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In Vivo Documentation of Stimulus Velocity Tuning of Mechanically Induced Reflex Cough

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Summary

In order to clear airways and lungs defensive reflexes are provoked rather by the dynamic phase of mechanical stimulus. It is speculated that provocation of defensive response depends not only on stimulus duration but also on stimulus velocity. Fourteen adult rabbits were anaesthetized and tracheotomized. Mechanical stimulus was provoked by a mechanical probe introduced through the tracheotomy and rotated by a small electrical motor using a rotational velocity of 40 rpm/s and 20 rpm/s. Threshold, incidence and intensity of cough reflex (CR) were analyzed for each animal. Statistical comparisons between two velocities were performed using Friedman nonparametric test for repeated measurements. Results are median (25-75 %). The threshold of CR was significantly increased ($p=0.005$) from 350 ms (300-500 ms) to 550 ms (350-1150 ms) and the incidence of cough reflex was significantly reduced ($p=0.002$) from 50 % (19-50 %) to 0 % (0-25 %) when the rotational velocity of the mechanical probe was reduced by half. The findings of this study are of interest as they show that protective reflex cough, an important mechanism that allows clearing airways even during sleep or anesthesia, is tuned by mechanical stimulus velocity.

Key words

Cough reflex • Mechanical stimulus • Stimulus velocity • Dynamic phase

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Introduction

Cough can take multiple forms. It serves as a vital reflex mechanism in a case of aspiration or during chronic respiratory disorders when clearing airways from foreign bodies, particulate matter or extensively produced mucus. It is an important symptom of respiratory disorders associated with inflammation processes. But cough can become a disease itself, especially when chronic, isolated and non-productive and occurring without any apparent cause. Recent studies focusing on peripheral terminals of the vagal afferent nerves subserving cough (Mazzone and Undem 2009) and on the central organization of cough neural pathways (Canning *et al.* 2014) reveal the complexity of cough regulation. Cough can be produced reflexively or behaviorally. Reflex cough is dependent only upon brainstem processing of incoming afferent input and requires no involvement of higher brain centers. It can be therefore provoked in anaesthetized humans and anaesthetized or decerebrated animals.

Among all vagal afferent neurons innervating the respiratory system, several studies (Canning *et al.* 2014, Mazzone and Undem 2016) identified two specific subtypes of vagal afferent neurons that are responsible for initiating cough, the low-threshold mechanosensitive A-δ fibers originating from nodose ganglia and chemically sensitive bronchopulmonary C-fibers, whose soma is situated in jugular ganglia. Among them, the low-threshold mechanosensitive A-δ fibers that terminate in the extrapulmonary airways (larynx, trachea and large

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bronchi) and whose soma is situated in nodose ganglia were recently named “cough receptors”. They are exquisitely sensitive to light touch and rapid change in pH and are thought to be responsible for mediating cough induced by mechanical stimuli and acid (Kollarik and Undem 2002). They lack the capacity to discriminate noxious mechanical stimuli and do not directly respond to chemical stimuli including inflammatory autacoids (Kollarik *et al.* 2007) so as they are indifferent to the physiological stimuli in the airways (airflow, changes in tracheal geometry during breathing) but are readily activated by the noxious stimuli associated with aspiration: the localized punctiform mechanical stimuli and acid (Kollarik and Undem 2002). Owing to the fact that stimulation of nodose mechanosensitive A-δ fibers leads to coughing even in anaesthetized animals and humans it seems likely that these fibers are responsible for reflex cough (Canning *et al.* 2014, Mazzone and Undem, 2016, Sun *et al.* 2017). They adapt rapidly to punctuate mechanical stimulation and their terminals are found in stereotypical position within airway mucosa where they are arranged along the circumferential axis of the airways.

