# Normobaric hypoxia induces mild damage to epithelium of terminal bronchioles in rabbits (ultrastructural study)

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**ABSTRACT**: We studied the ultrastructure of the epithelium of terminal bronchioles in rabbits exposed for 96 hours to hypoxia (10% $O_2$) in isobaric hypoxic chamber. Rabbits of the first control group (treated controls) were exposed for the same time in the same hypoxic chamber with atmosphere regulated at 21% $O_2$. In both groups, the temperature in the chamber was 23°C and humidity 100% during the whole experiment. The second control group (untreated controls) was kept under standard conditions. The target cells for the effect of high temperature, humidity and normobaric hypoxia were the secretory elements. Both in hypoxic animals and treated controls, isolated Clara cells revealed signs of pathological alteration. More degenerative changes of Clara cells and marks of their compensatory proliferation were ascertained after exposure to hypoxia. Electron dense secretory granules were observed in most Clara cells. This finding reflected the initial stage of their secretory product formation. The secretory granules were usually stored in cytoplasm; morphological signs of their evacuation were found only exceptionally. The ciliated cells were less damaged than the secretory ones. On their apical surfaces, formation of cytoplasmic protrusions, which sometimes led to degeneration of free cilia, was ascertained. The damage of the epithelium and mild secretory stimulation of its secretory elements could play a role in the lung injury caused by the subacute normobaric hypoxia.

**Keywords**: airways; Clara cells; ciliated cells; electron microscopy

The exposure of experimental animals to hypoxia has been widely used in many morphological and physiological studies. These studies dealt mostly with changes in the structure of pulmonary vessels (Hislop and Reid, 1976; Smith and Heath, 1977; Davies et al., 1985). Less attention was paid to the effect of hypoxia on the epithelial lining of airways or pulmonary alveoli. Nevertheless, the reaction of the epithelium can contribute to the development of changes observed in the pulmonary parenchyma. The expression of NO synthase, producing potent vasoactive substance NO, was demonstrated in airway epithelial cells of rats (Xue et al., 1994; for review see Hampl and Herget, 2000). The immunoreactivity of endothelin-1 (ET-1), that causes vasoconstriction of pulmonary vessels, was described in the epithelium of small airways in rats, too (Aguirre et al., 2000). In our previous studies, the stimulation of secretory elements to release their secretions and/or damage of epithelial cells leading to the loss of their integrity were described under various experimental conditions (Konradova, 1991; Uhlik and Tuma; 1998, Uhlik et al.; 1999, 2002). Thus, we hypothesised that intracellular factors liberated from

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the stimulated or damaged cells should influence surrounding tissues. We therefore decided to study the effect of hypoxia on the ultrastructure of the epithelium of rabbit airways. In this part of our study, we describe the reaction of the epithelium of terminal bronchioles.

## MATERIAL AND METHODS

Nine specified pathogen free (SPF) rabbits (New Zealand White, males, average body weight 2.4 kg, Charles River Deutschland, Sulzfeld, Germany) were used. Three rabbits were kept under standard conditions and served as untreated controls. Three animals (treated controls) were placed for 96 hours in a normobaric chamber containing room atmosphere (21% $O_2$) with 100% relative humidity and temperature 23°C. Three remaining rabbits spent 96 hours under the same conditions, but the atmosphere in the experimental chamber was maintained at 10% $O_2$. $CO_2$ concentration was less than 1.5%.

Immediately after removal from the chamber, animals were anaesthetised by i.m. administration of the mixture of ketamine (35 mg/kg b.w., Narkamon 5% inj. ad usum vet., Spofa, Prague, Czech Republic) and xylazine (5 mg/kg b.w., Rometar 2% inj., Spofa, Prague, Czech Republic). Ventral cervical region of the animals was infiltrated subcutaneously by procaine (Procain 1% inj., Leciva, Prague, Czech Republic) 10 minutes before preparation. Untreated control animals were anaesthetised by the same method. All experimental procedures were performed according to the certification of the Animals Protection Expert Commission of the 2nd Faculty of Medicine, Charles University, Prague, Czech Republic.

