Effect of Low Temperature on Tracheal Smooth Muscle Contractile and Relaxing Responses Evoked by Electrical Field Stimulation

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Objective. The aim of this work was to evaluate the effect of low temperature (LT) on the contractile and relaxing responses of rat tracheas (RTs) after electrical field stimulation (EFS).

Methods. Voltage-dependent (10-60V, 40Hz) and frequency-dependent (0.1-60 Hz, 40V) response curves were constructed at 37 and 18°C after the activation of tracheal intramural nerves with a Grass S88 stimulator. The EFS that produced half of the maximum contractile response (ES50) at 37 or 18°C was determined and considered as the dependent variable. The % relaxation of pre-contracted RTs (EFS; 5Hz, 40V) to sodium nitroprusside (SNP; 1x10^-3 – 1x10^-3 M) isoproterenol (ISP; 1x10^-9 – 1x10^-4 M) and to 20mM potassium chloride (KCl) after low-K⁺ inhibition of the Na⁺/K⁺ pump at 18 and 37°C were determined.

Results. We found that the tracheal responses elicited by EFS at 37 and 18°C were completely blocked with 1μM atropine. LT slightly increases the voltage-dependent ES50 from 33.7±4.0 to 37.8±4.8V, n=5 but decreases the frequency-dependent ES50 from 19.3±4.3 to 1.0±0.28 Hz, n=5, p<0.05. Relaxing responses to SNP, ISP and KCl at 37°C correspond to 43.5±6.1, 36.7±12 and 12.1±1.5 % respectively. No significant tracheal relaxations were elicited at 18°C. Our results indicate that in RTs, LT enhances tracheal sensitivity to EFS and decreases it in response to bronchorelaxants. The LT-dependent enhanced contractile response is observed only after a low frequency stimulation range (0.1-20Hz), that is very close to the frequency of vagal stimuli required for inducing bronchoconstriction in vivo. Furthermore, LT abolishes the sensitivity of RTs to exogenously added bronchorelaxants (NO and ISP). In addition, LT appears to decrease the Na⁺/K⁺ pump activity; this effect has been associated with increased tracheal hyperreactivity in vitro. ACH appears to be the main endogenous neurotransmitter involved in neurally mediated contractile responses at 37 and 18°C.

Conclusion. Low temperature enhances the contractile response of rat tracheas in response to endogenous ACH release. The effect of LT is limited to frequencies below 20Hz, which are within the physiological range required for bronchoconstriction. Furthermore, LT severely impairs the sensitivity of RTs to relaxant stimuli, either of endogenous or exogenous origin.

Key words: Cold-induced bronchoconstriction, Rat trachea, Airway cooling, Electrical field stimulation

The exposure of asthmatics to low temperature (LT) is linked to the onset and worsening of asthma (1). Increased pulmonary resistance characterizes the bronchoactive effect of LT, mostly due to narrowing of the central airways, up to a level that pulmonary function (FEV1) is compromised (2). The effect of LT on the airways has also been observed in vitro. For example, isolated tracheas of sheep, dogs, hamsters and rats develop an enhanced contractile response in conditions in which the temperature of the isolated organ is lowered from 37 to 18-20°C (3). Several hypotheses have been postulated to explain the enhanced contractile response induced by LT. Specifically, the bronchoactive effects of LT have been linked to the impairment of regulatory mechanisms present in the nerve terminals, epithelium, and smooth muscle of the airways (4-5).

In humans and rats, ACH release from parasympathetic nerve terminals has been recognized as the most important bronchoactive pathway in the airway (6). From a physiological standpoint, it is important to consider the possibility that LT could enhance the contractile response evoked by endogenous ACH release. Furthermore, the effect of LT upon the bronchorelaxant pathways of rat
tracheas (RTs) could be of importance in the bronchoactive effect of airway cooling. In view of the presence of the nitric oxide synthase in airway epithelial cells, it is possible that the airway epithelium may be a source for NO production and might be responsible for the regulation of airway smooth muscle (ASM) contractility. This idea is supported by the observation that the LT-dependent enhanced contractile response to exogenous ACh is potentiated in epithelium-denuded tracheas (7). In several species, including humans, NO release is also mediated by iNANC nerves in addition to the airway epithelium (8-10), therefore NO appears to play an important role in the regulation of ASM tone. It is possible that LT might inactivate some of the cascade of events linked to the activation of soluble guanylyl cyclase within the ASM (11-12).

