NO\(^+\) but not NO radical relaxes airway smooth muscle via cGMP-independent release of internal Ca\(^{2+}\)

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**Methods**

Preparation of tissues and cell dissociation. Segments of donor (i.e., nondiseased) human main stem bronchi were obtained from the Lung Transplant Program (Toronto, Ontario). Adult mongrel dogs were euthanized with pentobarbital sodium (100 mg/kg); their tracheae were excised and kept in physiological solution. Airway smooth muscle was isolated by removing connective tissue, vasculature, and epithelium and then cut into strips parallel to the muscle fibers (~1 mm wide). For single-cell studies, TSM strips (0.5–1.0 g wet wt) were transferred to dissociation buffer (see Solutions and chemicals for composition) containing collagenase (type IV; 2.7 U/ml), elastase (type IV; 12.5 U/ml), and BSA (1 mg/ml) and then were either used immediately or stored at 4°C for

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The article has been compared to two redox effects of two nitric oxide, NO\(^+\) (liberated by S-nitroso-N-acetylpenicillamine (SNAP)) and NO\(^-\) (liberated by 3-morpholinopropionimine (SIN-1) in the presence of superoxide dismutase), on cytosolic concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\); single cells) and tone (intact strips) obtained from human main stem bronchi and canine tracheals. SNAP evoked a rise in [Ca\(^{2+}\)]\(_i\) that was unaffected by removing external Ca\(^{2+}\) but was markedly reduced by depleting the internal Ca\(^{2+}\) pool using cyclopiazonic acid (10\(^{-5}\) M). Dithiothreitol (1 mM) also antagonized the Ca\(^{2+}\) transient as well as the accompanying relaxation. SNAP attenuated responses to 15 and 30 mM KCl but not those to 60 mM KCl, suggesting the involvement of an electromechanical coupling mechanism rather than a direct effect on the contractile apparatus or on Ca\(^{2+}\) channels. SNAP relaxations were sensitive to charybdotoxin (10\(^{-7}\) M) or tetraethylammonium (30 mM) but not to 4-aminopyridine (1 mM). Neither SIN-1 nor 8-bromoguanosine 3',5'-cyclic monophosphate had any significant effect on resting [Ca\(^{2+}\)]\(_i\), although both of these agents were able to completely reverse tone evoked by carbachol (10\(^{-7}\) M). We conclude that NO\(^+\) causes release of internal Ca\(^{2+}\) in a cGMP-independent fashion, leading to activation of Ca\(^{2+}\)-dependent K\(^+\) channels and relaxation, whereas NO\(^-\) relaxes the airways through a cGMP-dependent, Ca\(^{2+}\)-independent pathway.

Ca\(^{2+}\)-dependent K\(^+\) channels; redox forms of nitric oxide; electromechanical coupling

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**Inhibitory Neural Control** of tracheal smooth muscle (TSM) of many species is mediated in part by nitricergic nerves (2, 22). Nitric oxide (NO) can also be generated by the epithelium, invading inflammatory cells, and the smooth muscle itself. NO mediates bronchodilation through a variety of mechanisms. For example, it activates Ca\(^{2+}\)-dependent K\(^+\) channels in porcine TSM via guanylate cyclase and G kinase (25) as well as in bovine TSM through nitrothiosylation of some cellular target molecule (1). Likewise, NO-mediated relaxations in guinea pig and human airway smooth muscle are inhibited by blockers of Ca\(^{2+}\)-dependent K\(^+\) channels

(4). In all of these cases then, the ultimate effect is hyperpolarization of the membrane and consequent closure of voltage-dependent Ca\(^{2+}\) channels; the mechanism by which K\(^+\) channel activity is increased is not yet entirely clear. In addition to activating outward K\(^+\) currents, NO has been shown to suppress inward CI currents (21). NO may also act through nonelectromechanical coupling mechanisms. For example, acetylcholine- and caffeine-induced Ca\(^{2+}\) release are reduced by NO in porcine TSM (16). cGMP, the second messenger through which NO acts (26), reduces the Ca\(^{2+}\) sensitivity of the contractile apparatus (15) and modestly reduces cholinergically induced elevation of cytosolic concentration of Ca\(^{2+}\) (([Ca\(^{2+}\)]\(_i\)) (14) in canine TSM.

