pressure of oxygen in arterial blood.

What can be the role of noradrenergic glomus cells? The effects of NA on chemoafferent activity are controversial, probably because of differences between the species used and/or various experimental conditions. However, the dominant effect of the NA seems excitatory via beta-adrenergic receptors [23]. In addition, Milsom and Sadig [23] showed that the sensitivity of the CB to hypoxia, when it was abolished by treatment with reserpine, could be restored not only by the DA but also by the NA. Later, it was demonstrated that nicotine induce preferably a release of the NA from rabbit glomus cells [55]. One could therefore assume that the NA that would come from the glomus cells plays a role in the physiology of Chemoreception. However, the effects of hypoxia appear to affect the NA after a longer period compared to the DA [20]. This could be correlated with an increase after a delay of the sensitivity of the carotid body which contributes to the respiratory acclimatization to hypoxia [15]. Furthermore, our quantitative study shows that the CH increases the size of the *MS*c-CB. Our results confirm other previous data in humans and animals [18, 29, 45, 50, 52, 56, 57]. Increases the size of the CB described in our results (x 3.1) are similar to those usually obtained in rats [18, 31]. However, our data demonstrate that this increase is the result of a rearrangement of vascular and extravascular compartments. Thus, the total vascular volume and extravascular volume increased in parallel with the total volume of the CB. When these two parameters are expressed relative to the volume of the CB, there is no difference between the values of hypoxic and normoxic groups. However, the fact that the total vascular volume increases significantly (+305% for the *MS*h), while increasing the volume of small vessels is low (+20.5% in the *MS*h) suggests that it is rather large vessels that increase the total vascular volume. In fact, the increase in the volume of small vessels observed after hypoxia is only apparent since when expressed

relative to the volume of CB a decrease of about 25% was observed. In agreement with this, the numerical density of the small vessels and their endothelial wall surface expressed relative to the volume of CB, are both reduced. If all these changes can be explained by the increase in the total volume of the CB, mechanisms should be sought. It is well known that hypoxia stimulates capillary angiogenesis in tissues other than the CB [58]. In CB itself mitotic figures have been described in endothelial cells of hypoxic animals [21]. But in our study, despite the very dark coloring of the nuclei of endothelial cells by toluidine blue, except their crescent shape, we have not observed mitotic figures in capillary of hypoxic CB. However, lack of any visible mitotic spindle does not exclude the possibility that mitoses are produced in the early stages of hypoxia. It is therefore unlikely that angiogenesis occurred in the capillaries since smaller vessels have decreased in hypoxic CB.

Vascular changes observed in hypoxic CB could be due to a remodeling of the vasculature. This phenomenon is not new since it has already occurred in skeletal muscles of rats exposed to the CH. During the remodeling, the capillaries are associated with smooth muscle and become arterioles [59]. The possibility of this type of rearrangement in the CB is low because the capillaries irrigating groups of type I and type II cells are largely grouped in the caudal pole of the organ. Furthermore, the distribution of blood flow in the body is uneven and only a small proportion, about 8% of the total blood flow through the GC, pervades type I and type II cells, the rest is deflected by a widely arteriolar network that occupies the central parts of GC [60,61]. Changes in the arrangements of the arterioles in the central part of the body should appear in the serial histological sections, but in our observations we have not identified any variation of distribution of arterioles and capillaries within the tissue of hypoxic CB.

Remodeling of peripheral veins can he explain the increased volume observed in the large vessels? Our observations do not allow us to know if the increase in the volume of large vessels was due to the increase in the number of veins with more than 12 microns in diameter, or the increase in the diameter of small preexisting venous vessels in the organ. The first hypothesis is the most likely because the CH is accompanied by a rise in blood pressure in the veins [5]. This could contribute to the increase of peripheral veins size. This hypothesis is consistent with the fact that this vasodilatation is almost completely resorbed when the oxygen partial pressure (PaO_2) is brought back to normal [62]. However, some studies have shown that under the effect of CH, an increase in expression of growth factor from the vascular endothelium and its Flk-1 receptor could be attributed to an occurrence of new blood vessels [63,64]. This hypothesis is supported by the detection of endothelial cell divisions by the bromodeoxyuridine method of 1 to 7 days after initiation of hypoxia [46]. Morphological changes in endothelial cells may be due to a release of chemicals during the CH such as nitric oxide, adenosine or prostaglandins that cause relaxation of smooth muscle of the arterioles and veins [62, 65]. The result would be greater infiltration of the fluid in the interstitial space which could, in turn, contribute to the increase in the extravascular volume of the organ.

