

INTESTINE INFLAMMATION

Nitric oxide inhibitable isoforms of adenylate cyclase mediate epithelial secretory dysfunction following exposure to ionising radiation

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Background: Hyporesponsiveness of the intestinal epithelium to secretagogues occurs in different models of intestinal injury, including radiation enteropathy, and in human disease. While this impairment of barrier function has been linked to increased inducible nitric oxide synthase (iNOS) activity, the cellular target of NO in this phenomenon is not known, although recent studies suggest that some isoforms of adenylate cyclase are inhibited by NO.

Aims: To determine adenylate cyclase isoform distribution in colonic epithelial cells and, in particular, the physiological significance of NO inhibitable adenylate cyclase isoforms 5 and 6 in radiation induced epithelial secretory dysfunction.

Methods: Reverse transcription-polymerase chain reaction (RT-PCR), immunocytochemistry, and immunohistochemistry were used to examine adenylate cyclase expression. The responsiveness of mouse colon to secretagogues 72 hours post-15 Gy gamma radiation or following in vitro exposure to NO donors was measured in Ussing chambers. Also, cAMP, cGMP, and ATP levels were measured.

Results: RT-PCR, immunocytochemistry, and immunohistochemistry showed that adenylate cyclase 5 was expressed in mouse colon, and isoforms 5 and 6 were expressed in human biopsies and intestinal epithelium. Pharmacological studies showed that these isoforms are functionally important in chloride secretion. NO mediated hyporesponsiveness to secretagogues is primarily a result of decreased adenylate cyclase activity, and not G_i activation or decreased cellular ATP levels.

Conclusions: NO inhibitable isoforms of adenylate cyclase are expressed in mouse and human secretory colonic epithelia, and appear to be the target of radiation induced NO to reduce the responsiveness to cAMP dependent secretagogues.

Rapidly developing and persistent intestinal epithelial barrier defects are characteristic of abdominopelvic exposure to therapeutic doses of ionising radiation and may limit successful cancer treatment and contribute to chronic radiation enteropathy.^{1–3} Several studies have linked a luminal factor with the development of radiation enteritis, as well as inflammatory bowel disease, suggesting that breaches in the intestinal barrier trigger immune mediated inflammatory responses in genetically susceptible individuals.^{4–5} Studies in rats show that bacterial translocation from the lumen to the lamina propria occurs following exposure to ionising radiation.⁶ Clinically, radiation injury allows bacterial lipopolysaccharide (LPS) to cross the intestinal barrier and initiate a feedback loop in which LPS and inflammatory mediators cause further epithelial barrier dysfunction.⁷ Epithelial barrier dysfunction is therefore a common determinant of inflammatory conditions of the gastrointestinal tract, including radiation enteropathy.

The ability of crypt epithelial cells to respond to secretagogues with chloride and hence water secretion is an important component of the epithelial barrier that protects the intestine by preventing translocation of bacteria, bacterial products, and antigens to the lamina propria.⁸ Hyporesponsiveness to secretagogues has been established in different models of intestinal injury, including exposure to ionising radiation.^{9–11} We have linked decreased responsiveness to cAMP dependent secretagogues to increased inducible nitric oxide synthase (iNOS) activity in several animal models.^{10–12} Nevertheless, the cellular target of iNOS derived nitric oxide (NO) in the inhibition of chloride secretion which occurs post-irradiation is not known. Based on previous

observations that NO mediates hyporesponsiveness to cAMP but not Ca^{2+} dependent secretagogues,^{11–13} in this study we have focused on cAMP dependent secretion in radiation injury. The relative abundance of different adenylate cyclase (AC) isoforms plays a critical role in modulating cAMP dependent cellular responses.¹⁴ Of the nine forskolin sensitive membrane bound adenylate cyclase isoforms identified to date,¹⁴ isoforms 5 and 6 (AC5, AC6) are of particular interest because they are selectively inhibited by NO.¹⁵ Therefore, we hypothesised that epithelial AC5 and/or AC6 are the direct targets of iNOS derived NO and are thus the adenylate cyclase isoforms responsible for the decreased responsiveness to cAMP dependent secretagogues post-irradiation. To test this hypothesis, we determined the distribution of adenylate cyclase isoforms in intestinal mucosa and demonstrated pharmacologically that AC5 and/or AC6 were functionally significant in terms of colonic chloride secretion. Furthermore, we used a mouse model of acute radiation injury, as well as the NO donors sodium nitroprusside (SNP) and PAPA NONOate, to determine the mechanism under-

Abbreviations: Isc, short circuit current; AC, adenylate cyclase; SNP, sodium nitroprusside; PAPA NONOate, 1-propyl-1'-(aminopropyl)-2-hydroxy-2-nitrohydroxine; NO, nitric oxide; iNOS, inducible nitric oxide synthase; RT-PCR, reverse transcription-polymerase chain reaction; LPS, lipopolysaccharide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TBS, Tris buffered saline; PBS, phosphate buffered saline; 1400w, N-(3-(aminomethyl)benzyl)acetamide; L-NIL, L-N^ε-(1-iminoethyl)lysine; PKA, protein kinase A; PTX, pertussis toxin

lying NO dependent hyporesponsiveness to secretagogues in mouse colon.

MATERIALS AND METHODS

Cell culture

T84 cells (passages 40–64) were grown in a 1:1 mixture of HAM's F12 mixture and Dulbecco's modified Eagle's medium (Sigma, Oakville, Ontario, Canada) supplemented with 2.5 mM glutamine, 0.09 mg/ml tylosin, 110 units penicillin, 0.11 mg/ml streptomycin, and 10% fetal calf serum (Sigma). Cells were grown in 75 cm² flasks (Becton Dickinson Company, Franklin Lakes, New Jersey, USA) to 100% confluence before each experiment (5–7 days). Cells were maintained at 37°C in an atmosphere of 5% CO₂ in air. When confluent, cells were passaged using 1.5× trypsin and fed every 48 hours.

