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Hypoxia and the neonatal rabbit lung: neuroendocrine cell numbers, 5-HT fluorescence intensity, and the relationship to arterial thickness

IM KEITH, JA WILL

From the Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin, USA

ABSTRACT We assessed the dynamics of neuroendocrine (NE) cell numbers, the intensity of specific 5-HT fluorescence, and the arterial medial thickness in the lungs of neonatal rabbits in normoxia and acute and chronic hypoxia. Hypoxic neonates had significantly higher NE cell numbers and medial thickness of the pulmonary arteries at 5 days of age than did normoxic controls; 1- and 3-day-old young that died in hypoxia also had significantly higher cell numbers and medial thickness than did hypoxic survivors. A decline in these cell numbers was noted between 1 and 5 days of age among normoxic young, whereas there was no significant change among hypoxic young. Medial thickness was unchanged among normoxic young but increased between 1 and 5 days of age among hypoxic survivors. A 1-day exposure to normoxia of hypoxic young four days postpartum caused a decrease in NE cell numbers and medial thickness to more normal values. Serotonin (5-HT) fluorescence intensity levels of groups of NE cells or neuroepithelial bodies (NEBs) in this group were equal to those of normal controls although these levels were decreased in early chronic hypoxia. Medial thickness and NE cell numbers were inversely correlated with serotonin levels, suggesting that serotonin may be associated with medial hypertrophy and presence of argyrophil material. Medial thickness was positively correlated with NE cell numbers. The above findings led to the following summary: pulmonary NE cells respond to changes in airway oxygen levels; hypoxia or decreased oxygen is associated with decreased cellular 5-HT content and an increase in NE cell numbers by argyrophil stain and medial thickness of pulmonary artery walls. The change to normoxia from hypoxia results in higher cellular 5-HT content and decreased NE argyrophil cell numbers along with reduced pulmonary artery wall medial thickness.

Pulmonary vasoconstriction during hypoxia has been studied since 1946, when Von Euler and Liljestrand demonstrated that increased pulmonary arterial pressure was induced by acute hypoxia in the cat.[1] Hypoxically induced pulmonary hypertension has since been described concomitant with restrictive respiratory disorders[2] and in animals and man at high altitude.[3-6]

The mechanism is far from being explained. Involvement of the neuroendocrine cells of the lung has been implied in the hypoxic vasoconstrictor response,[7-9] whereas CNS reflexes, local pH, and lactic acid do not seem to be primary mechanisms.[10-13]

Address for reprint requests: Dr I M Keith, Department of Veterinary Science, University of Wisconsin, 1655 Linden Drive, Madison, WI 53706, USA.

Pulmonary NE cells are members of the APUD (amine precursor-uptake-decarboxylation) system described by Pearse.[14] They occur singly as neuroendocrine cells (NECs) or in groups as neuroepithelial bodies (NEBs) in the airway epithelium throughout the respiratory tract. Precursor uptake and cytoplasmic storage of the amines serotonin and dopamine in argyrophil dense-core vesicles (DCVs) has been demonstrated,[15 16] as has immunoreactivity to a bombesin-like peptide.[17] Changes in argyrophil stainability, dense-core vesicle appearance and serotonin concentration are known to occur with hypoxic stimulus.[18-21]

Our objective was to study the changes of pulmonary NE cells in relation to pulmonary vasoconstriction under short-term chronic hypoxia, with and

without normoxic recovery. This study describes changes of cell numbers and 5-HT fluorescence in these cells as well as changes in pulmonary arterial medial thickness.

Methods

One-, 3-, and 5-day-old rabbits, born and raised in hypoxia from New Zealand White does, and young born to normoxic does were studied. The hypoxic does were kept in a hypobaric chamber from the twentieth day of gestation at 520 mmHg for 23·5 h daily at 24°C and 50 ± 5% RH.

Two litters of 4-day-old hypoxic neonates were allowed to recover from hypoxia for one day. These, and a number of neonates that died in hypoxia on days 1, 3, and 5, were also included in the study. Treatments and sample sizes are given in tables 1-4.