NO is usually envisaged as an electrically neutral free radical (i.e., possessing an unpaired electron; NO\(^-\)), which stimulates generation of the second messenger cGMP through guanylate cyclase (19). However, NO also exists in an oxidized form (NO\(^+\)) and in a reduced form (NO\(^-\)), and all three redox forms can react with other free radicals and molecules (e.g., O\(_2\); H\(_2\)O\(_2\); CO), producing an increasingly bewildering variety of reactive species (19). These isoforms of NO, as well as the reactive species that they generate, can oxidize and covalently modify target protein(s) on the plasmalemma and/or within the cells, thereby altering cell function (e.g., Refs. 1 and 23).

In this study, we provide evidence for a novel mechanism whereby NO\(^+\), but not NO\(^-\), evokes relaxation in canine and human airway smooth muscle. In particular, we show that NO\(^+\) causes release of internally sequestered Ca\(^{2+}\) through an oxidative process independent of guanylate cyclase and cGMP, leading to activation of Ca\(^{2+}\)-dependent K\(^+\) channels and relaxation.

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use up to 48 h later; we have previously found that cells used immediately and those used after 48 h of refrigeration exhibit similar functional responses (i.e., contraction and activation of Ca\(^{2+}\)-dependent ion conductances). To liberate single TSM cells, tissues in enzyme-containing solution were incubated at 37°C for 60–120 min and then gently triturated.

Fura 2 fluorometry. Single cells were studied using a DeltaScan system (Photon Technology International; South Brunswick, NJ). After settling onto a glass coverslip mounted onto a Nikon inverted microscope, cells were loaded with fura 2 [fura 2-acetoxyethyl ester (AM), 2 \(\mu\)M for 30 min at 37°C] and then superfused continuously with Ringer buffer at 37°C (2–3 ml/min). Cells were illuminated alternately (0.5 Hz) at the excitation wavelengths, and the emitted fluorescence (measured at 510 nm) induced by 340-nm excitation (\(F_{340}\)) and that induced by 380-nm excitation (\(F_{380}\)) were measured using a photomultiplier tube assembly. \(F_{340}/F_{380}\) was converted to \([Ca^{2+}]_i\) using previously published methods (8). \(R_{\text{max}}\) and \(R_{\text{min}}\) (the fluorescence ratio values under saturating and Ca\(^{2+}\)-free conditions, respectively) were obtained previously, and the Ca\(^{2+}\)-fura 2 dissociation constant was assumed to be 224 nM (8). Agonists were applied by pressure ejection from a puffer pipette (Picospitzer, General Valve; Fairfield, NJ).

Organ bath studies. TSM strips were mounted vertically in a 3-ml organ baths using silk (Ethicon 4-0) tied to either end of the strip; one end was fastened to a Grass FT03 force transducer, whereas the other was anchored. Isometric changes in tension were digitized and recorded using an on-line program (DigiMed System Integrator, MicroMed; Louisville, KY). Tissues were bathed in Krebs-Ringer buffer (see Solutions and chemicals for composition) containing 10 \(\mu\)M indomethacin to inhibiting cyclooxygenase activity (in mM) 125 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 0.25 EDTA, 10 \(\ell\)-taurine, and 10 D-glucose, pH 7.4. Single cells were studied in Ringer buffer containing (in mM) 130 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 20 HEPES, and 10 D-glucose, pH 7.4. Intact tissues were studied using Krebs-Ringer buffer containing (in mM) 116 NaCl, 4.2 KCl, 2.5 CaCl\(_2\), 1.6 NaH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 22 NaHCO\(_3\), and 11 D-glucose, bubbled to maintain pH at 7.4.

