Bile acid induced colonic irritation stimulates intracolonic nitric oxide release in humans

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Abstract

Aim—To measure the intracolonic release of nitric oxide end products (nitrates plus nitrites) and eicosanoids in response to intraluminal irritation with deoxycholic acid (DCA).

Patients—Seven patients with irritable bowel syndrome.

Methods—The left colon was perfused with a solution with or without 3 mM deoxycholic acid. Aspirates were assayed for eicosanoids by specific radioimmunoassay, and for nitrates plus nitrites by the Griess reaction. To confirm that stimulated colonic mucosa can produce nitric oxide (NO), ancillary studies were performed in vitro using samples of normal mucosa obtained from five surgically resected colons. Samples were incubated for 30 minutes in Krebs's solution, 3 mM DCA or DCA with 1 mM L-nitro-arginine-methyl-ester (L-NAME) to inhibit the NO synthase. Finally, NO synthase activity was measured in five samples of human colonic mucosa.

Results—Intracolonic release of nitrates plus nitrites was basally undetectable in six of seven patients. Bile acid considerably increased the release of prostaglandin E₂ and nitrates plus nitrites ($p < 0.01$). By contrast, no increase in thromboxane and leukotriene was seen. In vitro mucosal incubation with DCA increased the production of NO synthase products, which was blocked by L-NAME. Activity of Ca⁺⁺ independent NO synthase was detectable in four of five samples of human colonic mucosa.

Conclusion—The human colonic mucosa responds to bile acid induced irritation by a surge in NO generation via NO synthase. (*Gut* 1996; 38: 719-723)

Keywords: nitric oxide synthase, colonic irritation, colonic perfusion, bile acids.

The intestinal mucosa is normally exposed to a variety of intraluminal irritants. The irritant effects of dihydroxy bile acids on both small bowel and colonic mucosa have been particularly well characterised and include secretion of water and electrolytes, increased mucus production, increased mucosal permeability, and damage of the epithelial surface.^{1,2} The precise mechanisms that mediate the mucosal response to intraluminal irritation are still not completely understood. Colonic mucosal production of eicosanoids increases after exposure to deoxycholic acid (DCA).³ However,

inhibition of either cyclooxygenase or thromboxane synthase not only fails to protect the mucosa from the effects of bile acids but even worsens the colonic damage,⁴ suggesting that non-prostaglandin mechanisms also participate. Lipooxygenase metabolites, such as the sulphidopeptidoleukotrienes, may also take part as these substances stimulate water secretion.⁵ We have also shown that human intrajejunal infusion of DCA increases intraluminal release of leukotriene C₄.⁶

It has been shown that bile acids increase the production of reactive oxygen in colonic mucosal scrapings and isolated colonic crypts.⁷ The degradation substances produced by the peroxidative process initiated by oxygen radicals can diffuse away causing oedema, increasing vascular permeability and inflammation.⁸ Nitric oxide (NO) may contribute to the actions of oxygen superoxide anion radical by forming peroxynitrite, which is decomposed into the potent oxidants OH[•] and NO₂.⁹ NO mediates many biological actions, such as smooth muscle relaxation, neurotransmission, inflammation or cytotoxicity.^{10,11} However, the possible role of NO in the colonic mucosal response to bile acid induced irritation has not been previously explored.

To investigate whether stimulation of NO synthesis participates in the response of colonic mucosa to bile acid irritation, we measured by a perfusion technique in humans the intracolonic release of NO derived products before and after colonic infusion of DCA in a group of patients with irritable bowel syndrome.

Methods

SUBJECTS

Perfusion studies were performed in seven patients (two men and five women, age ranging from 18 to 46 years) with irritable bowel syndrome. Diagnosis was based on standard clinical criteria (pain ceased after bowel movement, looser stools at onset of pain, more frequent bowel movements at onset of pain, abdominal distension, mucus per rectum, and feeling of incomplete emptying). Routine laboratory tests, upper gastrointestinal barium meal, and colonoscopic examination were normal in every subject. The study was approved by the Institutional Review Board, and all patients gave informed consent.

