Mechanism for substance P-induced relaxation of precontracted airway smooth muscle during development

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Mhanna, Maroun J., Ismail A. Dreshaj, Musa A. Haxhiu, and Richard J. Martin. Mechanism for substance P-induced relaxation of precontracted airway smooth muscle during development. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L51–L56, 1999.—Release of substance P (SP) from sensory nerve endings of the tracheobronchial system modulates airway smooth muscle contraction and may cause relaxation of precontracted airways. We sought to elucidate the effect of postnatal maturation on SP-induced relaxation of precontracted airways and determine the roles of endogenously generated nitric oxide (NO) and prostaglandins (PGs). Cylindrical airway segments were isolated from the midtrachea of rats at four different ages, 1, 2, and 4 wk and 3 mo, and contracted to 50–75% of the maximum response induced by bethanechol. SP was then administered in the absence and presence of the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME), the PG inhibitor indomethacin, or both. Relaxation of airways with SP decreased significantly with advancing postnatal age. SP-induced tracheal relaxation was consistently attenuated by pretreatment with L-NAME, indomethacin, or both. In a different group of animals, L-NAME significantly attenuated the relaxant response of airways to PGE2 exposure, but indomethacin had no significant effect on the relaxant response to exogenous NO. We conclude that SP induces a relaxant effect on precontracted airway smooth muscle, which decreases with advancing age and is mediated via SP-induced release of NO and/or PG.

TACHYKININ NEUROPEPTIDES such as substance P (SP) are released from sensory nerve endings in the respiratory tract and have potent effects on bronchomotor tone, epithelial cell function, airway secretions (3, 12), the bronchial circulation (20), and both inflammatory and immune cells (5). As a result, SP has been implicated in the genesis of reactive airway disease (4, 15). There is, however, limited information on the role of SP in modulating airway smooth muscle contraction in early postnatal life. Previous work from our laboratory (14) has demonstrated that airway contraction and submucosal gland secretion, induced by SP infusion, appear to be attenuated in piglets during early postnatal life. The mechanism underlying this attenuated contractile response to SP during early maturation is unclear (14). Because nitric oxide (NO) synthase (NOS) is abundantly expressed in lungs around term gestation in both pigs and rats (8, 24) and SP stimulates NO generation in tracheal epithelium of different species (17, 29, 30), SP-induced activation of nonadrenergic, noncholinergic relaxant pathways could contribute to the weak contractile response induced by SP in the newborn piglet.

In contrast to the enhanced contractile response exhibited by airway smooth muscle of many species in response to SP (5, 9, 11, 14, 18, 22, 31), an inhibitory response of precontracted airway smooth muscle to SP has been reported in adult Sprague-Dawley rats (27, 28). This relaxant response is mediated by activation of neurokinin (NK)-1 receptors on epithelium and the subsequent release of inhibitory prostaglandins (PGs) in mature rats (9a, 28). Furthermore, SP induces NO release from the airway epithelium of mature guinea pigs (10), although there are no data on the maturation of this response. We hypothesized that in early life there is an enhancement of the relaxant effect of SP on precontracted airway smooth muscle and that both PGE2 and NO could play a role in this response. Our objective, therefore, was to elucidate the effect of postnatal maturation on SP-induced relaxation of precontracted airway smooth muscle in maturing rats and characterize the underlying mechanisms.