Chemicals were obtained from Sigma Chemical, with the exception of fura 2-AM (Calbiochem; La Jolla, CA). All agents were prepared as aqueous solutions except for cyclopiazonic acid (CPA), fura 2, S-nitroso-N-acetylpenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), and penicillamine (all prepared in DMSO) and ryanodine (95% ethanol). SNAP and SIN-1 were prepared in DMSO and SNAP (10\(^{-5}\) M) also elevated [Ca\(^{2+}\)]\(_i\) (which reached a peak within seconds) and was generated more slowly and was sustained. In 2 other canine TSM cells studied in similar fashion, SNAP-evoked response was abolished by pretreatment with cyclopiazonic acid (CPA; 30 \(\mu\)M) to deplete internal Ca\(^{2+}\) store (B) but was apparently unaffected by removal of extracellular Ca\(^{2+}\) (C). \(F_{340}/F_{380}\), ratio of 340- to 380-nm fluorescence. D: mean changes in [Ca\(^{2+}\)]\(_i\), evoked by SNAP in canine airway smooth muscle cells in absence and presence (+) of external Ca\(^{2+}\) or CPA. Numbers in parentheses indicate number of animals studied.

RESULTS

SNAP elevates [Ca\(^{2+}\)]\(_i\), by releasing internally sequestered Ca\(^{2+}\). In freshly dissociated TSM cells, SNAP (10\(^{-5}\) M) triggered sustained elevations of [Ca\(^{2+}\)]\(_i\). Figure 1A provides a sample tracing of such a response as well as that evoked by caffeine (10 mM); the latter has been described in detail elsewhere (10). In both human and canine cells, the SNAP-induced elevation of [Ca\(^{2+}\)]\(_i\) developed much more slowly than the caffeine response (which reached a peak within seconds) and was generally much longer lasting; the mean magnitudes of these responses were 146 ± 42 nM (\(n = 9\)) in human cells and 163 ± 12 nM (\(n = 18\)) in canine cells (Table 1). Penicillamine (10\(^{-5}\) M); the carrier molecule for NO in SNAP) dissolved in vehicle (DMSO) had no effect on [Ca\(^{2+}\)]\(_i\); (\(n = 5\); data not shown); previously, we have demonstrated that DMSO alone does not alter [Ca\(^{2+}\)]\(_i\) in these cells (data not shown).
Table 1. Responses triggered by NO donors in airway smooth muscle

<table>
<thead>
<tr>
<th>NO Donor</th>
<th>Canine TSM</th>
<th>Human TSM</th>
<th>Relaxation in Canine TSM, % reversal*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAP (10^{-5} M)</td>
<td>163 ± 12 (n = 18)</td>
<td>146 ± 42 (n = 9)</td>
<td>92 ± 2 (n = 5)</td>
</tr>
<tr>
<td>+ DTT (10^{-3} M)</td>
<td>20 ± 12 (n = 7)†</td>
<td>70 ± 44 (n = 5)‡</td>
<td>77 ± 10 (n = 5)</td>
</tr>
<tr>
<td>SIN-1 (10^{-4} M + 3 U/ml SOD)</td>
<td>-25 ± 41 (n = 7)†</td>
<td>ND</td>
<td>90 ± 1 (n = 5)</td>
</tr>
<tr>
<td>8-BrcGMP (10^{-4} M)</td>
<td>-3 ± 22 (n = 5)‡</td>
<td>ND</td>
<td>102 ± 4 (n = 5)</td>
</tr>
</tbody>
</table>

Values are means ± SE; number of cells studied in parentheses. NO, nitric oxide; TSM, tracheal smooth muscle; [Ca^{2+}], cytosolic concentration of Ca^{2+}; SNAP, S-nitroso-N-acetylpenicillamine; DTT, dithiothreitol; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; 8-BrcGMP, 8-bromoguanosine 3’,5’-cyclic monophosphate; ND, not done. *Tissues preconstricted with carbachol (10^{-7} M). †SOD was added to prevent reaction of NO with superoxide. ‡Significantly different from response evoked by SNAP alone (P < 0.05).

