NEUROCHEMICAL EFFECTS OF LONG-TERM HYPOXIA ON NORADRENERGIC NEURONS IN NUCLEUS TRACTUS SOLITARIUS OF THE RAT

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RESUMEN: Efectos Neuroquímicos de la Hipoxia Prolongada en las Neuronas Noradrenérgicas del Núcleo del Tracto Solitario de la Rata

Para determinar los efectos de la hipoxia prolongada sobre la actividad catecolaminérgica en el núcleo del tracto solitario (NTS), se sometió a ratas machos a hipoxia normobárica (10% O_2 en nitrógeno) por 3 días, 1,2 o 3 semanas. La ventilación aumentó gradualmente durante la exposición hipóxica alcanzando una meseta luego de 10 días de hipoxia. La hipoxia prolongada estimuló el recambio de norepinefrina en las neuronas noradrenérgicas localizadas en el NTS caudal al obex, la región a la cual se proyectan fibras carotídeas aferentes. La quimiodenervación bilateral abolió los cambios centrales en la actividad noradrenérgica. La actividad y la cantidad de tirosina hidroxilasa (TH), la enzima limitante de la síntesis de catecolaminas, y el nivel de codificación de mARN para TH aumentaron en el NTS caudal en respuesta a la hipoxia prolongada, indicando inducción de nuevas moléculas de proteína TH. Estos cambios neuroquímicos ocurrieron solamente luego de hipoxia prolongada, sugiriendo que las neuronas noradrenérgicas están implicadas en la vía cental de quimioreceptores durante la hipoxia sostenida, pero que no son esenciales para las respuestas reguladoras a la hipoxia aguda. La norepinefrina liberada bajo condiciones de hipoxia prolongada podría jugar un rol neuromodulador en la aclimatación ventilatoria.

Palabras claves: Tronco encefálico, Células A2, Vía quimiorefleja, Tirosina hidroxilasa.

RÉSUMÉ: Effets neurochimiques de l'hypoxie prolongée sur les neurones noradrénergiques du noyau du tractus solitaire du rat.

Afin de déterminer les effets de l'hypoxie prolongée sur l'activité des catécholamines du noyau du tractus solitaire (NTS), des rats mâles adultes ont été soumis à une hypoxie normobare (10% O_2 dans l'azote) pendant 3 jours, puis 1, 2 ou 3 semaines. La ventilation respiratoire a augmenté graduellement pendant l'exposition hypoxique, jusqu'à atteindre un palier au bout de 10 jours. L'hypoxie prolongée a stimulé le remplacement de la norépinéphrine des neurones noradrénergiques localisés dans le NTS caudal par rapport à l'obex, région où se projettent les afférents carotidiens chimiosensoriels. La chimioénervation bilatérale a aboli les changements centraux au cours de l'activité noradrénergique. On a noté une augmentation de l'activité et de la quantité de l'enzyme limitative de la biosynthèse des catécholamines tyrosine hydroxylase (TH) et du niveau de mRNA pour la TH dans le NTS caudal, en réponse à l'hypoxie prolongée, indiquant l'induction de nouvelles molécules de la protéine TH. Ces changements neurochimiques ne se produisent qu'après une hypoxie prolongée, suggérant que les neurones noradrénergiques sont impliqués dans le trajet des chimiorécepteurs centraux pendant l'hypoxie soutenue, mais qu'ils ne sont pas essentiels pour les réponses régulatrices à l'hypoxie aiguë. La norépinéphrine libérée dans des conditions d'hypoxie prolongée joue un rôle neuromodulateur dans l'acclimatation respiratoire.

Mots-clés : Tronc encéphalique, Cellules A2, Trajet des chimiorécepteurs, Tyrosine hydroxylase.