PERFUSION PROCEDURE

Steady state intracolonic release of nitrates plus nitrites was measured by a double lumen colonic perfusion technique. After an

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overnight fast, a double lumen polyvinyl assembly was placed into the descending/sigmoid colon by sliding it over a guiding wire placed by prior colonoscopy. The oral lumen opened at 50 cm from the anal verge and was used for infusion of the perfusion solutions. The caudal lumen opened 30 cm caudally from the oral one and was used to recover the perfusates by siphonage. The correct position of the perfusion tube was assessed by fluoroscopy. Perfusion solutions were infused at 5 ml/min using a volumetric pump (IMED 927, Milton Trading Estate, Abingdon, UK).

The perfusion solution was an isotonic and neutral solution composed of 280 mmol/l mannitol and 2 g/l polyethylene glycol 4000 (PEG 4000) as a non-absorbable marker. Irritation was induced by the addition of DCA (Koch-Light Laboratories, Colnbrook, Bucks, UK) to the solution to a final concentration of 3 mmol/l. This concentration of DCA was chosen because it causes a very mild irritation while inducing a measurable secretory response in the human colon.³ The pH of the solution was adjusted to 7.8 with 0.1 mol/l NaOH. To prevent colonic water and electrolytes absorption or secretion induced by the solution, the perfusate did not contain glucose or electrolytes.

The perfusate was recovered by siphonage to preclude the local irritation that would otherwise occur with mechanical or manual aspiration.

EXPERIMENTAL DESIGN

Main studies

In the seven irritable bowel syndrome patients, two consecutive perfusion sequences each lasting 100 minutes were performed on a given day. Each sequence began with 30 minutes of colonic washing with the solution without DCA, followed by the test perfusion with or without DCA for 70 minutes (30 minute equilibration period and 40 minute test period). Because the secretory effects of DCA infusion on human colon are reversible,³ the order of the perfusions was randomised. During each perfusion period, perfusates were continuously collected on ice and pooled at 10 minute intervals.

Ancillary in vitro studies

To discover if the colonic mucosa produces nitric oxide via NO synthase, samples of histologically normal mucosa obtained from five surgically resected colons for neoplastic disease were incubated for 30 minutes in 10 ml Krebs's solution at 37°C. One aliquot of the sample, which served as control, was incubated in Krebs's solution; a second aliquot of the sample was incubated in Krebs's solution with 3 mM DCA, and a third aliquot of the sample with DCA and 1 mM L-nitro-arginine-methyl-ester (L-NAME, Sigma, St Louis, MO), an inhibitor of the NO synthase. The weight of tissue used in each incubation ranged from 310 to 520 mg.

To investigate whether the perfusion of DCA increases nitrates plus nitrites production by stimulation of the bacteria residing in the colon, we performed additional studies incubating human faecal samples with DCA. Faecal samples from subjects without intestinal disease and who had not received any antibiotic treatment were diluted 1:15 in saline and incubated with 3 mM DCA at 37°C. Nitrates plus nitrites were determined in aliquots at time 0 and sequentially up to two hours of incubation.

NO synthase activity was measured in mucosal samples from five patients undergoing colectomy for neoplasm. Specimens were always obtained from the distal margin of resection and in every instance, the margin was macroscopically and microscopically free of tumour.

ANALYTICAL PROCEDURES

Aliquots of 1 ml were stored at -20°C for later analysis of PEG, nitrates plus nitrites and eicosanoids. To prevent in vitro prostanoid synthesis, indomethacin (Sigma) was added to some aliquots up to a concentration of 50 µg/ml.

Samples were assayed for PEG by the Hyden method.¹² Nitrates were reduced to nitrites using a copper plated cadmium column, and nitrites were determined by the Griess method as previously described.¹³ Results are expressed as nitrites over nitrates (NO₂/NO₃). Prostaglandin E₂ (PGE₂), thromboxane B₂ (TXB₂), and leukotriene B₄ (LTB₄) were analysed by a previously validated specific radioimmunoassay.¹⁴

NO synthase activity was assayed in homogenates of the surgical samples of colonic mucosa as the transformation of L-arginine to L-citrulline.¹⁵ Aliquots of tissue homogenates were incubated in a medium containing 50 mM potassium phosphate buffer (pH 7.2), 60 mM valine, 1.2 mM citrulline, 120 µM NADPH, 24 µM arginine, 150 000 dpm ¹⁴C-arginine, 1.2 mM MgCl₂, 0.24 mM CaCl₂ for 10 minutes at 37°C. The reaction was stopped by dilution and removal of substrate by the addition of 50% Dowex 50W resin mix (200-400 mesh, 8% cross linked, Na⁺ form). The amount of ¹⁴C-citrulline formed was measured by scintillation counting. Aliquots with 1 mM L-NAME were used to subtract background citrulline formation, and addition of 1 mM EGTA to specific aliquots served to identify Ca²⁺ dependent activity. Protein content in the eluates was measured using a BCA protein assay from Pierce (Rockford, IL). Enzymatic activity was expressed as pmol of citrulline formed per mg of protein per minute.