Animals

Male C57BL/6 mice (Charles River, Montreal, Quebec, Canada), 4–6 weeks of age, were housed at a constant temperature (22°C) and photoperiod (12 hour light:12 hour dark cycle), and were given free access to standard lab chow and tap water. Mice were allowed 5–7 days to acclimatise to these conditions before the experiments commenced. The University of Calgary Animal Care Committee approved all procedures involving animals, and the experiments were conducted in accordance with the regulations established by the Canadian Council on Animal Care.

Irradiation protocol

Conscious mice were physically restrained during irradiation or sham treatment. A ¹²⁷Cs small animal irradiator (GammaCell 40 Exactor; MDS Nordion, Kanata, Ontario, Canada) was used to deliver a 15 Gy parallel opposed field, total body gamma irradiation at a dose rate of 1.1 Gy/min. Sham mice were treated similarly but were not exposed to the radioactive source. Mice were studied 72 hours after treatment, based on our previous observations of radiation induced secretory dysfunction.¹⁰

RT-PCR for adenylate cyclase isoform expression

Reverse transcription-polymerase chain reaction (RT-PCR) for expression of adenylate cyclase isoforms in mouse was conducted in mucosal scrapings from colons taken from both irradiated and sham treated mice, in colonic biopsy specimens from patients undergoing colonoscopy, and in T84 cells (a colonic carcinoma cell line). In mouse studies, the colon was excised, opened longitudinally, and placed on a glass microscope slide on ice. The mucosal layer was gently scraped away from the muscularis. Between 20 and 30 mg of the mucosal scraping and, in human studies, the biopsy samples, were frozen at –70°C prior to processing for RNA extraction using the RNeasy mini kit, according to the manufacturer's instructions (Qiagen Inc, Mississauga, Ontario, Canada). Briefly, tissue was thawed and homogenised in lysis buffer provided with the kit. The sample was spun for three minutes at maximum speed (20 800 *g*) and the supernatant was mixed with an equal volume of 70% ethanol. The sample was then loaded onto an RNeasy spin column. RNA was purified using a series of 15 second wash spins (9000 *g*) with the wash buffers provided. Finally, RNA was eluted from the column in 30 µl ultrapure RNAase free water. For studies in T84 cells, a confluent 25 cm² flask of cells was scraped directly into the lysis buffer provided with the RNeasy mini kit and cells were then shredded in a cell shredder column (Qiagen) and processed as described above. For all studies, the purity and concentration of the RNA was measured using a Gene Quant II nucleic acid analyser (Pharmacia Biotech, Uppsala, Sweden). For the RT reaction, RNA (2 µg) was

added to 2 µl of 10× PCR buffer, 2 µl of 10 mM dNTPs, 2 µl of N₆, 0.5 µl of RNAGuard, and 300 units of superscript enzyme, and the reaction (10 minutes at 20°C, 50 minutes at 42°C, 10 minutes at 95°C) was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, Alameda, California, USA). cDNA from this reaction was cooled to 4°C and stored at –20°C until the PCR step was carried out.

The resultant cDNA was amplified by PCR using previously published primer sequences for human AC1, AC2, AC3, AC4, AC5, AC7, AC8, and AC9.¹⁶ The primer sequences for human AC6 (forward: 5'-TGCCTCCATTGCCAACTTCT and reverse: 5'-GTGCTGTCCATACGACTAGA), mouse AC6 (forward: 5'-GTACTACCAGTCGTGTGAATG and reverse: 5'-GTGCTGTCCATACGACTGGA), and mouse AC4 (forward: 5'-GGAAGACGAGAAGGGCACCGCAA) were modified from sequences published by Chabardes and colleagues.¹⁷ Mouse AC5 forward and reverse primers and the AC4 reverse primer were identical to published primers.¹⁷ Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used as an internal control.¹⁸ The final PCR reaction mixture contained 1× PCR buffer, 200 µM of each dNTP, 0.5 µM each of the 5' and 3' primers, 2.5 units of HotStartTaq DNA polymerase (Qiagen), and 2 µl of the cDNA template. The reaction was cycled 40 times (one minute at 94°C, one minute at 55°C, and one minute at 72°C). A final extension step of 10 minutes at 72°C was used before the reaction was cooled to 4°C. Samples were separated on a 1% agarose gel containing ethidium bromide for five minutes at 100 V and 35 minutes at 80 V. The gel was photographed using a BioRad Gel Doc 2000 (BioRad Inc., Hercules, California, USA).

Immunocytochemistry

T84 cells were seeded at 6.25×10⁵ cells/chamber and grown for 3.5 hours on collagen coated chamber slides (Lab-tek, Naperville, Illinois, USA). The media was removed and cells were washed in 1× Tris buffered saline (TBS). Cells were fixed in ice cold methanol for 20 minutes, washed in TBS, incubated for 30 minutes in 0.1% sodium azide plus 0.3% H₂O₂, washed 3×five minutes in TBS, and blocked in 1% bovine serum albumin and 5% fetal bovine serum in modified Eagle's medium for 30 minutes. A primary antiadenylate cyclase 5/6 rabbit polyclonal antibody (1:50) (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) was used to determine protein expression of the NO inhibitable isoforms. The primary antibody was removed with 3×five minute washes in TBS. A swine antirabbit biotin labelled secondary antibody (1:250; Dako Diagnostics Canada Inc., Mississauga, Ontario, USA) was incubated with the cells for one hour before being washed (3×five minutes) in TBS. Streptavidin-biotin-horseradish peroxidase mixture was incubated with the cells for one hour and cells were washed 3×five minutes in TBS before being incubated with AEC substrate system (Dako Diagnostics Canada Inc.) for 15 minutes at room temperature. Cells were washed once in TBS and once in distilled water, and counterstained with Mayer's haematoxylin (Fluka Biochemika, Oakville, Ontario, USA). One drop of Aquaperm mounting medium was added to each chamber (Immunon Shandon, Pittsburg, Pennsylvania, USA). Slides were coverslipped using DPX (Fluka Biochemika) and viewed by an observer unaware of the treatments on a Zeiss Axioplan microscope.