SUMMARY: In order to determine the effects of long-term hypoxia on catecholamine activity in the nucleus tractus solitarius (NTS), male rats were subjected to normobaric hypoxia (10% O_2 in nitrogen) lasting for 3 days, 1, 2 or 3 weeks. Ventilation increased gradually during the hypoxic exposure before reaching a plateau after 10 days of hypoxia. Long-term hypoxia stimulated the norepinephrine turnover in noradrenergic neurons located in the NTS caudal to the obex, the discrete region to which the chemosensory carotid afferents project. Bilateral chemodenervation abolished the central changes in noradrenergic activity. The activity and quantity of tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine biosynthesis, and the level of mRNA coding for TH were increased in the caudal NTS in response to long-term hypoxia, indicating induction of new molecules of TH protein. These neurochemical changes occurred after long-term hypoxia only, suggesting that noradrenergic neurons are involved in the central chemoreceptor pathway during sustained hypoxia but are not essential for regulatory responses to acute hypoxia. Norepinephrine released under long-term hypoxia could play a neuromodulatory role in ventilatory acclimatization.

Key words: Brainstem, A2 Cell Group, Chemoreflex Pathway, Tyrosine Hydroxylase.

Various physiological adaptive responses take place in face of reduced oxygen concentration or pressure in inspired air. Among them, hyperventilation is probably the most obvious. When prolonging hypoxia, there is a progressive increase in hyperventilation even though the hypoxic stimulus remains at a constant level. Hyperventilation then reaches a plateau after a more or less long time depending on species. This phenomenon is known as ventilatory acclimatization to hypoxia. The mechanism underlying ventilatory acclimatization is still undefined, although it depends on peripheral arterial chemoreceptors (21).

Low blood pressure in oxygen is primarily sensed by peripheral arterial chemoreceptors. Stimulation of the carotid body chemoreceptors elicits an increase in firing rate of chemosensory neurons whose fibers course in the carotid sinus nerve and terminate in the caudal part of the nucleus tractus solitarius (NTS) within the brainstem (5, 6). The NTS contains respiratory neurons whose activity is modulated by central and peripheral afferents in response to environmental conditions. Thus, the NTS has been considered a structure of prime importance for the integration of chemosensory stimuli and control of respiration in response to afferent inputs. In the area to which the chemosensory afferents project, is located a cluster of noradrenergic neurons that constitute the A2 cell group (7). Norepinephrine in the NTS can depress the bulbar respiratory neurons (3).

The aim of our studies was to determine the effects of long-term hypoxia on catecholamine activity in the NTS during exposure to long-term hypoxia by investigating changes in norepinephrine turnover, content and activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis. In order to determine if the observed changes could be found at the gene level, in situ hybridization was employed to investigate the influence of long-term hypoxia on the level of mRNA coding for TH.

METHODS

Animals and hypoxia

Experiments were carried out on male Sprague-Dawley rats (IFFA Credo, L'Arbresle, France), a species that demonstrates human-like ventilatory acclimatization to hypoxia (13). The animals were placed for 3, 7, 14 or 21 days in a normobaric Plexiglas chamber. The chamber was supplied with a gas mixture consisting of 10% 02/90% N_2. The chamber air was recirculated in a continuous circuit. Incorporated into the circuit were a chilled tank and soda lime to trap expired water vapour and to absorb carbon dioxide, respectively. The CO_2 concentration inside the chamber was less than 0.1%. Control groups of normoxic rats were kept in normoxia and sacrificed at the same time as their respective hypoxic counterparts.

Chemodenervation was performed by cutting both carotid sinus nerves between the apical pole of the carotid body and the point of branching with the glossopharyngeal nerves. In these conditions no chemosensory reinnervation is possible. The rats were allowed to recover from anesthesia and surgery for one week before to be subjected to hypoxia. Sham-operated animals were used as controls.

Neurochemistry

Noradrenergic activity in the brainstem was assessed using different experimental approaches based on the assessment of catecholamine turnover by pharmacological blockade of their biosynthesis (22), *in vivo* and *in vitro* measurements of TH activity, the rate-limiting enzyme in catecholamine biosynthesis (23), assays of TH content (17, 18, 19), and *in situ* hybridization of mRNA coding for TH protein (4).