METHODS

Experimental preparation. Sprague-Dawley rats at 1 wk (n = 58), 2 wk (n = 57), 4 wk (n = 24), and 3 mo (n = 12) were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and killed, and their tracheae were removed. Under the dissecting microscope, each trachea was freed of adventitia and fat tissue. A cylindrical airway segment of 3-mm length was isolated from the midtrachea of each rat from the four different age groups and placed in a modified Krebs-Henseleit solution of the following composition (in mM): 118.2 NaCl, 25 NaHCO3, 4.6 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2.5 CaCl2, and 10% dextrose, with pH adjusted to 7.4. The medium was continuously aerated with 5% CO2 balanced with O2. Tracheal cylinders were suspended between a sturdy glass rod and a force displacement transducer (FT 03, Grass Instruments, Quincy, MA) connected to an amplifier as Agani et al. (2) described previously. Generated forces were continuously monitored and recorded on a rectilinear chart recorder. The cylinders were allowed to equilibrate in the organ bath (Radnoti Glass) for 40–45 min before any challenge. The optimal length at which maximal isometric force developed was obtained for each cylinder by 0.1-g increments of load until electrical field stimulation (5-V AC applied through platinum electrodes, 250 mA/cm2) applied for 10 s at 4-min intervals gave a reproducible maximal response. Experimental protocol. A cumulative concentration-response curve to bethanechol (3 × 10-8 to 10-3 M) was obtained for the four different age groups: 1 wk, 2 wk, 4 wk, and 3 mo. The concentration of bethanechol that elicited 50–75% of maximal response (ED50–75) was determined. The airway cylinders were then washed, equilibrated, and precontracted with bethanechol (ED50–75 between 3 × 10-6 and 10-5
M). SP at $10^{-6}$ M was added to each precontracted cylinder on only one occasion to prevent desensitization to SP (19). This concentration of SP was chosen because it evoked marked relaxant responses when previously used by Szarek et al. (28) in bronchi of mature rats. When distilled water (solvent for SP) was randomly administered to the precontracted trachea, no change in tension was ever seen. The percentage of relaxation from the precontracted state was then calculated for each cylinder. The percentage of relaxation in response to SP was defined as the reduction in tension (in g) in relation to baseline tension in the precontracted state, with each tracheal segment serving as its own control (before and after SP exposure).

In additional animals studied at 1 and 2 wk, we sought to block the relaxant effect of SP on precontracted airways. Airway segments were exposed to either the constitutional NOS inhibitor N^3-nitro-L-arginine methyl ester (L-NAME) at $10^{-4}$ M, the cyclooxygenase inhibitor indomethacin at $10^{-4}$ M, or both for 1 h before betahanechol followed by SP exposure as described above. Cylinders only exposed to betahanechol followed by SP served as controls. A different set of cylinders was also exposed to $10^{-4}$ M aminoguanidine, an inducible NOS inhibitor, before SP exposure. Finally, D-NAME, an L-NAME isomer, was added to a set of tracheal rings to serve as a control for L-NAME.

In another group of rats, we sought to determine whether the relaxation of precontracted airways induced by PGE2 is mediated via NO or vice versa. Dose-response curves to PGE2 and NO (5 $\times$ $10^{-5}$ M) that induced maximal relaxation of precontracted airway segments were used to elicit the interaction between PG and NO. Airway cylinders from 1- and 2-wk-old rat pups were incubated with L-NAME at $10^{-4}$ M for 1 h before betahanechol exposure (as described above), followed by $10^{-6}$ M PGE2. Another group of tracheal cylinders was incubated with indomethacin at $10^{-6}$ M for 1 h before betahanechol exposure, followed by NO administration. To confirm the reversibility of L-NAME, a control group of airway segments was incubated with L-NAME plus L-arginine (an NO precursor) at $10^{-4}$ M before SP exposure. Additional experiments were conducted in which tracheal segments were exposed to nitrogenated distilled water to serve as a control group for the NO donor solution. To determine the effect of NO on the viability of the tissues, NO exposure was followed by either SP administration or electrical field stimulation in separate experiments.