In order to trigger reflex cough as a response to aspiration of food, particles or gastric contents or when clearing of mucus from lower airways cough receptors seems to respond to dynamic rather than a static phase of mechanical stimulation. This notion is extremely important and demonstrated by experimental studies and clinical practice, when mechanical stimulus is capable to promote cough especially or even just when it’s in motion, i.e. during inhalation of foreign bodies, when moving the experimental probe in the trachea or when using techniques of nonpharmacological airway clearance in patients with altered mucus clearance (e.g. chest physiotherapy including postural drainage, chest wall percussion and vibration, forced expiration technique) (McAlexander *et al.* 1999, McCool and Rosen, 2006, Canning *et al.* 2014).

In a case of mechanically induced cough in anaesthetized animals, the defensive response depends strongly on duration of mechanical stimulus where increase of stimulus duration increases incidence of cough response (out of total number of stimulations) so as the number of coughs evoked by each stimulation (Canning 2007, Varechova *et al.* 2011).

Given the fact that reflex cough is rather produced by the dynamic phase of mechanical stimulus, it is speculated here that defensive response depends not only on the duration of mechanical stimulus but also on its velocity. Neurophysiologic study realized in vitro using guinea pig tissue preparation (trachea with intact nerves including sensory vagal ganglia) support this notion as the peak frequency of action potential discharge of nodose ganglion neurons was significantly reduced when the speed of the motor driving the mechanical probe was reduced by three quarters (McAlexander *et al.* 1999).

However, there is no *in vivo* study that highlighted such velocity tuning of cough reflex response to mechanical stimulation yet. In our experimental model, we used a validated and reproducible methodology of mechanical stimulation realized by rotating mechanical probe against the tracheal epithelium. The aim of the study was to compare the threshold of defensive response so as the incidence of defensive response elicited by mechanical stimulation of equal duration but 2 different rotational velocities. In order to evaluate the effect of stimulus velocity exclusively on reflex cough supposed to be mediated by A-δ fibers, C-fiber mediated cough was silenced using general anesthesia. Finally, ventilatory responses to mechanical stimulation of trachea were identified from airflow and EMG of abdominal muscles, enabling us to differentiate cough reflex from expiration reflex, another type of defensive reflex of the respiratory tract. Despite fundamental physiological and pharmacological differences between these two reflexes, their differentiation in the clinical setting is hardly possible.

Methods

Animals

Experiments were performed in 14 New Zealand adult rabbits (2725±478 g). Animal experiments were carried out in accordance with the French legislation in place at the time of the study. The study was conducted under the license (A54518-03409) from the French ministry of agriculture and fisheries and the Ministry of higher education and research and supervision by the regional veterinary services. Ethics committee approval was not required, in accordance with the French legislation in place at the time of the study.

Anesthesia, analgesia, and euthanasia

Induction of surgical anesthesia was performed by intravenous administration of pentobarbital (30 mg/kg) through the marginal ear vein. Preoperative local analgesia before a tracheotomy was performed by subcutaneous administration of Laocaine R 20 mg/ml (Intervet, Schering-Plough Animal Health, France). After induction, the depth of anesthesia and analgesia was assessed every 20 min by testing the pedal withdrawal reflex (footpad pinch on both hind feet) and respiratory rate. In the case of the animal responsiveness to painful stimuli and/or increase in respiratory rate, the animal was supplied with additional anesthesia and the evaluation of the depth of anesthesia was retested before continuing experimental procedures.

Euthanasia was achieved with intravenous administration of Doléthal R (Vétoquinol S.A., France) in a dose of 200 mg/kg.

Animal preparation

The anaesthetized animal was placed in the supine position on a heating pad to maintain body temperature between 38 and 39 °C and left to rest for 20 min. An upper cervical tracheotomy allowed the insertion of a tracheal cannula that was connected to a No. 0 Fleisch pneumotachograph (linear range ± 250 ml/s) and to the mechanical stimulation apparatus. The electromyogram of the transverse abdominal muscle was performed by insertion of bipolar insulated fine-wire electrodes according to Basmajian and Stecko (1963) to further characterize the active expiration of cough and expiration reflex.