Although the sympathetic innervation of upper airways is scarce, B2 agonists are an important tool for therapeutic bronchodilatation (13). The mechanism of action of B2 agonists results in the activation of Ca2+-activated K+ channels and myocyte hyperpolarization via cAMP second messenger synthesis. The tracheal response to B2 receptor activation in LT conditions could bring important information about the effect of LT on tracheal sensitivity to exogenous bronchodilators. In addition, membrane events required for and adequate contractile response appears to be affected by LT. Cooling of the airways has been linked to Na+/K+ pump inactivation, leading to ASM depolarization and enhancement of the contractile response (14). Therefore, the Na+/K+ pump seems to be a part of the response mechanism mediating the reaction of the airways to LT. Nonetheless, a study that integrates the inactivation of the Na+/K+ pump with other LT-activated mechanisms has not been carried out.

The above mentioned data indicates that NO, B2 receptor activation and the Na+/K+ pumps could be important elements in the response of the airway to LT. Therefore, this research work was designed to study the effect of LT on the bronchoactive and bronchodilator responses of RTs exposed to electrical field stimulation (EFS) and the response of EFS-stimulated RTs to bronchodilator and Na+/K+ pump inhibition. This study will contribute for a better understanding of the regulatory mechanisms that are impaired by LT at the cellular and organ level.

**Materials and Methods**

**Isolation of rat trachea (RT).** Male Sprague Dawley rats, (250-300g), supplied by Harlan Sprague Dawley, Inc., Indianapolis, Ind., were anesthetized with an intraperitoneal lethal dose of pentobarbital sodium (150 mg/kg). As soon as the animal fell into a deep anesthesia, an intact segment (8-10 mm) of the cervical trachea was surgically removed and cleaned of connective tissue. The tracheal segment was conserved intact and placed without further manipulation in a pre-oxygenated Krebs-Ringer Bicarbonate (KRB) solution with the following composition (mM): NaCl, 117.7; KCl, 4.7; CaCl2, 3.3; MgSO4, 2.4; NaHCO3, 24.8; KH2PO4, 1.2; and glucose, 10.6; pH = 7.4 and bubbled with a gas mixture of 95% O2 and 5% CO2. The pH of the KRB solution was maintained at 7.4.

**Isometric tension studies.** To determine changes in muscle tension, two tissue supporters made of stainless steel suspended the intact tracheal segment in a 25ml water-jacketed organ bath, supplied by Radnoti Glass, Monrovia, CA. Using SP205 Poly-viein surgical thread, the hook of one tissue supporter was secured to the tissue holder and the other hook was connected to a force displacement transducer attached to a DC preamplifier. The signal was fed into a personal computer, containing a data acquisition card to record the changes in isometric tension. According to the experimental protocol, the temperature of the KRB solution was maintained at 37°C or 18°C by changing the temperature of the external water-jacket. The intact trachea was set at an optimal length by equilibrating against a passive load of 2.0g at 37°C for 1 hour.

Electrical field stimulation and determination of ESO. Stimulation of tracheal intramural nerves was achieved by means of two custom-made platinum rings electrodes placed parallel to the trachea and connected to a Grass S88 stimulator, (Grass Medical Instruments, Quincy, Mass, USA.) Tracheas were stimulated sequentially with unipolar rectangular pulses of 0.5ms applied for 2 minutes. Frequency-response curves were generated, by setting the voltage to 40V, and changing the frequency between 0.1-60Hz. In additional experiments, voltage-response curves were generated, by setting the frequency to 40Hz, and changing the voltage between 10 to 70V. Pulse duration (5ms) was left unchanged in all the experiments. After electrical stimulation at 37°C, the tissue was washed, and stimulated again at 18°C until the concentration-response curve was completed. The electrical stimuli that produced half of the maximum contractile response (ES50) at 37 or 18°C was determined and considered as the dependent variable. In other set of experiments, tracheas were stimulated with a single electrical stimulus (5Hz, 40V) and the maximum change in contractile response (Emax) from baseline (g) was considered to be the dependent variable. Three minutes of resting time was allowed between each period of electrical stimulation to allow adequate replenishment of ACH stores. Changes in ESO were determined under multiple experimental conditions.