CALCULATIONS AND STATISTICAL METHODS

Results are expressed as mean (SEM). Results of NO₂/NO₃ and eicosanoids are expressed as rates of intracolonic release. Release rates were calculated by a standard formula using PEG as a non-absorbable marker. Data correspond to

Figure 1: Individual values of intracolonic release of NO_2/NO_3 before and after DCA stimulation. Basal release was undetectable in six patients and bile acid irritation significantly increased the release of NO breakdown products in five of seven patients.

the 20 to 40 minute period from control and DCA perfusion. Statistical differences in colonic perfusion studies were calculated for paired data by the Student's *t* test. In the *in vitro* studies the Mann-Whitney U test was used.

Results

EFFECTS OF DCA ON COLONIC NO_2/NO_3 RELEASE

Basal intracolonic nitric oxide release measured as luminal NO_2/NO_3 release was undetectable in six of seven patients, and barely detectable in one. As Figure 1 shows DCA perfusion considerably stimulated colonic release of NO_2/NO_3 in five patients. For the whole group of seven patients, the mean intracolonic release of NO_2/NO_3 was of 0.9 (0.9) and 45.2 (12) nmol/min before and after DCA stimulation respectively, $p < 0.01$.

EFFECTS OF DCA ON COLONIC EICOSANOID RELEASE

Eicosanoids were continuously released into the colonic lumen at detectable values under basal conditions. The mean basal release of PGE_2 , TXB_2 , and LTB_4 was 0.89 (0.1) ng/min, 0.98 (0.2) ng/min, and 0.75 (0.1) ng/min respectively.

Figure 2 represents the change in PGE_2 release induced by DCA perfusion. DCA increased intracolonic PGE_2 release to 4.98 (1.1) ng/min ($p < 0.01$ v basal) with a mean response index (response index = (stimulated - basal)/basal) of 5.1 (1). The mean intracolonic release of TXB_2 during stimulation with DCA was 1.76 (0.4) ng/min (Fig 2), which was not significantly different from the basal release, and represents a response index of only 1.2 (0.4). Similarly, intracolonic release of LTB_4 with DCA stimulation was 1.1 (0.1) ng/min (Fig 2), which was not significantly different from basal release, and represents a response index of only 0.9 (0.5).

EFFECT OF DCA ON IN VITRO COLONIC MUCOSAL PRODUCTION OF NO DERIVED METABOLITES

Mean concentration of NO_2/NO_3 in supernatants of control incubations of human colonic mucosa was 2.6 (0.3) nmol/ml. As Figure 3 shows, incubation of mucosal samples

Figure 2: Individual values of intracolonic release of PGE_2 , TXB_2 , and LTB_4 before and after DCA stimulation. All three eicosanoids were released at detectable values under basal conditions. PGE_2 release into the colonic lumen was considerably increased with DCA perfusion (response index of 5.1, $p < 0.01$). Intraluminal release of TXB_2 was only slightly stimulated by DCA (response index of 1.2, NS). LTB_4 release was not stimulated by DCA (response index of 0.9, NS).

Figure 3: Individual values of in vitro production of NO_2/NO_3 by samples of human colonic mucosa. The production of NO_2/NO_3 was increased in the presence of DCA, but the addition of L-NAME inhibited the response to DCA.

with DCA increased the concentration of NO_2/NO_3 in the medium ($13.2 (3.0) \text{ nmol/ml}$, $p < 0.01$ v control). However, in the presence of L-NAME, addition of DCA did not change NO_2/NO_3 concentrations ($3.14 (0.1) \text{ nmol/ml}$, $p < 0.01$ v DCA stimulated values).