Immunohistochemistry

Mouse colon was fixed in Zamboni's fixative for 15 hours and washed in phosphate buffered saline (PBS: 0.55 M K₂HPO₄, 0.01 M NaH₂PO₄, H₂O, 0.31 M NaCl) with 0.01% sodium azide. The colons were cryoprotected in PBS containing 20% sucrose, embedded in OCT, and frozen at –70°C.

Sections (10 μm) were cut on a Jung CM 3000 cryostat (Leica Microsystems, Richmond Hill, Ontario, Canada), placed on silane coated slides, and stained with the selective AC5/6 rabbit polyclonal antibody (described above) overnight at 4°C. A CY3 conjugated donkey antirabbit secondary antibody (1:500; Jackson ImmunoResearch Laboratories, Mississauga, Ontario, Canada) was incubated with the sections for 90 minutes. They were then washed three times in PBS, coverslipped, and visualised under epifluorescence using a Zeiss Axioplan microscope. Images were captured and digitised with a CCD video camera and Northern Exposure imaging software (Carsen Vision, Edmonton, Alberta, Canada).

Studies of chloride secretion

Mice were either irradiated or sham treated, as above, and killed by cervical dislocation 72 hours later. The colon was rapidly excised and cut along the mesenteric border, mounted in Ussing-type diffusion chambers (Harvard Apparatus, Saint Laurent, Quebec, Canada), and bathed on both the mucosal and serosal sides with Krebs buffer, as previously described.^{10, 18, 19} The short circuit current (Isc) required to electrically clamp the tissue to zero volts was used as a measure of active electrogenic ion transport across the epithelium.

Tissues were allowed to establish a stable baseline Isc (approximately 10 minutes) before being incubated with the NO donors sodium nitroprusside (SNP, sodium pentacyanonitrosylferrate) or PAPA NONOate (1-propyl-1'-(aminopropyl)-2-hydroxy-2-nitrohydraxine), the selective iNOS inhibitors 1400w (N-(3-(aminomethyl(benzyl)acetamide; 5 μM)²⁰ and L-NIL (L-N⁶-(1-iminoethyl)lysine (3 μM),²¹ or the appropriate vehicle control. After incubation with SNP for 25 minutes or with PAPA NONOate for 15 minutes, tissues were exposed serosally to a near maximal concentration (10 μM) of the direct adenylate cyclase activator forskolin, the calcium dependent secretagogue carbachol (10 μM), or the protein kinase A (PKA) activator 8-Br-cAMP (1 mM). Once the secretagogue was added, the maximal change in Isc from baseline (ΔIsc) was recorded and expressed as $\mu\text{A}/\text{cm}^2$. All drugs were added to the serosal side of the tissue.

In some experiments, the change in Isc in response to forskolin was tested alone or in combination with the adenylate cyclase regulators 10 μM 8-Br-cAMP (to activate PKA) or 10 μM carbachol (to increase intracellular Ca^{2+}) to determine the functionally significant adenylate cyclase isoforms involved. In these costimulation studies, responses to forskolin (10 μM) alone were compared with coadministration of 10 μM forskolin+10 μM carbachol or 10 μM forskolin+10 μM 8-Br-cAMP in both irradiated and sham treated mice, as well as colons preincubated with the NO donor SNP.

Adenylate cyclase and guanylate cyclase activities

Generation of cAMP and cGMP were determined in mouse mucosal scrapings or T84 cells. Mucosal scrapings were obtained as described above, weighed, and suspended in Krebs's buffer (10 mM glucose, pH 7.4, and protease inhibitors leupeptin, aprotinin, and pepstatin A). Alternatively, 1×10^7 T84 cells were scraped into 1 ml of fresh Dulbecco's modified Eagle's medium. T84 cells or colonic mucosal scrapings were then incubated with vehicle (H_2O), 50 mM SNP, or 30 mM PAPA NONOate for 25 minutes or 15 minutes, respectively, at 37°C. Adenylate cyclase activity was measured using 10 μM forskolin in the reaction mixture. A final concentration of 100 μM of the cAMP phosphodiesterase inhibitor 4-(3-butoxy-4-methoxy-benzyl)imidazolidin-2-one) (Sigma, Oakville, Ontario, Canada) was added to the reaction mixture to prevent cAMP degradation. The reaction

was stopped after four minutes by snap freezing in liquid nitrogen. Because NO can act at soluble guanylate cyclase, we also measured cGMP levels. Tissue or cell suspensions were thawed and homogenised. Ethanol (100%) was added to a final concentration of 66% of the total volume. The tissue was centrifuged at 8600 *g* for five minutes and the cleared supernatant was dried using a speed vac (SC110; Savant, Holbrook, New York, USA). The dried sample containing the cyclic nucleotides was resuspended in 250 μl of assay buffer. Undiluted samples were assayed for cAMP or cGMP using commercially available EIA kits according to the manufacturer's protocol (R&D Systems, Minneapolis, Minnesota, USA).

Measurement of ATP levels post-irradiation

To determine that radiation induced epithelial hyporesponsiveness to forskolin was not due to depletion of substrate for adenylate cyclase, ATP levels in the tissue were measured using a commercially available ATP assay kit (Calbiochem-Novabiochem Corporation, San Diego, California, USA) based on the luciferin-luciferase system. At 72 hours post-irradiation or sham treatment, mice were killed and the colon excised. Mouse mucosal scrapings in 1 ml of Krebs's buffer were incubated for 10 minutes with or without 1400w (5 μM). Experiments with 1400w were included to determine if any radiation induced changes in ATP levels were due to iNOS derived NO. Samples were assayed according to the manufacturer's protocol. Samples were measured for 70 seconds using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, Michigan, USA).

Drugs

BDH (Toronto, Ontario, Canada) was the supplier for routine buffer reagents, unless otherwise indicated. 1400w was obtained from Alexis Corporation (San Diego, California, USA). PAPA NONOate was obtained from Cayman Chemical (Ann Arbor, Michigan, USA).

