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Reduction of the Secretory Response to *Escherichia coli* Heat-Stable Enterotoxin by Thiol and Disulfide Compounds

RICHARD N. GREENBERG,[1]* JOHN A. DUNN,[1] AND RICHARD L. GUERRANT[2]

Division of Infectious Diseases, Department of Internal Medicine, St. Louis University School of Medicine, St. Louis, Missouri 63104,[1] and Divisions of Geographic Medicine and Infectious Diseases, Department of Internal Medicine, University of Virginia, Charlottesville, Virginia 22908[2]

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We examined the effects of disulfide and thiol compounds on *Escherichia coli* heat-stable enterotoxin (ST) and cyclic GMP-induced secretion. Both cystamine and cystine (disulfide compounds) reduced the secretory responses to submaximal doses of ST in suckling mice (at 0.5 μmol per mouse) and reduced ST activation of guanylate cyclase (by 33 to 73% at 1 mM). In higher doses, cystamine completely eradicated a maximally effective ST dose as well. In addition, the sulfhydryl (thiol) compounds cysteamine, cysteine, and acetylcysteine strikingly reduced the secretory response and the guanylate cyclase response to ST. Neither the disulfide nor the thiol compounds tested reduced cyclic GMP-induced secretion. These studies suggest that disulfide and thiol compounds both block ST-induced secretion before its activation of guanylate cyclase. Taken with the work of others, these findings suggest that disulfide compounds may alter the oxidation reduction state of a cell or act directly on the guanylate cyclase enzyme, whereas thiol compounds may inactivate ST itself by breaking its disulfide bridges, or it may alter guanylate cyclase activation by ST. Both families of compounds deserve further consideration among potential antisecretory agents for application in the control of ST-induced diarrhea.

The morbidity and mortality associated with *Escherichia coli* enterotoxigenic diarrheal illness should be reduced by pharmacological inhibition of the action of *E. coli* enterotoxins (both heat-stable [ST] and heat-labile enterotoxins). Methanol-soluble ST, active in suckling mice, specifically activates the particulate fraction of guanylate cyclase in intestinal mucosal cells (14, 23; J. M. Hughes, F. Murad, and R. L. Guerrant, Clin. Res. **26**:524A, 1978), resulting in increased mucosal cell cyclic GMP (cGMP) levels and a net increase in intestinal fluid secretion (26). A second *E. coli* ST (ST_b) has recently been described. ST_b is methanol insoluble, inactive in suckling mice, and has a mechanism of action which does not increase intestinal mucosal cell cGMP (7, 24; R. N. Greenberg, D. J. Kennedy, A. H. Stephenson, A. J. Lonigro, F. Murad, and R. L. Guerrant, Clin. Res. **30**:367A, 1982). *E. coli* heat-labile enterotoxin has a mechanism of action similar to that of choleratoxin (8, 12, 21, 22). In this study, we examined a possible pharmacological blockade of intestinal secretion induced by the suckling mouse-active ST.

Although the precise regulation of guanylate cyclase activity and cGMP accumulation in vivo are unknown, several studies suggest that free radicals activate guanylate cyclase and that guanylate cyclase activity is influenced by the oxidation reduction state of the cell and by intracellular levels of thiols and disulfides (5, 6, 9, 29, 32, 33, 40). Butylated hydroxyanisole, a free radical scavenger, significantly but incompletely reduces ST activation of guanylate cyclase and ST-induced intestinal fluid secretion (23). Hydroxylamine, an agent that activates guanylate cyclase by the production of free radicals (30), also causes intestinal ion transport alterations similar to those seen with ST (13). Recently, Brandwein et al. demonstrated that the disulfide compound cystamine could almost completely inactivate a highly purified preparation of guanylate cyclase (5). These findings may relate to the mechanism of action of ST. An alternative explanation is that thiols or disulfides might alter the binding of ST to its receptor or alter subsequent ST activation of particulate guanylate cyclase. Staples et al. have reported that their ST preparation lost its activity on treatment with thiol-reducing agents (37).

