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Hyperoxia Attenuated Nitrotyrosine Concentration in the Lung Tissue of Rats with Experimental Pneumonia

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Summary

Although nitrated proteins have been repeatedly used as markers of lung injury, little is known about their formation and metabolism under hyperoxia. We therefore measured 3-nitrotyrosine (3NTYR) concentrations in lung tissue and serum of rats with carrageenan-induced pneumonia exposed to hyperoxia. Twenty-nine Wistar male rats were assigned to one of 4 groups. Two experimental groups were treated by intratracheal application of carrageenan (0.5 ml of 0.7 % solution) and then one was exposed to hyperoxia for 7 days (F_IO_2 0.8), the other to air. Rats of two control groups breathed either hyperoxic gas mixture or air for 7 days. At the end of exposure the ventilation was determined in anesthetized, intubated animals in which 3NTYR concentrations were measured in the lung tissue and nitrites and nitrates (NO_x) were estimated in the serum. Carrageenan instillation increased 3NTYR concentrations in lung tissue (carrageenan-normoxic group 147±7 pmol/g protein, control 90±10 pmol/g protein) and NO_x concentration in the serum (carrageenan-normoxic group 126±13 ppb, control 78±9 ppb). Hyperoxia had no effect on lung tissue 3NTYR concentration in controls (control-hyperoxic 100±14 pmol/g protein) but blocked the increase of lung tissue 3NTYR in carrageenan-treated rats (carrageenan-hyperoxic 82±13 pmol/g protein), increased NO_x in serum (control-hyperoxic 127±19 ppb) and decreased serum concentration of 3NTYR in both hyperoxic groups (carrageenan-hyperoxic 51±5 pmol/g protein, control-hyperoxic 67±7 pmol/g protein, carrageenan-normoxic 82±9 pmol/g protein, control 91±7 pmol/g protein). The results suggest that hyperoxia affects nitration of tyrosine residues, probably by increasing 3NTYR degradation.

Key words

Nitrotyrosine • Hyperoxia • Experimental pneumonia • Carrageenan • Nitrites and nitrates

Introduction

3-nitrotyrosine (3NTYR) is formed in a biological process associated with NO and reactive oxygen species (ROS). It has therefore been repeatedly used as an indicator of increased oxidative stress, particularly in inflammation-related forms of lung injury like asthma (Hanazawa *et al.* 2000), cystic fibrosis (Balint *et al.* 2001), acute respiratory distress syndrome (Lamb *et al.* 1999) or airway inflammation in lung transplants (De Andrade *et al.* 2000). Interestingly enough, Banks *et al.* (1998) reported that plasma nitrotyrosine content was also increased in infants who have developed bronchopulmonary dysplasia and that its

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level correlated with the fraction of inspired oxygen that the infant was receiving. The latter finding suggests that 3NTYR could be a marker of possible adverse effects of high oxygen concentrations used during oxygenotherapy. However, because of the complicated and not fully understood pathogenesis of bronchopulmonary dysplasia, it is difficult to speculate whether increased 3NTYR levels were due to high inspired O_2 concentrations or to the severity of the disease necessitating higher O_2 concentrations during treatment. To elucidate the role of hyperoxia in 3NTYR formation, we exposed the rats with experimental pneumonia to hyperoxia lasting 7 days. We measured 3NTYR concentrations in lung tissue and serum after the exposure in these rats and in respective controls.

Methods

Studies were performed in adult male Wistar rats with initial body weight 228±3 g (mean ± SEM). All techniques used were compatible with the National Institute of Health Guidelines. The rats were divided into four groups and treated as follows:

Group	n	treatment
Car+O₂	6	carrageenan[1] + 7 days of breathing F_IO_2 0.78-0.84
Car+A	8	carrageenan[1] + 7 days of breathing air
O₂	7	7 days of breathing F_IO_2 0.78-0.84
C	8	7 days of breathing air

[1] 0.5 ml of 0.7 % solution intratracheally

Exposure to hyperoxia was performed in a normobaric chamber (Herget and Kuklík 1995) as described previously (Fišárková and Vízek 2003).

Measurements

The animals were anesthetized by thiopental (40 mg/kg, i.p.) and intubated (tracheal cannula ID 1.7 mm, OD 2.3 mm). Rats were then placed in the body plethysmograph (Maxová and Vízek 2001), the tracheal cannula was connected to an outer circuit ventilated with room air. Pressure changes in the plethysmograph were measured by pressure differential transducer (Elema-Schonander EMT 32). A specific computer program (Maxová and Vízek 2002) was used to calculate ventilatory parameters.

