cGMP-independent mechanism of airway smooth muscle relaxation induced by S-nitrosoglutathione

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Perkins, William J., Christina Pabelick, David O. Warner, and Keith A. Jones. cGMP-independent mechanism of airway smooth muscle relaxation induced by S-nitrosoglutathione. Am. J. Physiol. 275 (Cell Physiol. 44): C468–C474, 1998.—This study tested the hypothesis that the NO donor S-nitrosoglutathione (GSNO) relaxes canine tracheal smooth muscle (CTSM) in part by a cGMP-independent process that involves reversible oxidation of intracellular thiols. GSNO caused a concentration-dependent relaxation in ACh-contracted strips (EC50 = 1.2 µM) accompanied by a concentration-dependent increase in cytosolic cGMP concentration ([cGMP]i). The soluble guanylate cyclase inhibitor methylene blue prevented the increase in [cGMP]i, induced by 1 and 10 µM GSNO, but isometric force decreased by 10 ± 4 and 55 ± 3%, respectively. After recovery of [cGMP]i to baseline, GSNO-induced relaxation persisted during continuous ACh stimulation. Dithiothreitol caused a rapid recovery of isometric force to values similar to those obtained with ACh alone in these strips. We conclude that GSNO relaxes CTSM by reversible oxidation of intracellular protein thiols.

Nitric oxide; canine lung; canine trachea; guanosine 3′,5′-cyclic monophosphate; sulfhydryl reagents; DL-dithiothreitol

Nitric oxide plays a significant role in a number of physiological processes, including regulation of smooth muscle tone, neurotransmission, and platelet function, and pathophysiological states such as sepsis (21). In smooth muscle, NO is thought to mediate relaxation largely by activation of soluble guanylate cyclase (10, 14), which increases levels of cGMP. cGMP subsequently activates cGMP-dependent protein kinases (PKG), which ultimately decrease both the cytosolic calcium concentration ([Ca2+]i) and the amount of isometric force produced by a given [Ca2+]i, i.e., the Ca2+ sensitivity) (28) by phosphorylation of unidentified intracellular proteins (6). Although the role of NO in regulation of airway tone is unclear, airway smooth muscle (ASM) contains soluble guanylate cyclase (14) and is responsive to NO (32) and a variety of NO donors, including nitrovasodilators (34), 3-morpholinosydnonimine (SIN-1) (13), NO-nucleophile adducts (9), and S-nitrosothiols (11, 34).

The mechanisms by which NO donors relax ASM vary. For example, whereas the degree of relaxation of ASM induced by diethylnitrosoflazen-1-ium-2,2-dioxide (DEA-NO) or SIN-1 is correlated with an increase in cytosolic cGMP concentration ([cGMP]), sodium nitroprusside (SNP)-induced relaxation is not and is thus thought to relax ASM by cGMP-independent mechanisms (32, 34). Although little is known regarding these cGMP-independent mechanisms, there are at least two plausible mechanisms by which this could occur, both of which involve intracellular thiol oxidation. The first is S-nitrosylation of protein or other intracellular thiols, a mechanism that has been implicated in the NO-dependent regulation of proteins such as protein kinase C (PKC) (8) and glyceraldehyde-3-phosphate dehydrogenase (5). The second is oxidation of protein thiols without S-nitrosylation (31). The contribution of either of these thiol-related, cGMP-independent mechanisms to smooth muscle relaxation may be determined using a thiol reducing reagent, such as DL-dithiothreitol (DTT), which should reverse thiol oxidation-mediated effects.

S-nitrosothiols such as S-nitrosoglutathione (GSNO) are stable at 37°C and pH 7.4 in the presence of transition metal chelators (27). However, the presence of trace free transition metal ions, such as Cu2+/Cu+, stimulates the catalytic breakdown of GSNO to NO and glutathione disulfide (7, 27). S-nitrosothiols can also undergo nitrosation (NO−) transfer to other intracellular thiols. S-nitrosothiols increase [cGMP]i and cause relaxation of agonist-induced contractions in ASM (11, 32, 34). The extent to which S-nitrosothiol-induced ASM relaxation is contingent on increased [cGMP]i is unknown. The following hypotheses were tested: 1) GSNO relaxes canine tracheal smooth muscle (CTSM) by both cGMP-dependent and cGMP-independent mechanisms and 2) the cGMP-independent mechanism of relaxation is mediated by reversible oxidation of intracellular thiols.

