Effect of increased pressure on tracheal ciliary beat frequency

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Effect of increased pressure on tracheal ciliary beat frequency. J.H. Calvet, F. Verra, J.Maleine, M.C. Millepied, A. Harf, E. Escudier. ©ERS Journals Ltd 1999.
ABSTRACT: Effects of increased ambient pressure on mucociliary clearance have been poorly investigated.

The effects of increasing pressures on ciliary beat frequency (CBF) of guinea-pig tracheal rings were studied *in vitro*.

Increased pressures of 25 and 100 kPa induced a significant and equivalent enhancement of CBF from 30 min after the pressure increase. The increase in CBF observed after a pressure increase of 50 kPa (inspiratory oxygen fraction = 21%), was significantly greater than that observed with an equivalent oxygen tension at atmospheric pressure, *i.e.* with a gas mixture containing 30% oxygen. Addition of N^G-nitro-L-arginine methylester (L-NAME) inhibited the enhancement in CBF observed after the 25 kPa pressure increase. Addition of L-arginine reversed the effect of L-NAME.

These results demonstrate that a pressure increase applied to tracheal rings, *in vitro*, induces an enhancement of ciliary beat frequency and that generation of nitric oxide may be involved in this ciliary stimulation.
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Mucociliary transport in the respiratory tract plays an important role in lung defence mechanisms. The two main components of the mucociliary apparatus, cilia and mucus, are exposed to environmental or toxic factors which can impair their structure and functions. The effects of various physical factors such as temperature, relative humidity and pH on mucociliary clearance, have been previously studied [1–3]. The respiratory epithelium covering upper and lower airways can also be submitted to pressure variations. These variations can be modest, such as in cough or assisted ventilation, or major, as in diving. The effects of increased pressure on tracheal mucus clearance have received little attention in the literature [4]. Pressure variations can act either on mucus properties and/or on ciliary motility. In order to test the direct effects of increased pressure on ciliary beat frequency (CBF), a series of *in vitro* experiments on guinea-pig tracheal rings were conducted. The data demonstrated that increased pressure caused marked enhancement in tracheal CBF. As the generation of nitric oxide by airways has been shown to be involved in the modulation of CBF [5, 6], it was hypothesized that increased pressure could act on CBF *via* NO release. The effect of N^G-nitro-L-arginine methylester (L-NAME), an inhibitor of NO synthase (NOS), on CBF enhancement induced by a pressure increase was therefore studied.

Methods

Ciliary beat frequency measurement

Ciliary beat frequencies were measured on guinea-pig tracheal rings immersed in Tyrode's solution (composition in mM: 139.2 NaCl; 2.7 KCl; 1.8 CaCl$_2$; 0.49 MgCl$_2$·6H$_2$O; 0.4 NaH$_2$PO$_4$·H$_2$O; 5.5 glucose; 11.9 NaHCO$_3$, pH 7.4). The CBF measurement system included a video camera (Sony, Paris, France) connected to the triocular head of an inverted phase contrast microscope (Nachet, Dijon, France) using a ×40 objective enabling the image to be stored on videotape (Panasonic, Paris, France). Videotape sequences were analysed sequence by sequence, using automated images, according to the technique initially described by ZAHM *et al.* [7]. Briefly, the video output of the processor board was directed to a black and white monitor. The image was digitized by a digital signal processor board with 8-bit greyscale and 512 × 512 pixel size. The software developed was driven by a PC computer. The region of interest was outlined by a graphical square with a size of 66 × 66 pixels positioned over a ciliated cell on the digitized image using the computer mouse. The sample area was 150 μm^2 and corresponded to a single ciliated cell area, including ~200 cilia. A fast Fourier transform (FFT) algorithm was used to analyse the brightness variations according to the method of COOLEY and TUKEY [8]. From FFT analysis, the spectrum distribution of CBF was displayed on the computer monitor. For each spectrum, the mean value and standard deviation of the frequency was calculated and stored on the computer hard disk. Each measurement represents the mean value calculated from 15 different regions, randomly selected by pointing with the computer mouse.

Pressure increase

The pressure chamber was designed and built in the authors' institution. This chamber consisted of a solid metal block machined in a 70-mm stainless steel circle. Its

internal diameter was 28 mm and its height was 10 mm. The lower and upper round glass cover slips were made from microscope slides (1 mm thick) for microscopic examination. Tests were performed to ensure that the pressure chamber was sufficiently robust to support a pressure of 200 kPa. Pressure was induced by a pressure regulator (Brooks, 8601D, New York, NY, USA) and was controlled with a pressure manometer (Bourdon, Paris, France).