In all animals, the lungs were removed after thoracotomy and immediately perfused by 5% glutaraldehyde (Merck, Hohenbrunn bei Munchen, Germany) in cacodylate buffer (pH 7.2). From one pulmonary lobe, pieces of approximately 1 $mm^3$ of the tissue were collected, fixed for 90 minutes with the same fixative and then for 60 minutes with 2% $OsO_4$ (JMC, Hertfordshire, United Kingdom) in cacodylate buffer (pH 7.4). The material was dehydrated in graded series of alcohol and embedded in a Durcupan-Epon mixture (Fluka, Buchs, Switzerland). Terminal bronchioles were localised in semithin sections stained with toluidine blue. Ultrathin sections were prepared on Ultrotome Nova (LKB, Broma, Sweden), contrasted with uranyl acetate and lead citrate and examined under JEM 100 C electron microscope (Jeol, Tokyo, Japan).

For the quantitative evaluation, the total number of ciliated and Clara cells and the functional state of Clara cells were recorded in the electron microscope using the methods described in our previous paper (Uhlik, 1996). In untreated controls, treated controls and in rabbits after 4-day hypoxia, 601, 581, and 531 epithelial cells were evaluated, respectively. To compare the results in individual groups, the $\chi^2$ test of homogeneity in frequency tables was used. To specify categories causing deflections from the hypothesis of homogeneity, adjusted standardised deviations were employed.

Using the computer image analyser Lucia G (Laboratory Imaging, Prague, Czech Republic), details of Clara cells' cytoplasm were evaluated in digitalised electronmicrographs. The supranuclear cytoplasm of randomly selected Clara cells was marked and measured. In this area, secretory granules and mitochondria were delineated, counted and automatically gauged. In untreated controls, treated controls and in experimental group, 2 767.3 $\mu m^2$, 2 704.8 $\mu m^2$, and 2 854.4 $\mu m^2$ of Clara cells' cytoplasm with 426, 591, and 683 granules and 2 172, 2 721, and 2 381 mitochondria were analysed, respectively. The results were statistically evaluated by the one-way analysis of variance. The differences between individual groups were tested by the two-sample *t*-test (software NCSS version 6.0).

## RESULTS

### The ultrastructure of the epithelium of terminal bronchioles in untreated control rabbits

The terminal bronchioles of untreated control rabbits were lined by a simple epithelium where low columnar or cuboidal ciliated cells and high columnar Clara cells alternated almost regularly. Both types of the epithelial cells did not differ in details of the arrangement and ultrastructure from those described previously (Uhlik, 1996).

In the epithelium of the terminal bronchioles of untreated control rabbits, the Clara cells and the ciliated ones represented 52.7 ± 3.6% and 47.3 ± 3.6% of epithelial cells, respectively. Secretory granules were found in the majority of Clara cells (73.5 ±

Table 1. Quantitative evaluation of the epithelium of terminal bronchioles and functional state of Clara cells in rabbits after 4-day hypobaric hypoxia (relative values)

| | Untreated controls | Treated controls | Hypoxia |
|---|---|---|---|
| Ciliated cells (%) | 47.3 ± 3.6 | 48.7 ± 6.4 | 42.6 ± 3.4# |
| Clara cells (%) | 52.7 ± 3.6 | 51.3 ± 6.4 | 57.4 ± 3.4# |
| Clara cells with granules (%) | 73.5 ± 9.4 | 85.6 ± 3.7* | 86.9 ± 6.5* |
| Clara cells without granules (%) | 26.5 ± 9.4 | 14.4 ± 3.7* | 13.1 ± 6.5* |
| Number of granules per 1 μm$^2$ | 0.17 ± 0.07 | 0.22 ± 0.08 | 0.23 ± 0.14 |
| Area of granules (μm$^2$) | 0.15 ± 0.04 | 0.11 ± 0.01 | 0.15 ± 0.03 |
| Number of mitochondria per 1 μm$^2$ | 0.73 ± 0.35 | 1.02 ± 0.34 | 0.81 ± 0.23 |
| Area of mitochondria (μm$^2$) | 0.22 ± 0.08 | 0.15 ± 0.05 | 0.18 ± 0.08 |