An increase in the number of type I cells should contribute to increasing the volume of extravascular CB. But we have not seen signs of mitotic cells in the islets after CH. However, Wang and Bisgard [46] showed in rats that most divisions in type I cells were held before the 3rd day of hypoxia. A short period of cell proliferation, should not in itself explain the importance of the increased size of the organ during the CH. In addition, the reversible nature of the CB size after return to basic oxygen partial pressure [66] would be an argument in favor of increasing the volume of CB by dilatation rather than division of type I cells during the period of the CH. An increase in cell number is irreversible unless the number of cells is reduced by apoptosis, but we have seen that the proportion of degenerating cells is not increased by hypoxia. Thus, the return to the normal size of the CB would not be due to apoptosis but to reduce the size of vascular and extravascular compartments. Therefore, if the number of type I cells remains relatively constant, increasing the extravascular volume took place by hypertrophy of type I cells and an increase in the size of the interstitial space.

Regarding the mechanism underlying the increased sensitivity of the CB during ventilatory adaptation to the CH, some work has been able to demonstrate that hypoxia increases the electrical activity in the efferent fibers of the NSC [67]. However, it is unlikely that such activity is responsible for the gradual increase in discharges in the CB during acclimatization, since the effect that dominates the activity of efferent fibers is the inhibitory action on the CB [68]. Another widely proposed mechanism but the evidence is somewhat disputed, is represented by the inhibitory action of the DA mechanism [13]. The results of this study suggest an alternative explanation based both on a dynamic balance between the inhibitory action of the DA and the excitatory effect of the NA [for references, 6] and histological reorganization capacity in CB in response to a long period of hypoxia. Thus, it was established that under normoxic conditions mean PO_2 in the carotid body is about 25 mm Hg in the cat [69]. Then one must admit that whatever the signal transduction activity of the organ, represented by the discharge frequency in the afferent fibers of the NSC would be dependent on the level of tissue PO_2. The tissue PO_2 depends on many factors: arterial PO_2, oxygen consumption by the tissue, blood flow in the tissue and the diffusion distance between the capillary and the tissue [for references, 6]. We suggest that ventilatory adaptation related to carotid chemoreceptor can benefit, at

least in part, of progressive morphological changes, reported here, leading to the increase in extravascular volume, reducing the density of the small vessels, and increased extravascular reported by small vessel. This should have the effect of increasing the distance between the blood vessels and islets formed by type I and type II cells that would reduce the PO_2 in the tissue and from there an increase in the activity of the chemoreceptor. If, as we have proposed, increasing the extracellular volume is the result of the increase in the interstitial space and probably of cell type I and type II. This should increase further the capillary-tissue distance.

Furthermore, individuals born or living for several years in high altitude may have a loss of ventilatory response to acute hypoxia [70, 56]. This was due to a decrease in the sensitivity of the CB to hypoxia. In other reports, cats and rats showed an attenuation of ventilatory reaction and the response of the CB to acute hypoxia after a 3-4 week stay at 5500 meters above sea level or exposure to 10% O_2 [71, 72]. By using these animal models, the authors argue that the data obtained indicate a strong dopaminergic activity in the CB that would be responsible for the attenuation of the sensitivity of CB [73]. Now in the CB, the effect of dopamine is associated with changes in ion channels of type I cells, making it less excitable chemoreceptor [74, 75, 76]. If the main cause of this phenomenon is attributed to a strong dopaminergic activity, we do not yet understand how strong CB excitability during acclimation to the CH turns into attenuation. This, because a high dopaminergic activity seems to start well before the attenuation grows, that is while the response of the CB to acute hypoxia is still very strong. A contribution of morphological changes in a "desensitization" of the CB in the long-term hypoxia remains unclear. This, is most likely because Aaron and Powell [77] have not really been able to demonstrate a reduction in ventilatory response after 3-4 weeks of hypoxia in rats. A number of other animal species show no attenuation of the response to hypoxia after birth or after long exposure to high altitude hypoxia [70].

It is well known that back to sea level after living at high altitude, some hyperventilations persist temporarily (ventilatory reacclimatisation) [78]. CB sensitivity to acute hypoxia may remain high at least during the immediate period of reacclimatisation [14] before gradually returning to normality. Based on the observations reported here, we believe that this phenomenon can be attributed, at a first step, to the persistence of hypertrophy, and then to the gradual disappearance of morphological variations and NA contained in type I cells induced originally by long-term hypoxia.

In summary, the CH induced profound morphological and neurochemical changes in the CB. It causes hypertrophy of type I cells, an increase in the number of cells containing the NA, dilation of microvessels having a diameter greater than 12 microns and a flow of fluid into the interstitial space. These changes appear to be responsible for increasing the size of the CB and the increase in chemo-sensory activity during the long-term hypoxia. Further studies are needed to demonstrate how the CB could be desensitized after long-term exposure to hypoxia.

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