Blood samples (2 ml) were taken from the jugular vein, the animals were sacrificed by an overdose of anesthetic and the two lower lobes of the right lung were taken for determining 3-nitrotyrosine, nitrite and nitrate concentrations. The left lung was used to assess its wet and dry weight (Glogowska and Widdicombe 1973).

Nitrite and nitrate concentrations were analyzed using the chemiluminiscence determination of NO, based on its reaction with ozone (Hampl *et al.* 1996) using the Chemiluminiscence NO Analyzer ECO Physics CLD 77 AM. To convert nitrites and nitrates in serum to NO, vanadium, chloric acid and heating of the sample to 90 °C were used as described by Michelakis and Archer (1998)

ELISA estimation of 3-nitrotyrosine

Competitive ELISA for estimation of 3-nitrotyrosine in serum proteins was described by Herget *et al.* (2000). Briefly, polystyrene ELISA 96-well plates (Maxisorp, Nunc) were coated with BSA nitrated by TNM dissolved in PBS at a concentration 5 nM nitrotyrosine overnight. The plates were blocked by three 5 min incubation with PBS plus 0.05 %(v/v) Tween-20 (TPBS). Then 50 µl per well of 0.2 % gelatin in TBS (Tris buffered saline) pH 8.4 was pipeted and standard solution of nitrated BSA (prepared by peroxynitrite treatment) were serially diluted. Examined samples of rat serum were diluted 1:20 in the same buffer and 50 µl added to each well. Then 50 µl of 1:125 000 diluted ascites of monoclonal antibody NO-60-E3 (prepared in our laboratory) in the same buffer were added and mixture was incubated under gentle shaking at laboratory temperature for 60 min. After three washings with PBS the plates were incubated with 100 µl of antimouse Ig rabbit antibody conjugated with peroxidase (Sigma A-8924) diluted 1:1000 in 1 % BSA in PBS for 90 min. After five washings with TPBS (with duration of washings 15 min), the plates were developed with o-phenylenediamine and reaction was terminated by addition of sulphuric acid. Absorbance was read at 492 nm using a microplate reader.

All extraction and centrifugation steps were performed at 4 °C. About 100 mg of lung tissue (wet weight) was homogenized in 2 ml TBS with protease inhibitors (benzamidine, PMSF, EDTA) and centrifuged (48 000 g, 10 min). Supernatants were diluted 1:10 and concentration of 3-nitrotyrosine estimated by the same competitive ELISA. Concentration of proteins was measured by the bicinchoninic acid method (Smith *et al.* 1985).

Standard curves and concentrations of 3-nitrotyrosine in the samples were calculated according to Rodbard's four parameter equation (Rodbard and McClean 1977).

Data analysis and statistics

Each ventilatory variable was averaged over six consecutive respiratory cycles. Results are presented as means ± S.E.M. ANOVA and Fisher´s PLSD test were used for statistical evaluation of the data. $P<0.05$ was considered significant.

Results

Ventilation

Ventilatory parameters of all groups are summarized in Table 1. Minute ventilation (V'_E) of both groups of rats exposed to hyperoxia was somewhat lower than that of the controls, the decrease being significant only for *Car+O₂* group. The rats of *Car+A* group reached the same V'_E as controls, but had a higher rate of breathing and lower tidal volume.

Table 1. Minute ventilation (V'_E), breathing frequency (f_R) and tidal volume (V_T) in control group, group exposed to 7 days of hyperoxia (O_2), group breathing air after carrageenan application (Car+A) and group exposed to 7 days of hyperoxia after carrageenan application (Car+O_2).

	control	O_2	Car + A	Car + O_2
n	8	7	8	6
Body weight (g)	293±5	256±5 *	268±8 *	240±11 *
V'_E (ml/min)	208.2±14.3	172.9±8.7	200.5±13.4	163.9±15.4 *
f_R (c/min)	145±8	119±7	175±12 $^+$	131±15
V_T (ml)	1.4±0.1	1.5±0.1	1.2±0.1 *†	1.3±0.1 †

Data are means S.E.M., *$p<0.05$ from the control group, $^+p<0.05$ from all other groups, $^\dagger p<0.05$ from the O_2 group

Serum nitrites and nitrates

The concentrations of NO_x^- in the serum are shown in Figure 1. The application of carrageenan or the exposure to hyperoxia increased NO_x^- concentration, which remained unaffected by the combination of both interventions.