$n$ = 3, values are expressed as mean ± SD
values designated * differ significantly ($P < 0.01$) from untreated controls
values designated # differ significantly ($P < 0.05$) from treated controls

9.4%). During the computer image analysis of the Clara cell supranuclear cytoplasm, 0.165 ± 0.066 secretory granules and 0.73 ± 0.35 mitochondria were found per 1 μm$^2$. The average area was 0.15 ± 0.04 μm$^2$ in granules and 0.22 ± 0.08 μm$^2$ in mitochondria. The results of computer image analysis were summarised in Table 1.

### The ultrastructure of the epithelium of terminal bronchioles in rabbits after 4 days spent in the experimental chamber regulated at 21% $O_2$ (treated controls)

The terminal bronchioles of rabbits that spent 4 days in the chamber were lined with a simple columnar epithelium composed of 48.7 ± 6.4% of ciliated cells and 51.3 ± 6.4% of Clara cells. Apical junctional complexes were intact, intercellular spaces remained narrow and did not contain any free cells. All epithelial cells widely rested on a well-developed basal lamina. Cells of diffuse neuroendocrine system containing small, spherical, electron-dense granules were found in the epithelium only exceptionally.

In the ciliated cells' cytoplasm, mild marks of the pathological alteration were observed. On their apical surfaces, formation of small cytoplasmic protrusions was ascertained. The apical blebs interfered with the regular arrangement of cilia and sometimes incorporated free cilia, which subsequently led to their degeneration. Increase in number of small vesicles and multivesicular bodies was observed in deeper portions of the cytoplasm. Cisternae of Golgi complex were often slightly dilated. In some ciliated cells, altered mitochondria filled with an electron-lucent, inhomogeneous matrix were found.

Secretory Clara cells were usually intact. Their apical cytoplasm was filled with thin tubules of smooth endoplasmic reticulum, numerous mitochondria and relatively large, electron-dense secretory granules. The basal portion of these cells contained narrow cisternae of rough endoplasmic reticulum arranged in parallel pattern. Only isolated Clara cells revealed signs of the pathological alteration. They contained wider tubules of smooth endoplasmic reticulum filled with light content and altered mitochondria. Parts of their degenerated cytoplasm were recorded even free in the lumen of terminal bronchioles. Electron dense secretory granules were observed in 85.6 ± 3.7% of Clara cells. The granules were usually stored in the cytoplasm. Morphological signs of liberation of their content were found only exceptionally. Only a few granules were recorded lying in tight contact with the plasma membrane or inside the apical cytoplasmic protrusions. The computer image analysis showed increase in number of both the secretory granules and mitochondria per 1 μm$^2$ of the supranuclear cytoplasm to 0.22 ± 0.08 and 1.02 ± 0.34, respectively. Their average area was 0.11 ± 0.01 μm$^2$ and 0.15 ± 0.05 μm$^2$ (Table 1).