METHODS AND MATERIALS

Experimental Techniques

Tissue preparation. Mongrel dogs (15–23 kg) of either sex were anesthetized with an intravenous injection of pentobarbital (30 mg/kg) and exsanguinated. A 5- to 10-cm portion of extratracheal trachea was excised and immersed in chilled physiological salt solution (PSS) with a composition (in mM) of 110.5 NaCl, 25.7 NaHCO3, 5.6 dextrose, 3.4 KCl, 2.4 CaCl2, 1.2 KH2PO4, and 0.8 MgSO4. Fat, connective tissue, and the epithelium were removed with tissue forceps and scissors.

Mechanical responses. For cyclic nucleotide measurements, CTSM strips (width 2–3 mm, length 1.0–1.5 cm, and weight 25–67 mg) were suspended in 25-ml water-jacketed tissue baths that were filled with PSS (37°C) aerated with 94% O2 and 6% CO2 (pH = 7.4, PaO2 = 150 mm Hg, and PaCO2 = 36 mm Hg in the PSS). One end of the strip was anchored to a metal hook at the bottom of the tissue bath; the other end was attached to a calibrated force transducer (model FT03D, Grass Instrument, Quincy, MA). During a 3-h equilibration period, the strips were repeatedly contracted isometrically for 30 s every 5 min by supramaximal electrical field stimulation (400 mA, 25 Hz, and pulse duration of 0.5 ms). Electrical field stimulation was triggered by stimulator (model S88D, Grass Instrument) and delivered by a direct current amplifier (Section of
been used in this laboratory to demonstrate that NO donor-

ate cyclase inhibitor in this study because it has previously

this equilibration period, the strips were washed with fresh

SSS every 10 min.

Cyclic nucleotide measurements. CTSM strips were homog-

enized in 4 ml of cold (2°C) 95% ethanol using a ground glass

pestle and homogenizing tube. The precipitated pellet was

eparated from the soluble extract by centrifugation at 4,000

g for 10 min. The soluble extract was evaporated to dryness at

\( \sim 55^\circ C \) under a stream of nitrogen and then suspended in 0.3

ml of 4 mM EDTA (pH 7.5). \(^{3}H\)cGMP (0.4 µCi) or \(^{3}H\)cAMP

(1.25 µCi) was added as a tracer for cGMP or cAMP recovery
determinations, respectively. Commercially available RIA

kits were used to determine the concentrations of cGMP and
cAMP in the soluble extract (3). The protein content of the

precipitated pellet was determined by the method described

by Lowry et al. (17), using BSA dissolved in 1 N NaOH as the

standard. [cGMP] and cytosolic cAMP concentration ([cAMP])

were expressed as picomoles per milligram protein.

Experimental Protocols

Five experimental protocols were conducted, each on sepa-

rate sets of CTSM strips. All strips were incubated with 10

µM indomethacin to prevent the formation of prostanoids (12,

34). In previous studies, contraction of CTSM with ACh
during incubation with indomethacin had no effect on [cGMP],
or [cAMP], compared with unstimulated tissue (11).

Concentration-dependent effect of GSNO on isometric force.

For determination of the concentration dependence of GSNO-

induced relaxation, seven strips obtained from a single dog

were contracted with 0.1 µM ACh (approximately equal to the

EC\(_{50}\)) for 15 min, the time required for contractions to

stabilize. Six strips were then exposed to 0.3, 1.0, 3.0, 10, 30,
or 100 µM GSNO for 40 min; the seventh strip was not

exposed to GSNO and served as a control for the effect of time

on the ACh-induced contraction.

Time course for the effects of GSNO and DTT on isometric force, [cGMP], and [cAMP]. Eight strips were contracted with

0.1 µM ACh for 15 min (see Fig. 2A). Two strips were not

stimulated with GSNO and were flash frozen for cyclic nucleotide

measurement after contraction with ACh for 15 min (baseline)

and 75 min (time control for baseline). Five strips were then

contracted with 100 µM GSNO and flash frozen at 30 s, 1, 5, 10,
and 40 min for cyclic nucleotide concentration measure-

ments. To determine the extent to which thiol oxidation was

responsible for the GSNO-mediated relaxation of ACh-

induced contractions, the eighth strip was treated with 100

µM GSNO for 40 min, then was treated with 1 mM DTT for 10

min, and was flash frozen for cyclic nucleotide measurement.

