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Influence of hypercapnia on rabbit intrapulmonary neuroepithelial bodies: microfluorimetric and morphometric study

J.M. Lauweryns, A. Tierens, M. Decramer

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ABSTRACT: The present investigation was undertaken to investigate the influence of hypercapnia on intrapulmonary neuroepithelial bodies (NEB). Rabbits were mechanically ventilated with a hypercapnic gas mixture (7% carbon dioxide, 20% oxygen, 73% nitrogen). Lung samples were examined by a microspectrographical analysis of the NEB formaldehyde-induced fluorescence to quantify the cytoplasmic 5-hydroxy-tryptamine (5HT) content and by electron microscopy to determine morphometrically the extent of the secretory exocytosis at the basal poles of the NEB epithelial cells. In contrast to our earlier studies on the effects of hypoxia and/or vagal stimulation, hypercapnia did not alter significantly the NEB cytoplasmic fluorescence nor did it affect the corpuscular epithelial exocytosis. NEB appear not to be influenced by hypercapnia to discharge their contents of 5HT and peptides. This investigation appears to support a high selectivity of the intrapulmonary NEB to local hypoxia and changes in vagal efferent output. *Eur Respir J., 1990, 3, 182–186.*

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Since our original description in 1972 of neuroepithelial bodies (NEB) in the human infant [1] and mammalian lung [2], they have been identified in the intrapulmonary airway mucosa of a large variety of vertebrates including amphibians, reptiles and birds [3–11]. They consist of nonciliated cylindrical cells with a clear, slightly eosinophilic cytoplasm reaching from the basement membrane to the airway lumen [1]. Ultrastructurally the corpuscular NEB cells are characterized by the presence of numerous intracytoplasmic dense-cored vesicles and by a prominent innervation with morphologically afferent and efferent nerve endings [12, 13]. By means of different vagotomy procedures the cell bodies of the afferent nerve endings have been localised to the nodose ganglion [12]. Unilateral vagal stimulation, activating motor nerves to the NEB, showed that they may play an important role in modulating the NEB sensitivity and reaction to various stimuli [14]. Moreover, marked changes in the intensity of staining and number of calcitonin gene-related peptide (CGRP) - immunoreactive endocrine cells in the rat lower respiratory tract following capsaicin treatment and vagal ligation suggest that these cells may be under neuronal influence [15].

Microspectrographical and immunohistochemical methods showed that NEB contain 5HT [14, 16], various peptides such as bombesin, calcitonin and somatostatin [17–19] and the enzyme marker neuron-specific enolase [20]. Recently, chromogranin [20], CGRP [22] and protein gene product 9.5 (PGP 9.5) [23] were also revealed. They may exert their effects through an endocrine or paracrine pathway.

Finally, aromatic L-amino acid decarboxylase (AADC) was demonstrated immunocytochemically in mouse, rat and human NEB, revealing that besides amine-storage the synthesis of amines occurs in these cells [24].

Earlier studies on the effects of acute and chronic hypoxia under various experimental conditions [25–29] have supported our hypothesis that NEB could represent an intrapulmonary neuro(chemo)receptor.

As one of these earlier investigations suggested some sensitivity of neonatal rabbit NEB to hypercapnia [25], the present study was undertaken to evaluate these preliminary results under more systematic and carefully controlled conditions.

In this study 3 to 4 week old rabbits were artificially ventilated with a hypercapnic gas mixture, the blood and respiratory variables being strictly monitored.

Materials and methods

Fourteen young rabbits (3 to 4 weeks old) were anaesthetized by an intramuscular injection of Hypnorm (initial dose 0.05–0.1 ml per 100 g body wt). A tracheostomy was performed and a straight metal cannula

inserted into the trachea. The animals were mechanically ventilated using a Harvard rodent ventilator. Tracheal pressure, airflow and tidal volume were recorded with a Hewlett Packard 7754 B four channel hot pen recorder. Seven animals served as controls, being mechanically ventilated with room air; the other seven rabbits received a hypercapnic gas mixture (7% carbon dioxide, 20% oxygen and 73% nitrogen). At the end of each experiment, which lasted 20 min, arterial blood samples were drawn from the left ventricle. The rabbits were then killed by an overdose of Hypnorm and a thoracotomy performed. Lungs and heart were dissected out. Samples were taken from the lungs and prepared for further histochemical and ultrastructural investigations.