Mechanical stimulation of trachea

The mechanical stimulation of the trachea (at the level of the carina) was performed when breathing was regular. It was realized using a semi-rigid wire (nylon fishing line) rotated by a23HSX-102 universal stepper motor, an electromagnetic device that converts digital pulses into mechanical shaft rotation (Rare Earth Magnet Stepper Motors ref. 23HSX-102, McLennan Servo Supplies Ltd., Ash Vale, UK) piloted via microcontroller that achieved a square wave stimulus automatically triggered 50 ms from the beginning of inspiration. The beginning of inspiration was detected electronically as soon as the flow signal reached a positive value. The stimulation time could be varied in a stepwise manner between 100 and 2400 ms. The initial stimulus was set to 600 ms, a duration that has been shown to readily elicit a response in adult rabbits (Varechova *et al.* 2011). Each animal underwent 8 stimulations with initial 600 ms stimulus, 4 stimulations using the rotational velocity of 20 rev/sec and 4 stimulations using the rotational velocity of 40 rev/sec in a pseudo-random manner. In order to asses a threshold of defensive responses for each rotational velocity the stimulation duration was altered stepwise-down to 100 ms or up to 2400 ms (according to the protocol detailed in Coutier-Marie *et al.* 2017). Each stimulation step using stimulation duration other than 600 ms was tested 4 times for each rotational velocity. At least 1 min was allowed to elapse between two mechanical stimulations. The signals of airflow, integrated volume and stimulus were fed to the LabChart recorder (ADinstruments, Oxford, UK), digitized at 1000 Hz and used for on-line and off-line analysis.

Ventilatory responses to tracheal stimulation

- *Types of defensive responses*

The defensive responses triggered by mechanical stimulation of the trachea were identified from the change in tidal volume (VT), peak expiratory flow (V'Epeak) and rectus abdominis EMG of the respiratory cycle undergoing stimulation (stimulation breath) compared to the preceding respiratory cycles (reference breath) (Varechova *et al.* 2012). Cough reflex (CR) was defined as an increase of VT followed by an increased V'Epeak associated with a burst of activity in the rectus abdominis EMG (Fig. 1A). Expiration reflex (ER) was defined as a brief V'Epeak of variable amplitude associated with a burst of activity in the rectus abdominis EMG, without a prior increase in VT (Figure 1B). In order to take into account, the spontaneous between-breath variability, an unbiased differentiation of CR from ER was achieved by a statistical evaluation of VT between stimulation and reference breath. The tidal volume of reference breath was determined as the mean of 3 breaths prior to stimulation and its upper limit as mean + 3 standard deviations. The cough reflex was identified when VT of stimulation breath was higher than the upper limit of reference VT.

When a complex defensive response was elicited, consisting of a bout of several CR and/or ER, it was counted as CR response whenever such motor pattern was present in the bout.

Data analysis

Statistical analysis was performed using SYSTAT 13 Package (San Jose, CA, USA).

Following quantitative parameters were analyzed for each animal:

a) The threshold of defensive responses (CR or ER), the threshold of CR, and threshold of ER-defined as the shortest stimulus necessary to evoke at least one defensive response, one CR or one ER, respectively,

b) Incidence of defensive responses (CR and ER), the incidence of CR, and incidence of ER-defined as a number of defensive responses (CR and ER), CR and ER expressed as a percentage of the total number of stimulations,

c) The intensity of maximal expiratory effort of CR was calculated from the maximum V'Epeak of stimulation breath and was expressed as a percentage of the reference breath.

When no response occurred using 2400 ms (longest) stimulation, the threshold was arbitrarily set to 2400 ms. Normal distribution of data within each age group was tested using the Kolmogorov-Smirnov test. Data are expressed as median (25th to 75th percentile) owing to non-normal distribution even after logarithmic transformation. Statistical comparisons of quantitative parameters between two rotational velocities were performed using Friedman nonparametric test for repeated measurements. Differences were considered significant at $p < 0.05$.