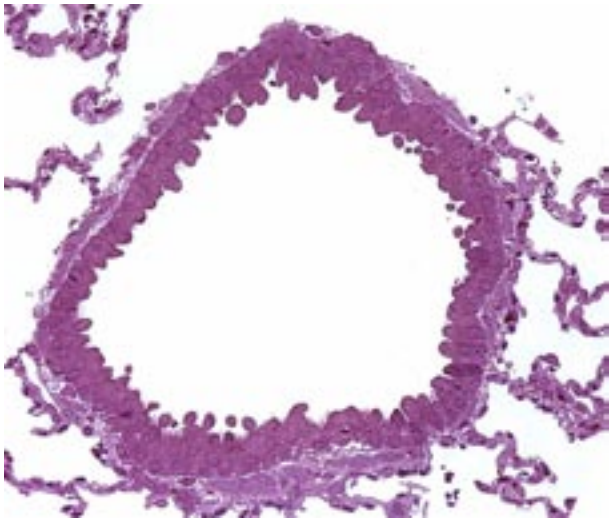

Figure 1. Terminal bronchiole lined with a typical simple columnar epithelium composed of lower ciliated and higher Clara cells. Rabbit, 4-day hypoxia, semithin section, toluidine blue, light microscope, obj. 20×

### The ultrastructure of the epithelium of terminal bronchioles in rabbits after 4-day hypoxia (10% $O_2$)

The terminal bronchioles of rabbits exposed to 4-day hypoxia were lined with a simple columnar epithelium composed of 42.6 ± 3.4% of ciliated cells and 57.4 ± 3.4% of Clara cells (Figure 1). Apical junctional complexes, intercellular spaces and basal surface of the epithelium were unchanged (Figure 2). Exceptional presence of diffuse neuroendocrine system cells was noticed, too.

The cytoplasm of ciliated cells revealed more pronounced marks of the pathological alteration. On their apical surfaces, the same apical blebbing as in the previous group was visible (Figure 3). In deeper portions of cytoplasm, the increase in number of larger vesicles, multivesicular bodies and lysosomes with variable size and content was observed (Figure 4). Cisternae of Golgi complex were more dilated in comparison with the previous group. Marks of the mitochondrial alteration were more pronounced, too.

Signs of Clara cells pathological alteration were more expressed than in the treated controls. In some cells, wide tubules of smooth endoplasmic reticulum and altered, dilated mitochondria almost entirely filled the cytoplasm (Figure 5). As in the previous group, parts of the degenerated cytoplasm were found in the lumen of airways (Figure 6). Secretory granules were ascertained in 86.9 ± 6.5%

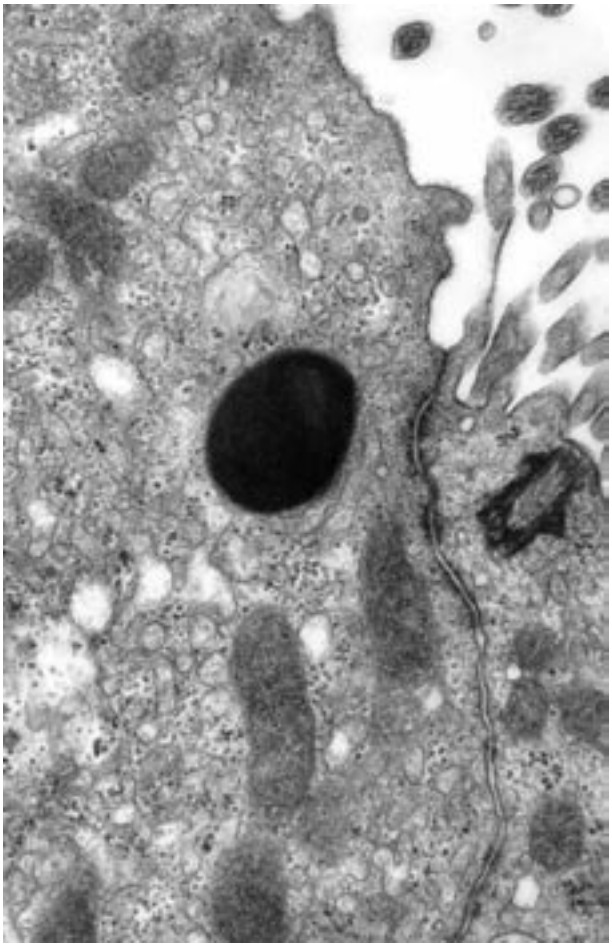

Figure 2. Intact apical junctional complex between a ciliated and Clara cell. Rabbit, 4-day hypoxia, electron microscope, 50 000×