Statistics

Sample size per group for experiments using cell lines was 3–4, and for experiments in tissue, 5–17 per group. Statistical analyses of the data were conducted using GraphPad Instat software (version 3.0, San Diego, California, USA). One way ANOVA with a post hoc Tukey test was used to compare more than two groups. An unpaired Student's *t* test was used when comparing two treatment groups. Data are expressed as means (SEM) with a probability (*p*) value of less than 0.05 considered significant.

RESULTS

Adenylate cyclase isoform expression

As determined by RT-PCR, human colonic biopsy samples expressed mRNA for all isoforms except AC1 and AC8 (fig 1). T84 cells expressed AC3, 4, 5, 6, and 7. In another study, a mixed population of differentiated HT-29 cells expressed AC2, 3, 4, 5, 6, 7, and 9 (data not shown). AC4 and AC5 expression was demonstrated in mouse mucosal scrapings using mouse specific primers (fig 1). There was no difference in expression of mouse adenylate cyclase mRNA in colonic mucosal scrapings from irradiated mice compared with sham treated controls (fig 1). Samples were negative when assayed for contamination by genomic DNA (data not shown). AC5/6 immunoreactivity was located specifically within epithelial cells of the mouse colonic mucosa, with the most intense staining in the crypt region (fig 2). Positive immunoreactivity for AC5/6 was also observed in T84 cells (fig 3).

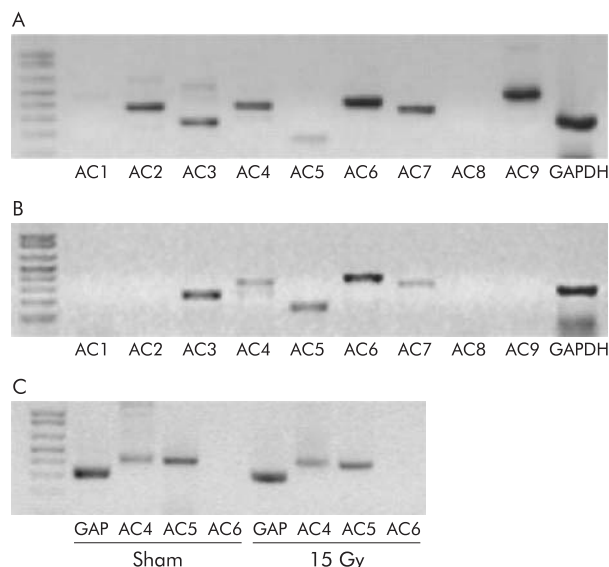


Figure 1 Distribution of adenylate cyclase isoforms 1–9 (AC1–9) in intestinal epithelium was identified by reverse transcription-polymerase chain reaction. cDNA from total RNA was amplified for 40 cycles by polymerase chain reaction. (A) Adenylate cyclase isoform mRNA expression in human biopsy samples ($n = 2$). (B) Adenylate cyclase isoforms in cultured intestinal T84 epithelial cells ($n = 4$). (C) Mouse specific primers were designed only for AC4, 5, and 6. AC4 and 5 were found in mucosal scrapings from colons taken from sham treated and irradiated mice ($n = 4$). There were no qualitative differences in expression of these adenylate cyclase isoforms three days post-irradiation. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Nitric oxide mediated hyporesponsiveness is specific for cAMP dependent chloride secretion post-irradiation

The Isc responses to forskolin, which activates adenylate cyclase, and carbachol, which stimulates secretion through increases in intracellular Ca^{2+} , were determined in experiments in which colons from unirradiated mice were incubated with the NO donors SNP or PAPA NONOate.

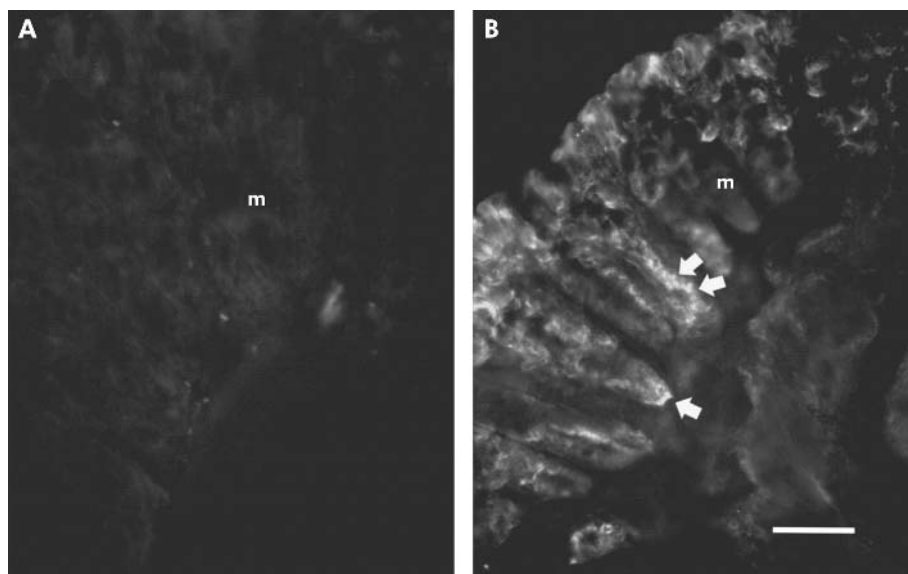


Figure 2 Immunoreactivity of adenylate cyclase isoforms AC5/6 was observed in epithelial cells of mouse colonic mucosa. Mouse colon was fixed and stained for AC5/6, as described in the methods section. (A) Section of mouse colonic mucosa incubated with only the secondary antibody (negative control). (B) The intestinal mucosa (m) exhibited positive AC5/6 immunoreactivity. The most intense staining was seen in the crypt epithelium (arrows). Some AC5/6 immunoreactivity was also observed in the submucosa. Scale bar 10 μm . Representative picture from a group of five.