To ascertain the degree to which Ca^{2+} influx contributed to this fluorimetric response, canine TSM cells were studied in Ca^{2+}-free medium containing 0.1 mM EGTA. Under these conditions, SNAP still evoked large elevations of [Ca^{2+}], the mean magnitudes of which were not significantly different from control (Fig. 1, C and D). When the internal Ca^{2+} store was depleted using CPA (30 µM; see Refs. 10, 12, and 13), however, the fluorimetric responses to SNAP were markedly and significantly smaller than control (Fig. 1, B and D).

SNAP-induced elevation of [Ca^{2+}] is nonetheless accompanied by relaxation. Elevation of [Ca^{2+}] in smooth muscle is usually thought to result in contraction. We therefore established that SNAP did in fact act as a relaxant in these tissues. In canine TSM preconstricted with carbachol (10^{-7} M), SNAP evoked relaxations at concentrations ranging from 10^{8} to 10^{-4} M (Fig. 2A); these were transient at concentrations less than 10^{-6} M but tended to be sustained at higher concentrations (Fig. 2A). The mean dose-response relationship for SNAP under these control conditions is given in Fig. 2B; the mean EC_{50} was 0.2 ± 0.02 µM (n = 6).

NO acts via an oxidative process. NO can exist as a free radical that acts through oxidation and covalent modification of certain target protein(s) (19). We used the reducing agent dithiothreitol (DTT, 1 mM) to test whether this was involved in the relaxations seen in these airway muscle preparations; SNAP was used as a source of NO (19).

Figure 2C shows a typical SNAP-induced elevation of [Ca^{2+}] as well as the complete reversal of this response on addition of 1 mM DTT. In 11 canine and 7 human cells tested in this way, the SNAP response was reversed 115 ± 9 and 53 ± 17%, respectively, by DTT. In other cells, which had first been pretreated with DTT (1 mM), subsequent addition of SNAP evoked only a small rise in [Ca^{2+}], which was markedly and significantly smaller than the control response (Table 1).

Likewise, in intact tissues pretreated with DTT (1 mM), the SNAP concentration-response relationship was significantly displaced a full log unit to the right, with an EC_{50} of 12 ± 0.3 µM (n = 6). NO acts through stimulation of guanylate cyclase. The electrically neutral free radical form of NO acts through stimulation of guanylate cyclase to generate the second messenger molecule cGMP (26). We tested whether this pathway was also involved using SIN-1 because decomposition of this agent tends to produce NO; unless indicated otherwise, superoxide dismutase was added to prevent reaction of NO with superoxide.
(3 U/ml) was used to prevent reaction of NO· with O2, leading to the more reactive peroxynitrite species.

Surprisingly, SIN-1 (10⁻⁴ M) had no significant effect on [Ca²⁺]i in single cells (Fig. 3A; Table 1). Despite having little effect on [Ca²⁺]i, cholinergic contractions could be fully reversed by SIN-1 in a dose-dependent fashion, with an EC₅₀ of 1.0 ± 0.3 µM (n = 6; Fig. 3B and C). DTT (1 mM) had no statistically significant effect on SIN-1-evoked relaxations; the EC₅₀ for SIN-1 in the presence of DTT was 2.0 ± 0.1 µM (n = 5; Fig. 3C).

Similarly, 8-bromoguanosine 3',5'-cyclic monophosphate (10⁻⁴ M) had very little effect on [Ca²⁺]i but markedly reversed cholinergic tone (Fig. 4, A and B, respectively; Table 1).

SNAP relaxations involve electromechanical coupling. The signaling pathway(s) through which NO mediates its relaxant effect was examined first by comparing cholinergic contractions in the absence and presence of 10⁻⁵ M SNAP; carbachol was applied at concentrations ranging from 10⁻⁹ to 10⁻⁴ M, added in cumulative fashion, because different excitation-contraction coupling mechanisms predominate at different degrees of cholinergic stimulation (6). SNAP was most effective against the lower concentrations of carbachol, where electromechanical coupling mechanisms predominate (6) but had relatively little effect against the higher concentrations of carbachol (Fig. 5).