Histochemical procedure: formaldehyde-induced fluorescence technique

To quantify the 5HT content of the NEB in the left lung tissues were investigated with the histochemical fluorescent amine technique of Falck and Owman [30] as described in previous studies [14, 25, 26, 30]. The biopsies (1 mm^3) were quenched in liquid nitrogen and lyophilized for 3 days at temperatures increasing from -80 to 30°C. One experimental lung and one control lung were always studied at the same time. Next the tissues were treated for 1 h with formaldehyde vapour with a relative humidity of 49% at 80°C. After embedding in paraffin, 7µm sections were cut and examined with a Leitz MPV fluorescence microscope. This technique demonstrates the presence of 5HT and catecholamines, which can be distinguished from one another since their fluorophores display different colours after excitation with ultraviolet light, *i.e.* yellow for 5HT and blue for catecholamines. The yellow fluorescence emission of rabbit NEB corresponds microspectrographically with the emission maximum of 5HT [17, 30]. A close relation between the 5HT concentration and the fluorescence intensity has been established [31]. The emission of the NEB was limited to a circular region of 2 µm diameter; thus the emission of the same amount of basal cytoplasm was recorded for all NEB studied. The fluorescent intensities were electronically recorded and expressed in relative units. A Leitz fluorescence standard served for the calibration of the microscope photometer. The registrated millivoltages recorded from the hypercapnic lungs were compared to the control lungs, using Student's t-test. Mean±SD are presented in the text.

Ultrastructural and morphometric procedures

For electron microscopy, the right lung lobes were immediately fixed by a gentle intrabronchial perfusion of 2.5% glutaraldehyde at 4°C until grossly expanded. The lung lobes were then cut into small cubes (1 mm^3), immersed for another 2 h and postfixed in 1% OsO_4 (0.1 M in phosphate buffer, pH 7. 2) for 1 h at 4°C. After dehydration the biopsies were embedded in Epon and 1µm sections cut and stained with toluidine blue for light microscopical investigation. Whenever NEB were seen, the block was carefully trimmed. The corresponding ultrathin sections were mounted on copper grids and stained with uranyl acetate and lead citrate for further investigation with a Philips EM 300 A. To obtain a representative sample three sections of each NEB were mounted on successive grids and selected for photography. Each section was systematically screened and micrographs (final magnification ×17,000) were taken of the basal pole of all NEB epithelial cells. To quantify the basal degranulation we counted on each micrograph the dense-cored vesicles (DCV) undergoing exocytosis at the base of the epithelial cells [14]. The DCV thus counted include three categories: those at a distance less than their diameter from the basal epithelial cell membrane; those in contact with the basal epithelial cell membrane; exocytotic profiles at the level of the basal epithelial cell membrane. Their number was divided by the total length of the basal epithelial cell membrane, measured with a semiautomatic Leitz ASM image analysis system. Indexes were expressed in DCV per micron of basal epithelial cell membrane. For each animal a mean exocytosis index was determined. 373 micrographs were measured. They revealed 2485 µm basal cell membranes belonging to 71 NEB of 14 animals. 1234 dense-cored vesicles undergoing exocytosis were counted.

Results

Blood gas analysis

An increase in $Paco_2$ and systemic acidosis were observed in the animals mechanically ventilated with the hypercapnic gas mixture. The blood gas values of the control animals were within the normal range. These results are summarized in table 1.