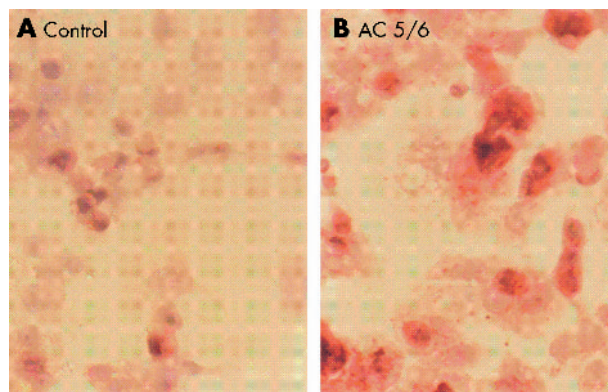


Figure 3 Immunoreactivity of adenylate cyclase isoforms AC5/6 was observed in a human intestinal epithelial cell line. T84 cells were fixed in methanol and stained for AC5/6 immunoreactivity. Cells were counterstained with haematoxylin. A red colour was indicative of positive staining for adenylate cyclase. (A) As a negative control, cells were incubated with only the secondary antibody. (B) Cells were incubated with a rabbit polyclonal anti-AC5/6 antibody. Representative micrographs from a total of three in each group.

There was a concentration dependent decrease in the Isc response to 10 μM forskolin following a 25 minute incubation period with SNP or a 15 minute incubation period with PAPA NONOate (fig 4). The response to carbachol was unchanged in the presence of the NO donors (fig 4).

Effect of irradiation or NO on cyclic nucleotide generation

There was a significant decrease in forskolin stimulated cAMP in mouse tissue and T84 cells treated with the NO donors SNP and PAPA-NONOate (fig 5). No changes in cGMP concentration were observed in mouse colon or T84 cells exposure to NO donor. When measured at 0, 1, 5, and 10 minutes after exposure to NO donor, cGMP levels in mouse colon were (in nM): 0.13 (0.01), 0.09 (0.01), 0.16 (0.04), and 0.10 (0.01), respectively. Levels of cGMP in T84

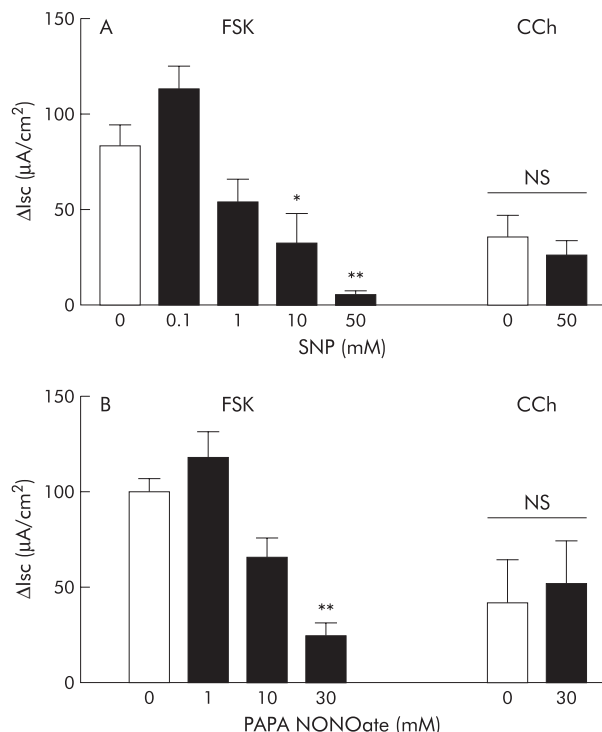


Figure 4 Nitric oxide (NO) inhibited cAMP dependent chloride secretion in a dose dependent manner but did not alter calcium dependent chloride secretion. Unirradiated mouse colon was mounted in Ussing chambers and incubated with (A) sodium nitroprusside (SNP 0.1–50 mM) for 25 minutes or (B) 1-propyl-1'-(aminopropyl)-2-hydroxy-2-nitrohydroxine (PAPA NONOate 1–30 mM) for 15 minutes before the change in short circuit from baseline (Δ Isc) was measured in response to 10 μ M forskolin (FSK), an activator of adenylate cyclase, or to 10 μ M carbachol (CCh), a cholinergic agonist. * $p < 0.05$; ** $p < 0.01$ compared with colonic samples not receiving pretreatment with the NO donor; NS, not statistically significant compared with colonic samples not receiving the NO donor. $n = 5$ –14 per group.

cells were (in nM): 8.74 (2.92), 9.00 (3.43), 7.50 (2.32), and 8.00 (2.31) at the same time points.

Determination of the adenylate cyclase isoform(s) mediating NO induced hyporesponsiveness

Due to the lack of availability of agonists and antagonists selective for the different adenylate cyclase isoforms, we took advantage of recent reports which showed that AC5 and 6 can be differentiated functionally from other isoforms based on the fact that their activities are reduced by both PKA activation and increases in intracellular calcium.^{14,22} 8-Br-cAMP was added as an activator of PKA and carbachol was used to increase intracellular calcium. There was a significant decrease in the Isc response to forskolin when tissues were costimulated with 10 μ M forskolin and either 10 μ M 8-Br-cAMP or 10 μ M carbachol, compared with the Isc response elicited by forskolin alone ($p < 0.05$) (fig 6). At the concentrations used, both 8-Br-cAMP and carbachol caused only modest changes in Isc when added on their own (data not shown).