A gas mixture with a high oxygen concentration was generated with an air/oxygen mixture (A01; Bennett, Los Angeles, CA, USA). Gas composition, especially oxygen pressure in the chamber, was determined from samples analysed with a blood gas analyser (ABL 300; Radiometer SA, Caen, France).

Experimental protocol

The experimental procedure and specific protocol were approved by the Committee on Animal Care of the authors' Institution. Male Hartley outbred guinea-pigs (Charles River, Saint Aubin Les Elbeuf, France) weighing 250–300 g, housed at 22°C with food (UAR, Villemoisson, France) and water freely available, were used in this study. Guinea-pigs were anaesthetized with 50 mg·kg body weight^{-1} *i.p.* of pentobarbital sodium and exsanguinated by severing the abdominal aorta. The thorax was opened and the trachea was immediately removed and sectioned in rings which were immersed in Tyrode's solution aerated with 95% O_2 and 5% CO_2 for 30 min. Each experiment was performed on a group of six animals. Before all experiments, it was ensured that the CBF of a tracheal ring, immersed in the pressure chamber closed and maintained at atmospheric pressure, was not modified for 4 h. As variations of pH and temperature are also known to modify CBF, these two parameters were checked at the beginning and at the end of each experiment.

The influence of the pressure on CBF was determined by studying the course of the CBF of tracheal rings when the pressure was set at 25 and 100 kPa for 150 min. Reversibility was studied on the same tracheal rings after return to atmospheric pressure, either in the same solution or after washing in new Tyrode's solution. When pressure increases with a constant gas composition, the partial pressure of the various gases, especially oxygen, also increases. In another series of experiments, the respective effects of increasing pressure were therefore compared with the effects of increasing oxygen concentration, *i.e.* air at a pressure of 50 kPa with a mixture containing 31% oxygen at atmospheric pressure (n=6).

Lastly, to demonstrate the pathway involved in CBF stimulation, the effects of L-NAME (10^{-4} M), an inhibitor of NOS, on CBF enhancement observed on a tracheal ring submitted to a pressure increase of 25 kPa for 30 min were studied. The effects of L-arginine (10^{-4} M), a precursor of NO, were also studied on the same preparation. L-NAME or its solvent were added once the CBF stimulation was observed, by opening the chamber and so their effects were evaluated on preparations, just after return to atmospheric pressure. L-arginine or its solvent were added 15 min after the addition of L-NAME.

Reagents

L-NAME was obtained from Sigma (Saint Quentin Fallavier, France) and L-arginine was obtained from Bioblock (Illkirch, France). These compounds were dissolved in Tyrode's solution.

Statistical analysis

Data are expressed as mean±SEM. Results of the various experiments were compared using one way analysis of variance followed by a *post hoc* Bonferroni-Dunn test. A p-value <0.05 was considered to be statistically significant.

Results

Influence of ambient pressure on ciliary beat frequency

In order to evaluate the effects of pressure and the time course of this effect, two different levels of pressure were studied during a 150-min period. The effects observed at pressures of 25 and 100 kPa were not significantly different (fig. 1). For each pressure value, an increase in CBF, compared to control values, was demonstrated 30 min after the onset of the pressure increase (from 7.66±0.21 Hz to 8.32±0.27 Hz for 25 kPa and from 7.49±0.15 Hz to 8.94±0.26 Hz for 100 kPa, p<0.05). A plateau was reached after 90 min (10.58±0.35 Hz for 25 kPa and 10.98±0.26 Hz for 100 kPa) and was maintained for 150 min. CBF measured after 150 min at 25 kPa was not modified after return to atmospheric pressure and persisted for 60 min, whether the tracheal ring remained in the same Tyrode's solution (10.51±0.39 Hz after 150 min at 25 kPa and 10.57±0.48 Hz, 60 min later) or was placed in a new solution of the same composition (10.72±0.47 Hz after 150 min at 25 kPa and 10.35±0.22 Hz, 60 min later) (fig. 2).
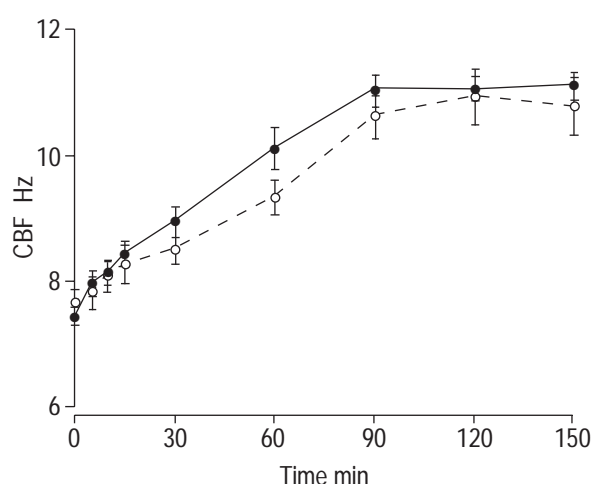