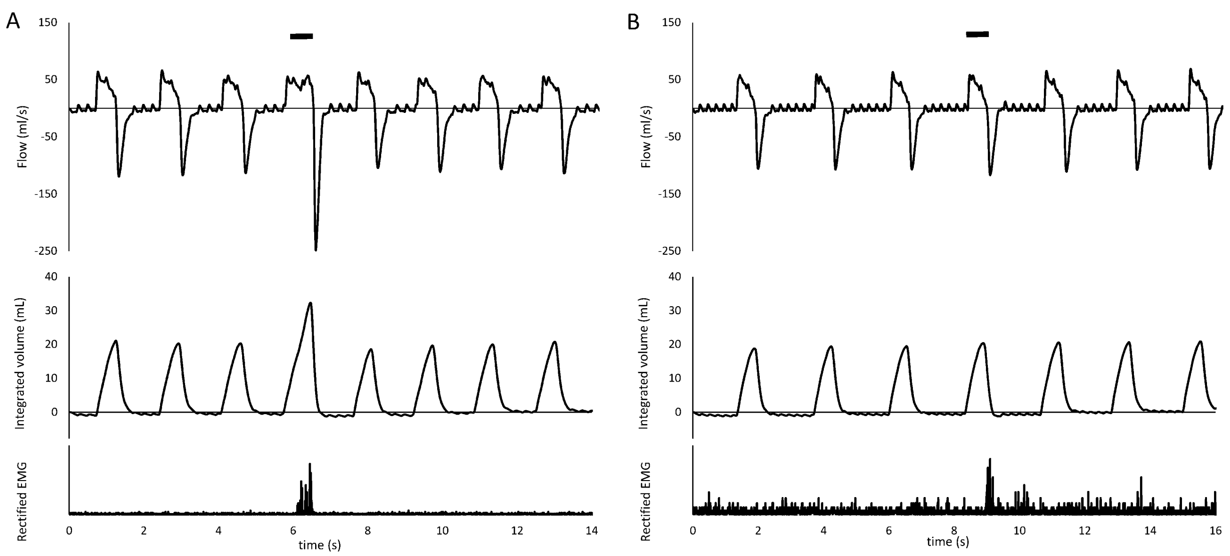

Fig. 1. Airflow, tidal volume and abdominal EMG recordings illustrating cough reflex (**A**) and expiration reflex (**B**) to mechanical stimulation of the trachea. The increased expiratory flow is preceded by an increased tidal volume in the cough - but not expiration reflex.

Results

The threshold of defensive responses

The threshold of overall defensive responses was significantly increased when rotational velocity of the mechanical probe was reduced by half (40 rev/s: 300 ms (200-500 ms) vs. 20 rev/s: 500 ms (300-600 ms), p=0.03). This was uniquely caused by the increase of CR threshold (40 rev/s: 350 ms (300-500 ms) vs. 20 rev/s: 550 ms (350-1150 ms), p=0.005). Threshold of ER was not significantly changed by reduction of rotational velocity (40 rev/s: 600 ms (200-1800 ms) vs. 20 rev/s: 600 ms (350-750 ms), p=0.7).

Incidence of defensive responses

The overall incidence of defensive responses was not found significantly different between two rotational velocities (40 rev/sec: 75 % (37.5-100 %) vs. 20 rev/sec: 50 % (12.5-75 %), p=0.3).

However, incidence of cough reflex responses was significantly reduced (p=0.005) from 25 % (12.5-75 %) to 12.5 % (0-25 %) when the rotational velocity of the mechanical probe was reduced by half. On the other hand, incidence of expiration reflex responses was not significantly changed with rotational velocity reduction (40 rev/s: 25.0 % (0.0-25.0 %) vs. 20 rev/s: 25.0 % (0.0-50.0 %), p=0.2 (Fig. 3).

Intensity of maximal expiratory effort of CR

The intensity of maximum expiratory effort of CR was not significantly different between two rotational velocities (40 rev/s: 190.1 % (113.7-291.7 %) vs. 20 rev/s: 160.4 % (136.1-210.6 %), p=0.7). The intensity of maximum expiratory effort of ER was not significantly different between two rotational velocities (40 rev/s: 102.3 % (96.5-112.9 %) vs. 20 rev/s: 102.3 % (97.4-107.7 %), p=0.8).