All live neonates were killed using 1 cc pentobarbital IP for rapid anaesthesia, and decapitation was performed before respiration was impaired. The hypoxic neonates were killed within 15 minutes after the chamber pressure was normalised. The lungs were instilled with Bouin's fixative through a tracheal cannula at a pressure of 11·4 cm water in an open-chest preparation. All six lobes per animal were embedded in paraffin and 7 μ-thick serial sagittal sections were silver-stained by the Grimelius method[22] to locate NECs and NEBs. Miller's elastic-Van Gieson counterstain[23] was used for morphometry, and haematoxylin and eosin for morphology. An average surface area of 1·95 cm^2 per animal was scanned, using 450× magnification, and the counted neuroendocrine cells were expressed as the number of NECs and NEBs seen per cm^2. Actual surface area for each section was determined as follows: the entire section was projected on a sheet of paper with known weight per cm^2 (enlargement 100×). The contours of the image were outlined and cut, and the weight of this paper image was multiplied by a conversion factor [1 cm^2/(weight of 1 cm^2 × 100)]. Tests of significance were based on log-transformed cell numbers so that normal statistics could be applied. A two-way ANOVA and Duncan's multiple range test were used to determine significant differences.[24]

To determine pulmonary artery changes we calculated medial thickness (MT) from outer (OD) and inner (ID) diameter at two perpendicular levels in cross-sectional arteries from the right inferior lobe. Ten arteries per animal were measured, ranging in OD from 50 to 100 μ. A combination of paper-tracing and a computerised morphometry system was used for this procedure. Medial thickness was expressed relative to outer diameter, here called arterial index (MT/OD). Statistical tests are based on mean arterial index for each animal.

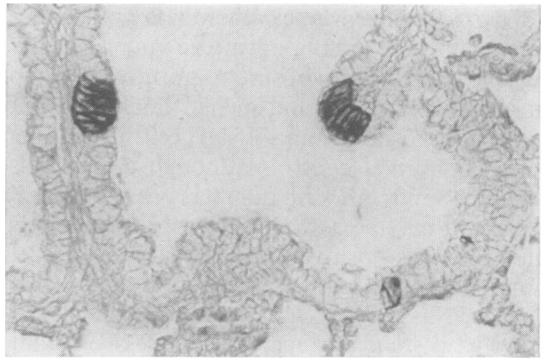

Fig 1 *NEBs at bronchiolar bifurcations. Grimelius stain, original magnification × 375.*

Table 1 *Means and standard errors of neuroendocrine cell numbers per cm^2 in lung sections of 1-, 3-, and 5-day-old rabbits subjected to different in utero and postpartum oxygen treatments*[1]

Number of days postpartum	*Does normoxic through gestation, young normoxic postpartum*	*Does hypoxic from day 20 of gestation, young hypoxic postpartum*	
	Young killed	*Young killed*	*Young died*
1	A 72·0 ± 14·0 (6)	B 93·5 ± 10·3 (6)	C 171·2 ± 20·2 (19)
3	D 48·0 ± 4·7 (7)	E 113·3 ± 11·2 (4)	F 283·9 ± 34·1 (13)
5	G 25·6 ± 2·9 (26)	H 61·7 ± 12·7 (7)	103·1 ± 25·3 (6)
Duncan's multiple range test, means[2]	G D H A B I E C F		

[1] Number of young rabbits per treatment is given in parentheses.
[2] Analysis of variance disclosed significant differences within days and within treatments; means from groups underscored by the same line were not significantly different at $p \leq 0{\cdot}05$. The means are rearranged in ascending order.

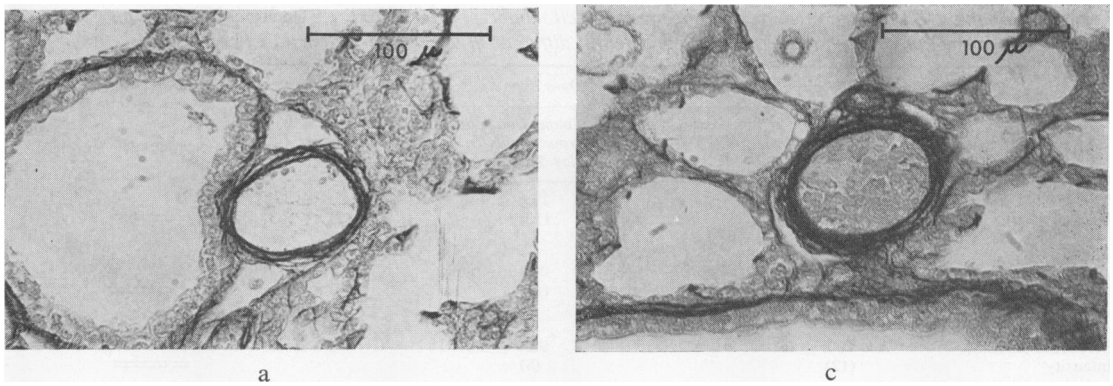

Fig 2 (a) *Small pulmonary artery from a 5-day-old normoxic rabbit.* (b) *Small pulmonary artery from a rabbit that died in hypoxia at 5 days of age. Note the separation of elastic laminae indicating persistence of fetal elastic morphology.* (c) *Small pulmonary artery from a 5-day-old rabbit killed after one day of normoxic recovery from hypoxia. Original magnifications* × *450.*