Fig. 1. – Comparative effect of a pressure increase of 25 kPa (- - - -) and 100 kPa (——) on ciliary beat frequency (CBF). Values are mean±SEM. After 30 min, the increase in CBF was significant for the two pressure levels compared to control values (p<0.05, Bonferroni-Dunn test, n=6).
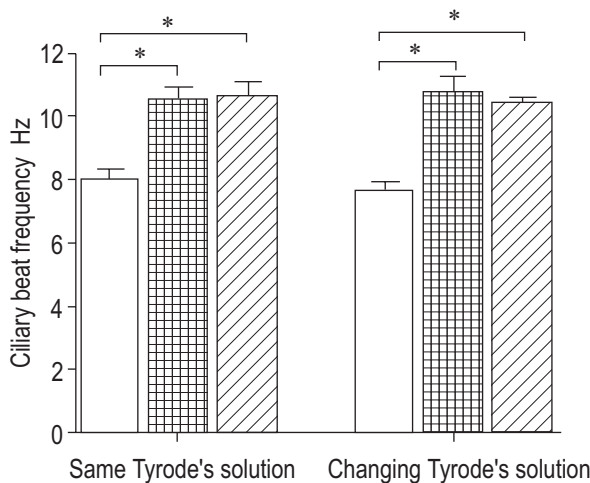
Fig. 2. – The course of a 25 kPa pressure increase. Values are mean± SEM. The tracheal ring was either maintained in the same Tyrode's solution when returning atmospheric pressure or the solution was changed after 150 min, *i.e.* when returning at atmospheric pressure. No reversibility was observed in either situation. □: control; ▦: 25 kPa for 150 min; ▨: 25 kPa for 150 min followed by 0 kPa for 60 min. *: p<0.05, Bonferroni-Dunn test, n=6.

Respective effects of pressure and oxygen tension

The increase in CBF of tracheal rings submitted to a pressure of 50 kPa for 60 min was significantly greater than the increase in CBF observed in tracheal rings of the same trachea placed in a gas mixture containing 31% O_2 at atmospheric pressure (from 8.53±0.46 to 10.40±0.63 Hz and from 8.85±0.51 to 11.89±0.60 Hz, respectively, p<0.05).

Effect of nitric oxide synthase inhibitor

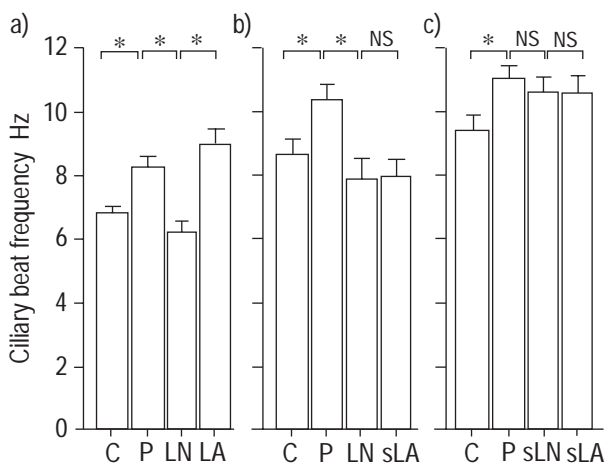The CBF of tracheal rings exposed to a pressure of 25 kPa increased from 6.76±0.24 Hz to 8.16±0.49 Hz (p<

Fig. 3. – The effect of nitric oxide synthase inhibitor. Values are mean± SEM. a) N^G-nitro-L-arginine methylester (LN) inhibits ciliary beat frequency (CBF) enhancement observed after a pressure increase of 25 kPa for 30 min and L-arginine (LA) inhibits the effects of LN; b) the solvent of LA (sLA) did not inhibit the effects of LN; and c) the solvent of LN (sLN) and sLA had no effect on the CBF enhancement observed after a pressure increase of 25 kPa. C: control; P: 25 kPa for 30min. *: p<0.05, Bonferroni-Dunn test, n=6.

0.05). Addition of L-NAME induced a decrease in CBF to 6.19± 0.35 Hz within 5 min (p<0.05) (fig. 3). Addition of L-arginine to these same tracheal rings induced an increase in CBF to 8.9±0.52 Hz, within 5 min (p<0.05). For these two products, solvents alone did not induce any significant variation of CBF. Addition of L-NAME or L-arginine alone did not induce any significant variations of basal CBF (data not shown).

Discussion

These results demonstrate that a pressure increase applied to guinea-pig tracheal rings *in vitro* induces enhancement of CBF and that NO generation may be involved in this ciliary stimulation.