Table 1. – Blood gas values: rabbit

	Hypercapnic lungs	Control lungs
pH	7.21±0.01	7.39±0.02
Pco_2, Torr	78.3±1.86	45.0±2.14
Po_2, Torr	104.8±2.93	94.1±5.59
HCO_3^- mmol·l^{-1}	31.7±0.84	27.48±0.56

Formaldehyde-induced fluorescence

No significant difference in fluorescence intensity of NEB was recorded between the normocapnic control and the hypercapnic lungs: 100% *versus* 101.58 ± 11.1% (fig. 1).

Ultrastructural studies

No obvious change in NEB ultrastructure was noted

on the electron micrographs. Morphometrical investigation of the number of DCV undergoing exocytosis at the base of the NEB cells confirmed the absence of a significant difference in the mean exocytosis index of basal DCV degranulation between the hypercapnic and the normocapnic lungs: 0.47±0.138 *versus* 0.45±0.089 DCV·μm^{-1} basal membrane (t–value: 0.32) (fig. 2).

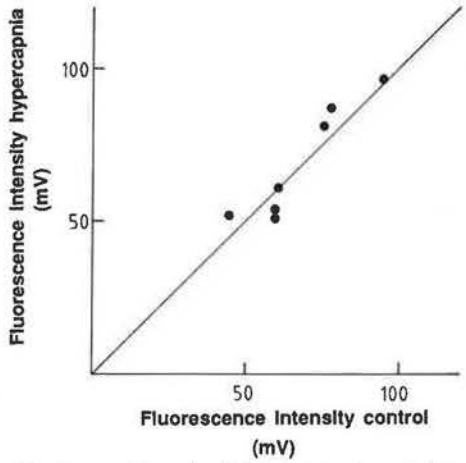

Fig. 1. – Fluorescence intensity (mV) in experimental (hypercapnic) lung *vs.* control lung. Data points are values from individual animals. Solid line is identity line.

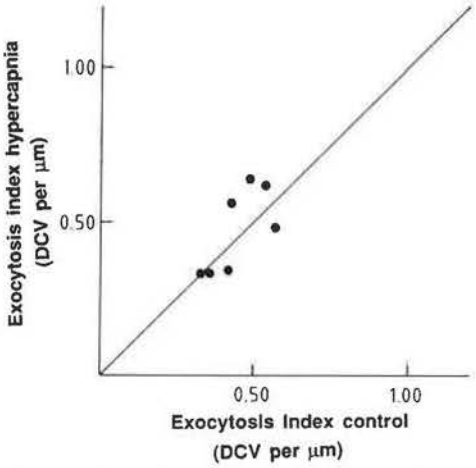

Fig. 2. – Exocytosis index in experimental (hypercapnia) lung *vs.* control lung (dense-cored vesicles (DCV) per micron (μm)). Data points are values from individual animals. Solid line is identity line.

Discussion

In the present study hypercapnia altered significantly neither the NEB cytoplasmic fluorescence intensity nor the basal epithelial degranulation. The histochemical and ultrastructural results suggest that the secretory activities of the NEB corpuscular cells are not markedly influenced by local hypercapnia to discharge 5HT and/or peptides. Since in our earlier studies an identical experimental exposure to acute hypoxia [26] or to vagal stimulation [14] induced acute morphological changes in the secretory exocytosis of the NEB, we conclude that during hypercapnia no considerable secretion of 5HT and/or peptides occurs at the basal pole of NEB cells.

These observations differ from our previous and preliminary results [25] which suggested some sensitivity of the intrapulmonary NEB to CO_2. Several factors may have contributed to this discrepancy. In our previous studies [25] spontaneously breathing newborn rabbits were exposed for 20 min in an air-locked cage to a gas mixture with a constant composition (5% CO_2, 75% N_2, 20% O_2). In the present investigation 3 to 4 week old rabbits were mechanically ventilated with a hypercapnic gas mixture of slightly different composition (7% CO_2, 73% N_2, 20% O_2).