When tissues were pretreated with SNP, the ability of both 8-Br-cAMP and CCh to reduce the response to FSK was lost (fig 6), suggesting that it is the NO inhibitable isoforms of adenylate cyclase that are blocked by costimulation. Similarly, when irradiated colons were costimulated with forskolin together with either 8-Br-cAMP or carbachol, no further inhibition of the Isc response to forskolin was observed (fig 6). Furthermore, the ability of costimulation

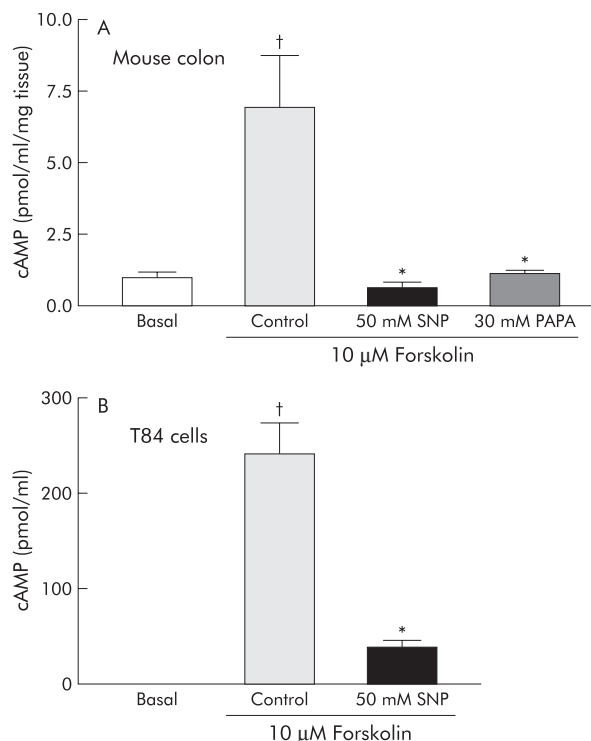


Figure 5 Adenylate cyclase activity, as measured by generation of cAMP in the presence of a phosphodiesterase inhibitor, was decreased in mouse colon and T84 cells following treatment with nitric oxide (NO) donors. (A) Segments of mouse colon were incubated with 50 mM sodium nitroprusside (SNP), 30 mM 1-propyl-1'-(aminopropyl)-2-hydroxy-2-nitrohydroxine (PAPA), or vehicle (control) before adenylate cyclase activity was stimulated with forskolin. The amount of cAMP is expressed as the concentration per ml of homogenate, normalised to the mass of the tissue sampled. (B) T84 cells were incubated with 50 mM SNP or vehicle (control) before addition of 10 μ M forskolin. The amount of cAMP is expressed as the concentration in the medium in which the cells were suspended. Accumulation of cAMP in both experiments was measured by a commercially available EIA kit. Basal levels of cAMP in the presence of NO donor were not different from untreated controls (data not shown). * $p < 0.05$ versus control; † $p < 0.05$ versus basal (not treated with forskolin). $n = 5$ –8 per group.

with 8-Br-cAMP or carbachol to reduce forskolin stimulated Isc was returned in colons from irradiated mice treated for 10 minutes with the iNOS inhibitor L-NIL (fig 6). L-NIL had no effect in sham treated mice.

Effect of radiation induced NO on responses to 8-Br-cAMP

To test the hypothesis that radiation induced NO was acting directly on adenylate cyclase, and not downstream from the enzyme, to inhibit cAMP dependent secretion, the mouse colonic secretory response to a secretory concentration (1 mM) of the cAMP analogue 8-Br-cAMP (1 mM) was assessed in irradiated colons or in the presence of the NO donor PAPA-NONOate (30 mM). This dose of 8-Br-cAMP was chosen because in control mice it produced a response comparable with that elicited by 10 μ M forskolin (96 (12) ν 100 (25) μ A/cm², respectively). Following irradiation, the response to 8-Br-cAMP was reduced by 50.5% in colons from mice exposed to 15 Gy gamma radiation compared with responses elicited in sham treated mice ($p < 0.05$), and this effect was not altered by pretreatment with the iNOS inhibitor L-NIL (59.8% reduction, sham ν 15 Gy in the presence of L-NIL; $p < 0.05$). Furthermore, the response to 8-Br-cAMP was not significantly reduced in the presence of

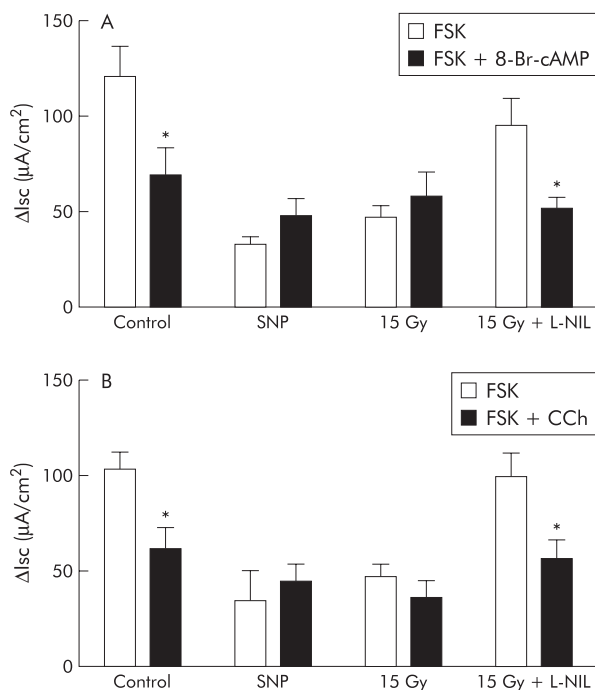


Figure 6 Change in short circuit current (Δ Isc) in response to administration of forskolin (FSK). Tissues from sham treated (Control) mice were exposed to the nitric oxide donor sodium nitroprusside (SNP 10 mM) prior to being exposed to FSK alone or FSK plus either (A) 8-Br-cAMP or (B) carbachol (CCh). In addition, tissues from irradiated (15 Gy) mice were exposed to FSK alone or FSK plus either (A) 8-Br-cAMP or (B) carbachol (CCh) in the presence or absence of the inducible nitric oxide synthase inhibitor L-N⁶-(1-iminoethyl)lysine (L-NIL). Coadministration of either 8-Br-cAMP or CCh reduced the response to FSK in colons from control mice. This was not observed in tissues exposed to SNP or in tissues from irradiated mice. However, the ability of coadministration of 8-Br-cAMP and CCh to reduce the response to FSK was restored in L-NIL pretreated colons from irradiated mice. * $p < 0.05$ versus FSK alone. $n = 6-17$ per group.

the NO donor PAPA-NONOate (30 mM) but rather was increased 1.6-fold ($p < 0.05$).