Serotonin levels, as determined by fluorescence emission intensities, were measured in NEBs of normoxic and hypoxic neonates. Twenty NEBs were measured from the left inferior lobe in each animal using methods previously described.[21 25] Treatments and sample sizes are given in tables 3 and 4.

Results

NEUROENDOCRINE CELL NUMBERS

Single and groups of neuroendocrine cells appeared in the epithelium of all airways studied. The trachea and larynx were not included. NEBs were frequently noted at bifurcations (fig 1).

There was no significant difference in neuroendocrine cell numbers between killed normoxic or hypoxic 1-day-old rabbits (table 1). There were, however, significantly higher cell numbers among killed hypoxic young by days 3 and 5. Neonates that died in hypoxia on days 1, 3, and 5 had significantly higher cell numbers than normoxic controls, and those that died on days 1 and 3 also had significantly

Table 2 *Means and standard errors of arterial indices (medial thickness/outer diameter) in lung sections of 1-, 3-, and 5-day-old rabbits subjected to different in utero and postpartum oxygen treatments*[1]

Number of days postpartum	*Does normoxic through gestation, young normoxic postpartum*	*Does hypoxic from day 20 of gestation, young hypoxic postpartum*	
	Young killed	*Young killed*	*Young died*
1	A 0·104 ± 0·005 (22)	B 0·115 ± 0·006 (25)	C 0·183 ± 0·007 (19)
3	D 0·125 ± 0·010 (7)	E 0·125 ± 0·005 (4)	F 0·166 ± 0·009 (13)
5	G 0·095 ± 0·007 (16)	H 0·161 ± 0·008 (7)	I 0·159 ± 0·008 (6)
Duncan's multiple range test, means[2]	G A B D E I H F C		

[1] Number of young rabbits per treatment is given in parentheses.
[2] Analysis of variance disclosed significant differences within days and within treatments; means from groups underscored by the same line were not significantly different at $p \leq 0{\cdot}05$. The means are rearranged in ascending order.

Table 3 *Means and standard errors of neuroendocrine cell numbers, arterial indices, and specific serotonin fluorescence in the lungs of 5-day-old rabbits subjected to different in utero and postpartum oxygen treatments*[1]

	Does normoxic through gestation	*Does hypoxic from day 20 of gestation*		*Duncan's multiple range test, means*[2]
	Young 5 days in normoxia	*Young 4 days in hypoxia plus 1 day (day 5) in normoxia*	*Young 5 days in hypoxia*	
Number of NECs and NEBs per cm²	A 25·6 ± 2·9 (26)	B 19·6 ± 4·6 (13)	C 61·7 ± 12·7 (7)	B A C
Arterial index (medial thickness/ outer diameter)	A 0·095 ± 0·007 (16)	B 0·119 ± 0·006 (13)	C 0·161 ± 0·008 (7)	A B C
Serotonin emission intensity (relative units)	A 90·8 ± 6·8 (12)	B 92·0 ± 3·5 (8)	C No data	A B

[1] Number of young rabbits per treatment is given in parentheses.
[2] Analysis of variance disclosed significant differences between treatments; means from groups underscored by the same line were not significantly different at $p \leqslant 0{\cdot}05$. The means are rearranged in ascending order.

higher cell numbers than their hypoxic survivors.

There was a significant decrease in cell numbers between days 1 and 5 in normoxic young, but no significant change among hypoxic young. Animals that died in hypoxia on day 3 had significantly higher cell numbers than young dying on days 1 and 5.

PULMONARY VASCULAR CHANGES

Early thickening of the media by medial smooth muscle cells separated the elastic layers and decreased the lumen (fig 2b). The folding of endothelium and elastica interna was observed in arteries of hypoxic rabbits, and oedema sometimes occurred around vessels where constriction was pronounced.