The NEB reactivity may also be age dependent. Preliminary evidence indicates that NEB nerve endings are not fully developed in neonatal rabbits [34]; although precise morphological and morphometric investigations have not yet been performed. This could affect NEB excitability to various stimuli. Previous experiments have revealed a diminished NEB reactivity to hypoxia in older rabbits [26].

Stimulation of upper airway receptors, modifying the output from peripheral chemoreceptors, could have occurred in our earlier studies with spontaneously breathing animals. The sensitivity of the intrapulmonary NEB, as a peripheral chemoreceptor, might be decreased by an influence from the upper airway receptors. Especially in spontaneously breathing rabbits, receptors in the nose, nasopharynx or larynx may be stimulated by local airflow. This phenomenon was avoided in the present study with mechanically ventilated animals. It may be responsible for the discrepancy between our earlier and the present investigations.

It is well known that stress and anxiety influence sympathetic and vagal tone. The nervous strain of unanaesthetized newborn animals placed in an air-locked cage on the one hand and of the anaesthetized 3 to 4 week old rabbits on the other hand was probably quite different and may have contributed to different NEB responses.

Anaesthesia does not influence the NEB fluorescence intensity and mean exocytosis index under normoxic and normocapnic conditions [34]. There is no evidence that anaesthesia may have changed the NEB sensitivity and reactivity to hypercapnia.

Finally, the blood gas values and other respiratory variables were not controlled during our previous preliminary investigations [25]. In the present study, on the contrary, all these variables were carefully controlled. Thus the present results are due to the effect of hypercapnia only.

From the present study it appears unlikely that NEB play an important physiological role as intrapulmonary CO_2-sensitive chemoreceptors. The best known routes through which the CO_2-H^+ complex exerts its effects are *via* the intracranial and arterial chemoreceptors [35]. However afferent pathways, including myelinated and non-myelinated vagal fibres originating in the tracheobronchial tree and lungs, also respond to changes to $Paco_2$ [36]. But we do not know whether a primary sensitivity to changes in CO_2 in the physiological range is indeed a feature of any particular category of afferent

nerve endings. It is interesting that there are morphologically unidentified CO_2-receptors in birds and lizards [36].

In conclusion NEB appear not to be stimulated by hypercapnia to discharge their contents of 5HT and peptides; they seem to respond to one specific stimulus, namely local hypoxia. Thus the present negative findings corroborate indirectly our original proposal that the NEB are "secretory hypoxia-sensitive neuro(chemo)receptors" [1, 2].

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Influence de l'hypercapnic sur les corps neuroépitheliaux du lapin: étude microfluoro-métrique et morphométrique. J.M. Lauweryns, A. Tierens, M. Decramer.

RÉSUMÉ: Dans cette étude nous avons examiné l'effet d'une hypercapnie sur les corps neuroépithéliaux intrapulmonaires (CNE). A cet effet, des lapins de 3 à 4 semaines ont été ventilés avec un mélange gazeux hypercapnique (7% dioxide de carbone, 20% d'oxygène, 73% d'azote). D'une part la sérotonine cytoplasmique des cellules épithéliales des CNE a été évaluée par une analyse microspectrographique de leur fluorescence indirecte (technique de Falck) et d'autre part l'index de l'exocytose sécrétoire au pôle basal des CNE a été déterminé par une étude morphométrique au microscope électronique. A l'encontre de nos études précédentes au sujet de l'influence d'une hypoxie et/ou d'une stimulation du nerf vague, l'hypercapnie n'influence dans cette étude ni l'intensité de la fluorescence cytoplasmique, ni l'index de l'exocytose. Ainsi l'hypercapnie ne semble pas influencer les CNE qui ne paraissent pas sécréter leur contenu cytoplasmique de sérotonine et de peptides. Cette étude confirme ainsi indirectement la grande sensibilité des CNE à une hypoxie intrapulmonaire locale et aux effets du nerf vague.
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