Estimation of catecholamine turnover

•-Methyl-para-tyrosine injected intraperitoneally at 250 mg/kg b.w., 2.5 hours before sacrifice allowed the estimation of norepinephrine turnover by blocking the catecholamine biosenthesis. Each experimental group was divided into two half groups: one receiving α-methyl-para-tyrosine, the other receiving the same volume of vehicle (0.9% NaCl). The norepinephrine content was measured in the NTS of saline-treated and α-methyl-para-tyrosine-treated animals. After injection of α-methyl-para-tyrosine, the decline of norepinephrine is exponential. The slope of norepinephrine decrease was calculated and multiplied by the mean of norepinephrine content of saline-treated rats to ahtain the turnover rate.

Estimation of tyrosine hydroxylase activity

In vivo activity of TH was estimated by measuring L-DOPA accumulation after the inhibition of L-amino acid decarboxylase by NSD 1015 (3-hydroxybenzylhydrazine dihydrochloride). Rats were injected intraperitoneally with either NSD 1015 (100 mg/kg b.w.) or the same volume of vehicle (0.9% saline) 20 min before sacrifice. Tyrosine hydroxylation rate was estimated by subtracting the content of DOPA in the structures of saline-treated rats from the content of DOPA in the NTS of NSD-treated rats. DOPA and norepinephrine were assayed by high performance liquid chromatography coupled with electrochemical detection.

Tissue dissection for biochemical analyses

The brain was rapidly removed, frozen on dry ice and stored at -80 °C. The brainstem was cut into serial frontal slices of 48(), µm in thickness. The noradrenergic cell group A2 was punched out according to the dissection procedure described by Palkovits and Brownstein (5). The A2 cell group was subdivided into two parts, respectively caudal and rostral to the calamus scriptorius (22).

In situ hybridization

Frozen horizontal sections (15 µm thick) of NTS were cut serially on a cryostat (Leitz). In situ hybridization was carried out using a labelled 35S probe complementary to the rat TH mRNA (4). The TH protein was located on adjacent sections by immunocytochemistry and revealed by autoradiography (9).

Ventilatory measurements

Ventilation was measured every 3 days for a period of 18 days in animals exposed to hypoxia (10% O_2 in nitrogen). The tidal volume and respiratory frequency were measured by plethysmography using the barometric method (2). In brief, the spirogram of each rat was obtained from a differential transducer (Validyne) interfaced between the chamber flushed with humidified hypoxic air and a reference box of same size. The tidal and minute volumes, and the respiratory frequency were measured and the product of these two parameters gave the minute volume.

RESULTS

The minute volume increased gradually during the first 7 to 14 days of hypoxic exposure, and thereafter, stabilized, thus indicating that ventilatory acclimatization was achieved.

The turnover rate of norepinephrine in the caudal portion of the A2 cell group was increased about 5 fold after 2 weeks of hypoxia. In striking contrast, the norepinephrine turnover remained unaffected in the rostral portion of A2. In order to compare the influence of alteration in barosensory activity, a second group of rats was given daily for 2 weeks the hypotensive drug dihydralazine (20 mg/kg b.w.). The pharmacological treatment induced a selective increase in norepinephrine turnover in the rostral portion of A2 (+104 ±12% above control level) whereas the caudal portion was unaffected. Bilateral transection of the carotid sinus nerves carried out one week before the hypoxic exposure abolished the hypoxia-induced changes in norepinephrine turnover observed in the caudal NTS.

Regarding the influence of hypoxia on catecholamine biosynthesis, the in vivo activity of TH was found to be enhanced after 1 week of hypoxia (±60 ± 8% above control level), although it was unaffected by shorter hypoxic exposures (3 days). Long term hypoxia elicited a delayed increase in TH content (±36 ± 4% above control level) that was apparent after 2 weeks of exposure.

In situ hybridization of the THmRNA in the NTS revealed that hypoxia lasting for 2 weeks elicited a marked increase in THmRNA expression within the caudal NTS. Hypoxia elicited both an increase in the number of grains per cell and a rostral extension of the labeled area. The TH immunoreactivity in the caudal NTS was also increased but more rostrally than THmRNA.

DISCUSSION

Our main finding was that long-term hypoxia induced the stimulation of noradrenergic A2 neurons in the caudal NTS, the primary site of projection of peripheral chemosensory afferents within the brainstem (5, 6).