These observations suggest that NO is acting largely through a mechanism involving electromechanical coupling, that is, triggering membrane hyperpolarization.
leading to closure of voltage-dependent Ca\textsuperscript{2+} channels and subsequent cessation of contraction. To test this, we examined the ability of SNAP to alter contractions evoked by KCl, which acts largely through depolarization and opening of Ca\textsuperscript{2+} channels. Contractile responses in intact tissues exposed to KCl at concentrations ranging from 15 to 60 mM (increased in 15 mM increments) were recorded both before and after exposure to SNAP (10\textsuperscript{-5} M) (Fig. 6A). SNAP markedly and significantly attenuated the contractile response evoked by 15 mM KCl but had much less effect against the higher concentrations of KCl (Fig. 6). These observations are consistent with a mechanism involving SNAP-induced opening of K\textsuperscript{+} channels and rule out the possibilities that NO is acting directly on voltage-dependent Ca\textsuperscript{2+} channels or on the contractile apparatus; however, they do not necessarily rule out the possible additional involvement of other pathways such as enhanced Ca\textsuperscript{2+} uptake in the sarcoplasmic reticulum.

Finally, we examined the sensitivity of SNAP-evoked relaxations to the Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel blockers charybdoctoxin (10\textsuperscript{-7} M) and tetraethylammonium chloride (TEA; 30 mM) as well as to the voltage-dependent K\textsuperscript{+} channel blocker 4-aminopyridine (4-AP; 1 mM). Canine TSM tissues were treated with these agents for 15 min, which tended to lead to elevation of tone, and then further preconstricted with carbachol (10\textsuperscript{-7} M). SNAP (10\textsuperscript{-6} M) evoked substantial relaxations in control tissues as well as in those pretreated with 4-AP, whereas those evoked in tissues pretreated with charybdoctoxin or TEA were markedly and significantly reduced (Fig. 7). The mean magnitude of the relaxant response (expressed as percent reversal of overall tone produced by K\textsuperscript{+} channel blocker plus carbachol) evoked by 10\textsuperscript{-6} M SNAP was 62 ± 13% under control condi-
tions and 68 ± 14% in the presence of 4-AP compared with 25 ± 6 and 20 ± 7% in the presence of charybdotoxin and TEA, respectively (n = 5 animals for all 4 groups). At higher concentrations, SNAP was able to completely reverse tone under all four conditions (Fig. 7).

**DISCUSSION**

Although there already have been several studies of the mechanisms underlying NO-mediated relaxation of airway smooth muscle (see the introduction), we report here for the first time that this effect is accompanied by elevation of [Ca^{2+}] in human and canine airway smooth muscle. Although this finding might be counter-intuitive (elevation of [Ca^{2+}] is usually thought to result in contraction), it has been shown previously in bovine TSM cells that β-adrenergic agonists also elevate [Ca^{2+}] in the peripheral regions of the cytosol but decrease that in the deeper cytosol (24).

Moreover, we have shown that this Ca^{2+} response is mediated by NO but not by NO• or cGMP (which actually cause a net decrease in [Ca^{2+}]) and involves oxidation of some target molecule, leading to membrane hyperpolarization and consequent suppression of voltage-dependent Ca^{2+} influx. Our observation that the SNAP-induced Ca^{2+} release was markedly reduced by CPA but was relatively unaffected by removal of external Ca^{2+} suggests that the source of this Ca^{2+} was the sarcoplasmic reticulum. The cellular target which NO• modifies to produce these changes might include the ryanodine receptor on the sarcoplasmic reticulum, as is the case in canine cardiac muscle (23); the ryanodine receptor in that tissue contains ~84 thiol groups and nitrosylation of up to 12 of these (i.e., 3 thiols on each of the 4 subunits) causes progressive channel activation, which can be reversed using a reducing agent. Xu et al. (23) also showed that oxidation of the ryanodine receptor under certain conditions was not reversible; this might explain why we found that DTT was much less able to reverse the SNAP-induced Ca^{2+} responses in human ASM cells compared with those of the dog.