Drugs. All dilutions of drugs were prepared on the day of the experiments. Stock solutions were made by dissolving SP (Sigma) in distilled water at 1 mM. Betahanechol, L-NAME, D-NAME, L-arginine, and aminoguanidine were dissolved in distilled water. Indomethacin was dissolved in 0.1 M sodium carbonate solution. PGE2 was dissolved in 0.1 M phosphate buffer solution. The NO solution was prepared by bubbling nitrogen gas into 10 ml of distilled water in a vacuum container for 30 min, followed by addition of NO gas for a subsequent 30 min. Using an electrochemical method, we determined that the concentration of NO in the final solution saturated with NO was ~1 mM (6).

Statistical analysis. One-way ANOVA was used for statistical analysis of the maturational response of precontracted airway smooth muscle to SP between 1 wk and 3 mo of age. For individual comparison between groups, the Newman-Keuls test was used. Unpaired t-tests were used to analyze the response to SP with either indomethacin, L-NAME, or both at 1 and 2 wk of age. Unpaired t-tests were also used to determine the response to NO or PGE2 in the presence or absence of indomethacin or L-NAME, respectively, at 1 and 2 wk of age. All data are means ± SE.

**RESULTS**

Response to SP with advancing age. Before SP exposure, betahanechol caused a dose response-dependent increase in tracheal tension in all four groups. As expected, this contractile response was age dependent: the maximum contraction was 0.85 g at 1 wk, 1.5 g at 2 wk, 2.08 g at 4 wk, and 2.57 g at 3 mo. However, the estimated ED50–75 of betahanechol did not differ among ages. Exposure of precontracted tracheae to SP caused a relaxant response that decreased significantly with age (P < 0.001) between 1 wk and 3 mo of age. SP induced a relaxation of 21.7 ± 2.9% (n = 13 airway segments) at 1 wk, 13.4 ± 1.7% (n = 12) at 2 wk, 13.6 ± 2.5% (n = 12) at 4 wk, and 1.5 ± 0.6% (n = 12) at 3 mo of age (Fig. 1). For individual comparison among age groups, Newman-Keuls analysis showed a significant difference between 1 and 2 wk (P < 0.05), 1 and 4 wk (P < 0.05), 1 wk and 3 mo (P < 0.001), and 2 wk and 3 mo (P < 0.01). There was no significant difference between 2 and 4 wk, but the response at 4 wk was greater than that at 3 mo (P < 0.01). Exposure of precontracted airways to distilled water alone (control group for SP) induced no relaxation. Fully relaxed tracheae also had no response to SP.

Response to SP with and without L-NAME, indomethacin, or both. When precontracted airway segments were incubated with L-NAME before SP exposure, L-NAME caused a significant decline in the degree of relaxation induced by SP. At 1 wk, pretreatment with L-NAME reduced the relaxant response to SP to 11.4 ± 2.3% (n = 6), which was significantly less than the response to SP alone (P < 0.01 for SP alone vs. SP + L-NAME). Similarly, at 2 wk of age, SP-induced relaxation was attenuated to 5.1 ± 1.7% after pretreatment with L-NAME (n = 6), which was significantly less than the response to SP alone (P < 0.04; Fig. 2A). Addition of L-NAME plus L-arginine elicited a 20.9 ±
6.3% (n = 6) relaxation to SP, which was significantly greater than the SP-induced relaxation after incubation with L-NAME alone (P < 0.01) and not different from SP alone. When tracheal segments were incubated with D-NAME (n = 6) or aminoguanidine (n = 6), there was no significant effect on the relaxant response to SP.

Indomethacin also caused a significant attenuation in the percentage of relaxation induced by SP to 9 ± 3.5% (n = 7), which was less than the response to SP alone (P < 0.01 for SP alone vs. SP + indomethacin). At 2 wk, SP-induced relaxation fell to 3.7 ± 2% (n = 6) after indomethacin pretreatment compared with SP alone (P < 0.04; Fig. 2B). In the presence of both L-NAME and indomethacin, the percentage of relaxation to SP was also significantly attenuated. At 1 wk, the percentage of relaxation of trachea was reduced to 11.9 ± 3.2% (n = 8) in response to SP plus indomethacin plus L-NAME compared with SP alone (P < 0.03 for SP alone vs. SP + indomethacin + L-NAME). At 2 wk, SP-induced relaxation was reduced to 4.7 ± 2.5% (n = 8) in the SP plus indomethacin plus L-NAME-pretreated group (P < 0.01; Fig. 2C). The percentage of relaxation to SP after L-NAME or indomethacin alone was comparable with that after pretreatment with L-NAME plus indomethacin at either age.