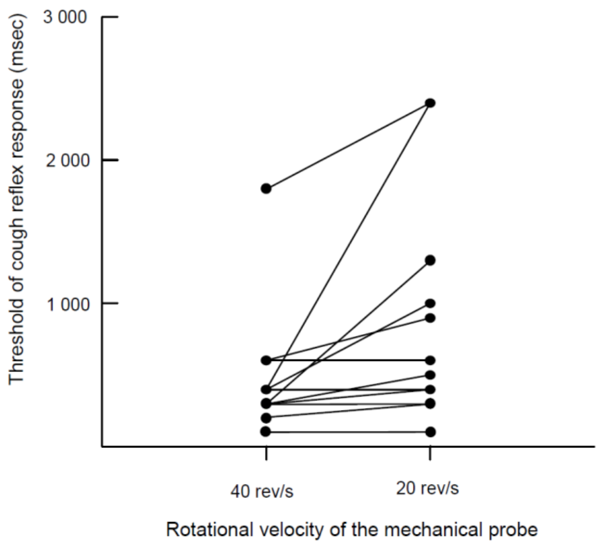

Fig. 2. Individual values of cough reflex threshold (the shortest stimulus duration capable to provoke at least one cough reflex) when using two rotational velocities of mechanical probe, 40 rev/s and 20 rev/s.

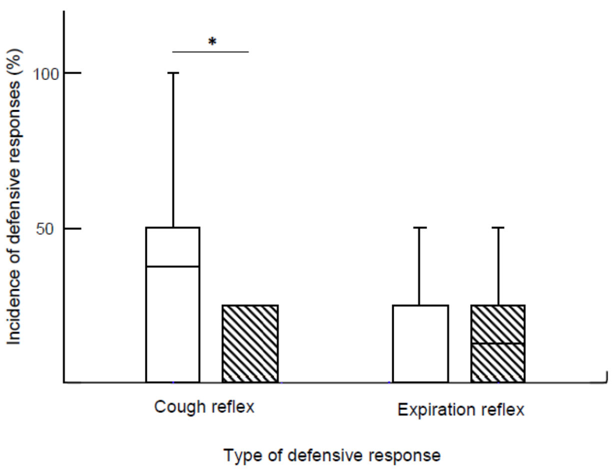

Fig. 3. Incidence of defensive responses (expressed as percentage of the total number of stimulations) provoked by a 600 mec stimulus when using two rotational velocities of mechanical probe, □ 40 rev/s and ▧ 20 rev/s. *p<0.05.

Discussion

This study is the first one to investigate the effect of mechanical probe velocity on cough reflex response in an animal model *in vivo*. Threshold and incidence of mechanically induced cough reflex, but not the intensity, were significantly altered when the rotational velocity of the mechanical probe was reduced by half. Unlike mechanically induced cough, change in rotational velocity did not induce significant modulation of expiration reflex response.

Velocity tuning of vagal sensory nerves subserving reflex cough was little studied. There is only one study, performed in a guinea-pig model *in vitro*, that described velocity tuning of rapidly adapting nodose ganglion-derived fibers, but not jugular-derived ones, that conducted in the Aδ range (McAlexander *et al.* 1999). As cough reflex is supposed to be mediated by the same type of vagal sensory fibers the results of this study strongly supported our hypothesis of stimulus velocity tuning of the mechanically induced cough reflex.