Effect of irradiation on colonic ATP levels

The decreased cAMP levels observed following irradiation could be an indirect result of decreased substrate availability for adenylate cyclase. However, the amount of ATP in the tissue was not different between sham and irradiated mice (2.8 (1.2) v 1.4 (0.4) nmol/mg tissue; $p > 0.05$), and was not affected by inhibiting iNOS activity with 1400w (sham, 2.0 (0.4) v 15 Gy, 2.1 (0.3) nmol/mg tissue; $p > 0.05$).

Role of G_i in NO mediated hyporesponsiveness to secretagogues

An alternate explanation for the hyporesponsiveness observed following irradiation or exposure to NO donors could be that an inhibitory signalling pathway, such as G_i, is activated by NO to reduce cAMP activity and thus chloride secretion.²³ To test this possibility, we first assessed the effect of G_i activation on the Isc response for forskolin. Indeed, the response to forskolin was significantly reduced when forskolin (10 μ M) and somatostatin (1 μ M), which acts through G_i, were added simultaneously to the chamber bath (fig 7). This effect was reversed by preincubation of the tissue with 250 ng/ml of the G_i inhibitor pertussis toxin (PTX). This experiment confirmed that forskolin stimulated chloride secretion in mouse colon is sensitive to modulation by G_i. However, blocking G_i with PTX had no effect on the

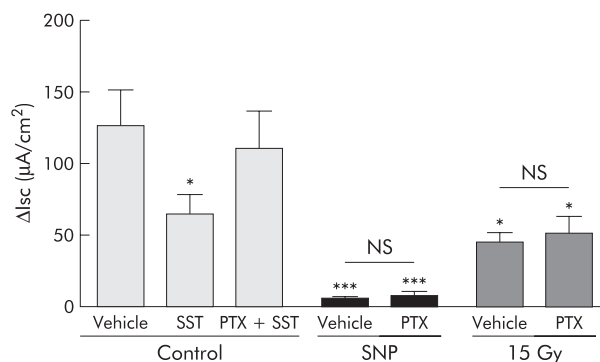


Figure 7 Nitric oxide mediated reduction of adenylate cyclase activity was independent of an inhibitory G protein coupled mechanism. In Ussing chambers, the change in short circuit current (Δ Isc) was measured in response to 10 μ M forskolin in the presence 1 μ M somatostatin (SST), an activator of the G_i signalling cascade, and a G_i inhibitor, pertussis toxin (PTX 250 ng/ml). Costimulation with forskolin and SST significantly reduced the secretory response compared with forskolin alone. This effect was reversed when the tissue was incubated for 25 minutes with PTX. When tissue from sham treated mice was incubated with 50 mM SNP \pm PTX for 25 minutes (black bars), or when tissue from irradiated mice (15 Gy) was incubated with PTX (grey bars), there was a significant hyporesponsiveness to FSK which was not reversed by PTX. * $p < 0.05$, *** $p < 0.001$ versus forskolin in the presence of vehicle; NS, not significantly different. $n = 5-12$ per group.

hyporesponsiveness to FSK observed either after exposure to SNP or in colons from irradiated mice (fig 7).

DISCUSSION

We have previously shown in a rat model of colitis that loss of responsiveness to secretagogues is associated with increased bacterial translocation,⁸ suggesting that a lack of responsiveness to secretagogues contributes to a deficiency in epithelial barrier function. Exposure to ionising radiation also predisposes animals to the transepithelial movement of bacteria,⁶ and we have demonstrated an NO dependent component to radiation induced epithelial hyporesponsiveness to secretagogues.¹⁰ NO, derived from iNOS, inhibits cAMP dependent but not Ca²⁺ dependent secretion,¹⁰ so an understanding of the mechanisms of the effect of NO on adenylate cyclase activity is key in understanding radiation induced epithelial barrier dysfunction and, ultimately, radiation enteropathy.

We have demonstrated here that the mucosa from human and mouse colon expresses mRNA for several adenylate cyclase isoforms. Of relevance to this study, the NO inhibitable isoforms AC5 and AC6¹⁵ were expressed in T84 cells and colonic biopsies, and AC5 was expressed in mouse colon. Furthermore, data from T84 cells and from immunohistochemistry of mouse colon suggest that AC5 and/or AC6 are also expressed in chloride secreting colonic epithelial cells. These isoforms are therefore in a position to participate in cAMP dependent secretion, and to be negatively regulated in situations where mucosal NO levels are elevated.

Having determined that the NO inhibitable isoforms of adenylate cyclase were expressed in secretory epithelia, we then considered whether these isoforms are responsible for radiation induced hyporesponsiveness to secretagogues. Our technical inability to conduct ion transport studies in biopsy specimens precluded us from pursuing studies in human tissue. Thus because we showed expression of the mRNA for AC5 in mouse colon, and that AC5 expression was not affected by exposure to ionising radiation, we used mouse for subsequent functional studies. To determine if NO inhibitable adenylate cyclase was involved in intestinal chloride secretion, we used an approach that took advantage of differences in regulatory properties of several of the adenylate cyclase

isoforms responsive to forskolin.¹⁴ A similar approach has been used in human airway smooth muscle cells to show that although cells express seven of nine adenylate cyclase isoforms, AC5 had a dominant functional role.¹⁶ We showed that coadministration of forskolin and either the PKA activator 8-Br-cAMP or the Ca²⁺ activator carbachol resulted in significantly lower forskolin induced chloride secretion compared with the secretory response to forskolin alone, consistent with the effects of these compounds on forskolin induced activation of AC5 or AC6, but not other adenylate cyclase isoforms.^{14 16 22 24–26} Observations similar to those made with carbachol coadministration were made in studies in which forskolin was coadministered with thapsigargin, which also increases intracellular Ca²⁺ (data not shown).