Response to PGE2 with and without L-NAME and to NO with and without indomethacin. L-NAME caused a significant attenuation in the relaxant response of airway segments to PGE2. At 1 wk, the percentage of relaxation to PGE2 alone was 50.9 ± 8.3 (n = 6) vs. 16.8 ± 6.6% (n = 6) when PGE2 was administered after L-NAME (P < 0.03 for PGE2 alone vs. PGE2 + L-NAME). At 2 wk, PGE2 alone induced a 44 ± 10.5% (n = 7) relaxation vs. 14.3 ± 6.5% (n = 6) in the PGE2 plus L-NAME group (P < 0.03 for PGE2 alone vs. PGE2 + L-NAME; Fig. 3A). Indomethacin had no significant effect on the relaxant response to NO (Fig. 3B). The response to NO was brief; i.e., the precontracted tracheal cylinders had a 5- to 10-s duration of relaxation before returning to their baseline contractile state. The...
responses to SP and electrical field stimulation were comparable before and after NO administration, confirming the viability of the tissue after exposure to NO. Nitrogenated distilled water alone (solvent for NO) had no effect on the precontracted airway smooth muscle, confirming that the relaxation was secondary to NO administration.

DISCUSSION

The current study clearly demonstrates that SP induces relaxation of precontracted tracheae in rat pups, which decreases with advancing age and is absent in adult rats. This is consistent with the previous observation by Haxhiu-Poskurica et al. (14) that SP-induced constriction of piglet trachea is attenuated during early postnatal life. The current study also demonstrates that the relaxant effect induced by SP is mediated by a mechanism involving PG and NO. Szarek et al. (28) previously reported that SP elicited a 57–80% relaxation of preconstricted bronchi of adult Sprague-Dawley rats, and the relaxant response was mediated by epithelium-derived PGs. In our study, the airway segments were isolated from the midtrachea instead of from the bronchi, and this is the likely explanation for the relaxant response observed by Szarek et al., but not by us, in adult rats. This is also consistent with a prior study (11) that demonstrated a greater effect of tachykinins on distal rather than proximal airways. Work remains to be done to characterize the effect of postnatal maturation on contractile responses of large and small airways to SP.

A previous in vitro study (26) demonstrated that SP stimulates ciliary activity in human nasal mucosa as a result of secondary production and release of endogenous PGs and NO. Szarek et al. (28) showed in Sprague-Dawley rats that neuropeptides released from capsaicin-sensitive sensory nerves have potent inhibitory effects on airway ciliary movements mediated, in part, by activation of NK1 receptors on epithelium and the subsequent release of an inhibitory PG. Although a nonspecific NK1-receptor antagonist (CP-99994) failed to block the relaxant effect of SP in this study, when a selective rat NK1-receptor antagonist (RP-67580) was used, it induced an attenuation of this relaxant response. Because PGE2 derived from the epithelium is a likely mediator of SP-induced relaxation responses in Sprague-Dawley rats (28), we incubated airway segments of 1- and 2-wk-old animals with indomethacin, a cyclooxygenase inhibitor, before SP exposure. The ability of indomethacin to reduce the tracheal relaxation induced by SP implicates PGs as mediators of this airway relaxation in rat pups. There is strong evidence that SP induces NO release in different organs and species (7, 23, 25). In particular, SP stimulates NO generation in tracheal epithelium (17, 29, 30) and relaxes guinea pig trachea via stimulation of NK1 receptors and NO release (10). SP induces the generation and release of NO by canine cultured tracheal epithelium (29) and increases NO production in a dose-dependent manner in rabbit trachea (30). Because of the role of NO in airway relaxation and the abundant of NOS in airway epithelium around term gestation in pigs and rats (8, 24), we sought to test the hypothesis that SP induces precontracted airway segment relaxation by NO release in addition to the effect of SP on release of PGs. The significant attenuation in the degree of relaxation induced by SP after incubation of airway segments with L-NAME and the reversibility with L-arginine confirm this hypothesis. These findings are consistent with prior studies (7, 17, 23, 25, 29, 30, 32) that have documented NO release by SP. The enhanced relaxant response in the rat pups vs. mature animals is consistent with the abundance of NOS-containing nerve endings in the newborn airway (8) and previous data by Jakupaj et al. (16) that cholinergically induced airway smooth muscle contraction causes release of endogenous NO in piglets.