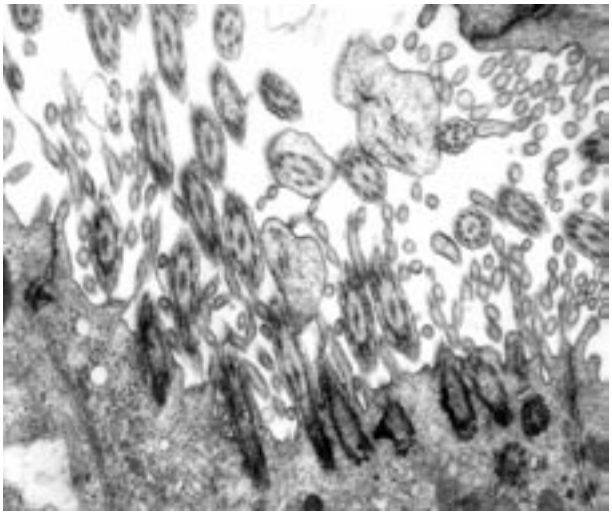

Figure 3. Apical portion of a ciliated cell with small cytoplasmic protrusions containing disintegrating axonemes of kinocilia. Rabbit, 4-day hypoxia, electron microscope, 37 500×

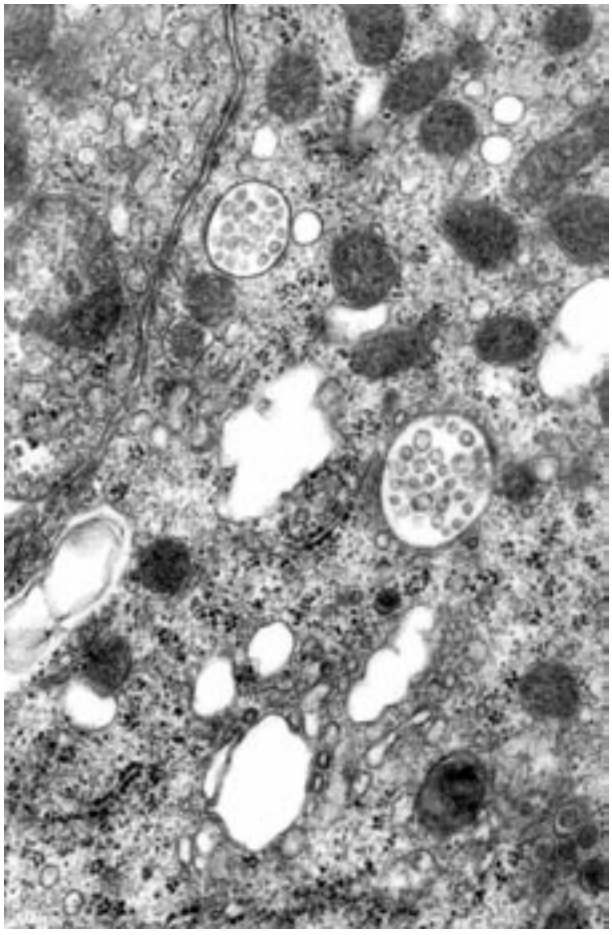

Figure 4. Two multivesicular bodies, small lysosome and dilated spaces of Golgi complex in the cytoplasm of an altered ciliated cell. Rabbit, 4-day hypoxia, electron microscope, 50 000×