**Statistical analysis.** Statistical analysis was done with
the curve fitting program Graph Pad Prism, v2.0. Statistical significance of the differences between experimental and control groups was analyzed using paired and unpaired t-tests, as appropriate. P values below 0.05 were considered to represent significant changes in the magnitude of the contractile response.

**Results**

**Tracheal response to EFS and the development of voltage-response curves in low temperature conditions.**

Figure 1 represents a portion of a typical tracheal response to EFS. In these experiments, RTs were stimulated with EFS (12-60 V, 40 Hz) and the temperature was alternated between 37 or 18°C for each stimulus. At 37°C the tracheal contraction pattern is characterized by a sustained increase in tension, that remains steady until the EFS is turned off. Lowering the temperature to 18°C, followed by a similar EFS pulse, induces a contractile response that persists for a few seconds after the pulse is turned off. From this tracing it can be observed that the tracheal response at 18°C is decreased, as compared to the response observed at 37°C. The decreased contractile response at 18°C was significantly lesser along the entire range of applied voltages. For example, the contractile response evoked by EFS (36 V) corresponds to 0.46±0.07 at 37°C vs 0.16±0.05 at 18°C, p=.005, n=3. Figure 2 represents the changes in voltage-response curves evoked by LT. It is apparent that LT induced a rightward shift in tracheal sensitivity to EFS. Although the \( ES_{30} \) values (from 33.7±4.0 at 37°C vs 37.8±4.8 at 18°C, n=5) do not reach significant figures, it highly suggests that LT decreases the sensitivity of RTs in response to EFS stimuli. Furthermore, tracheal contractility was significantly decreased in the voltage range tested. The contractile response induced by EFS at 37 and 18°C was blocked with 1μM ATR, thus indicating that ACH is the principal bronchonconstrictor released by EFS.

**Tracheal response to EFS and the development of frequency-response curves in low temperature conditions.**

Due to rightward displacement of the voltage-response curve induced by LT, we decided to study the effect of LT in conditions in which the voltage is set to 40 V and the frequency of the stimuli ranges from 0.1 to 60 Hz. In these sets of experiments the contractile response at 37 and 18°C was determined after EFS with frequencies ranging from (0.1-60Hz, 40V). Figure 3 represents the effect of LT on the contractile response elicited by EFS, from 0.1 to 20Hz. In this experimental conditions LT induce a significant enhancement of the contractile response. For example, delivery of EFS (2Hz) at 37°C induces a response of 0.11±0.02 vs 0.48±0.08 at 18°C, p=.015, n=5. Indeed, figure 4 shows that LT induces a significant decrease in \( ES_{30} \) from 19.3±4.3 at 37 to 1.0±0.28 Hz at 18°C, p=.003, n=5. Interestingly, the enhanced contractile response begins to reverse when frequency reaches 20Hz. This latter finding is very similar to the results obtained in the voltage-response curves. As seen previously 1μM ATR blocked the tracheal response, thus confirming its cholinergic
response curve at 37 and 18°C. Figure 5a shows the leftward displacement in ES_{50} due to tracheal cooling. In this group of experiments, LT induces an 83% increase in tracheal sensitivity (from 5.2±0.6 Hz at 37°C to 9.1±0.43 Hz at 18°C, p<.03, n=4 ). Figure 5b reveals that the changes in ES_{50} were still present after pre-treatment with KTN (6.0±1.0 Hz at 37°C to 1.3±0.12 Hz at 18°C, p<.04). Therefore, blockade of HT receptors has no significant effect on the frequency-response curve, and suggests that 5-HT does not modulate tracheal sensitivity to ACH.