In this study, we evaluated the effect of cystamine and related sulfhydryl and disulfide compounds on (i) ST-induced intestinal fluid secretion, (ii) ST activation of guanylate cyclase, and (iii) 8-bromo cGMP-induced intestinal fluid secretion. These studies will show whether any of

these compounds block ST effects, where the block occurs (i.e., before or after activation of guanylate cyclase), and whether any of these agents warrants further investigation as pharmacological inhibitors of ST.

(These findings were presented in part at the annual meeting of the American Society for Microbiology, Atlanta, Ga., March 1982 [R. N. Greenberg, L. Halterman, and R. L. Guerrant, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B136, p. 40].

MATERIALS AND METHODS

Tissue preparation for guanylate cyclase assay. Sprague-Dawley rats (weighing 200 to 300 g each) were sacrificed by decapitation. The lower half of the small bowel was removed and rinsed with ice-cold saline. The small intestinal tract was everted to allow scraping of the mucosa. Mucosa was homogenized at 4°C in 0.25 M sucrose containing 50 mM Tris-hydrochloride (pH 7.9), 1 mM EDTA, and 1 mM dithiothreitol. Homogenates were centrifuged at 105,000 × *g* at 4°C for 1 h. Particulate fractions were stored at −60°C and used within 1 month.

Guanylate cyclase assay. Guanylate cyclase activity was determined as previously described (18). The cGMP formed was measured by radioimmunoassay with acetylation of samples (New England Nuclear Corp., Boston, Mass.) (5, 25). Protein was solubilized in 1 N NaOH and quantitated by the method of Lowry et al. (31) with bovine serum albumin as the standard.

Suckling mouse assay. The suckling mouse assay was performed as previously described (19). Test solutions were prepared in either 50 mM Tris-hydrochloride (pH 7.6) or water. The pH of all test solutions before the addition of ST was between 7 and 8. The ratio of intestinal weight to remaining body weight was determined after 3 h (10, 16). In some instances, 0.05 ml of test agent was given subcutaneously (s.c.) before or at the time of an intragastric (i.g.) injection of ST or 8-bromo cGMP. The osmolality of the i.g. solutions given to the mice ranged from 15 to 202 mosmol/liter. Solutions made in water ranged in osmolality from 15 to 50 mosmol/liter (water only or with maximum test amount of agents in water, respectively). Solutions made in Tris-hydrochloride buffer ranged in osmolality from 63 to 202 mosmol/liter (Tris buffer only or with maximum test amount of agents in buffer, respectively).

ST preparations. A semipurified ST preparation was prepared as described previously (20). A more purified ST made from the same material was used in the guanylate cyclase assays. Following the protocol of Alderete and Robertson, the material was subjected to acetone fractionation and preparative gel electrophoresis (1). The preparation was then further purified by gel filtration chromatography with a Bio-Gel P-6 column. The resulting effective dose of the preparation was 100 ng of protein.

Reagents. Reagent-grade cystamine, cysteamine, D- and L-cystine, D- and L-cysteine, cystathionine, 8-bromo cGMP, sucrose, EDTA, and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo. Acetylcysteine was bought from Mead Johnson & Co., Evansville, Ind. Other reagents were obtained as described previously (26).

Statistics. The standard error of the mean was calculated by pooling data from all experiments. The two-tailed Student's *t* test was used to test the significance of the differences between the mean values.

RESULTS

Inhibition of ST activation of guanylate cyclase. Table 1 summarizes the effects of the various sulfhydryl and disulfide compounds on basal and ST-activated (6 ST units/ml) intestinal guanylate cyclase activity. This concentration of ST resulted in more than a twofold increase of guanylate cyclase activity. Cystamine (1 mM) significantly inhibited ST activation of guanylate cyclase and also significantly inhibited basal activity. Cystamine (10 mM) produced even greater inhibition of ST-induced guanylate cyclase activity (from 150 ± 13 to 35 ± 5 pmol of cGMP per mg of protein per 10 min) and basal enzyme activity (from 72 ± 4 to 34 ± 1 pmol of cGMP per mg of protein per 10 min). At the lower concentration of 0.1 mM, ST-induced guanylate cyclase activity was reduced to 102 ± 9 pmol of cGMP per mg of protein per 10 min, with a reduction in basal

TABLE 1. Inhibition of basal and ST-stimulated guanylate cyclase activity by selected disulfide and thiol compounds

Drug	Basal guanylate cyclase activity[a]			ST (6 U/ml)-stimulated guanylate cyclase activity[a]		
	Without drug	With drug	(*P* value)	Without drug	With drug	(*P* value)
Cystamine	72 ± 4	39 ± 5	<0.001	150 ± 13	41 ± 5	<0.001
L-Cystine	76 ± 6	69 ± 3	NS[b]	221 ± 3	105 ± 8	<0.001
D-Cystine	73 ± 4	68 ± 3	NS	179 ± 9	119 ± 4	<0.001
Cysteamine	73 ± 3	68 ± 5	NS	201 ± 13	158 ± 8	<0.04
Acetylcysteine	55 ± 3	60 ± 8	NS	139 ± 18	103 ± 13	<0.04
L-Cysteine	63 ± 5	63 ± 22	NS	160 ± 22	118 ± 17	<0.02
D-Cysteine	63 ± 5	54 ± 7	NS	160 ± 22	114 ± 46	NS
Cystathionine	82 ± 2	85 ± 3	NS	205 ± 8	199 ± 5	NS

[a] Values are in picomoles of cGMP per milligram protein per 10 min ± standard error of the mean. All compounds were 1 mM in the guanylate cyclase reaction mixture. (n = 4 to 10 experiments per value).

[b] NS, Not significant ($P > 0.05$).

Cystamine $NH_2 - CH_2 - CH_2 - S - S - CH_2 - CH_2 - NH_2$

Cysteamine $NH_2 - CH_2 - CH_2 - SH$

Cystine $HOOC - (NH_2)\ CH - H_2C - S - S - CH_2 - CH(NH_2) - COOH$

Cysteine $HOOC - (NH_2)\ CH - CH_2 - SH$

Acetylcysteine $HOOC - (NHCOCH_3)\ CH - CH_2 - SH$

Cystathionine $HOOC - (NH_2)\ HC - H_2C - H_2C - S - CH_2 - CH(NH_2) - COOH$

FIG. 1. Chemical formulae of compounds used in the study. The disulfide compounds are cystamine and cystine. Cysteamine is a thiol derivative of cystamine. Cysteine and acetylcysteine are thiol derivatives of cystine. Cystathionine is neither a thiol nor a disulfide compound.

activity to 61 ± 5 pmol of cGMP per mg of protein per 10 min. Two other disulfide compounds, L-cystine and D-cystine, were also tested. Because of the precipitation of these reagents, concentrations greater than 1 mM could not be studied. At 1 mM, neither drug affected basal activity, but both L-cystine and D-cystine inhibited ST-induced activity of guanylate cyclase.

Among the thiol compounds, cysteamine (1 mM) inhibited ST-induced guanylate cyclase activity but not basal enzyme activity. At a higher concentration (10 mM), cysteamine did not significantly reduce basal activity but further reduced ST-induced activity (from 201 ± 13 to 75 ± 2 pmol of cGMP per mg of protein per 10 min). None of the other sulfhydryl compounds (1 mM) affected basal enzyme activity, but each (except D-cysteine) significantly reduced ST-induced enzyme activity. At 10 mM, these compounds did not significantly alter basal activity but did inhibit ST-activated enzyme activity by 38 to 57%. At 10 mM, D-cysteine also acted like the other thiol compounds: it did not significantly alter basal activity but did inhibit ST-activated enzyme activity by 46% ($P < 0.001$).

Cystathionine, a compound consisting of serine and homocysteine linked by a sulfur molecule (Fig. 1), had no effect on either basal or ST-induced enzyme activity. Cystathionine was examined because of its structural resemblance to the disulfides and sulfhydryl agents, with the important difference of not having its sulfur as a disulfide or thiol.

Inhibition of ST-induced fluid secretion in suckling mice. We found significant inhibition of ST-induced fluid secretion by all sulfhydryl and disulfide compounds but not by cystathionine (Table 2). Each agent was examined both by s.c. and i.g. administration.

Cystamine. Among the disulfides studied, cystamine demonstrated the most inhibition of ST-mediated intestinal fluid secretion. At 0.5 μmol per mouse, cystamine given s.c. significantly reduced ST-induced intestinal fluid secretion by either 1.6 or 6.4 ST units (Fig. 2). Cystamine at 0.5 μmol per mouse i.g. did not significantly affect ST-induced secretion. However, at the 1-log-higher dose (5 μmol per mouse) either s.c. or i.g., cystamine significantly reduced ST-induced secretion (Fig. 2). In prior experiments, we have found that certain agents such as chlorpromazine alter unstimulated (no ST added) gut to remaining body weight ratios (19). These results suggest to us that there is a "basal" fluid level in the gut that may be affected by various agents. In this set of experiments, cystamine at 5 or 0.5 μmol per mouse either s.c. or i.g. did not significantly alter basal intestinal fluid levels. Mortality with cystamine was noted only with doses exceeding 5 μmol per mouse; our mean lethal dose was 10 μmol per mouse s.c.

D and L-cystine. D- and L-cystine could not be studied at amounts greater than 0.5 μmol per mouse because of solubility problems. At 0.5 μmol per mouse s.c., neither compound reduced intestinal secretion induced by either dose of ST. When administered i.g., both compounds significantly reduced intestinal fluid secretion induced by 1.6 ST units but not by the larger dose of 6.4 ST units. Neither agent given s.c. or i.g. significantly altered the basal fluid level.

Cysteamine. Among the thiol agents, cysteamine showed the greatest inhibitory effect. As little as 0.05 μmol per mouse i.g. reduced the intestinal fluid ratio caused by 1.6 ST units. Cysteamine given i.g. did not alter the basal fluid level at 0.05, 0.5, and 5.0 μmol per mouse but reduced secretion induced by the larger amount of 6.4 ST units at 0.5 and 5.0 μmol per mouse ($P < 0.001$ in each instance) (Fig. 3).

Cysteamine (5 μmol per mouse), administered s.c., reduced intestinal secretion induced by either dose of ST. At the much lower amount (0.5 μmol per mouse, s.c.), the 1.6 ST unit ratio was not significantly changed. Basal fluid levels were not affected by either 5 or 0.5 μmol per mouse, s.c.

Acetylcysteine. At 0.5 μmol per mouse i.g., ST-induced responses by 1.6 and 6.4 ST units

TABLE 2. Inhibition of ST-induced fluid secretion in suckling mice by selected disulfide and thiol drugs[a]

Drug, amt (μmol per mouse), and route of administration	ST dose	Secretory response (gut/remaining body wt ratio) ± SEM		% Reduction in secretion
		Without drug	With drug	
Cystamine				
0.5, s.c.	1.6	0.107 ± 0.003	0.094 ± 0.003	38[b]
0.5, s.c.	6.4	0.130 ± 0.002	0.115 ± 0.006	25[c]
D-Cystine				
0.5, i.g.	1.6	0.111± 0.002	0.103 ± 0.004	20[b]
0.5, i.g.	6.4	0.133 ± 0.003	0.120 ± 0.004	22
0.5, s.c.	1.6	0.111 ± 0.002	0.109 ± 0.011	1
L-Cystine				
0.5, i.g.	1.6	0.111 ± 0.002	0.094 ± 0.004	43[b]
0.5, i.g.	6.4	0.133 ± 0.003	0.128 ± 0.003	7
0.5, s.c.	1.6	0.111 ± 0.002	0.112 ± 0.006	0
Cysteamine				
0.5, i.g.	1.6	0.107 ± 0.003	0.082 ± 0.002	70[c]
0.5, i.g.	6.4	0.130 ± 0.002	0.080 ± 0.005	85[c]
0.05, i.g.	1.6	0.107 ± 0.003	0.080 ± 0.005	75[c]
5.0, s.c.	6.4	0.130 ± 0.002	0.084 ± 0.005	78[c]
5.0, s.c.	1.6	0.107 ± 0.003	0.077 ± 0.003	83[c]
Acetylcysteine				
0.5, i.g.	1.6	0.107 ± 0.003	0.080 ± 0.005	77[c]
0.5, i.g.	6.4	0.130 ± 0.002	0.106 ± 0.005	41[c]
5.0, s.c.	1.6	0.107 ± 0.003	0.080 ± 0.003	77[b]
D-Cysteine				
0.5, i.g.	1.6	0.111 ± 0.002	0.077 ± 0.002	86[c]
0.5, i.g.	6.4	0.133 ± 0.003	0.112 ± 0.005	34[b]
L-Cysteine				
0.5, i.g.	1.6	0.111 ± 0.002	0.068 ± 0.001	109[c]
0.5, i.g.	6.4	0.133 ± 0.003	0.088 ± 0.006	74[b]

[a] Basal fluid level is 0.071 ± 0.002 (n = 6 to 143 mice per value).
[b] $P < 0.05$.
[c] $P < 0.001$.

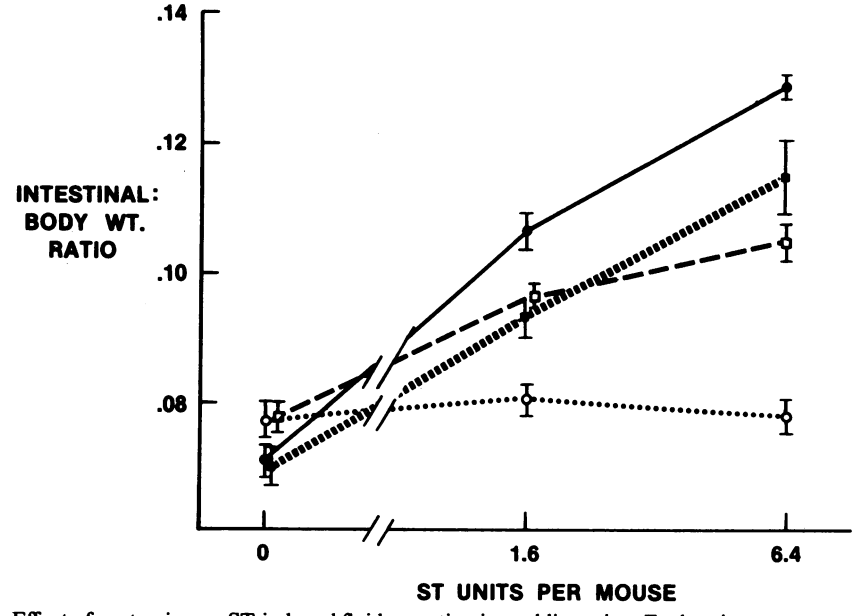

FIG. 2. Effect of cystamine on ST-induced fluid secretion in suckling mice. Each point represents the mean ± standard error of 12 to 143 mice. Symbols: ●, no cystamine; ■, cystamine at 0.5 μmol per mouse, s.c.; □, cystamine at 5.0 μmol per mouse, i.g.; ○, cystamine at 5.0 μmol per mouse, s.c. In the dose of 1.6 ST units per mouse, $P < 0.01$ for 0.5 μmol, s.c., and 5.0 μmol, i.g., and $P < 0.001$ for 5 μmol, s.c. In the dose of 6.4 ST units per mouse, $P < 0.001$ for all cystamine administrations.

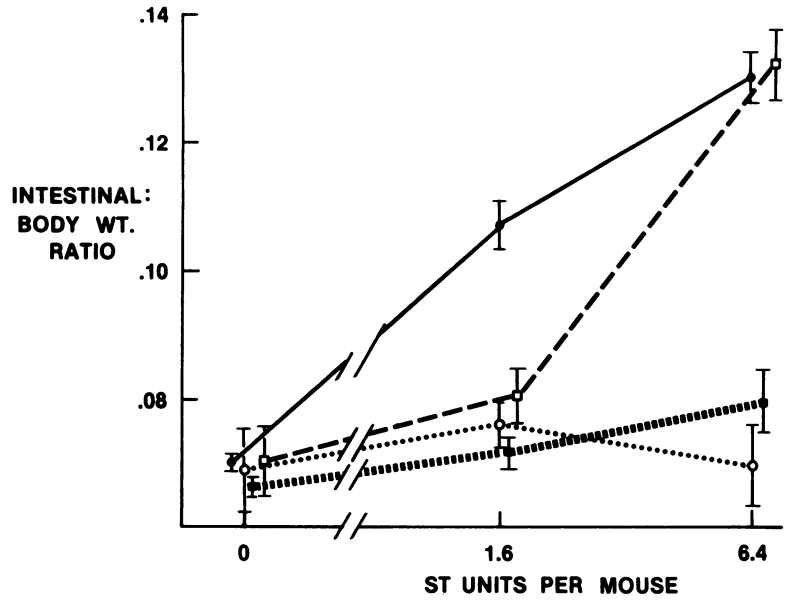

FIG. 3. Effect of cysteamine on ST-induced fluid secretion in suckling mice. Each point represents the mean ± standard error of 6 to 43 mice. Symbols: ●, no cysteamine; □, cysteamine at 0.05 μmol per mouse, i.g.; ■, cysteamine at 0.5 μmol per mouse, i.g.; ○, cysteamine at 5.0 μmol per mouse, i.g. In the dose of 1.6 ST units per mouse, $P < 0.001$ for all cysteamine regimens. In the dose of 6.4 ST units per mouse, $P < 0.001$ for 0.5- and 5.0-μmol cysteamine administrations.

were significantly reduced. The basal fluid level was not affected by either 0.5 or 5 μmol per mouse, given s.c. or i.g. No significant inhibition was evident with a lower amount (0.05 μmol per mouse). When given s.c., the 1.6 ST unit response was significantly reduced by a dose of 5 μmol per mouse; however, there was no inhibition of the larger 6.4 ST unit dose response. The basal fluid level was not altered by 5 μmol per mouse, s.c.

D- and L-cysteine. Both D- and L-cysteine, in a dose of 0.5 μmol per mouse i.g., reduced secretion induced by 1.6 and 6.4 ST units. No effect on the basal fluid level was noted at this dose or at 5 μmol per mouse when the agents were given either i.g. or s.c. At 5 μmol per mouse s.c., neither D- nor L-cysteine inhibited ST-induced fluid responses caused by either 1.6 or 6.4 ST units.

Cystathionine. Cystathionine, when given i.g. or s.c. at 0.5 μmol per mouse, did not inhibit ST-induced fluid secretion or alter the basal fluid level. Because of solubility problems, higher concentrations of the drug were not studied, and we cannot be sure that at a higher concentration inhibitory effects would not be evident.

Lack of inhibition of 8-bromo cGMP-induced intestinal secretion. We examined the effects of the disulfide agents, thiol compounds, and cystathionine on 8-bromo cGMP-induced intestinal fluid secretion in suckling mice (26). As reported in Table 3, no agent given either s.c. or i.g.

TABLE 3. Lack of inhibition of 8-bromo cGMP-induced intestinal fluid secretion in suckling mice

Drug	Dose (μmol per mouse)	Intestinal/remaining body wt ratio + SEM[a]		
		8-Bromo cGMP	8-Bromo cGMP + drug	
			i.g.	s.c.
Cystamine	5.0	0.111 ± 0.002	0.119 ± 0.003	0.112 ± 0.003
D-Cystine	0.5	0.110 ± 0.002	0.105 ± 0.004	0.100 ± 0.005
L-Cystine	0.5	0.110 ± 0.002	0.119 ± 0.004	0.104 ± 0.004
Cysteamine	5.0	0.111 ± 0.002	0.111 ± 0.010	0.107 ± 0.005
Acetylcysteine	5.0	0.111 ± 0.002	0.103 ± 0.003	0.102 ± 0.003
D-Cysteine	0.5	0.110 ± 0.002	0.119 ± 0.006	0.116 ± 0.005
L-Cysteine	0.5	0.110 ± 0.002	0.110 ± 0.004	0.105 ± 0.006
Cystathionine	0.5	0.110 ± 0.002	0.104 ± 0.002	0.118 ± 0.004

[a] Each value is the mean ± standard error of the mean of 4 to 59 mice.

significantly altered 8-bromo cGMP-induced intestinal fluid secretion.

DISCUSSION

The purpose of this study was to examine the effects of thiol and disulfide compounds on the activity of ST. Staples et al. have reported that their purified ST loses biological activity promptly on exposure to the reducing reagents 2-mercaptoethanol and dithiothreitol. They have suggested that the activity of ST depends on the presence of disulfide bridges. Their particular ST contains six half-cystine amino acids, suggesting the presence of multiple disulfide bridges (37). Most other purified preparations of ST contain multiple residues of half-cystine (1, 15, 28).

It is also possible that thiol or disulfide reagents affect the binding of ST to its receptor or inhibit steps beyond toxin binding that result from mucosal cell exposure to ST. The enterotoxin effects of ST have been recently reviewed, and many investigators have noted that ST causes an increase in intestinal cell cGMP concentrations (17). It has been shown that disulfide compounds such as cystamine and thiol compounds have very marked effects on guanylate cyclase activity (6, 32). Investigators have speculated that these agents alter the oxidation reduction state of cellular components (possibly by free radical scavenging) and thus alter guanylate cyclase activity. In addition, Brandwein et al. have shown that when [^{35}S]cystine and purified soluble guanylate cyclase are incubated together, the radioactivity is incorporated into the enzyme, and there is a reversible loss of enzyme activity (5).

In this study, we examined the effects of various disulfide and thiol compounds on ST-induced intestinal secretions in suckling mice, ST activation of guanylate cyclase, and cGMP-induced intestinal secretions in suckling mice. Our results show that the disulfide compound cystamine alters guanylate cyclase activity. The fact that cystamine inhibits ST-activated guanylate cyclase significantly more than basal activity suggests that cystamine limits maximal guanylate cyclase activity or that it may have an additional effect on ST or on ST binding. Cystamine has no effect on 8-bromo cGMP-induced intestinal secretion. Similar but less effective results were seen with D- and L-cystine. The inhibition of ST-induced secretion by the disulfides cystamine and cystine clearly occurs before the formation of cGMP.

The sulfhydryl compounds (cysteamine, acetylcysteine, and D- and L-cysteine, each at 1 mM) do not alter basal guanylate cyclase activity; each, however, reduces ST-stimulated guanylate cyclase activity and ST-induced fluid secretion in the suckling mouse. None of the thiol agents could alter 8-bromo cGMP-induced intestinal secretion. Thiols seem to be more specific inhibitors of ST than disulfides, as none showed significant alteration of basal guanylate cyclase activity. Like the disulfide agents, thiol compounds inhibit ST effects before the formation of cGMP. Thiol compounds probably reduce the disulfide bonds of ST which are necessary for its biological activity. The clearance and metabolism of these agents, although not well understood, probably relate to local redox conditions and pH and may explain why thiol derivatives inhibit best when given i.g. and disulfide agents inhibit best when given s.c.

The sulfur-containing compound cystathionine shows no alteration of basal or ST- or cGMP-induced responses. These results further suggest that it is the sulfhydryl group or the presence of a disulfide bond that seems necessary for compounds to inhibit ST activity. Until now, research with thiols and disulfides as therapeutic agents has centered on their radioprotective properties (3, 4, 34, 39) and their efficacy in the treatment of acetaminophen overdoses (2, 11, 27, 35, 36) and nephropathic cystinosis (38, 41).

In summary, selected disulfide and thiol compounds inhibit ST-mediated fluid secretion before ST-induced formation of cGMP. Cysteamine seemed the most effective inhibitory agent. Cysteamine may directly alter the toxin. In contrast, the disulfide compound cystamine clearly appeared to have a nonspecific inhibitory effect, perhaps by altering the cyclase enzyme directly. Future research, evaluating disulfide and thiol compounds as inhibitors of intestinal secretion induced by ST and possibly by other enterotoxins, may lead to an effective antisecretory agent for enterotoxin-induced diarrhea.

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