Interaction between PG and NO has been described previously (1, 13, 21). In newborn pigs, PG has a role in the autoregulation of retinal and choroidal blood flow mediated, in part, through the release of NO (13). NO also stimulates PGE2 production in the acute phase of allergic conjunctivitis in guinea pigs (21). To determine the sequence of events and the interaction between NO and PGs, we incubated airway segments of 1- or 2-wk-old animals with L-NAME or indomethacin before exposure to exogenous PGE2 or NO in pharmacological doses. The degree of relaxation was attenuated when airway segments were incubated with L-NAME before PGE2 exposure, but there was no difference in the relaxation when the airway segments were incubated with indomethacin before NO exposure. At the pharmacological doses employed in this study (PGE2 at $10^{-6}$ M and NO at $5 \times 10^{-5}$ M), they induced a greater percentage of relaxation than SP but at lower concentrations; PGE2 (at $5 \times 10^{-9}$ M) and NO (at $10^{-5}$ M) had a modest relaxant effect similar in response to SP at $10^{-6}$ M. These data suggest that PGE2 induces airway relaxation mainly via NO release, whereas NO-induced airway relaxation appears complete in the absence of PG. In vivo and in vitro studies to quantify the amount of PG and NO released in response to SP are needed to substantiate our conclusions. Differences between our data and previous findings of others (13, 21) regarding the interaction between PG and NO could be related to interspecies differences, variation in responses among organ systems, or incomplete blockade of PG.

We have shown in our study that SP induced no contraction of fully relaxed tracheae in vitro, which is consistent with previous findings in that species (28). In contrast, SP does enhance airway contractile re-

![Fig. 4. Summary of proposed pathways whereby SP elicits airway smooth muscle relaxation in rat pups. NOS, nitric oxide synthase.](http://ajplung.physiology.org/ by 10.220.33.1 on October 28, 2017)
responses in vivo by stimulating ACh release in adult rats. The ability of SP to induce airway constriction appears to differ between species and is mediated via both a direct contractile response and an enhanced cholinergic effect. Relaxation of precontracted bronchi has been described in mature rats, and we are now reporting that SP induces relaxation of precontracted tracheae of maturing rats, which decreases with advancing age. Interspecies and maturational differences appear to exist in the ability of SP to elicit contractile responses in relaxed airways and yet induce relaxant responses in the precontracted state. Further studies are needed to examine the ability of SP to relax airways of different species and maturational ages before conclusions can be made regarding human disease.

In conclusion, we have shown that SP induces a relaxant effect on precontracted tracheal smooth muscle of rats, which decreases with age and is negligible by 3 mo of age. This relaxation appears to be mediated by a mechanism involving PG and NO, as summarized in Fig. 4. We speculate that this relaxant response serves to protect the neonatal airway from airway contractile and secretory responses induced by release of SP from sensory nerve endings. Damage to the airway epithelial layer may remove this protective effect and enhance neonatal airway injury.

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