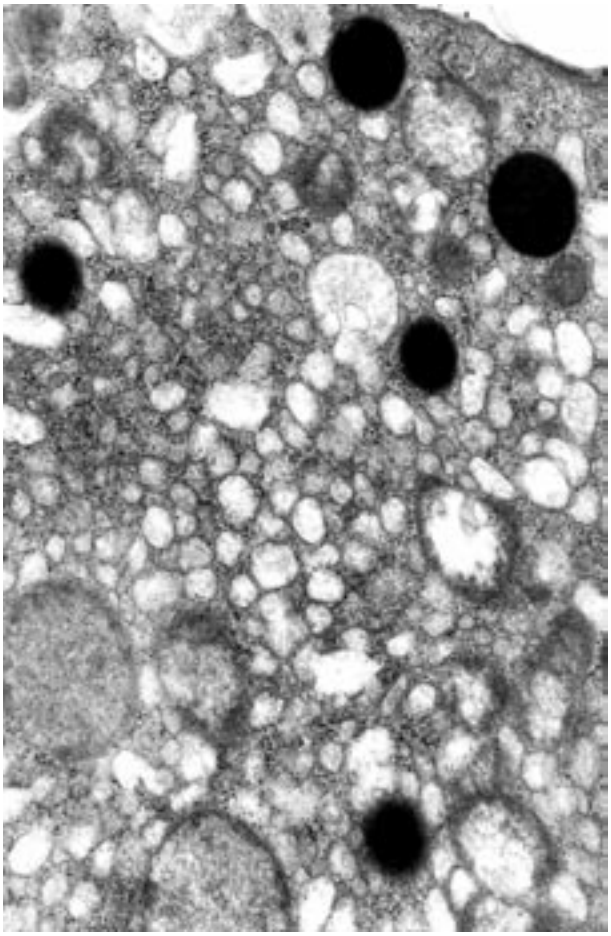

Figure 5. Dilated tubules of a smooth endoplasmic reticulum and altered mitochondria in a Clara cell cytoplasm. Rabbit, 4-day hypoxia, electron microscope, 37 500×

of Clara cells. The morphological signs of evacuation of their content were found rarely. The results of computer image analysis of the supranuclear cytoplasm were 0.23 ± 0.14 granules and 0.81 ± 0.23 mitochondria per 1 $\mu m^2$. The granules measured 0.15 ± 0.03 $\mu m^2$, the average area of mitochondria was 0.18 ± 0.08 (Table 1).

## DISCUSSION

To our knowledge, the morphological studies of the airway or alveolar epithelium in animals exposed to any type of hypoxic conditions are not frequent. There is a problem to compare the results of these studies because their design usually differed. The hypoxia was achieved either by the decrease of atmospheric pressure (hypobaric) or by the regulation of $O_2$ content in the inhaled gas mixture under the normal pressure (normobaric). The length of the exposure varied from 6 hours to 60 days.

In an ultrastructural study, Sulkowska (1997) observed the degranulation of type II pneumocytes after short-time (3 days) hypobaric (380 mm Hg) hypoxia in rats. After the longer hypoxia (10 and 30 days), hyperplasia of the same cells occurred. After short-time (16 h) normobaric hypoxia (9% $O_2$), the decrease of number and size of type II pneumocytes granules was described in rabbits (Reffy et al., 1977). In the tracheal epithelium of hypoxic (normobaric, 13% $O_2$, 6 h) young rabbits, decrease in number of cells of the diffuse neuroendocrine system was found (Echt et al., 1982). The reduction of sodium transport through the nasal epithelium of hypoxic (hypobaric, 383 mm Hg, 24 h) rats Tomlinson (Tomlinson et al., 1999) explained by the damage of the epithelium.