Figure 4. Effect of LT on the frequency-response curve to EFS: Lowering the temperature to 18°C induced a leftward shift on the frequency-response curve to EFS (0.1-60 Hz, 40V). ES_{50} values decreased from 19.3±4.3 at 37°C vs 1.0±0.28 at 18°C, n=5. The bronchoactive effect of LT was observed at low-frequency stimulation, from 0.1 to 20Hz. Higher frequency stimulation (>20Hz) resulted in a decreased contractile response. ATR treatment completely blocked the EFS evoked response. *p<0.05.

Figure 5. Tracheal responses to EFS before and after the blockade of 5-HT receptors. a: LT enhanced the tracheal sensitivity and contractility at 18°C at low frequency stimulation (0.5Hz-20Hz). b: tracheal sensitivity and contractility were unchanged in the presence of KTN. ES_{50} values corresponded to 5.2±0.6 Hz at 37°C vs 9.1±0.43 Hz at 18°C, n=4. After KTN, the respective values were 6.0±1.0 and 1.3±12Hz at 37 and 18°C. *p<0.05.

EFS: Relaxation responses to SNP on precontracted tracheas.

Our previous findings indicate that the sensitivity of RTs to contractile agonists is enhanced after the exposure to LT. However very little has been mentioned about tracheal responses to relaxing agents, i.e. NO, ISP, etc.
after EFS. To determine the degree of tracheal relaxation at 37 and 18°C, RTs were precontracted with EFS (5Hz, 40V) and exposed to increasing SNP concentrations (0.1μM to 1mM) at 37°C and 18°C.

Figure 6a represents the typical relaxing responses to SNP at 37 and 18°C. At 37°C, tracheal stimulation with EFS (5Hz, 40V), induces a sustained response that lasts until SNP is added to the bathing solution. The tracheal response to SNP is characterized by a rapid relaxing response that approximates 43% of the E\(_{\text{max}}\). EFS stimulation at 18°C induces a large tracheal contraction that is insensitive to SNP addition. Furthermore, SNP was unable to induce a significant relaxation of RTs, even when high concentrations (1mM) were used. Figure 6b demonstrates the % relaxation of RTs induced by SNP addition. The E\(_{\text{max}}\) for SNP at 37°C corresponds to \(-6.6\pm0.31\) (25μM) and the maximal relaxation of precontracted tracheas corresponds to a 48.5±5.8%. At 18°C SNP induced a maximum relaxation of about 3%, which does not change even at maximal SNP concentrations. NO\(_2\) (as a marker on SNP conversion to NO) was confirmed to be present in the organ chamber at 37 and 18°C (31μM and 36μM NaNO\(_2\), respectively).

**EFS: Relaxation responses to isoproterenol.**

In view of the functional antagonism of B\(_2\) agonists on the contractile response, the response of RTs to B\(_2\) receptor activation at 18°C was evaluated. Isoproterenol (ISP) is the agent of choice because it shows a high affinity to B\(_2\) receptor and is linked to tracheal relaxation of human airways. To accomplish this goal, precontracted RTs (5Hz, 40V) were exposed at 37 and 18°C to increasing concentrations of ISP (1x10\(^{-9}\) to 1x10\(^{-5}\) M) and the corresponding relaxation responses were recorded. Figure 7 is a typical recording of the effects of ISP on tracheal contractility. At 37°C, EFS (5Hz, 40V) induced a sustained contractile response, which was almost unchanged in the presence of low concentrations of ISP. Only when the ISP concentration reached 1x10\(^{-4}\)M a large relaxing response is observed. Further addition of ISP has no significant effects. After turning off the electrical stimulus, tracheal temperature was lowered to 18°C. Under these conditions, the addition of ISP, up to 1x10\(^{-4}\)M was not linked to tracheal relaxation. Furthermore, the increase on tracheal responses, induced by LT, (from 0.83g to 1.42g) was insensitive to the relaxing effects of ISP. In a set of
determinations, the relaxing effect of ISP to 1 x10^{-6} M reaches 36.7±12%, n=3, at 37°C. No relaxing responses were observed at 18°C.