Isometric force was measured continuously up to the time the

muscle strips were flash frozen.

Effect of methylene blue on GSNO-induced relaxation and increase in [cGMP]. To determine the extent to which GSNO-

induced relaxation is contingent on increased [cGMP], strips

were treated with the soluble guanylate cyclase inhibitor

methylene blue. Four pairs of strips from a single dog were

prepared. One strip from each pair was treated with 10 µM

methylene blue for 10 min before the onset of contraction. All

strips were contracted with 0.1 µM ACh for 15 min. Then each

pair of strips was exposed to either 0 (baseline), 1, 10, or 100

µM GSNO for 5 min (time required to reach peak increase in

[cGMP]). Methylene blue was selected as the soluble guany-

late cyclase inhibitor in this study because it has previously

been used in this laboratory to demonstrate that NO donor-

mediated relaxation with DEA-NO and SIN-1is cGMP contin-
gent in this tissue (9, 13). The concentration of methylene

blue used in this study is the highest concentration that does

not cause spontaneous contractions and has no effect on

ACH-induced contractions in this tissue (13).

Effect of GSNO washout on isometric force and [cGMP],

recovery. In the tissue bath experiments described in the first

two protocols, GSNO was present for the duration of the

experiment. In the following two protocols, the use of superfu-
sion wells (for [cGMP], measurements) and of a tissue superfu-
sion chamber (for isometric force measurements) permitted

rapid washout of residual GSNO and determination of whether

GSNO effects on isometric force and [cGMP], persisted after

washout. In addition, this protocol permitted testing whether

GSNO-mediated relaxation persisted after recovery of [cGMP],
to baseline values. Seven strips obtained from a single dog

were placed in superfusion wells (see Fig. 4A). All strips were

first stimulated with 0.1 µM ACh for 15 min. One of these

strips was not treated with GSNO (strip 1, baseline). Two

strips were then superfused with 100 µM GSNO for 1 or 5 min

(strips 2 and 3, respectively), and the remaining four strips

were superfused with 100 µM GSNO for 5 min and then with

0.1 µM ACh only for 1, 3, 5, or 15 min (strips 4–7, respectively)

to washout GSNO. Strips were flash frozen at the times
described for [cGMP] measurements. Isometric force was

measured in a strip obtained from the same animals using

the same protocol. The half time for superfuse exchange

was \( \sim 19 \) s, resulting in \( >99\% \) equilibration with superfuse

within 95 s.

Effect of GSNO and DTT on isometric force. Three strips in

a set were contracted with 0.1 µM ACh for 15 min. Two strips

were then relaxed by addition of 100 µM GSNO to the

superfusate for 5 min, and the third strip continued to be

superfused with ACh alone. Thereafter, GSNO was discon-

tinued for 15 min and 1 mM DTT was added to the superfusate

of one of the GSNO-treated strips to determine whether

reversible thiol oxidation played a role in relaxation that persisted

after washout of GSNO. DTT was added to the strip

superfused with ACh alone at the same time as the GSNO-treated

strip to determine the effect of DTT alone on ACh-induced

contraction. The second GSNO-treated strip was not treated

with DTT for measurement of spontaneous force recovery. In

a separate set of experiments, strips were contracted as

described above and then relaxed by addition of 1, 10, or 100

µM GSNO for 5 min. GSNO was then discontinued for 15 min,

at which time 1 mM DTT was added to the superfusate.

Changes in isometric force induced by GSNO were expressed

as a percent change from the stable ACh-induced responses

before addition of GSNO. For each experiment, a strip from a
different animal was used.

Materials

RIA kits for cGMP and cAMP measurements were pur-

chased from Calbiochem (Arlington Heights, IL). GSNO and

all other drugs and chemicals were purchased from Sigma

Chemical (St. Louis, MO). All drugs and chemicals were

dissolved in distilled water.

Statistical Analysis

Data are expressed as means ± SD; \( n \) represents the

number of dogs. Initial forces of strips contracted with ACh
during incubation with or without methylene blue were

compared by unpaired Student’s t-test. The effects of GSNO

and DTT on isometric force, [cGMP], and [cAMP], were

assessed by repeated measures ANOVA with post hoc analy-
sis using Duncan’s multiple-range test. Concentration-
Isometric Force, [cGMP]i, and [cAMP]i

Time Course for the Effect of GSNO and DTT on Isometric Force (Fig. 1). GSNO to the tissue baths. The EC50 values for GSNO-induced relaxation were 1.2 ± 0.6 µM, consisting of a rapid onset followed by a partial recovery. The sustained relaxation was defined as that remaining 20 min after addition of GSNO to the tissue baths. The EC50 values for GSNO-induced peak and sustained relaxation of ACh-induced contraction were 1.2 ± 0.2 and 1.2 ± 0.6 µM, respectively (Fig. 1B).

Time Course for the Effect of GSNO and DTT on Isometric Force, [cGMP], and [cAMP].

In addition to relaxation (Fig. 2A), 100 µM GSNO significantly increased [cGMP], which was maximal at 5 min but then slowly decreased to sustained levels greater than baseline (Fig. 2B). In contrast, GSNO had no effect on [cAMP], (Fig. 2B). The baseline and time control [cGMP] were not significantly different (0.73 ± 0.14 and 0.69 ± 0.12 pmol/mg protein, respectively). Subsequent addition of 1 mM DTT to strips relaxed with 100 µM GSNO caused a transient decrease in isometric force, followed thereafter by a rapid recovery to 91 ± 6% of the time control isometric force (Fig. 2A). The peak isometric force reduction with 100 µM GSNO was 93 ± 2% and the sustained isometric force reduction was 57 ± 6% (Figs. 1B and 2A). Addition of DTT resulted in recovery of [cGMP], to levels not significantly different from either baseline or time control but had no effect on [cAMP]I (Fig. 2B). The transient decrease in isometric force following addition of DTT was associated with a transient increase in [cGMP], (data not shown). Addition of 1 mM DTT to strips contracted with 0.1 µM ACh and not treated with GSNO had no effect on isometric force (Fig. 2A).

Effect of Methylene Blue on GSNO-Induced Relaxation and Increase in [cGMP].

GSNO caused a concentration-dependent decrease in isometric force and concentration-dependent increase in [cGMP]. Methylene blue had no effect on baseline force or on initial isometric force induced by 0.1 µM ACh. Methylene blue significantly increased the EC50 for GSNO from 2.5 ± 0.7 to 5.2 ± 0.2 µM (Fig. 3B). Methylene blue abolished the increase in [cGMP]I induced by 1 and 10 µM GSNO (Fig. 3A) but only partially inhibited GSNO-induced relaxation; 1 and 10 µM GSNO decreased isometric force by 10 ± 4 and 55 ± 3%, respectively, in the presence of methylene blue (Fig. 3B). Methylene blue significantly attenuated, but did not abolish, the increase in [cGMP]I induced by 100 µM GSNO (Fig. 3A).

RESULTS

Concentration-Dependent Effect of GSNO on Isometric Force

Contractions induced by 0.1 µM ACh were stable within 15 min. GSNO added to the tissue baths decreased isometric force in a concentration-dependent manner (Fig. 1, A and B). GSNO-induced relaxation consisted of a rapid onset followed by a partial recovery with GSNO concentrations >0.3 µM (Fig. 1A). Peak relaxation was attained within 2–5 min, taking longer at lower concentrations. The sustained relaxation was defined as that remaining 20 min after addition of GSNO to the tissue baths. The EC50 values for GSNO-induced peak and sustained relaxation of ACh-induced contraction were 1.2 ± 0.2 and 1.2 ± 0.6 µM, respectively (Fig. 1B).

Time Course for the Effect of GSNO and DTT on Isometric Force, [cGMP], and [cAMP].

In addition to relaxation (Fig. 2A), 100 µM GSNO significantly increased [cGMP], which was maximal at 5 min but then slowly decreased to sustained levels greater than baseline (Fig. 2B). In contrast, GSNO had no effect on [cAMP], (Fig. 2B). The baseline and time control [cGMP] were not significantly different (0.73 ± 0.14 and 0.69 ± 0.12 pmol/mg protein, respectively).

Subsequent addition of 1 mM DTT to strips relaxed with 100 µM GSNO caused a transient decrease in isometric force, followed thereafter by a rapid recovery to 91 ± 6% of the time control isometric force (Fig. 2A). The peak isometric force reduction with 100 µM GSNO was 93 ± 2% and the sustained isometric force reduction was 57 ± 6% (Figs. 1B and 2A). Addition of DTT resulted in recovery of [cGMP], to levels not significantly different from either baseline or time control but had no effect on [cAMP]I (Fig. 2B). The transient decrease in isometric force following addition of DTT was associated with a transient increase in [cGMP], (data not shown). Addition of 1 mM DTT to strips contracted with 0.1 µM ACh and not treated with GSNO had no effect on isometric force (Fig. 2A).

Effect of Methylene Blue on GSNO-Induced Relaxation and Increase in [cGMP].

GSNO caused a concentration-dependent decrease in isometric force and concentration-dependent increase in [cGMP]. Methylene blue had no effect on baseline force or on initial isometric force induced by 0.1 µM ACh. Methylene blue significantly increased the EC50 for GSNO from 2.5 ± 0.7 to 5.2 ± 0.2 µM (Fig. 3B). Methylene blue abolished the increase in [cGMP]I induced by 1 and 10 µM GSNO (Fig. 3A) but only partially inhibited GSNO-induced relaxation; 1 and 10 µM GSNO decreased isometric force by 10 ± 4 and 55 ± 3%, respectively, in the presence of methylene blue (Fig. 3B). Methylene blue significantly attenuated, but did not abolish, the increase in [cGMP]I induced by 100 µM GSNO (Fig. 3A).
Effect of GSNO Washout on Isometric Force and [cGMP], Recovery

Addition of 100 µM GSNO to the superfusate containing 0.1 µM ACh resulted in nearly complete relaxation (Fig. 4A) and a significant increase in [cGMP] (Fig. 4B). After washout of GSNO during continuous superfusion with 0.1 µM ACh, isometric force slowly recovered, with only 45% isometric force recovery after 15 min. By contrast, [cGMP] recovered to baseline values within 5 min of GSNO washout (Fig. 4B).

Effect of GSNO and DTT on Isometric Force

Superfusion with 0.1 µM ACh caused a sustained increase in isometric force (Fig. 5). Subsequent treatment with 100 µM GSNO for 5 min resulted in a 95.3 ± 1.1% reduction in isometric force (n = 11) that only slowly recovered over 15 min (Fig. 5). Addition of DTT after 15-min washout of GSNO resulted in a rapid recovery in isometric force to 85.7 ± 7.5% of that initially induced by ACh. The recovery of isometric force was greater in GSNO-treated strips following exposure to DTT than in strips allowed to spontaneously recover over the same time interval (46.9 ± 3.9 vs. 6.4 ± 1.2%, respectively; P < 0.001). Addition of 1 mM DTT to strips superfused with ACh alone resulted in a 4.6 ± 1.1% reduction in isometric force.

Whereas 1, 10, and 100 µM GSNO each caused a decrease in isometric force, the effects of GSNO washout during continuous superfusion with 0.1 µM ACh varied with GSNO concentration (Fig. 6). Whereas the recovery of isometric force was complete during washout of 1 µM GSNO, the recovery of isometric force during washout of 10 or 100 µM GSNO was not. After 15-min washout of 1, 10, or 100 µM GSNO during continuous superfusion with 0.1 µM ACh, 1 mM DTT caused a transient decrease in isometric force (Fig. 6). In strips treated with 1 µM GSNO, DTT had no effect on isometric force aside from the transient effects described above (Fig. 6A). However, in strips treated with 10 or 100 µM GSNO, DTT caused a rapid recovery of isometric force to that initially induced by 0.1 µM ACh before superfusion with GSNO (Fig. 6, B and C).

DISCUSSION

The major findings of this in vitro study are that during ACh-induced contraction of CTSM, the S-nitrosothiol out of 1 µM GSNO, the recovery of isometric force during washout of 10 or 100 µM GSNO was not. After 15-min washout of 1, 10, or 100 µM GSNO during continuous superfusion with 0.1 µM ACh, 1 mM DTT caused a transient decrease in isometric force (Fig. 6). In strips treated with 1 µM GSNO, DTT had no effects on isometric force aside from the transient effects described above (Fig. 6A). However, in strips treated with 10 or 100 µM GSNO, DTT caused a rapid recovery of isometric force to that initially induced by 0.1 µM ACh before superfusion with GSNO (Fig. 6, B and C).

DISCUSSION

The major findings of this in vitro study are that during ACh-induced contraction of CTSM, the S-nitrosothiol
GSNO caused relaxation mediated by both cGMP-dependent and cGMP-independent mechanisms. The cGMP-independent mechanism involves reversible oxidation of intracellular thiols on proteins that regulate smooth muscle contraction or on the contractile proteins.

The formation and decay of S-nitrosothiols, such as GSNO, may represent a mechanism for the storage or transport of NO (24). According to this hypothesis, S-nitrosothiols are produced in vivo by reaction of NO with a thiol (30) and subsequently diffuse to the site of action. S-nitrosothiols then mediate their effects either by decomposition to yield free NO or by transnitrosylation of specific protein thiols (29, 31). Although the physiological relevance of S-nitrosothiols remains to be established, these agents have been used as NO donors in smooth muscle and have been proposed as therapy for diseases such as hypertension, asthma, and uterine hypertonia. Synthetic S-nitrosothiols relax agonist-induced contraction in both vascular smooth muscle and ASM (11, 15).

In vascular smooth muscle, cGMP appears to mediate the relaxant effects of both NO (23) and S-nitrosothiols, although cGMP-independent mechanisms have not been ruled out (22). The mechanisms by which S-nitrosothiols relax ASM are not fully known. In CTSM contracted with 1 µM methacholine, S-nitroso-N-acetyl-penicillamine (SNAP) caused a relaxation that was accompanied by an increase in [cGMP], (34). The increase in [cGMP], and relaxation were significantly inhibited by methylene blue, suggesting that ASM relaxation induced by SNAP is in part caused by increased [cGMP]. In the same study, methylene blue had no effect on the relaxation induced by the nitrovasodilator SNP. A similar result has been reported with SNP and SIN-1 in porcine ASM (32). On the basis of these results, it has been suggested that some NO donors may relax ASM by mechanisms that do not involve activation of soluble guanylyl cyclase and increases in [cGMP]. Similar observations have been made in vascular smooth muscle (2).

To investigate the relative importance of cGMP in mediating GSNO-induced relaxation of CTSM, we determined 1) whether the relaxation induced by GSNO was accompanied by a concentration- and time-dependent increase in [cGMP], and 2) the effect of methylene blue, a putative soluble guanylate cyclase inhibitor, on both the GSNO-mediated increase in [cGMP], and relaxation. GSNO relaxed CTSM strips that had been contracted with ACh in a concentration-dependent manner, with an EC50 comparable to that reported in guinea pig trachea, ~1 µM (12). The relaxation was accompanied by both a time- and a concentration-dependent increase in [cGMP], and no change in [cAMP], a result consistent with those obtained in CTSM using the NO donors SIN-1 and DEA-NO (9, 13). Although methylene blue attenuated both the amount of relaxation and the increase in [cGMP], induced by GSNO, significant relaxation was observed at 1 and 10 µM GSNO, concentrations at which methylene blue completely inhibited the increase in [cGMP] (Fig. 3). These data indicate that, in general, CTSM relaxation induced by GSNO is mediated in part by both cGMP-dependent and cGMP-independent mechanisms. At approximately the EC50 for GSNO, cGMP-independent mechanisms account for less than one-half of the observed relaxation, but, at higher GSNO concentrations (greater than or equal to the 90% effective concentration), cGMP-independent mechanisms account for the majority of the observed relaxation. The tissues were frozen for determination of cyclic nucleotides at the time peak increases in [cGMP], were observed in the time course studies, making it unlikely that these results were due to the kinetics of cGMP production. Further evidence supporting a role for cGMP-independent relaxation for GSNO was provided by the superfusion chamber experiments, in which washout of 100 µM GSNO resulted in relaxation that persisted after recovery of [cGMP] to baseline (Fig. 4). However, small or highly localized increases in [cGMP], cannot be ruled out by these experiments.

The rate-limiting step in cGMP-mediated relaxation in smooth muscle ultimately involves PKG phosphorylation and phosphatase-mediated dephosphorylation of largely uncharacterized protein targets (28). It is therefore possible that [cGMP], does not perfectly correlate with the degree of relaxation observed in smooth muscle. A direct comparison of the kinetics of [cGMP] vs. PKG target phosphorylation or of dephosphorylation vs. isometric force has not to our knowledge been performed. The rapid recovery of isometric force following administration of NO or NO donors to contracted vascular smooth muscle and CTSM (9), however, suggests that the phosphorylation status of the PKG targets involved in regulating smooth muscle contractile state is relatively fast.

To determine whether reversible thiol oxidation was involved in mediating GSNO-induced relaxation, the ability of the thiol reducing agent DTT to reverse
GSNO-induced relaxation was investigated. In the tissue bath studies, the addition of DTT to strips caused a transient additional relaxation followed by a rapid recovery of both isometric force to time control levels and [cGMP] to baseline. These results may have been due to one of three causes: 1) DTT both stimulated and scavenged NO released from GSNO, thereby decreasing or eliminating NO transport to the heme of soluble guanylate cyclase, 2) DTT served as a competitive source of free thiol in transnitrosation reactions, or 3) DTT reduced reversibly oxidized thiols, possibly nitrosylated by NO from GSNO. However, in superfusion chamber experiments, following GSNO washout and recovery of [cGMP], to baseline, addition of DTT in the absence of GSNO in the tissue bath also caused a rapid recovery of isometric force. The absence of GSNO in the superfusion experiment eliminates the possibility of a direct interaction between DTT and GSNO. This rules out the first two possible causes described above and indicates that the cGMP-independent, DTT-reversible component of GSNO-mediated relaxation involves reversible oxidation of thiols.

In the current study, addition of DTT to ACh-contracted strips that had not been relaxed by GSNO had only a small effect on isometric force. In contrast, addition of DTT to ACh-contracted strips following washout of 1 µM GSNO and complete recovery of isometric force caused a transient, much greater relaxation. Similar transient effects were observed following washout of 10 and 100 µM GSNO. Two plausible explanations for these observations are 1) that there is residual GSNO in the cell even though it has been completely removed from the superfusion chamber and 2) that NO from GSNO is stored in the tissue at functionally silent sites, possibly protein thiols, and is released via thiol-mediated release of NO from S-nitrosothiols in the presence of metal ions. The finding that [cGMP] had completely recovered to baseline following washout of GSNO suggests that the first possible explanation is unlikely. However, conclusive distinction between the two possibilities is beyond the scope of the current investigation.

The specific proteins that underwent thiol oxidation by GSNO are unknown but may include proteins involved in the PKG pathway, the contractile proteins (actin or myosin), proteins involved in the regulation of [Ca^{2+}], or proteins that regulate the amount of isometric force produced for a given [Ca^{2+}], such as smooth muscle myosin light chain kinase or phosphatase. There are several recent examples of NO donor-induced reversible thiol oxidation of intracellular proteins, with resultant changes in the activity of the protein, including PKC (8), creatine kinase (33), and the ryanodine receptor Ca^{2+}-release channel (1, 20). The common theme in each of these instances is that the protein targets have reactive thiols that are critical for their activity. In each of the examples cited, an NO donor decreased activity and the effect was reversed by treatment with a thiol-reducing agent such as DTT. The NO donor may modify the protein either by S-nitrosylation or by driving the formation of an intramolecular disulfide between vicinal thiols, as reported for the N-methyl-D-aspartate receptor (16). An attractive potential protein target that could explain the results is myosin. The smooth muscle myosin head contains reactive cysteine thiols, which, when oxidized or covalently modified, inhibit myosin ATPase activity (4). The NO donor SNP inhibits the actomyosin ATPase in rabbit psoas single fibers, an effect that is also reversible with the thiol reducing agent DTT (26). Such a modification would decrease isometric force independently of [Ca^{2+}]. Yet another possible molecular target is the smooth muscle myosin light chain kinase, a protein that is critical in inducing force development and that contains multiple cysteines (25). The ultimate molecular targets of GSNO in CTSM are, however, unclear at this time.

Another potential cGMP-independent GSNO effect would be to decrease ACh activation of muscarinic receptors, thereby decreasing the amount of isometric force developed at a given ACh concentration. This possibility was tested by determining the effect of GSNO on contractions induced by membrane depolarization with isotonic KCl, which does not result in activation of muscarinic receptors. As with ACh-contracted CTSM, GSNO resulted in a rapid reduction in KCl-induced contraction, an effect that persisted following washout of the NO donor (Fig. 7). It is thus unlikely that the cGMP-independent relaxation observed in CTSM is due to decreased ACh activation of muscarinic receptors.

In summary, the results of this study demonstrate that GSNO relaxes CTSM contracted by ACh. This
results indicate a previously undescribed mechanism of relaxation involves reversible oxidation of thiols on proteins that regulate smooth muscle contraction or on the contractile proteins, such as the myosin head. The results indicate a previously undescribed mechanism for regulation of CTSM contraction that requires further investigation.

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