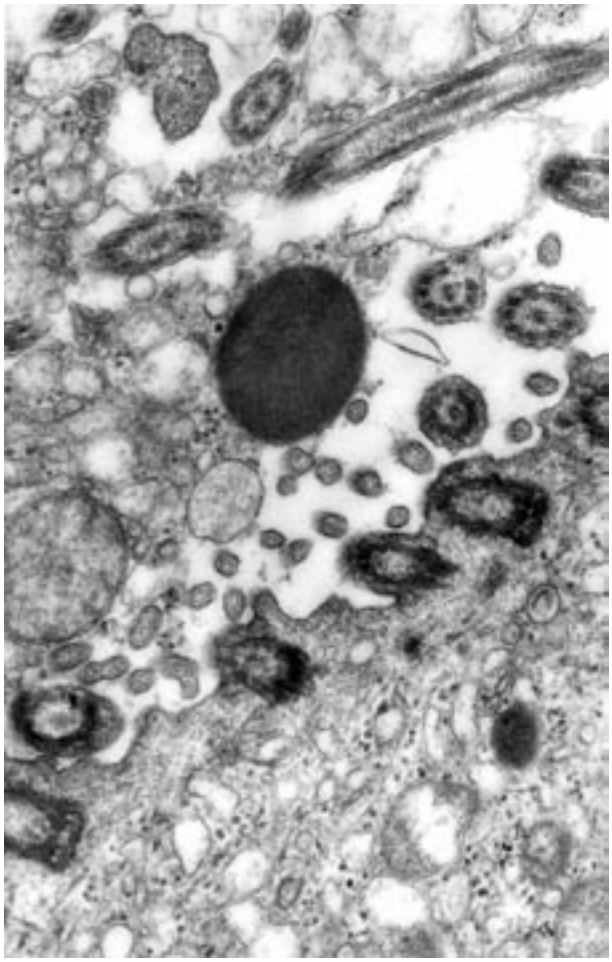

Figure 6. Degenerated cytoplasm of a Clara cell in a lumen of a terminal bronchiole. Rabbit, 4-day hypoxia, electron microscope, 50 000×

The ultrastructure of Clara cells in rats exposed for 12 h to hypobaric hypoxia (265 mm Hg) was studied by Smith and his co-workers (Smith et al., 1974). They described pathological changes of Clara cell cytoplasm leading even to the degeneration of the whole cells. After 5-day stay in the hypobaric chamber (310–340 mm Hg), Tsagareli and his co-workers described stimulation of rat Clara cells to release their secretion. The longer exposure (15–60 days) induced the accumulation of the secretion in the cytoplasm (Tsagareli et al., 1988). The 21-day exposure of rats to normobaric (10% $O_2$) hypoxia was followed by the increased ET-1 immunoreactivity in almost all cells lining the small airways (Aguirre et al., 2000).

In our previous paper dealing with the ultrastructure of the tracheal epithelium of the same rabbits used in the current study (Konradova et al., 2002), the overstimulation and degeneration of secretory goblet cells with highly significant marks of massive compensatory differentiation of new secretory elements were encountered. This process was accompanied with their hyperplasia leading to the formation of intraepithelial mucous glands.

The currently described ultrastructural changes of the epithelium of terminal bronchioles were not so prominent, but they corresponded with the findings described in the tracheal epithelium (Konradova et al., 2002). Pathological alteration was found both in ciliated and Clara cells, but degenerative changes were observed only in some Clara cells. Observed significant increase ($P < 0.05$) of Clara cell relative number in hypoxic rabbits could reflect their compensatory proliferation. The significantly higher ($P < 0.01$) proportion of Clara cells containing secretory granules denoted the initiative stage of their stimulation to produce secretion in both treated controls and hypoxic animals. Also the computer image analysis of the Clara cell supranuclear cytoplasm showed an increase in number of granules in both groups.

The results of current study support our previous findings in the airway epithelium of rabbits exposed to various experimental procedures (Uhlik and Tuma, 1998; Uhlik et al., 1999; 2002). The damage to the tracheal and bronchiolar ciliated cells was always similar, but the secretory elements responded differently at these two airway levels. The reaction of the mucus-secreting tracheal goblet cells was rapid and pronounced, whereas the bronchiolar Clara cells, that secrete less voluminous secretion rich in proteins and play also other roles in airway biology, revealed much more discreet changes. Nevertheless, their damage and mild secretory stimulation could play a role in the lung injury caused by the subacute normobaric hypoxia.

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