Airways can be exposed to environmental physico-chemical factors. Variations in these factors can modify airway mucociliary function [3]. The influence of some of these factors such as temperature, humidity and pH on mucus transport has been previously studied [1–3]. As mucociliary clearance depends on interactions between ciliary activity and mucus properties, effects of modifications of these physical factors have been separately studied on each of these two components. The effects of changing temperature, pH and humidity on CBF have consequently been studied *in vivo* and *in vitro* [9, 10]. For these parameters, a good correlation was found between the effects observed on mucociliary clearance and the effects observed on CBF [4]. To the authors' knowledge, the direct effect of increasing pressure on CBF has not been studied.

The present results demonstrate that an increase in pressure can enhance CBF. When pressure exceeds 25 kPa, a significant enhancement of CBF is observed within 30 min, a plateau is reached 90 min after the onset of the pressure increase and CBF remains relatively constant for at least 150 min. Enhancement of CBF is not reversible after returning to atmospheric pressure whether the tracheal ring remains in the same solution or is placed in a new physiological saline solution. This result suggests an intrinsic mechanism.

In the experiments performed with room air, when pressure increased, with constant inspiratory oxygen fraction (F_{I,O_2}), oxygen tension (P_{O_2}=pressure $\times F_{I,O_2}$) also increased. Several studies have dealt with the effect of hyperoxia on CBF [4]. As HARRISON *et al.* [11] demonstrated that short-term local hyperoxia could stimulate CBF, the respective effects on CBF of an increase in pressure and an increase in P_{O_2} were determined. In this way, the effect observed with air (~21% O_2) at a pressure of 50 kPa was compared to the effect observed with a mixture containing 31% O_2 at atmospheric pressure, *i.e.* two conditions with the same equivalent P_{O_2} (~33 kPa). The enhancement of CBF observed with increased pressure was significantly greater than that observed with hyperoxia alone. This result indicates that an important part of the enhancement of CBF observed in response to a pressure increase is owing to the increase in P_{O_2} associated with this pressure increase and another part is directly due to the pressure *per se*. This result is in accordance with the classical observation that ciliated cells are mechanosensitive [12]. When the cell surfaces or cilia of cultured ciliated epithelial cells from rabbit

trachea are stimulated with a small glass microneedle, CBF increases [13]. However, in this case, in contrast to the response observed in the present study, SANDERSON and DIRKSEN [13] demonstrated that the CBF increases within milliseconds to seconds of the stimulus and this response is calcium dependent.

Many mechanisms are involved in CBF modulation. Among the mediators that are known to upregulate CBF, NO, a multipurpose messenger molecule largely present in the airways [14, 15], has been shown to enhance CBF in epithelial cells in culture, in response to isoproterenol or salbutamol [5, 6]. Based on the time-course of the increase in CBF observed in the present study, occurring within 30 min and lasting for longer than 60 min, this effect resembles that observed with isoproterenol. It was therefore hypothesized that NO could also be involved in the effect of high ambient pressure on CBF. These results support this hypothesis. This conclusion is based on the following observations: 1) the NOS inhibitor, L-NAME, rapidly slowed CBF stimulation induced by the pressure increase; 2) this effect was reversed by L-arginine, a precursor of NO; 3) solvents of these products did not have any effect *per se*; and 4) L-NAME had no significant effect on the basal CBF. The exact pathways of transduction involved in the NO-induced increase in ciliary motility have not been completely elucidated. Epithelial cells contain large amounts of constitutive NOS (cNOS), as demonstrated on histological sections by immunostaining in previous studies [14]. However, they are capable of expressing inducible NOS (iNOS). Based on the time-course of the effect, it is more probable that the pressure increase may activate iNOS.

An increased pressure along the airways can be observed in cough, assisted ventilation or diving. Pressures generated in cough and assisted ventilation are ~100 cmH_2O, *i.e.* 10 kPa, for brief periods. Only a part of these pressures is dissipated in shearing forces at the wall, *i.e.* epithelial cells. In cough or assisted ventilation, the direct effects of pressure on epithelial cell functions are probably limited. In diving, a significant hydrostatic pressure is inseparable from the aquatic environment in which the ambient pressure increases by ~10 kPa for a depth of 1 m. Pressures observed between 1 and 10 m in depth which represent the depths of most diving expeditions, are between 10 and 100 kPa. These pressures are in the range of the pressures applied in this study and their time of application can be as long as in this experiment. These results suggest that pressures applied on epithelial cells during diving may influence ciliary beat.

This *in vitro* study demonstrated that an increased pressure may improve ciliary beat, possibly *via* a mechanism involving nitric oxide. Other studies are needed to evaluate the effect of pressure on mucus properties, the other major component of mucociliary clearance.

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