**EFS: Relaxation responses to KCl addition to a K^±-free medium:**

Based on the failure of SNP and ISP to induce relaxing responses on tracheas exposed to LT, the relaxation of precontracted tracheas after KCl addition to a K^±-free solution was investigated. The degree of relaxation observed in smooth muscle after the addition of KCl in a K^±-free medium has been considered as a measure of Na^+/K^+ activity in ASM cells [15]. Therefore, this technique will provide us information on whether or not the contractile responses induced by EFS are affected by tracheal hyperpolarization at 37 an 18°C.

Figure 8 represents the response of precontracted tracheas to KCl in a K^±-free solution at 37 and 18°C. In control conditions (5mM KCl), EFS (5Hz, 40V) elicited contractile responses that were further increased after the addition of 15mM KCl (20mM total KCl). Tracheal stimulation at 18°C induced an enhanced relaxation that appears to be further incremented after the addition of 15mM KCl. After a resting period, tracheas were exposed to a K^±-free solution at 37°C. Under these conditions, addition of 20mM KCl at the plateau induces a significant relaxation, which corresponds to 12.1±1.5%, n=4. This relaxing effect was not observed after the addition of 20mM KCl at 18°C.

**Discussion**

The EFS-dependent release of endogenous neurotransmitter allows us to procure a more physiological approach to the study of tracheal responses to LT. This is of primordial importance because ACH is the most important endogenous bronchoconstrictor released from the nerve terminals in many species, including humans and rats.

This study indicates that the exposure of RTs to LT induces a rightward shift in the voltage-response curve, which is consistent with a decreased sensitivity and contractility to EFS (see figure 2). These experiments were done at a relatively high frequency (0.1-60 V, 50 Hz), therefore, it might be possible that neural mechanisms of feedback inhibition were activated. For example, in vivo vagal stimulation (20Hz) of cat airways has been associated to activation of iNANC nerves and NO production (16). In addition, in horse airways, the pre-junctional effect of neuromodulators is apparent only within a certain range of frequency and voltage stimulation (17).

At variance with the inhibitory effect on the voltage-response curves at high frequency (50Hz), LT induced a significant enhancement in tracheal sensitivity at low-frequency stimulation (0.1-60 Hz, 40V), see figure 4. The bronchoactive effect of LT was limited to the 0.1-20Hz-stimulation range. Tracheal response to low-frequency stimulation at 18°C induces a similar leftward shift as the contractile responses to exogenous ACH in LT conditions, as described previously by Ishii and Shimo (1985). This finding is of outmost importance because it demonstrates that the enhancement of the contractile response induced by LT is not only an effect of pharmacological stimulation of the airways. This finding confirms that the effects of LT are also present after the endogenous release of bronchoactive neurotransmitters, mainly ACH. We have observed that the enhancement of the contractile response induced by LT at low frequency stimulation is within the range of in vivo vagal stimulation (5-15 Hz) required for the development of bronchoconstriction in rabbit airways (18). Thus, the bronchoactive effect of LT might well be present within the range of physiological stimulation of nerve terminals. This is of great importance because it has been suggested that excessive ACH release from nerve terminals is linked to airway hyperresponsiveness (19). In addition, the enhanced contractile response induced by LT at low frequency stimulation (0.1-20Hz) began to reverse when the EFS increased over 20Hz. This latter observation confirms the previous findings related to the inhibition of the LT effects at high frequency stimulation and suggests that frequency-dependent mechanisms of neurally mediated regulation of ASM response are present in RTs.
In addition, the EFS-dependent tracheal sensitivity and contractility at 37 and 18°C was unchanged after the blockade of 5HT, receptors with KTN, but was completely abolished in the presence of ATR, figure 5. This finding confirms the idea that the contractile response to EFS at low frequencies is completely mediated by endogenous ACh release. Furthermore, it suggests that 5-HT, a bronchoconstrictor released from mast cells (20) is not released from tracheal intramural nerve terminals. The effects of LT are not exclusively related to an enhanced endogenous contractile response, but we found that LT diminishes the relaxing responses of precontracted tracheas using low-frequency EFS. At 37°C, precontracted tracheas (5Hz, 40V) relax in response to SNP in a concentration-dependent manner, figure 6a. However, the responsiveness of precontracted tracheas to SNP was markedly decreased after lowering the temperature to 18°C as shown in figure 6b. SNP donates NO in vascular and airway tissue by a photochemical reaction (21,22). Therefore the absence of a relaxing response at 18°C could be secondary to a decreased NO production from SNP. However, we confirmed the presence of NO products in the organ chamber at both 37 and 18°C by means of the Griess reaction (23). These observations indicate that the sensitivity of the ASM to the SNP-derived NO was markedly impaired in LT conditions.

The decreased effect of bronchorelaxants in tracheas exposed to LT was further confirmed in the next set of experiments. From the tracing in figure 7, it is observed that precontracted tracheas failed to show a relaxing response to SNP, a B2-specific agonist. In the same tracing, a significant relaxing response is observed at 37°C. These findings are of great clinical significance because B2 receptors are important modulators of the ASM tone during exercise (24). Furthermore, hyperpnea and airway cooling are triggers of exercise-induced bronchoconstriction (25). In addition, B2 receptor stimulation is an important tool for therapeutic bronchodilatation.

Changes in the activity of the ASM Na+/K+ pump seem to act as a regulatory element in the contractile status of the airways. Relaxing responses mediated by membrane events, specifically Na+/K+ pump activation, might well have been impaired in LT conditions, as seen in figure 8. This finding is of critical importance, because it may indicate that the activity Na+/K+ dependent Ca++ extrusion mechanisms in the ASM membrane could be partially or completely inactivated in cooling conditions, thus increasing cytosolic Ca++ within the ASM, as recently suggested by Mustafa and coworkers (26).

In conclusion, our results clearly indicate that LT enhances the contractile response of rat tracheas in response to endogenous ACh release. The effect of LT is limited to frequencies below 20Hz, which are within the physiological range required for bronchoconstriction. Furthermore, LT severely impairs the sensitivity of RTs to relaxant stimuli, either of endogenous or exogenous origin thus promoting a higher level of contraction of the ASM.

Resumen

Este trabajo fue diseñado para evaluar el efecto de la baja temperatura en las respuestas contractiles y relajantes de la tráquea de rata bajo estimulación eléctrica. Se construyeron curvas de voltaje vs respuesta contractil y frecuencia vs respuesta contractil a 37 y 18°C luego de la estimulación de los nervios traqueales intramurales por mediación de ondas DC aplicadas a través de la técnica de campo eléctrico en frecuencia fija (40Hz) y frecuencia variable (5-40Hz). También se determinó el porcentaje de relaxación inducido por isoproterenol, cloruro de potasio y nitroprusiato sódico en tráqueas precontraídas eléctricamente (5Hz, 40V) a 37 y 18°C. Encontramos que las respuestas contractiles inducidas por estimulación eléctrica fueron completamente bloqueadas con atropina. La baja temperatura disminuyó la sensibilidad de la respuesta voltaje-dependiente, y aumentó la sensibilidad de la respuesta frecuencia-dependiente. A 37°C hubo respuesta relajante de la tráquea a isoproterenol, cloruro de potasio y nitroprusiato de sodio. A 18°C no hubo respuesta a los agentes relajantes utilizados. Concluimos que la baja temperatura aumenta la contractilidad de la tráquea de rata en respuesta a la liberación de acetilcolina endógena. Este efecto se limita a frecuencias por debajo de 20Hz, las cuales están dentro del margen fisiológico requerido para el desarrollo de broncoconstricción. La baja temperatura también disminuye significativamente la sensibilidad de la tráquea a estímulos broncoplejantes, de origen endógeno o exógeno.

References