The changes in noradrenergic activity cannot be explained by a reduced availability in oxygen for A2 neurons. Indeed, increased capillary density in the brain, increased cerebral blood flow and polycythemia are common features observed in response to long-term hypoxia, that may maintain the tissue oxygen concentration close to basal level (8, 10). In contrast, the activation of A2 noradrenergic neurons appear clearly dependent on the integrity of chemosensory afferents since the prior bilateral chemodenervation abolished the changes in norepinephrine turnover induced by longterm hypoxia (22). In the rat, the carotid bodies are the major peripheral arterial chemoreceptors whereas functional aortic chemoreceptors are absent (16).

Hypoxia failed to stimulate the norepinephrine turnover or to increase the TH content in the rostral part of A2 cell group (18, 22). This area corresponds to the site of projection of barosensory nerve fibers in the rat (6). In contrast, chronic hypotension induced by dihydralazine induced a selective increase in

noradrenergic activity in the rostral A2 subset while the caudal A2 subset remained unaltered (19, 22). Taken together, the data provide evidence for the functional heterogeneity of A2 neurons according to their location, caudal or rostral to the obex. The caudal cells are part of the chemoreceptor pathway, whereas the rostral cells are influenced by barosensory inputs.

From a neurochemical point of view, stimulation of noradrenergic neurons by hypoxia is accompanied by a sustained release of norepinephrine (20, 22). The neuronal norepinephrine stores can be replenished, first by an increase in the TH activity (23) and then by an increase in the content of TH protein (18). Our data also showed that the changes in TH content did not result from alterations of the catabolism of the protein but from increased expression of the gene coding for biosynthesis of TH. Indeed, the TH mRNA expression was strikingly enhanced in the caudal NTS of long-term hypoxic rats (4). Thus, the data reveal an hypoxia-induced plasticity of noradrenergic neurons at the gene level in the caudal NTS.

Acute hypoxia increases the firing rate in chemosensory afferent fibers. During sustained hypoxia, the carotid body afferent discharge is increased resulting in a parallel increase in ventilatory output (1, 12). These functional changes are associated with a progressive increase in catecholamine activity in the carotid body as reflected by gradual increases in dopamine and norepinephrine turnover, and in TH content and activity (15, 17). The increased carotid chemoreceptor afferent input during sustained hypoxia could be expected to influence gradually the neuronal activity in the NTS. However, in contrast to the carotid body, the norepinephrine activity in the NTS was largely delayed as the early noradrenergic alterations in this area appeared only after 1 week of exposure (23). This finding shows that norepinephrine is not the first-order neurotransmitter involved in the immediate physiological responses to hypoxia. In fact, glutamatergic neurons are possible candidates to play the role of primary first-order neurons involved in the integration of chemosensory inputs within the NTS. Glutamate is released in the caudal NTS in response to acute hypoxia (11, 24). Instead, norepinephrine might act as a neuromodulator rather involved in the processes of acclimatization to hypoxia. In this context, it is worthwhile to mention that norepinephrine injected locally in the caudal NTS can depress the central respiratory neurons, leading to a decreased discharge of phrenic nerve fibers (3). Within the NTS are located the neurons of the dorsal respiratory group which are adjacent to the A2 noradrenergic neurons. The anatomical vicinity of both types of neurons and the ability of norepinephrine to alter the activity of respiratory neurons led to the suggestion that activation of A2 noradrenergic neurons induced by long-term hypoxia might participate in the stabilization of the hypoxic stimulation of respiratory neurons in the NTS (20). This hypothesis is further supported by the time coincidence between the occurrence of ventilatory acclimatization and noradrenergic activation. Both respiratory and neurochemical changes were indeed observed between 1 and 2 weeks of hypoxia. In addition, a significant correlation was found between the level of minute ventilation after ventilatory acclimatization and the amount of TH protein (20). This strengthens the suggestion of a functional relationship between A2 neurons and ventilatory acclimatization to hypoxia (20).

In conclusion, noradrenergic neurons in the caudal NTS are involved in the central chemoreceptor pathway during sustained hypoxia but are not essential for regulatory responses to acute hypoxia. Caudal A2 neurons could contribute to the ventilatory acclimatization to hypoxia through the inhibitory effects of norepinephrine on the dorsal respiratory group.

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