The differences in arterial index show a pattern similar to that of the neuroendocrine cells (table 2). There were no significant differences in arterial index between killed normoxic or hypoxic neonates on days 1 and 3, although killed hypoxic young had a higher index by day 5. Neonates that died in hypoxia had higher arterial indices than normoxic controls on days 1, 3, and 5, and higher than hypoxic survivors on days 1 and 3. Arterial index did not change over the first five days among normoxic controls or those dying in hypoxia, whereas it increased significantly among hypoxic survivors.

Table 4 *Means and standard errors of serotonin emission intensity of NEBs in neonatal rabbit lungs*[1]

Number of days postpartum	*Does normoxic throughout gestation young normoxic postpartum*	*Does hypoxic from day 20 of gestation young hypoxic postpartum*
	A 89·6 ± 7·4 (5)	B 57·0 ± 5·6 (8)
3	C 113·6 ± 3·8 (4)	52·7 (1)
Duncan's multiple range test, means[2]	B A C	

[1] Number of young per treatment given in parentheses.
[2] Analysis of variance disclosed significant differences within and between treatments; means from groups not underscored by the same line were significantly different from one another at $p \leqslant 0{\cdot}05$. The means are rearranged in ascending order. Unlabelled means not included.

SHORT-TERM CHRONIC HYPOXIA WITH NORMOXIC RECOVERY

Neonates that were in hypoxia for four days postpartum and killed after normoxic recovery for one day showed a significant decrease in neuroendocrine cell numbers and arterial indices when compared with hypoxic young killed five days postpartum (table 3). Cell numbers in this recovery group were equal to those of normoxic 5-day-old controls. Arterial indices decreased to a value equal to that in normoxic 5 day-olds, and significantly lower than in any other treatment. The inner elastic lamina had a smooth appearance resembling the normal state (fig 2a, c).

SEROTONIN FLUORESCENCE OF NEBS

Serotonin levels were significantly lower in hypoxic 1-day-old rabbits than in normoxic controls (table 4). We also noted a significant increase in serotonin emission on day 3 in normoxic rabbits. Furthermore, in the hypoxic group with normoxic recovery for one day, serotonin fluorescence was equal to that of normoxic controls (table 3).

RELATIONSHIP OF NEUROENDOCRINE CELL NUMBERS AND SEROTONIN LEVELS TO ARTERIAL INDEX

A regression analysis of individuals in tables 1 and 3 disclosed a significant correlation between neuro-

endocrine cell numbers and arterial indices ($r = 0{\cdot}62$; $n = 97$; $p < 0{\cdot}01$; $y = 0{\cdot}0199 \cdot \ln(x) + 0{\cdot}0567$). Another regression analysis disclosed a significant negative correlation between serotonin emission values and arterial indices ($r = -0{\cdot}48$; $n = 38$; $p < 0{\cdot}01$; $y = -0{\cdot}0006x + 0{\cdot}171$). Furthermore, there was a significant negative correlation between neuroendocrine cell numbers and serotonin emission means ($r = -0{\cdot}52$; $n = 38$; $p < 0{\cdot}01$; $y = -1{\cdot}243x + 168{\cdot}14$).

OTHER FINDINGS

Hypoxic neonates frequently had interstitial haemorrhage in ears, nose, tail and feet, and petechiae were observed on lung surfaces and in the gastrointestinal tract. Muscularisation of small arterioles (20-30 μm in diameter) was noticed in hypoxic lungs, especially among young that died. Veins in hypoxic lungs appeared to have thicker walls.

Discussion

Higher neuroendocrine cell numbers in chronic hypoxic lungs were previously reported by Taylor[26] in his study of rabbits killed at high altitude. Increased airway oxygen tension appears to be a strong stimulus for reduction in the number of detectable neuroendocrine cells. This was demonstrated in our chronic hypoxic neonates with one day of normoxic recovery (table 3). Reduction of argyrophil substance and arterial index appears to occur rapidly and was already noted within 15 minutes after hypoxia. For example, in contrast to above results, Hernandez-Vasquez *et al*[20] reported that hypoxic 1- and 5-day-old rabbits had significantly *lower* cell numbers than normoxic controls. This probably resulted from inadvertently prolonged exposure of hypoxic young to normoxic conditions before killing.

The decline in argyrophil cell numbers after birth, as reported in the normoxic neonates, has been previously demonstrated in rats and rabbits.[18 20] This suggests that the neuroendocrine cells may play their most important role during the early neonatal period, and may therefore be involved in adaptation to and maturation of respiration.