Taking this approach from cell free systems and applying it to our studies is complicated by the fact that the signalling systems which feedback on AC5 and 6 are also systems which mediate chloride secretion. Thus our data present the paradox that the forskolin stimulated change in Isc is reduced by mediators which, themselves, increase Isc. One can reconcile this paradox by considering that the observed response to forskolin alone is the net effect of both the stimulated chloride secretory response and inhibitory feedback mechanisms. Indeed, if these compounds did not have an inhibitory effect on secretion in addition to their secretory effect, one would expect an additive Isc response when they were coadministered with forskolin.

The next step was to determine if the hyporesponsiveness to forskolin following an NO donor or following exposure of mice to ionising radiation was occurring through inhibition of AC5. To do this, we repeated the costimulation studies (forskolin+8-Br-cAMP or forskolin+carbachol) in the presence of SNP or in colonic samples taken from irradiated mice. In each case, the response to forskolin alone was reduced by SNP or irradiation, but was not further reduced by coadministration of either 8-Br-cAMP or carbachol, suggesting that NO and coadministration of 8-Br-cAMP or carbachol are working through a similar mechanism. We anticipated that if NO was acting through an AC5 independent pathway, then the antisecretory effects of AC5 inhibition and the NO donor or irradiation would be additive. This was not the case, strengthening our contention that NO inhibits cAMP induced secretion through inhibition of AC5. Furthermore, in irradiated colon, pretreatment with the iNOS inhibitor L-NIL restored the ability of coadministration with 8-Br-cAMP or carbachol to reduce the Isc response to forskolin. If one considers these results in the context of the studies which showed that increased PKA and Ca²⁺ inhibit AC5 and AC6,^{24–26} then it can be inferred from our data that radiation induced hyporesponsiveness of mouse colon to cAMP dependent secretagogues is occurring through inhibition of AC5 by iNOS derived NO.

We next conducted experiments to determine the mechanism of NO mediated inhibition of secretion. Firstly, we assessed adenylate cyclase activity by measuring generation of cAMP. NO donors inhibited forskolin stimulated cAMP formation in both T84 epithelial cells and mucosal scrapings from mouse colon. These observations are in agreement with those made in isolated cholangiocytes.¹³ The effect of NO was not through soluble guanylate cyclase as we did not observe an increase in cGMP levels following exposure to the NO donors. We attempted to test the role of cGMP further by measuring the effect of NO donors on the Isc response of segments of mouse colon to the heat stable enterotoxin from *Escherichia coli*, which elicits secretion through cGMP. However, as observed by others, heat stable enterotoxin had little secretory effect on its own in segments of mouse distal colon²⁷ (data not shown).

We also assessed the ability of NO to act downstream of adenylate cyclase to affect cAMP dependent chloride secretion by determining the Isc response to a relatively high concentration (1.0 mM) of the PKA activator 8-Br-cAMP, in irradiated and sham treated tissue. We observed that irradiated tissue exhibited a significantly smaller response to 1 mM 8-Br-cAMP compared with tissue from sham treated animals. The degree of hyporesponsiveness was less than that observed for forskolin.¹⁰ Interestingly, hyporesponsiveness to 8-Br-cAMP was not reversed by pretreatment of the tissue with the iNOS inhibitor L-NIL. This suggests that the hyporesponsiveness observed post-irradiation was to some degree multifactorial, having NO dependent (likely at adenylate cyclase) and NO independent (downstream from adenylate cyclase) components. In contrast with our observations, Morel *et al* showed that while the secretory response of rat colon to forskolin was reduced post-irradiation, the responses to 8-Br-cAMP and Db-cAMP were not different between sham and irradiated tissue, suggesting that the process was being inhibited at adenylate cyclase or further upstream.²⁸ Differences between that study and ours may be due to differences in the species used (rat *v* mouse) or the dose of radiation delivered (10 *v* 15 Gy). Nevertheless, it is clear from our studies that NO inhibits adenylate cyclase activity as we demonstrated unequivocally that preincubation with NO donors significantly decreased forskolin stimulated adenylate cyclase activity and did not alter the response to 1 mM 8-Br-cAMP. More work is necessary to fully delineate the more subtle effects of radiation on cellular events downstream from adenylate cyclase in our model.

To further investigate the mechanism underlying radiation induced hyporesponsiveness to cAMP dependent secretagogues, we sought to determine the role of radiation induced iNOS on intracellular ATP. In agreement with Taylor *et al* who demonstrated that following hypoxia, reduced cAMP stimulated ion transport was independent of reduced ATP levels,²⁹ there was no difference between sham and irradiated colon (in the presence or absence of the iNOS inhibitor 1400w), indicating that NO was not inhibiting substrate availability. Similarly, in agreement with Tao *et al* who demonstrated that NO mediated inhibition of cAMP accumulation in forskolin stimulated membranes was unaffected by PTX,²³ we determined that NO dependent inhibition of forskolin induced secretion was independent of G_i activation as PTX was unable to reverse the SNP induced hyporesponsiveness to forskolin. This was despite the fact that forskolin stimulated chloride secretion was sensitive to inhibition by somatostatin, an activator of the G_i pathway.³⁰

In summary, our studies have extended previous evidence showing that iNOS derived NO inhibits cAMP dependent secretion^{10 11 13} by showing that adenylate cyclase isoforms AC5 and/or AC6 mediate chloride secretion in the intestinal epithelium, are the targets of NO derived from iNOS induced by exposure to ionising radiation, and mediate radiation induced secretory dysfunction. NO mediated reduction in chloride secretion may predispose the gut to increased LPS and bacterial translocation that may initiate a positive feedback loop at the time of initial radiation injury that permanently alters the epithelial microenvironment. NO mediated hyporesponsiveness to secretagogues may be a link between acute and chronic inflammation. Selective inhibitors that maintain epithelial barrier function during acute injury may represent an important new therapeutic strategy to prevent radiation enteropathy. Future studies will need to delineate the cellular source of iNOS derived NO that affects adenylate cyclase activity, and to determine the mechanism of action of NO on the cyclase.

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