3-nitrotyrosine in the serum

The application of carrageenan did not change the 3NTYR concentration in serum (Fig. 2), but its concentration decreased in both groups exposed to 7 days of hyperoxia. The differences between controls and *O_2 group* and between *Car+A* and *Car+O_2* groups were significant.

3-nitrotyrosine in the lung tissue

As expected, lung tissue concentration of 3NTYR was increased in carrageenan-treated rats breathing air (Fig. 3). The concentrations of 3NTYR in the control rats exposed to hyperoxia alone and rats exposed to carrageenan and hyperoxia did not differ. This means that exposure to hyperoxia blocked the carraggenan-induced increase, while it did not affect the 3NTYR concentration in normal lungs.

There was no correlation between concentration of 3NTYR in lung tissue and in serum.

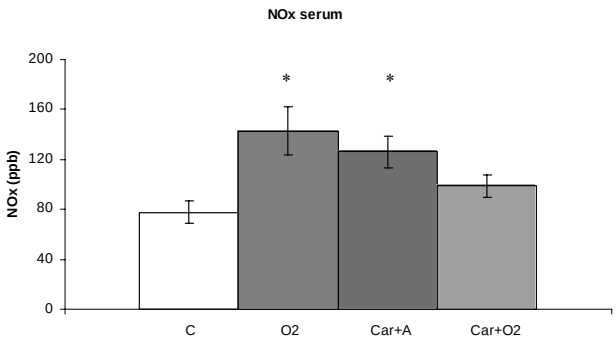

Fig. 1. Concentration of nitrites and nitrates (NOx) in serum of control rats (C), rats exposed to 7 days of hyperoxia (O_2), rats breathing air for 7 days after carrageenan application (Car+A) and rats exposed for 7 days to hyperoxia after carrageenan application (Car+O_2). *$p<0.05$ from the control (C) group

Weight of the left lung

The application of carrageenan increased both wet and dry weight of the left lung (*C group* 0.41±0.02; 0.09±0.01 g, *O_2 group* 0.42±0.01; 0.09±0.01 g, *Car+A group* 0.76±0.04; 0.16±0.01 g, *Car+O_2 group* 0.68±0.08;

0.15±0.02 g). There were no differences in dry to wet weight ratios of our groups.

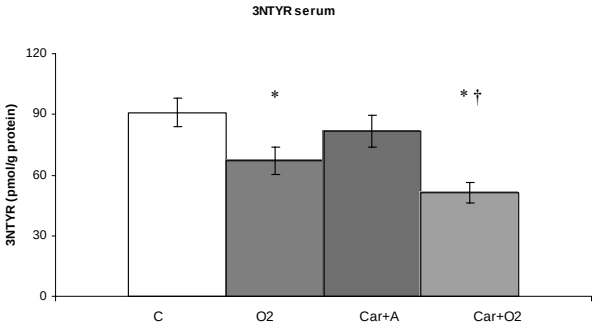

Fig. 2. Concentration of 3-nitrotyrosine (3NTYR) in serum of control rats (C), rats exposed to 7 days of hyperoxia (O_2), rats breathing air for 7 days after carrageenan application (Car+A) and rats exposed for 7 days to hyperoxia after carrageenan application (Car+O_2). *$p<0.05$ from the control (*C*) group, $^{\dagger}p<0.05$ between values in air and hyperoxia of the carrageenan treated rats.

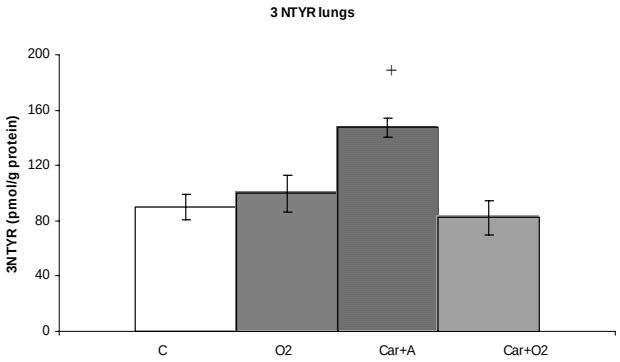

Fig. 3. Concentration of 3-nitrotyrosine (3NTYR) in the lung tissue of control rats (C), rats exposed to 7 days of hyperoxia (O_2), rats breathing air for 7 days after carrageenan application (Car+A) and rats exposed to hyperoxia for 7 days after carrageenan application (Car+O_2). $^{+}p<0.05$ from all other groups

Discussion

This study was designed to test: 1) whether breathing of hyperoxic gas mixture affects 3NTYR concentrations in lung tissue and serum of rats with experimental pneumonia, and 2) whether concentration of 3NTYR in serum reflects changes in protein nitration in the lungs. High oxygen concentration surprisingly decreased 3NTYR levels in serum of control as well as carrageenan-treated rats and blocked the increase of 3NTYR concentration in lung tissue found in carrageenan-treated rats breathing air. The 3NTYR concentration in the serum and in the lung tissue did not correlate.