Incubation of diluted faecal samples with DCA did not show any generation of NO_2/NO_3 , which was undetectable both in basal aliquots and in the sequential determinations up to two hours of incubation. These data suggest that DCA did not stimulate the production of nitrate plus nitrite by bacterial populations present in the colonic lumen.

Total citrulline formation by homogenates of human colonic mucosa was $4.70 (0.64) \text{ pmol/mg/min}$; in the presence of EGTA, the amount of citrulline formed was $4.66 (0.72)$, and in the presence of L-NAME, $4.15 (0.60) \text{ pmol/mg/min}$. Thus, activity of the Ca^{2+} dependent fraction of the NO synthase was undetectable in most samples of colonic mucosa. In contrast, the Ca^{2+} independent fraction was detectable in four of five samples (median 0.12 pmol/ml/min , range 0.00 to 0.15).

Discussion

Our colonic perfusion studies show that under basal conditions release of NO_2/NO_3 into the human colonic lumen is undetectable by the Griess reaction. However, after mild irritation with a weak dihydroxy bile acid solution, the release of NO metabolites noticeably increases in most subjects. In addition, irritation induced the release of PGE_2 into the colonic lumen.

It is known that PGE_2 has cytoprotective properties in the colonic mucosa, preventing damage induced by toxin producing clostridia, ethanol, immune complexes or trinitrobenzenesulphonic acid.^{16,17} In vitro studies have shown that PGE_2 exerts a direct protective

action on duodenal luminal cell membrane vesicles incubated with deoxycholate.¹⁷ These findings suggest that the mucosal eicosanoid response to intraluminal irritants is a protective response. Nearly all human cells can metabolise arachidonic acid to prostaglandins, and it has been shown that intestinal epithelial cells produce PGE_2 .¹⁸

The source of the intracolonic NO_2/NO_3 measured in our study is unlikely to be other than the colonic mucosa. Conceivably, ingested food particles could be a complementary source but the absence of nitrates and nitrites during the basal perfusion and its presence only after the irritative stimuli would exclude this possibility. To corroborate that the origin of nitrates and nitrites is the NO produced by the colonic mucosal NO synthase, in vitro studies incubating normal colonic mucosa with DCA with or without L-NAME were performed. These ancillary studies provide evidence showing that DCA is able to stimulate the mucosal production of nitrates and nitrites and the response is blocked by L-NAME. This finding suggests that nitrates plus nitrites are generated by the colonic mucosa, and in fact our experiments with mucosal homogenates showed that inducible NO synthase activity is present in human colonic mucosa. In addition, the experiments with faecal samples showed that bacteria present in the colonic lumen did not release NO at detectable values in response to DCA. However, it is not possible to establish the precise cellular source of NO_2/NO_3 ; epithelial cells and macrophages as well as vascular and neural elements may contribute.

The functions of NO in the gastrointestinal tract are diverse, and include regulation of motility, non-adrenergic, non-cholinergic neural transmission, and preservation of mucosal blood flow. Because the nitrovasodilator NO donors attenuate the endotoxin induced intestinal damage,¹⁹ it has been suggested that constitutive NO exerts a protective effect on intestinal mucosa. However, the mechanisms of such putative protection have not been elucidated. On the other hand, pretreatment with dexamethasone prevented the delayed changes induced by lipopolysaccharide by inhibiting the induction of calcium independent NO synthase activity.²⁰ These results suggested that NO formed by the inducible NO synthase does not have protective properties. Inducible NO synthase generates large amounts of NO with powerful cytotoxic and antimicrobial effects.¹⁰ Cytotoxicity of neutrophils as well as macrophages is related, at least in part, to generation of NO via an NO synthase induced by endotoxins and cytokines.²¹⁻²³ Thus, we postulate that the NO mediated response to intracolonic irritants seen in our study may represent an active mechanism of mucosal defence by which the mucosa attempts to destroy intraluminal bacteria by releasing NO.

In summary, our studies of human colonic mucosal response to bile acid irritation with in vivo colonic perfusions and in vitro mucosal studies show a mucosal response to irritation entailing increased release of PGE_2 and NO

breakdown products. This response may represent an attempt to clear the intraluminal irritants releasing cytotoxic NO, and increasing the synthesis of cytoprotective PGE₂.

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