Effect of enterotoxin on glutathione status in the intestinal mucosa

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The effect of luminal exposure of enterotoxins on the intestinal mucosal glutathione (GSH) was studied in rat. Cholera toxin induced fluid secretion and decreased mucosal GSH by 35% without altering oxidized glutathione (GSSG) level. Toxin induced fluid secretion was tested after mucosal GSH depletion by compounds such as diethyl maleate (DEM) and buthionine sulfoximine (BSO) and thiol supplementation with N-Acetyl cysteine (NAC). Fluid secretion was not altered by prior thiol depletion or supplementation. Exposure of intestinal lumen to bacterial endotoxin resulted in 25% decrease in mucosal GSH with two fold increase in GSSG. Luminal exposure of Shiga toxin did not alter the mucosal thiol. The level of other low molecular weight thiols, cysteine and cystine was not altered by luminal exposure of any of these toxins. These results show that although cholera toxin decreased the mucosal GSH level, prior modulation of thiol status of the mucosa may not have any effect on toxin-induced fluid secretion.

Toxins produced by enteric bacteria are a well established cause of diarrhoea. Fluid secretion is related to the enzymatic effect of enterotoxins on intestinal cells, often through specific receptors and increased cyclic nucleotide concentration. Cholera toxin increases cAMP concentration, thereby inducing secretion, whereas Shiga toxin induces diarrhoea by selectively destroying absorptive epithelial cells\textsuperscript{1,2}. Endotoxins are bacterial cell wall components which elicit inflammation by priming inflammatory cells\textsuperscript{3}.

Intracellular thiols are known to modulate cellular function. Many cellular proteins require the presence of reduced thiol groups and oxidation or mixed disulfide formation with glutathione (GSH) or cysteine can alter their function\textsuperscript{4}. Intestinal epithelial brush border membranes are involved in transport and it has been shown that presence of free thiol groups are important for their transport function\textsuperscript{5}. In renal brush border membranes, it has been shown that maintenance of thiol groups in the reduced state keeps transport activity normal\textsuperscript{6}. The present study looks at the role of thiol compounds such as glutathione and cysteine on enterotoxin induced fluid secretion by intestinal mucosal cells.

\textbf{Materials and Methods}

Glutathione (oxidized and reduced form), cholera toxin (CT), 1-fluoro-2,4-dinitrobenzene (FDNB), buthionine sulfoximine (BSO), NADPH and bovine serum albumin were all obtained from Sigma Chemical Co. Shiga toxin was a kind gift from Dr. Gerald Keusch of Tufts New England Medical Centre, Boston, USA. Endotoxin (LPS) used in the experiments was prepared from overnight broth cultures of Salmonella M strain by the phenol/water extraction technique\textsuperscript{7}. All other chemicals used were of analytical grade.

\textbf{Luminal exposure of toxins}

Overnight fasted rats weighing 150-200g were anaesthetised with pentobarbitone (50 mg/kg body wt). The abdomen was opened with a midline incision, a 20 cm segment of the small intestine was isolated and gently flushed with normal saline. This was cannulated proximally and distally, the segment was returned to the abdomen and closed. Physiological saline containing various toxins was instilled in the lumen of the intestinal segment and was clamped at both ends. The toxins used were: (1) Cholera toxin 2 \(\mu\)g/cm length for 2 hrs, (2) Shiga toxin 4 \(\mu\)g/cm length for 3 hr, and (3) Endotoxin 0.5 mg/100 g body weight for 2 hrs. In addition the effect of endotoxin was separately tested by adminis-
tering intraperitoneally (0.5 mg/100 g body wt) and intravenously (1 mg/100 g body wt). The body temperature was maintained with an overhead lamp during the experiment.

Assessment of fluid secretion—Krebs-Henseleit buffer pH 7.3 containing polyethylene glycol-4000 (2.5 g/l), a nonabsorbable volume marker, was perfused at a flow rate of 0.5 ml/min using a Vickers' Medical Tronic IP4 syringe pump through the segment of the intestine already exposed to enterotoxin. After an initial equilibrium period of 1 hr, the perfusate was collected for 1 hr at 15 min intervals. Fluid secretion was assessed by estimating concentration of PEG-4000 in toxin treated and untreated perfusate as described. Following incubation with toxin, the solution was flushed out and the intestine was immediately washed and the mucosa scraped. For thiol estimation, the mucosa was immediately homogenized in trichloroacetic acid (final concentration 5%) to prevent oxidation of thiols. For enzyme activity measurements, a 10% homogenate of the mucosal scrapings was made in 0.15M phosphate buffer pH 7.0.

Thiol depletion and supplementation—Effect of cholera toxin was done after thiol depletion with luminal incubation of a solution containing 5 mM diethyl maleate for 30 min. Mucosal thiol was also depleted by giving two bolus intraperitoneal injection of BSO (4 nmole/kg body wt) at interval of 3 hr prior to toxin exposure. BSO is a specific inhibitor of GSH synthesis. For thiol supplementation, intestinal segment was exposed to 1 mM N-acetyl cysteine (NAC) for 1 hr intraluminally prior to toxin exposure.

Thiol estimation—Glutathione in the acid supernatant of the mucosal tissue was quantitated using HPLC after derivatisation as described. The derivatisation procedure include reaction of iodoacetic acid with thiols to form S-carboxymethyl derivatives followed by chromophore derivatisation (DNP) of primary amino group with 1-fluoro-2,4-dinitrobenzene (FDNB). DNP derivatives were separated on Ultrasil-NH2 column using a gradient of methanol and sodium acetate and detected at 365 nm. Cysteine and cystine were estimated colorimetrically using acid ninhydrin reagent.

Enzyme assays—The supernatant obtained from centrifugation of homogenate at 10,000×g for 15 min was used for enzyme measurements. GSH peroxidase was assayed by following the change in absorbance at 340 nm using H2O2 as substrate. GSH transferase activity was measured using 1-chloro,2,4-dinitrobenzene as substrate. GSH reductase activity was determined by change in absorbance of NADPH at 340 nm using GSSG as substrate. Protein was estimated using bovine serum albumin as standard.

Results

Figs 1 and 2 show the effect of cholera toxin and cellular thiol modulating agents on fluid secretion by the small intestine and glutathione status in the intestinal mucoza. Luminal exposure (for 2 hrs) of cholera toxin, induced fluid secretion and decreased mucosal GSH by 35% without altering GSSG concentration. The other low molecular weight thiols, cysteine and cystine were not altered (data not shown).
shown). To test whether prior depletion of cellular GSH would affect cholera toxin induced secretion, mucosal GSH was depleted by direct conjugation or by inhibiting GSH synthesis. As shown in Fig. 2, these agents decreased the GSH level by nearly 30-40% but cholera toxin induced secretion was not altered by prior mucosal thiol depletion (Fig. 1). GSH depletion by chemical agents per se did not induce fluid secretion. Since cholera toxin treatment decreased mucosal GSH content, we intended to study whether thiol supplementation by providing one of its precursor would modify secretion. N-acetyl cysteine which is known to act as a precursor for GSH synthesis was incubated in the lumen for 1 hr prior to cholera toxin treatment. As shown in Fig. 2, N-acetyl cysteine as such did not increase the mucosal GSH or cysteine in control animals. N-acetyl cysteine treatment followed by cholera toxin still showed 75% mucosal GSH as compared to control and decrease in mucosal GSH by cholera toxin was not replenished by N-acetyl cysteine. Cholera toxin induced fluid secretion was not altered by prior supplementation of N-acetyl cysteine.

Another bacterial toxin, Shiga toxin was tested for its effect on mucosal thiol status. Shiga toxin was instilled in the lumen for 3 hr and mucosal thiol was quantitated. This toxin did not induce fluid secretion (data not shown) and did not alter the mucosal thiol levels (Fig. 3).

Bacterial endotoxins are known to alter certain cellular function. When intestinal lumen was exposed to endotoxin, there was a 25% decrease in mucosal GSH with a two fold increase in GSSG concentration (Fig. 3). Endotoxin administration intravenously or intraperitoneally did not alter the mucosal GSH concentration (data not shown). Cysteine and cystine levels were not altered in response to endotoxin administration. Endotoxin is known to stimulate free radical generation and glutathione and related enzymes are known to participate in protecting the tissue from the radical damage. Some of the glutathione dependent enzymes were measured in the mucosa after exposure to various toxins and no significant alteration in their activity was observed (data not shown).

**Discussion**

Glutathione along with cysteine form the major cellular low molecular weight thiols which are ubiquitous in nature and are implicated in many cellular function. Thiol compounds are essential for intestinal epithelial cell function and a deficiency of these compounds leads to severe degeneration of these cells. Many cellular proteins require the presence of free sulphydryl groups for their normal function. Intracellular Ca²⁺ pump is sensitive to sulphydryl reagents and physiological Na⁺ pump, Na⁺/K⁺-ATPase, has a critical thiol which regulates its activity. Intestinal epithelium has immense se-
Toxins produced by enteric bacteria act on the mucosal epithelium causing fluid secretion. Cholera toxin (CT) causes adenosine diphosphate ribosylation of an adenylate cyclase regulating protein leading to formation of cAMP\(^9\). Increased cAMP phosphorylates many cellular proteins and increases chloride secretion. This study has shown that cholera toxin can induce fluid secretion and this was associated with a decrease in mucosal thiol. Earlier studies have shown stimulation of GSH efflux and inhibition of GSH synthesis by cholera toxin in isolated hepatocytes\(^1\). It is possible that in enterocytes also cholera toxin may enhance the efflux of GSH which may be responsible for the observed decrease in GSH in the mucosal cells. This study has also suggested that alteration in the mucosal thiol is not directly related to fluid secretion since thiol depleting agents did not induce fluid secretion. Endotoxin is known to stimulate free radical generation and the decrease in GSH and increase in GSSG observed after luminal exposure may be due to consumption of GSH during oxidative stress. Exposing intestinal lumen to Shiga toxin did not result in fluid secretion or alter mucosal thiol and this may be due to the absence of specific receptors for this toxin in rat small intestine (unpublished observation). In conclusion this study has shown that cholera toxin decrease mucosal GSH but prior modulation of thiol status of mucosa does not influence fluid secretion induced by cholera toxin.

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