No differences in cell numbers and arterial indices were found between 1-day-old rabbits killed in hypoxia or normoxia whereas those dying in hypoxia had higher cell numbers and arterial indices. At day 5 postpartum both hypoxic groups had higher cell numbers and arterial indices than normal controls. This suggests that it is the postnatal oxygen condition that determines the changes seen in hypoxic neonates. These findings are supported by those of Naeye in his studies of pulmonary arteries in normoxaemic infants and infants hypoxaemic secondary to hyaline membrane disease.[27]

A large proportion of the hypoxic young in this study died. They had significantly higher neuroendocrine cell numbers and thicker pulmonary artery walls than their normoxic controls at all ages; they sometimes also differed from their hypoxic survivors. These findings may indicate hypoxic distress. It is known that the response to hypoxia is individual. Adaptation occurs in humans and other mammals but the ability to adapt varies with species, stimulus strength, and genetic disposition.[28-30]

Previous reports of rats and rabbits exposed to acute hypoxia disclosed a decrease in pulmonary neuroendocrine dense-core granular material, increased exocytosis of dense-core vesicles (DCVs) toward the basement membrane, and decreased formalin-induced fluorescence.[18 19 31] We earlier demonstrated that the reduced fluorescence was caused by a loss of serotonin specifically, and that this reduction was not accompanied by a significant change in argyrophil neuroendocrine cell numbers.[21] This leads to the probability that 5-HT is not responsible for the argyrophilia. This probability is supported by an earlier microspectrographic study which suggested that a polypeptide was present and by our localisation of the tetradecapeptide bombesin.[17 32] The evidence in the present study supports the view that serotonin is not the argyrophil substance because fluorescence is low in early chronic hypoxia even though the cell numbers by silver stain methods remain constant.

In the present study high neuroendocrine cell numbers and arterial indices rapidly decreased to normal range when hypoxic neonatal rabbits were rendered normoxic. Serotonin levels found to be low in hypoxia, also returned to normal. The normalisation is consistent with the time course of recovery of patients with high-altitude pulmonary hypertension and oedema when treated with supplementary oxygen.[33] Medial hypertrophy in the pulmonary arteries is reversible but speed and degree of normalisation depends on the duration of the hypoxic stimulus.[34]

The hypothesis of an oxygen-dependent *active* relaxation of pulmonary arteries was first suggested by Weir.[35] He postulated that pulmonary vasoconstriction in hypoxia was caused by the absence of oxygen-dependent relaxation rather than a hypoxia-induced constriction. Oxygen ventilation increases blood levels of the pulmonary vasodilator bradykinin, a nonapeptide. Inhibition of bradykinin metabolising enzyme also elevates bradykinin levels and has been shown to prevent pulmonary artery wall hypertrophy in chronic hypoxia.[36] Although bradykinin may not be the specific mediator of active normoxic pulmonary vasodilation, there is considerable evidence for this mechanism, and the results of our study

indicate that the NE cells of the lung may act as modulating O_2 sensors.

The changes in NE cell numbers and cytoplasmic 5-HT fluorescence induced by changes in airway oxygen levels, as recorded in our study, suggest that these cells are oxygen sensors responding to airway hypoxia rather than to hypoxaemia. In a cross-circulation experiment, where alveolar and arterial oxygen levels were altered independently, alveolar hypoxia and not hypoxaemia caused secretory changes in rabbit NEBs.[7] Hauge similarly noted that hypoxia, not hypoxaemia, elicited the most brisk vasoconstrictor response and concluded that the receptor for hypoxic pulmonary vasoconstriction was closer to the airway than to the vasculature.[37] Furthermore, Naeye found that prolonged unilateral hypoventilation in dogs, calves, and human infants caused hypertrophy and hyperplasia of small arteries on the ipsilateral side.[38]

It would seem logical and beneficial for the lung to react to local alveolar hypoxia (atelectasis, emphysema, inflammation, and so on) through local or regional arterial constriction, thus shunting blood to better ventilated areas, and making the ventilation-perfusion ratios more uniform. Hypobaric hypoxia or hypoventilation—that is, all airways hypoxic—would then be an extreme situation.

The significant correlations of (1) neuroendocrine cell numbers with arterial indices, and (2) cell numbers and arterial indices with serotonin levels, suggest that the NE cells may be involved in the mechanism of pulmonary vasoconstriction and relaxation. These correlations, under hypoxic and normoxic conditions are not, however, proof of a cause-and-effect relationship. The nature of the interaction between monoamines and polypeptides in the lung is not firmly established and their functions are not fully understood, although evidence of this interaction in many organs exists, and it appears to be only a matter of time before the significance of this relationship to control of the lung is more fully elucidated.

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