Roles of muscarinic receptor subtypes in small intestinal motor dysfunction in acute radiation enteritis

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1Discipline of Medicine and 2Discipline of Physiology, University of Adelaide, and 3Department of Radiation Oncology and 4Nerve-Gut Research Laboratory, Department of Gastroenterology, Hepatology and General Medicine, Royal Adelaide Hospital, Adelaide, South Australia

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Frisby CL, Fraser RJ, Schirmer MB, Yeoh EK, Blackshaw LA. Roles of muscarinic receptor subtypes in small intestinal motor dysfunction in acute radiation enteritis. Am J Physiol Gastrointest Liver Physiol 293: G121–G127, 2007. First published May 3, 2007; doi:10.1152/ajpgi.00469.2006.—Administration of abdominal radiotherapy results in small intestinal motor dysfunction. We have developed a rat radiation enteritis model that, after exposure in vivo, shows high-amplitude, long-duration (HALD) pressure waves in ex vivo ileal segments. These resemble in vivo dysmotility where giant contractions migrate both antegradely and retrogradely. Mediation of these motor patterns is unclear, although enteric neural components are implicated. After the induction of acute radiation enteritis in vivo, ileal segments were isolated and arterially perfused. TTX, hexamethonium, atropine, or the selective muscarinic antagonists pirenzepine (M1), methoctramine (M2), and 1,1-dimethyl-4-diphenylacetoxyipiperidinium iodide (4-DAMP; M3) were added to the perfusate. The baseline mean rate per minute per channel of HALD pressure waves was 0.35 ± 0.047. This was significantly reduced by TTX (83.3%, P < 0.01), hexamethonium (90.3%, P < 0.03), and atropine (98.4%, P < 0.01). The HALD pressure wave mean rate per minute per channel was significantly reduced by pirenzepine (81.1%, P < 0.03), methoctramine (96.8%, P < 0.001), and 4-DAMP (93.1%, P < 0.03) compared with predrug baseline data. As an indicator of normal motility patterns, the frequency of low-amplitude, short-duration pressure waves was also assessed. The mean rate per minute per channel of 5.15 ± 0.98 was significantly increased by TTX (19%, P < 0.05) but significantly reduced by pirenzepine (35.1%, P < 0.02) and methoctramine (75%, P < 0.0003). However, the rate of small-amplitude pressure waves was not affected by hexamethonium, atropine, or the M3 antagonist 4-DAMP. The data indicate a role for neuronal mechanisms and the specific involvement of cholinergic receptors in generating dysmotility in acute radiation enteritis. The effect of selective M1 receptor antagonists suggests that M1 receptors may provide specific therapeutic targets in acute radiation enteritis.

ACUTE RADIATION DAMAGE to the bowel is an important cause of morbidity during radiotherapy for treatment of gynecological and pelvic malignancy. Therapeutic irradiation commonly results in symptoms such as nausea, vomiting, diarrhea, and abdominal pain (35) and is frequently associated with weight loss and malabsorption (33, 34). Acute toxicity is another major dose-limiting factor in the delivery of radiotherapy (15). Current drug therapy for acute radiation enteritis is usually symptomatic and of variable effectiveness in part because of limited understanding of the underlying pathogenetic mechanisms. Although symptoms have previously been attributed to mucosal injury (19, 32), there is substantial evidence indicating that mucosal damage often occurs independent of intestinal dysmotility (25). Furthermore, many symptoms can be directly related to changes in intestinal motor patterns (8).

Studies in both humans (33, 34) and animals (31) have shown that acute radiation damage is associated with accelerated small intestinal transit. The motor activities responsible for this remain unknown in humans, although in animals, abdominal irradiation has profound effects on small intestinal motor function (9, 24, 25, 29, 30). Precise investigation of the mechanisms that alter intestinal transit in vivo is difficult due to the complex interactions between the enteric and central neurotransmitter mechanisms in intact animals. Treatment targeted at specific motor disturbances would, however, provide significant potential benefits for patients with acute radiation enteritis.

The development of an organ bath technique to assess the effects of radiotherapy on small intestinal motility allows additional insights into the mechanisms underlying motor dysfunction (3, 9). Our group has established this approach and previously shown that abdominal irradiation in vivo results in specific motor events that were termed high-amplitude, long-duration (HALD) pressure waves in subsequently isolated arterially perfused loops from the rat ileum (9–11). These migrate in both antegrade and retrograde fashion similar to specific motor events mediated by enteric control pathways and humoral mechanisms in intact animals. Treatment targeted at specific motor disturbances would, however, provide significant potential benefits for patients with acute radiation enteritis.

METHODS

Animals. Experiments were performed with 34 adult male Sprague-Dawley rats ranging between 300 and 350 g in weight. Animals were individually housed in metabolic cages for the duration of the experiments, and, during this time, rats had free access to both food and water. The Animal Ethics Committees of the University of Adelaide

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and the Institute of Medical and Veterinary Science approved the study.

**Induction of acute radiation enteritis.** Rats were anesthetized by halothane (2–3%) prior to abdominal irradiation with a single dose of 10 Gy ionizing radiation administered at 0.67 Gy/min through a half-value layer of 2-mm copper focused to a skin distance of 50 cm via a Phillips Deep X-Ray Unit (RT 250, Phillips, Eindhoven, The Netherlands). Dose accuracy was calculated to be 89% at a depth of 3 cm to equal that administered clinically with modern accelerator beams. Lead shielding was placed at the level of the pelvis and sternum to ensure shielding of nonabdominal tissues. Rats were allowed to recover fully from the anesthetic before being returned to their cages. This dose of radiation was chosen to reflect that which allowed to recover fully from the anesthetic before being returned to an organ bath for equilibration. Arterial perfusion of fluorocarbon solution (FC-43, Green Cross, