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Hyperosmolarity-induced relaxation and prostaglandin release in guinea pig trachea in vitro

Josephine Hjoberg [a,*], Gert Folkerts [b], Sandra B.E. van Gessel [b], Marieann Högman [a], Göran Hedenstierna [a], Frans P. Nijkamp [b]

[a] *Department of Medical Sciences, Clinical Physiology, University Hospital, S-751 85 Uppsala, Sweden*
[b] *Department of Pharmacology and Pathophysiology, Faculty of Pharmacy, Utrecht University, Utrecht, Netherlands*

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Abstract

In this study, a tracheal perfusion apparatus was used to investigate the nature of the relaxing factor released by hyperosmolarity on the epithelial side of guinea pig trachea. NaCl induced concentration-dependent relaxation. This relaxation was not affected when the trachea was preincubated with a vasoactive intestinal peptide (VIP) receptor antagonist or with the nitric oxide synthesis inhibitor N^G-monomethyl-L-arginine (L-NMMA). When the prostaglandin synthesis was prevented by preincubation with the phospholipase A_2-inhibitor quinacrine, or the cyclooxygenase inhibitor indomethacin, the maximal relaxation induced by NaCl was suppressed by 50% ($P < 0.05$). Moreover, the prostaglandin E_2 concentration was four times higher ($P < 0.05$) in the organ bath during the relaxations, whereas the nitric oxide concentration remained unchanged. In conclusion, increased osmolarity on the airway surface leads to the release of prostaglandins, which are involved in part in the hyperosmolarity-induced relaxation of airway smooth muscle. This might be relevant for asthmatic patients since prostaglandin may modulate the bronchoconstrictive response to hyperosmolar stimuli and exercise.

Keywords: Hyperosmolarity; Prostaglandin; Nitric oxide (NO); Vasoactive intestinal peptide (VIP); Tracheal perfusion; (Guinea-pig)

1. Introduction

An increase in the osmolarity of the airway surface liquid due to respiratory water loss is thought to be the stimulus for exercise-induced bronchoconstriction (Anderson, 1998). Bronchoconstriction is known to be induced in asthmatic patients when the osmolarity of the airway surface liquid is increased by nebulisation with hypertonic saline (Anderson et al., 1983). Often, there is a period of refractoriness following the bronchoconstriction induced by exercise or hypertonic saline (Belcher et al., 1987; O'Byrne, 1998). This refractory period can be shortened by pretreatment with the cyclooxygenase inhibitor indomethacin (O'Byrne and Jones, 1986; Margolskee et al., 1988; Hawksworth et al., 1992), suggesting that prostaglandin E_2 may play an important role in attenuating the bronchoconstriction during the refractory period.

The trachea acts as an osmotic sensor (Willumsen et al., 1994) and is sensitive to very small increases in osmolarity on both the epithelial and the serosal side (Fedan et al., 1999). Hyperosmolarity on the epithelial side of guinea pig trachea in vitro leads to airway relaxation, which is thought to be mediated by the so-called epithelium-derived relaxing factors (Munakata et al., 1988, 1989, 1990; Fedan et al., 1990, 1999; Folkerts and Nijkamp, 1998; Folkerts et al., 1995). The investigators reported different factors as being responsible for the relaxation induced by increased osmolarity, and the nature of this factor is not yet settled. One relaxing substance released by these cells in response to various stimuli is prostaglandin E_2 (Noah et al., 1991; Salari and Chan-Yeung, 1989; Delamere et al., 1994; Churchill et al., 1989), and another is nitric oxide (for review see Folkerts and Nijkamp, 1998).

In the present study, a guinea pig tracheal perfusion model was used in an attempt to identify the factor or factors underlying the relaxing effect of intraluminal hyperosmolarity, induced by the application of NaCl. We focused this study on the effects of nitric oxide and

* Corresponding author. Tel.: +46-18-664132; fax: +46-18-664135.
E-mail address: josephine.hjoberg@medsci.uu.se (J. Hjoberg).

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inhibitory prostaglandins, in particular, prostaglandin E_2. Another potent relaxant of airway smooth muscle in vitro, vasoactive intestinal peptide (VIP) (Barnes, 1998), was also evaluated in the search for the relaxant substance released by intraluminal hyperosmolarity.

2. Materials and methods

2.1. Perfused trachea preparation

Specific pathogen-free male Dunkin Hartley guinea pigs (400–450 g, Harlan Olac, Bicester, UK) were used for the experiments. Their care was in accordance with the guidelines and approval of the Dutch Committee of Animal Experiments (Utrecht, The Netherlands). The animals were given an overdose of pentobarbitone sodium (Euthesate®, Apharmo, Arnhem, The Netherlands; 1.25 mg 100 g^{-1} body weight intraperitoneally). The tracheas were dissected free of adherent tissues, and two hooks were then inserted through the tracheal wall, one on each side of the trachea, with the smooth muscle between them. Another similar pair of hooks was then attached in the same way and the trachea was divided into two parts, between the pair of hooks. This preparation is a modification of a method described by Pavlovic et al. (1989). The tracheas were mounted in an organ bath containing a modified Krebs–Henseleit buffer kept at 37°C and gassed with 95% O_2 and 5% CO_2. The tissues were extended to their approximal in situ length. The inside of the trachea was perfused (2 ml min^{-1}) with Krebs–Henseleit buffer in a recirculating loop. One of each pair of hooks was attached to a fixed point at the bottom of the organ bath and the other one was connected to an isometric force transducer (Harvard Bioscience, Kent, UK) and the preload on the trachea was set to 2 g. During an equilibration period of at least 60 min, when the trachea was washed by exchanging the intraluminal and extraluminal buffers for fresh buffer every 15 min, the preload was readjusted until a stable tone was reached.

2.2. Protocols

The relaxation from baseline tone in response to intraluminal hyperosmolarity was assessed by adding NaCl to the intraluminal bath in increasing concentrations (control, $n = 5$). The osmolarity of the modified Krebs–Henseleit buffer was thereby increased from the normal 290 to 330, 370, 450 and 530 mOsm.

To investigate the role of VIP in the hyperosmolarity-induced relaxation, tracheas were pretreated with a VIP receptor antagonist. Thirty minutes before the NaCl challenge, the VIP receptor antagonist was added to the intraluminal side (8 μM, $n = 6$). Since the epithelium can act as a barrier for several agents, experiments were also performed with the VIP receptor antagonist added to the extraluminal side (2 μM, $n = 6$).

The role of nitric oxide was assessed by pretreating tracheas ($n = 6$) with the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA; 120 μM), added to the intraluminal bath 30 min before the NaCl challenge.

To test whether prostaglandins were responsible for the relaxation, the cyclooxygenase inhibitor indomethacin or the phospholipase A_2 inhibitor quinacrine were used to block the production of prostaglandins. Indomethacin (1 μM) was given on the intraluminal side 30 min before the NaCl challenge ($n = 5$). Quinacrine was administered both on the intraluminal side (300 μM) and on the extraluminal side (100 μM) 30 min prior to NaCl challenge ($n = 5$).

2.3. Measurements of prostaglandin E_2 and nitric oxide release

To determine whether intraluminal hyperosmolarity induced the release of prostaglandin E_2, samples were taken from the intraluminal bath at the baseline and after addition of the different doses of NaCl ($n = 6$). Samples and prostaglandin E_2 standard (P-4172, Sigma Chemical, St. Louis, MO, USA) were incubated overnight at 4°C with assay buffer, prostaglandin E_2 tracer (DuPont NEN-products, Boston, MA, USA) and antibody (JP 7 rabbit, kind gift from Jansen Pharmaceutica, Beerse, Belgium). To separate free and bound prostaglandin E_2, samples were incubated with a suspension of charcoal and dextran on ice for 15 min, after which they were centrifuged at 5000 rpm (10 min, 4°C). The supernatant was decanted and diluted in scintillation fluid and then counted in a β-counter (Philips PW4700 liquid scintillation counter, Philips, Eindhoven, The Netherlands) for 5 min. The sensitivity of the assay was 8 pg 100 μl^{-1}, and the degree of cross-reactivity between antibody and prostaglandin $F_{1\alpha}$ was 0.6%, and for prostaglandin $F_{2\alpha}$, it was $< 0.01\%$.

To determine whether nitric oxide was released by the hyperosmolarity, samples were taken from the intraluminal bath fluid directly after the equilibration period, 30 min later immediately before NaCl challenge, and after the hyperosmolar challenge. One hundred microlitres of the fluid was injected into a gas-stripping apparatus containing 2 ml of a 1% solution of NaI in glacial acetic acid which was connected to a Sievers 270B nitric oxide analyser (Sievers, Boulder, CO, USA). Data are presented as area under the curve. The sensitivity of the nitric oxide analyser is < 10 pmol ml^{-1} with a linearity of three orders of magnitude. Calibrations were made with standard solutions of sodium or potassium nitrite in accordance with the manufacturer's instructions (Menon et al., 1991).

2.4. Chemicals

The modified Krebs–Henseleit buffer contained (in mM) NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.7 (Onderlinge Pharmaceutische Groothandel, Utrecht, the Netherlands), and was gassed

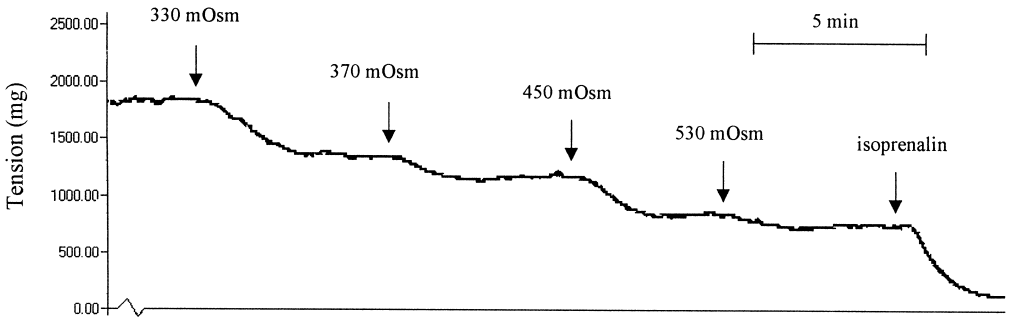

Fig. 1. Relaxation responses created by applying NaCl in increasing doses on the epithelial side of a guinea pig trachea.

with 95% O_2 and 5% CO_2. Indomethacin (Sigma) was dissolved in 96% ethanol (0.02‰ v/v), and the vehicle was added to control baths. The VIP receptor antagonist (Prod. no.: 24219, [Lys[1], Pro[2,5], Arg[3,4], Tyr[6]] VIP, Anaspec, San José, CA, USA.), L-NMMA (Welcome Research Laboratories, Beckham, Kent, UK) and quinacrine (Sigma) were dissolved in water.

2.5. Statistical analysis

Data are presented as mean ± standard error of mean (S.E.M.). Statistical analysis was performed with Statistica software (StatSoft., Tulsa, OK, USA). For differences between groups, a one-way analysis of variance (ANOVA) and Duncan's post hoc test were used, and where appropriate, Student's *t*-test. A statistical result with $P < 0.05$ was considered to be significant.

3. Results

Application of NaCl in increasing concentrations, giving osmolarities of 330, 370, 450 and 530 mOsm to the intraluminal side of the trachea produced concentration-dependent relaxation in the control situation (Fig. 1). Pretreating the trachea with a VIP receptor antagonist either on the intraluminal or extraluminal side did not alter this relaxing effect of hyperosmolarity. The VIP receptor antagonist had no effect on the baseline tone.

Pretreatment with the nitric oxide synthase inhibitor L-NMMA did not alter the baseline tone. Neither intraluminal nor extraluminal pretreatment with L-NMMA affected the response to the increased intraluminal NaCl concentration.

Quinacrine pretreatment had no effect on the baseline tone. This pretreatment did not affect the relaxation induced by 330 mOsm. At 370, 450 and 530 mOsm, the relaxations amounted to 51 ± 10%, 49 ± 11% and 53 ± 14%, respectively, which were significantly smaller responses than in the untreated control ($P < 0.05$) (Fig. 2).

Pretreatment of the tracheas with indomethacin relaxed the trachea from baseline ($P < 0.01$), and the tone had to be manually readjusted to zero and allowed to stabilise. Indomethacin pretreatment did not affect the relaxation produced by 330 or 370 mOsm, but significantly reduced that caused by 450 and 530 mOsm to 57 ± 9% and 52 ± 7% of the control, respectively ($P < 0.05$) (Fig. 2). The relax-

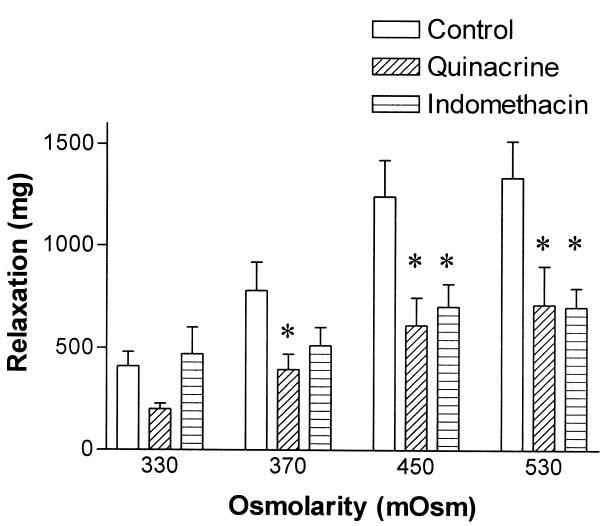

Fig. 2. Relaxations from baseline produced by increased osmolarity in control situation and after pretreatment with indomethacin or quinacrine.

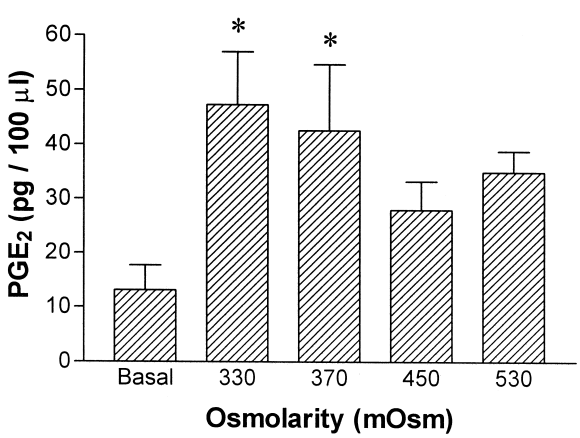

Fig. 3. Samples collected from the intraluminal bath during the NaCl challenge were evaluated by radioimmunoassay. The concentration of prostaglandin E_2 was assayed after increasing the osmolarity on the epithelial side to 330, 370, 450 and 530 mOsm. $^{*}P < 0.05$ compared to basal concentrations.

ation did not differ between indomethacin- and quinacrine-pretreated tracheas at any osmolarity.

Radioimmunoassay of the buffer perfused during the NaCl challenge revealed that prostaglandin E_2 was released in response to intraluminal hyperosmolarity. The basal concentration of prostaglandin E_2 was 13.1 ± 4.6 pg 100 μl^{-1}, and this increased markedly after the intraluminal osmolarity had been increased to 330 and 370 mOsm ($P < 0.05$). A further increase in osmolarity to 450 and 530 mOsm did not cause any further significant release of prostaglandin E_2 (Fig. 3). The nitric oxide content in the perfused buffer in control tracheas showed no differences when measured directly after the equilibration period, 30 min later, and after the NaCl challenge (areas under the curve: 14.2 ± 2.0, 11.9 ± 1.7 and 10.6 ± 0.9 mV s^{-1}, respectively; $P > 0.05$).

4. Discussion

This study has shown that the concentration-dependent relaxation induced by hyperosmolar concentrations of NaCl on the epithelial side of the guinea pig trachea in vitro is partly mediated by inhibitory prostaglandins, most likely, prostaglandin E_2. VIP and nitric oxide appear not to be involved in the relaxation induced by hyperosmolarity produced by NaCl.

Increased osmolarity in the airway lumen leads to shrinkage of the epithelial cells (Willumsen et al., 1994) and release of factors that may act on the smooth muscle of the airways, thereby, affecting airway tone (Boucher, 1994). The nature of these factors released by intraluminal hyperosmolarity was investigated in this study. Airway epithelial cells have been shown to reduce airway smooth muscle contraction through the release of epithelium derived relaxing factors (EpDRFs), such as prostaglandins and nitric oxide (Folkerts and Nijkamp, 1998). Prostaglandins are produced from arachidonic acid by the action of the enzymes cyclooxygenase and phospholipase A_2. In the present study, cyclooxygenase was inhibited by indomethacin, and phospholipase A_2 was blocked by quinacrine. The fact that indomethacin markedly decreased the baseline tension and that the tension had to be manually readjusted could be factors affecting the response to NaCl. However, the effects of indomethacin and quinacrine in reducing the relaxation produced by luminal hyperosmolarity were identical, and quinacrine did not affect the baseline tone and no readjustment was needed. This suggests that metabolites of arachidonic acid are partially responsible for the relaxation induced by increasing the luminal osmolarity with NaCl. The metabolite responsible is most likely to be prostaglandin E_2, which, in this study, was shown to be released upon hyperosmolar stimulation by NaCl on the epithelial side. In contrast, relaxations induced by KCl have been found to be unaffected by blocking the cyclooxygenase pathways (Munakata et al., 1988; Fedan et al., 1999), suggesting that the relaxation induced by NaCl and KCl may be mediated by different mechanisms. In the present study, the peak concentration of prostaglandin E_2 did not coincide with the maximal relaxation and one possible explanation for that might be that there is a reservoir of prostaglandin E_2 that is depleted after releasing a big amount of prostaglandin E_2. Alternatively, the reservoir of the substrate for prostaglandin E_2, arachidonic acid, may have been depleted after the first release of prostaglandin E_2. Other possible explanations are that some time might be required to receive this relaxing effect of prostaglandin E_2, or that the concentration of prostaglandin E_2 in the tissue is not directly comparable to the concentration in the perfused buffer.

Intraluminal hyperosmolarity caused by various agents such as KCl, NaCl, mannitol and urea caused concentration-dependent relaxation of carbachol pre-contracted guinea pig tracheas (Munakata et al., 1988, 1990). In these studies by Munakata et al. (1988, 1990), no relaxation was observed when the osmolarity was increased on the extraluminal side or when the epithelium was removed. They also found that neither the nitric oxide synthase inhibitor L-NMMA nor the inhibitor of soluble guanylate cyclase, methylene blue, affected the relaxation produced by hyperosmolarity, suggesting that the EpDRF was not nitric oxide. However, a previous study by Folkerts et al. (1995) showed that KCl did not induce relaxation in guinea pig tracheas pretreated by the nitric oxide synthase inhibitor L-NAME, suggesting that the mediator was nitric oxide. The use of different methods, such as isometric vs. pressure measurements of airway reactivity and differences in perfused volume and perfusion flow, could provide one explanation for these conflicting results.

In the present study, L-NMMA did not affect the relaxation induced by NaCl hyperosmolarity. Nor did intraluminal application of NaCl affect the production or release of nitric oxide. Thus, nitric oxide does not appear to be the factor mediating relaxation produced by NaCl-induced hyperosmolarity. The effect of inhibition of nitric oxide synthase on hyperosmolarity-induced relaxation seems to vary according to the agent causing the increased osmolarity. Since our experiments were performed with the same method as those in the study by Folkerts et al. (1995), the conclusion that NaCl and KCl mediate the relaxation by different mechanisms is further strengthened. Recently, Fedan et al. (1999) found that NaCl-induced relaxation was slightly reduced by L-NAME; however, nitric oxide production was not measured under their experimental conditions.

There is a possibility that an increase in nitric oxide was counteracted in our study by the release of prostaglandin E_2 that has been shown to reduce nitric oxide in exhaled air (Kharitonov et al., 1998). Further support for our finding that nitric oxide is not the relaxing mediator released by an increased NaCl concentration is the report by Nogami et al. (1998) where endogenous nitric oxide did

not modulate hyperventilation-induced bronchoconstriction in guinea pigs.

We have found marked concentration-dependent relaxations resulting from intraluminal hyperosmolarity induced by NaCl. In contrast, KCl caused concentration-dependent relaxations at low concentrations, which returned to baseline at higher concentrations (Folkerts et al., 1995). The maximal relaxation produced by KCl was about 500 mg at 368 mOsm, while in the present study, the maximal relaxation produced by NaCl was 1335 $\pm$ 181 mg at 530 mOsm. Thus, the magnitude and nature of the relaxation induced are also affected by the kind of substance producing the hyperosmolarity.

In conclusion, hyperosmolarity produced by adding NaCl to the intraluminal side of guinea pig tracheas in vitro produces a concentration-dependent relaxation that is partly mediated by prostaglandin E_2. These findings offer further support to studies suggesting that prostaglandin E_2 is the factor producing the refractory period after bronchoconstriction induced by increased osmolarity on the airway surface following provocation by exercise or hypertonic saline.

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