Our finding that SNAP releases internal Ca^{2+} and thereby elevates [Ca^{2+}] in single canine TSM cells is not inconsistent with a previous report that SIN-1 reduced cholinergically evoked [Ca^{2+}] transients in intact canine TSM strips (14). NO• (produced by SIN-1, acting through cGMP, could promote uptake of Ca^{2+} from the deep cytosol (16), whereas NO• (produced by SNAP) could trigger its release into the subsarcoplasmal space for subsequent extrusion and activation of Ca^{2+}-dependent K+ channels in the plasmalemma. We have shown that Ca^{2+} release through ryanodine-gated channels in canine TSM is preferentially directed toward the plasmalemma (where the Ca^{2+}-dependent K+ channels are found) rather than the deep cytosol (10). This then would explain in part the membrane hyperpolarizing effect of NO in airway smooth muscle (1, 4, 25). This mechanism is also consistent with that proposed to account for vasodilation (18); that is, relaxants act in vascular smooth muscle by triggering localized bursts of Ca^{2+} via ryanodine receptors on the sarcoplasmic reticulum ("Ca^{2+} sparks"), with consequent brief openings of Ca^{2+}-dependent K+ channels on the plasma-membrane (spontaneous transient outward currents). SNAP did not elevate [Ca^{2+}], in porcine TSM cells (16); this may be related to the exposure of those cells to DTT for 2 h (to activate papain) during enzymatic dissociation, or it may indicate a species-related difference.

Thus it may be that relaxants such as NO (and β-agonists) act by increasing [Ca^{2+}] (in the subplasmalemmal space) and altering Ca^{2+}-dependent K+ channel function directly much in the same way that many spasmodens act by both elevating [Ca^{2+}] (in the deep cytosol) and increasing the Ca^{2+} sensitivity of the contractile apparatus.

Our data do not speak against the dogma that NO also evokes relaxation in a cGMP-dependent fashion (4, 14, 25). In fact, the data indicate that NO is acting through more than one pathway. For example, SIN-1 (which produces NO•) and cGMP both triggered relaxation but did not elevate [Ca^{2+}]. Also, SNAP in the presence of DTT (which might reduce NO• to NO) was still able to fully relax the tissues even though the rise in [Ca^{2+}] was prevented. Finally, whereas charybdo-toxin and TEA were effective against low concentrations of SNAP (10−6 M), they were much less effective against the higher concentrations of SNAP used. The parallel contributions of these two distinct pathways would rationalize previously paradoxical findings such as the differential effects of guanylate cyclase inhibitors (methylene blue and hemoglobin) on relaxations triggered by different NO donors (7, 20, 26).

These findings may also shed light on the changes accompanying airway hyperresponsiveness. Our laboratory and several others have been puzzled by the finding that allergen inhalation produces airway hyper-responsiveness, which can be measured in vivo, but that airway tissues excised from these same animals and studied in vitro exhibit hyporesponsiveness (11, 17). Although PGE_{2} contributes to part of this reduced responsiveness, a considerable portion is evidently due to some nonprostanoid factor (11). More recently, NO synthase has been localized to the narrow space between the sarcoplasmic reticulum and the plasmalemma in canine TSM cells (5); this is the same cytosolic region in which we have documented the preferentially directed release of Ca^{2+} toward the plasmalemma rather than toward the deep cytosol (10). It may be that allergen-induced airway inflammation somehow results in altered Ca^{2+} handling, which in turn would alter the activity of NO synthase, generation of NO, and the inhibitory effects described in the present study.

In conclusion, we have shown that in addition to the well-described cGMP-mediated inhibitory actions of NO, there is an additional cGMP-independent pathway, which involves oxidation of some target molecule by NO•, leading to release of internally sequestered Ca^{2+} (3). The cellular target, which is modified by NO• to produce these changes in airway smooth muscle, is as yet unclear. Together with our earlier study of the superficial buffer barrier in airway smooth muscle (10),
these data suggest that this Ca$^{2+}$ flux is preferentially directed toward the plasmalemma, resulting in activation of Ca$^{2+}$-dependent K$^+$ channels and electromechanically coupled inhibition as well as extrusion of Ca$^{2+}$ from the cell by the plasmalemmal Ca$^{2+}$ pump.

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