An increased production of nitrogen and oxygen related reactive species in carrageenan-induced inflammation have been described in previous studies (Oh-ishi *et al.* 1989, Salvemini *et al.* 1996, Cuzzocrea *et al.* 1997). The higher concentrations of nitrates and nitrites (NO_x) in serum as well as 3NTYR in the lung tissue after intratracheal application of carrageenan were therefore expected in our rats. In addition, the pattern of breathing of carrageenan-treated rats during air breathing was similar to that described by Wachtlová *et al.* (1975).

The fact that hyperoxia increased NO_x^- concentration but decreased 3NTYR concentration in the serum suggests that hyperoxia might have enhanced oxidation of NO to NO_2 and NO_3. If so, the NO produced by endothelial cells was oxidized to NO_x^- which restricted formation of peroxynitrite and 3NTYR.

In general, hyperoxia is believed to increase formation of ROS, however, this effect depends on its level and also on the duration of the exposure. In rats, Crapo *et al.* (1980) found marked injury after exposure to 100 % oxygen, but lesser changes at 85 % O_2. The pronounced signs of lung injury (and ROS production) were reported after 48-72 h of exposure to hyperoxia and were concomitant with the infiltration and activation of phagocytes (Narasaraju *et al.* 2003). We tested the changes after 7 days of hyperoxia when its effects probably abated.

Data about the effects of hyperoxia on NO production in the lungs are controversial. Schmetterer *et al.* (1997) found an increase in exhaled NO levels in human and Arkovitz *et al.* (1997) found an increase in NO_x^- concentration in the bronchoalveolar lavage fluid during hyperoxia, while Cucchiaro *et al.* (1999) showed that hyperoxia induced iNOS expression in the rat lung, but did not affect NO concentration in the exhaled air and 3NTYR concentration in lung tissue.

It is difficult to explain the effects of the combination of carrageenan administration and hyperoxia, in particular the fact that 3NTYR concentrations decreased. Formation of 3NTYR was originally proposed as a relatively specific marker of peroxynitrite formation (Beckman 1996). However, other reactions, e.g. a reaction of nitrite with hypochlorous acid (Eiserich *et al.* 1998) and reaction of hydrogen peroxide with NO_2^- catalyzed by myeloperoxidase (Van der Vliet *et al.* 1997, Narasaraju *et al.* 2003), could also be involved. The increased concentration of 3NTYR in the lung tissue of our carrageenan-treated rats probably resulted from the activation of all these pathways.

Because hyperoxia is known to enhance reactions mediated by free radicals in the rat lung (Freeman and Crapo 1981), the expected result of the combination of inflammation with hyperoxia would be an increase in 3NTYR formation. However, hyperoxia surprisingly attenuated 3NTYR concentration in the lung tissue and serum of carrageenan-treated rats. Such decrease in 3NTYR concentration could be a result either from reduced nitration of proteins or from faster breakdown of 3NTYR or 3NTYR containing proteins. Although we cannot exclude an effect of hyperoxia on 3NTYR formation, it is difficult to envisage a metabolic pathway activated by hyperoxia, which would turn NO to substance(s) other than NO_x^- or 3NTYR. Therefore, an increase in breakdown of 3NTYR seems to be more likely. In our experiments, the amount of 3NTYR was calculated per gram of protein. This indicates denitrification of tyrosine or an increase in degradation of 3NTYR containing proteins, rather than the increased breakdown of all proteins. There are some data about enzymatic denitrification of 3NTYR-containing proteins (Gow *et al.* 1996, Kamisaki *et al.* 1997), but it is not known whether this process can be enhanced by hyperoxia.

3NTYR concentration in the serum did not correlate with that in the lung tissue, which suggests that changes in 3NTYR production localized to the lungs were too small to modify the 3NTYR concentration in the serum.

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