Inhibition of Sphincter of Oddi Function by the Nitric Oxide Carrier S-nitroso-N-acetylcysteine in Rabbits and Humans

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Abstract

Nitric oxide (NO) is an inhibitor of gastrointestinal smooth muscle. Model systems of the gut predict that NO will complex with biological thiol (SH) groups, yielding S-nitrosothiols (RS-NO), which may limit the propensity to form mutagenic nitrosamines. The inhibitory effects of NO and its biologically relevant adducts on sphincter of Oddi (SO) motility have been inferred from animal studies; however, their importance in regulating human SO is not known. The objectives of this study were to (a) provide histologic confirmation of nitric oxide synthase (NOS) in human SO; (b) characterize the pharmacology of S-nitroso-N-acetylcysteine (SNAC), an exemplary S-nitrothiol, on SO motility in a rabbit model; and (c) study the effects of topical SNAC on SO motility in humans.

Immunocytochemical and histochemical identification of NOS was performed in human SO. The pharmacologic response of SNAC was defined in isolated rabbit SO using a standard bioassay. Topical SNAC was then applied to the duodenal papilla in patients undergoing endoscopic retrograde cholangiopancreatography (ERCP) and biliary manometry.

NOS was localized to nerve fibers and bundles of the SO in rabbits and humans. SNAC inhibited spontaneous motility (frequency and amplitude) as well as acetylcholine-induced elevations in SO basal pressure in the rabbit model. In patients undergoing ERCP and biliary manometry, topical SNAC inhibited SO contraction frequency, basal pressure, and duodenal motility.

NOS is localized to neural elements in human SO, implicating a role for NO in regulating SO function. Supporting this concept, SNAC is an inhibitor of SO and duodenal motility when applied topically to humans during ERCP. Our data support a novel clinical approach using local NO donors to control gastrointestinal motility and regulate sphincteric function. (J. Clin. Invest. 1994. 94:1792–1798.) Key words: nitric oxide • S-nitroso-N-acetylcysteine • nitrothiol • endoscopic retrograde cholangiopancreatography • sphincter of Oddi

Introduction

Nitric oxide (NO)1 and derivatives thereof have been demonstrated to be potent inhibitors of gastrointestinal smooth muscle (1–5). Nitric oxide synthase (NOS), the enzyme responsible for generating NO via the oxidation of the guanidino nitrogen of L-arginine, has been localized to a population of neurons in the myenteric plexus of rodent small bowel and in the myenteric and mucosal ganglia of rodent and canine colon (6–9). NO has been proposed as the inhibitory transmitter responsible for nonadrenergic noncholinergic (NANC) neural activity in gastrointestinal smooth muscle (10–18).

The high reactivity of NO with a variety of biologically relevant molecules (19–23) and its resulting short half-life under physiologic conditions have lead several groups to postulate that NO-like activity may be stabilized in vivo (20–23). In particular, S-nitrosothiols (RS-NO), formed from the nitrosation of SH groups of low molecular weight thiols and proteins, are a class of relatively stable NO adducts that form under physiologic conditions and retain smooth muscle inhibitory activity in gastrointestinal (GI) and non-GI tissue (3, 13, 20–25). Moreover, the formation of RS-NO in the GI tract has been suggested to represent a protective mechanism that limits the generation of mutagenic nitrosamines (19). Indeed, experiments in model systems reproducing conditions found in the gut indicate that a significant portion of NO bioactivity may exist as RS-NO (26). S-nitroso-N-acetylcysteine (SNAC) is a well characterized model S-nitrothiol that is particularly stable under physiologic conditions and has documented smooth muscle relaxant properties (22, 23, 25); its thiol component, N-acetylcysteine, is used clinically as a mucolytic agent and as treatment for acetylaminophen toxicity.

There have been no studies localizing NOS in sphincter of Oddi (SO) and few studies exploring the effect of NO on SO function. A neuronal (constitutive; nc-NOS) NOS has been demonstrated in guinea pig SO homogenates, inhibition of which results in enhanced resting and stimulated tonic and phasic activity, as well as attenuation in field stimulation–induced SO relaxation (27–29). Kaufman et al. (30) administered systemic N-nitro-L-arginine methyl ester (an analogue of L-arginine that specifically inhibits NOS) intravenously to prairie dogs and demonstrated elevated sphincter of Oddi and duodenal motility.
indices as measured by in situ manometry. Baker et al. (29) identified neuronal cell bodies and nerve fibers with positive diaphorase staining, indicative of NOS activity, in circular strips of oppossum SO. These investigators found that N-nitro-L-arginine methyl ester inhibited electrical field stimulation--induced relaxations and that this inhibition was attenuated by L-arginine and oxyhemoglobin (an NO scavenger) and mimicked by sodium nitroprusside (an NO donor). These studies support the concept that endogenous NO acts as an inhibitory modulator of resting SO tone and duodenal motility in animals.

In this study, NOS, the source of endogenous NO generation, was localized to neural elements in the submucosa of human and rabbit SO, using histochemical and immunohistochemical techniques. These findings, suggesting important functions for NO, were extended to an ex vivo rabbit SO preparation in which the pharmacological response of SNAC exhibited potent inhibitory properties. Because inhibition of motor activity in the duodenum and SO are therapeutic goals during endoscopic retrograde cholangiopancreatography (ERCP), we postulated that topical SNAC might be an effective agent in facilitating ERCP via direct action on the ampulla and periampullary duodenum. SNAC was applied locally to the ampulla and peri-ampullary duodenal mucosa of patients undergoing ERCP and biliary manometry and was shown to both inhibit duodenal motility and lower sphincter of Oddi contraction frequency and basal pressure. These findings demonstrate that NO is an inhibitor of sphincter of Oddi function in humans and provide the basis for the development of novel, topically active agents to control intestinal motility and sphincteric functions.

**Methods**

**Histochemical and immunocytochemical localization**

Rabbit sphincter of Oddi were isolated as described below. A human sphincter of Oddi was obtained at surgery for nonmalignant disease under a "discarded materials" protocol approved by the Institutional Committee on Human Subjects and Research. The tissue was snap frozen, and cryostat sections (8 μm) were prepared for immuno or histochemical staining.

**NADPH diaphorase staining.** Sections were fixed in fresh 2% buffered paraformaldehyde for 10 min and rinsed with phosphate-buffered saline (PBS). The slides were then covered with a reaction mixture consisting of 0.25 mg/ml nitroblue tetrazolium, 1 mg/ml NADPH, and 0.5% Triton X-100 in 0.1 M Tris buffer, pH 7.6. Preliminary experiments indicated that 1 h provided optimal staining, and this duration of incubation was used in subsequent experiments reported below. After washing in PBS, sections were covered with glycerol mounting medium and coverslips were applied. Controls included omission of substrate and inclusion of 15 mM p-nitrophenylphosphate to inhibit endogenous phosphatases that can convert NADPH to NADH and cause false positive staining by NADH dehydrogenases (31).

**NOS immunolocalization.** Polyclonal rabbit anti-nc-NOS (neuronal) was used after affinity purification as previously described (6). Immunohistochemistry was performed on human tissue only, using a standard avidin solution immunoperoxidase procedure (32) followed by hematoxylin counterstaining. Controls included use of buffer alone or dilutions of nonspecific purified rabbit IgG in the primary layer.

**Isolation of rabbit sphincter of Oddi**

Male New Zealand White rabbits (3–3.5 lb; Milbrook Farms, Amherst, MA) were anesthetized with intravenous pentobarbital after cannulation of a marginal ear vein. After deep anesthesia was obtained, the abdomen was opened at the midline, and the gastric antrum and contiguous duodenum were isolated, removed en block, and transferred to ice-cold oxygenated Krebs buffer at pH 7.4. The Krebs buffer consisted of (mM): NaCl 118, NaHCO3 25, dextrose 11, KCl 4.7, CaCl2 2.5, MgSO4 1.2, and KH2PO4 1.2. The duodenum was opened along the antimesenteric border as was the adjacent pylorus and gastric antrum. From the luminal surface, the ampulla of Valter was identified, and the SO was isolated on ice under a microscope by sharp dissection.

**Preparation of SNAC**

SNAC was prepared at 25°C by reacting equimolar (200 mM) concentrations of N-acetylcysteine (NAC) (Sigma Chemical Co., St. Louis, MO) diluted in 0.5 N HCl with sodium nitrate (NaNO3) (Fisher Scientific, Fairhaven, NJ), diluted in deionized H2O. The reaction between NaN3 and NAC under acidic conditions is stoichiometric and essentially complete as confirmed by the method of Saville (22, 33–36). The product SNAC demonstrated a reddish hue with absorption maxima at 335 and 554 nm (22). For animal studies, stock solutions (100 mM) of SNAC were freshly prepared for each experiment, and dilutions were made into 10 cm3 of warm oxygenated Krebs buffer in organ baths.

For human studies, freshly prepared stock solutions of SNAC were passed across a 0.45-μm Millex filter (Millipore Corp., Bedford, MA) and diluted 1:1,000 into sterile saline for a final concentration of 100 μM. The final concentration was confirmed by both spectroscopic analysis and by the method of Saville. Stability of 100 μM SNAC in saline was monitored spectrophotometrically by absorption at 335 nm and demonstrated no appreciable decay over 3 h.

**Ex vivo bioassay**

Each isolated rabbit SO was cannulated in a retrograde fashion sequen- tially with a triangular stirrup and a fixed bar of a ring-type tissue mount and connected to a force-displacement transducer (Grass Instrument Co., Quincy, MA). The tissue was suspended in 10 cm3 of continuously oxygenated (95% O2, 5% CO2) Krebs buffer at 37°C. After 60 min of equilibration under a load of 100 mg, the load was increased to 250 mg, and circular contractions were recorded as changes in isometric force on a polygraph (model 7E; Grass Instrument Co.). Varying concentrations of SNAC were added to the organ bath, and the effects on unstimulated SO contraction frequency and amplitude were monitored. Acetylcholine (ACH; Sigma Chemical Co.) was prepared as a 1 M stock solution in deionized H2O and stored frozen. Varying concentrations of ACH were added to organ baths, and effects on SO basal pressure were monitored. In some experiments, SO were preincubated with varying concentrations of SNAC, and the effects of SNAC on ACH-induced basal pressure increases were monitored. After each experimental observation was complete, the sphincters were washed at least twice with 10 cm3 of fresh Krebs buffer and allowed to equilibrate for at least 20 min.

**Human studies**

After obtaining institutional approval and an Investigational New Drug Application from the Food and Drug Administration, five patients who were previously scheduled to undergo diagnostic ERCP were enrolled in a protocol following informed consent. Patients taking medications known to affect SO manometry were excluded, and sedation was achieved using intravenous midazolam only. After duodenal intubation with an Olympus EVIS 100 video duodenoscope, the major duodenal papilla was identified and cannulated, without the use of glucagon, with a 200-cm-long 1.8-mm–outer diameter triple-lumen catheter, the distal ports of which are spaced 10 mm apart (Arndorfer Medical Specialties, Greendale, WI). The position of the catheter in the common bile duct was verified fluoroscopically and by aspiration of bile. SO manometry was obtained using standard techniques perfusing each lumen at 0.25 ml/min using a low compliance hydraulic capillary infusion system (Arndorfer Medical Specialties) equipped with strain-gauge transducers (Hewlett-Packard Co., Burlington, MA). Tracings obtained in all three channels were displayed on a dynograph recorder (Sensor Medics, Anaheim, CA) as the catheter was slowly withdrawn after deep cannulation.
(station pull-through) (37). The position of the catheter was stationed at a point where the middle perfusion port was in the sphincteric zone. From this position, the proximal port lay in the duodenal lumen, immediately adjacent to the papilla. The proximal port was then disconnected from the recorder, and, after a stable baseline was obtained, 10 ml 0.5 N HCl diluted 1:1,000 in normal saline was infused over 20 s as a control. Recordings were taken for 2-4 min, after which 10 cm³ 10⁻⁴ M SNAC was infused over 20 s, and recordings continued for up to 10 min. The length of recording varied depending on patient cooperation and the ability to maintain the catheter fixed in position. Subjective assessments of duodenal motility were monitored on video during the procedure. In one patient in whom deep cannulation with the manometry catheter could not be obtained, duodenal motility was recorded before and after administration of 10 ml 10⁻⁴ M SNAC by advancing the perfusion catheter more distally in the duodenum and withdrawing the duodenoscope into the stomach. Patients' heart rate, blood pressure, and percent oxygen saturation were monitored noninvasively (Propaq; Protocol Systems, Inc., Beaverton, OR) throughout the procedure. Following the protocol, diagnostic ERCP was performed.

Statistics
SO contraction frequency and basal pressure before and after SNAC were compared using the paired t test.

Results

Histochemical and immunocytochemical studies. Immunohistochemical labeling of cryostat sections of human sphincter of Oddi with anti-neuronal (type I)-NOS demonstrated abundant nc-NOS antigen in nerve fibers and bundles interspersed among the muscle comprising the sphincter (Fig. 1, a and b). Positive staining fibers with the sphincter smooth muscle support localization of NOS within nerve elements in the rabbit sphincter. Similar results were seen using histochemical staining of human and rabbit SO using NADPH dehydrogenase activity (not shown). When human sphincter of Oddi was stained with antibodies against the inducible (type II) isoform of NOS, no positive staining was seen.

Ex vivo bioassay. When equilibrated in oxygenated Krebs at 37°C and loaded with 100 mg tension, isolated rabbit SO demonstrate intrinsic rhythmic contractility. After increasing the load to 250 mg, the average contraction frequency and amplitude were 12.9±0.9/min and 180.8±33.4 mg (mean±SEM, n = 12), respectively. SNAC inhibited both contraction frequency and amplitude in a concentration-dependent fashion (Fig. 2). The effects of SNAC were noted within seconds and persisted for up to 1 h (10⁻⁴ M SNAC). Data were obtained for the 60-s interval starting 30 s after the addition of SNAC.

Addition of ACH resulted in a concentration-dependent increase in basal pressure (Fig. 3). This effect was noted within seconds and decayed over 5 min. Preincubation (2 min) with SNAC resulted in a concentration-dependent inhibition of ACH (10⁻⁴ M) induced elevations in basal pressure (Fig. 4). The addition of SNAC during peak elevations in basal pressure induced by ACH resulted in abrupt and concentration-dependent decreases in pressure (data not shown). Longer periods of preincubation did not result in increased inhibition by SNAC. The addition of NAC or NaNO₂ alone at concentrations up to 10⁻³ M had no effect on SO contraction frequency and amplitude or 10⁻⁴ M ACH-induced elevations in basal pressure (data not shown).

Human studies. Sphincter of Oddi mean contraction fre-
concentration-dependent

Figure 3. Elevation in isolated rabbit sphincter of Oddi basal pressure by acetylcholine. Values are presented as mean±SEM of n = 4 experiments during which peak elevations in basal pressure were recorded. Acetylcholine induces concentration-dependent increases in basal pressure that are statistically significant (P < 0.01) by ANOVA.

Figure 4. Effect of SNAC on 10^{-5} M acetylcholine-induced elevations in isolated rabbit sphincter of Oddi. Values are presented as mean±SEM for four experiments. SNAC was preincubated 2 min before acetylcholine, and then peak elevations in basal pressure were recorded. The concentration-dependent inhibition by SNAC is statistically significant (P < 0.002) by ANOVA.

Figure 5. Inhibition of sphincters of Oddi contraction frequency (top) and basal pressure (bottom) in patients undergoing ERCP and sphincter of Oddi manometry. X are the mean±SEM of data recorded every minute for 4 min, before administration of SNAC (Pre-SNAC), and up to 10 min after SNAC (Post-SNAC) (n = 5). X_max are mean±SEM of maximal effects seen after SNAC (n = 5). *P = 0.08; †P = 0.05; ‡P = 0.001 by paired t test.

Frequency and basal pressure were calculated every 60 s for each patient. The recorder used in this study monitors analogue data only, and the sensitivity was selected to optimize recording basal pressure. Some peak contraction amplitudes went off scale, and thus, mean data for contraction amplitude were not obtained. After control infusion, the average SO contraction frequency and basal pressure were 2.45/min and 22.5 mmHg, respectively. After infusion of SNAC, these values decreased to 1.58/min and 16.96 mmHg (P = 0.05 and P = 0.08, respectively) (Fig. 5). Because the time to peak inhibition of both contraction frequency and basal pressure by SNAC varied among patients from 1 to 5 min, comparisons were also made (Fig. 5) between controls and average peak inhibition (X_max) of concentration frequency (0.8 min, P = 0.001) and basal pressure (12.5/mmHg, P ≤ 0.05). An apparent marked inhibition of duodenal motility was also noted on video within 30 s of SNAC infusion in all patients. In one patient whose ampulla could not be cannulated, duodenal motility was monitored manometrically after 10 cm3 sham infusion of 10^{-4} M SNAC. The pronounced decrease in duodenal motility after SNAC administration is shown in Fig. 6.

Heart rate, blood pressure, and percent oxygen saturation varied during the procedure in relation to the level of sedation and dosage of midazolam administered. No changes were noted after SNAC administration, no significant hemodynamic or heart rate changes were observed, and all patients tolerated the procedure well. On follow-up, no complications or unusual symptoms were reported.

Discussion

This study is the first to demonstrate the presence of nc-NOS activity in nerve fibers that course through the submucosa of
both human and rabbit SO, providing localization for the source of endogenous NO generation in this organ. To define further the role of NO in regulation of SO function, the pharmacologic response of the exogenous NO donor SNAC was explored in an ex vivo rabbit model and demonstrated potent inhibition of SO motility. As a corollary to these findings, SNAC was administered topically to humans during ERCP; SNAC inhibited duodenal motility and significantly lowered SO basal pressure and contraction frequency. Thus, this first in vivo application of an S-nitrosothiol to examine physiologic responses in humans provides the groundwork for the development of locally administered agents to modulate gastrointestinal function and assist during endoscopic procedures.

The recent interest and characterization of S-nitrosothiols stems from three broad areas of research, namely the mechanism of action of the NO-generating vasodilators, (i.e., glyceryl trinitrate [TNG], sodium nitroprusside, and isosorbide dinitrate), the identification of the endogenous vasodilator endothelium-derived relaxing factor, and the paradoxical servoregulatory and cytotoxic properties of NO. As a result of previous studies, it appears that the active metabolite of the nitrogen-containing vasodilators is NO or RS-NO and that endothelium-derived relaxing factor has similar properties (38–40). NO, or a closely related compound, then activates guanylyl cyclase, resulting in elevation of cellular cyclic GMP, an inhibitor of smooth muscle contraction (41–43). At the same time, NO and related donors may exhibit considerable toxicity, particularly in settings of oxidant stress and in acidic environments as found in the gut (44, 45). In this context it has been suggested that the reaction of NO, with tissue sulfhydryl groups may serve to limit formation of mutagenic nitrosamines as well as potential deleterious reaction of NO with reactive oxygen intermediates (19, 44). Indeed, S-nitrosothiols have been identified in human airways and in vivo in states predisposing to O_2-dependent toxicity (44, 45), and a much earlier literature strongly suggests that they form in the GI tract (26, 45).

Of interest, sodium nitroprusside has been used in animal studies to obliterate SO motility, although its mechanism of action has not been addressed (46). In humans, systemically administered TNG has been demonstrated to lower SO basal pressure and contraction amplitude measured manometrically in patients undergoing ERCP, and gallstones have been removed from an intact papilla after TNG-induced “chemical sphincterotomy” (47–49). In these studies, TNG did not affect SO contraction frequency, and effects on duodenal motility were not evaluated. Moreover, the therapeutic value of TNG may be compromised by systemic hypotension. In our study, we saw a significant inhibition in both SO basal pressure and contraction frequency. Owing to technical limitations, contraction amplitude was not quantitated. It is likely that the inhibition in contraction frequency seen with topical SNAC and the absence of such inhibition with systemic TNG relate to the mode of application and location of NO delivery, as both drugs are postulated to inhibit smooth muscle via NO-mediated mechanisms. Animal studies suggest that the SO “pacemaker” may be located at or near the duodenal luminal surface and that interdigestive intestinal and biliary motility are temporally linked by the migrating myoelectric complex (50–52). These observations notwithstanding, it remains to be shown whether local instillation of SNAC over the duodenal luminal surface of the papilla represents the optimal modality for inhibiting SO basal pressure. The modest decrease in basal pressure seen might be enhanced by direct instillation into the ampullary orifice. In addition, improving the efficacy of NO delivery by altering the composition of the administered solution (i.e., pH) or the carrier thiol, may result in improved smooth muscle relaxation.

The onset of SNAC-mediated inhibition of SO motility was within 5 s in the ex vivo animal model. In the human studies, the apparent inhibition of duodenal motility was seen within 30 s of SNAC administration while the onset of SO motility inhibition was variable among patients. The delay and variation seen in vivo likely relate to variability in tissue penetration and NO delivery by SNAC. Clearly, S-nitrosothiols can liberate NO by a nonenzymatic process; however, the kinetics of spontaneous NO production by S-nitrosothiols does not correlate with their potency for smooth muscle relaxation in either blood vessels or airways (22, 23). It is more likely that metabolism of RS-NO involves transfer of the NO moiety (as NO^+) to thiol functionalities on the cell surface (23, 53, 54). This reaction would be critically influenced by endogenous redox conditions and pH in the proximity of the mucosa (55), potentially contributing to the observed patient variability. Further experiments aimed at elucidating these mechanisms are ongoing.

When administered intravenously to rabbits, SNAC results in a profound decrease in systemic blood pressure within seconds; this hypotensive effect lasts several minutes (51). In superfusion studies using isolated GI and non-GI muscle, the duration of action of S-nitrosothiols is on the order of minutes (3, 25). In our animal studies, the inhibitory effects of SNAC were present at 1 h, and over 90% of the initial concentration of SNAC, as measured by the method of Saville, remained nonmetabolized in the bath (data not shown). SO motility returned within minutes of washing the tissue with fresh Krebs buffer, suggesting that the rate-limiting step in our model is delivery of NO (or a related redox form) to the smooth muscle cells. In human studies, manometric data were obtained only up to 10 min, as recordings beyond this time point were technically limited, and, thus, data on duration of action were not obtained.

**Figure 6.** Inhibition of duodenal motility in a patient undergoing ERCP by topical SNAC. The biliary manometry catheter was placed in the duodenum. Motility was recorded with the duodenoscope in the stomach after infusion of NaCl with 0.5 mM HCl (sham) and 10^-4 M SNAC. Diminished contractions can be seen 1 min after SNAC infusion.
The inhibition of duodenal motility, assessed visually and on video, persisted for up to 10 min, and again no comment can be made on duration of action.

In summary, this study provides the first localization of NOS activity in the sphincter of Oddi of humans. Consistent with a role for endogenous NO or some biologically relevant adduct on SO function, SNAC, an exogenous NO-containing compound was demonstrated to be a potent inhibitor of SO motility in an ex vivo rabbit model. Based on these observations, SNAC was administered topically to the ampulla and periampullary duodenal mucosa of humans undergoing ERCP and demonstrated a significant inhibition of SO contraction frequency and basal pressure, as well as an apparent marked inhibition in duodenal motility. Topical SNAC offers advantages over systemic nitrates and other anti-motility agents in that NO delivery can be targeted to the specific organ of interest, duodenal motility is simultaneously inhibited thus facilitating SO cannulation, and systemic effects (i.e., hypotension) can be minimized. Simultaneous relaxation of both duodenal and SO motility makes SNAC an ideal agent to assist ERCP. Studies aimed at the role of endogenous S-nitrosothiols in the regulation of SO motor function are in progress.

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