According to recent studies, initiation of cough reflex depends on the specific encoding of primary afferent information (Canning 2007). Here, only high firing rates along cough receptor axons allow the reconfiguration of the brainstem respiratory pattern generator in such a way that normal respiratory rhythm is replaced by a cough motor pattern (Mazzone *et al.* 2011, Canning and Mori 2011). It was suggested that except the duration of mechanical stimulation, the intensity of afferent nerves activation (impulses/sec) and/or the number of their peripheral terminals activated may determine the threshold for cough initiation (Canning 2007). We suppose that in our study, the change in rotational velocity of the motor driving the mechanical probe directly influenced the number of activated afferent nerve terminals and thus influenced the firing rates along axons of Aδ-fibers. When the rotational motor was set in motion, the mechanical probe span in the trachea in a way that the tip of the probe provoked numerous punctuate stimulations of tracheal mucosa. Therefore, the number of punctuate stimulations during mechanical probe rotation depended not only on stimulus duration but also on the rotational velocity of the motor driving the mechanical probe. This was attested by results showing that when using identical stimulus duration (600 ms), the incidence of cough reflex response decreased significantly when stimulus velocity was reduced by half (Fig. 3). High frequency firing along axons of Aδ fibers required to initiate cough reflex could be reached either by sufficient mechanical stimulus duration or by the increase of stimulus velocity. The threshold of cough reflex (shortest stimulus duration required to produce at least one CR) significantly decreased when stimulus velocity was doubled (Fig. 2). This observation may be of importance as using non-calibrated repeated mechanical stimulation (e.g. manually moving mechanical probe up and down in the trachea) may provoke biased study results.

It has been shown that cough receptors express the alpha3-containing isozymes of Na^+/K^+ ATPase essential in sustaining high frequency firing along afferent axons that allow specific encoding of cough reflex (Mazzone *et al.* 2009). However, relatively little is known regarding mechanisms of transduction of mechanical stimulus so as the mechanisms by which the dynamic phase of mechanical stimulus provokes cough initiation. Using new techniques of intravital labelling with FM2-10 and visualization through water-immersion optics confirmed adherence of Aδ-fiber terminals to the subepithelial matrix, where sensing physical disturbances is signalled through the structure of the matrix (Mazzone *et al.* 2009). The mechanotransduction, therefore, seems to work like a spider web (Canning *et al.* 2006). Spider web is used as the signalling medium that propagates vibrations from potential predators, prey or mates to the spider and that enable spider to differentiate between them (Mortimer, 2019). In airways, impacting inhaled particulates, accumulated mucus or punctuate mechanical stimulation provoke specific vibrations or movements within the extracellular matrix that may become a primary stimulus for cough (Canning, 2006). Of note, recently discovered Piezo 2 channels responding to punctate stimuli in the somatosensory system are strong candidates for ion channels involved in mechanotransduction in the respiratory tract (Mazzone and Undem 2017, Wu *et al.* 2017). This channel seems to be activated by the changes in membrane tension in the absence of other cellular components but may also be transmitted by tethering the channel to the extracellular matrix or the cytoskeleton.

These observations are in agreement with the study of McAlexander and coworkers published 20 years ago (McAlexander *et al.* 1999) that focused to the mechanisms of adaptation of airway afferent neurons to mechanical stimulation and their results suggested that the dynamic phase of the mechanical stimulus provokes cough initiation rather through the afferent nerve terminal-tissue interaction.

Given the supposed importance of extracellular matrix or cytoskeleton in the transduction of punctuate mechanical stimulus, there is a need to perform further studies in order to determine the effect of airway wall modifications on stimulus velocity tuning of the cough reflex. It may be implied in the mechanisms by which cough reflex threshold is modulated by disease processes of the respiratory tract that are associated with substantial remodelling of the airway wall, like asthma or chronic obstructive pulmonary disease.

Conclusions

The findings of this study are of interest as they show that protective reflex cough, an important defense mechanism that allows clearing airways even during sleep or anesthesia, is tuned by mechanical stimulus velocity. The data suggest that the mechanically sensitive vagal afferent neurons initiating cough in rabbit share the same properties as those identified in guinea pigs myelinated fibers by *in vitro* preparation (McAlexander *et al.* 1999). Finally, our study describes a potentially useful and simple model to explore stimulus velocity tuning of the mechanically induced cough reflex and its role in health and disease.

Conflict of Interest

There is no conflict of interest.

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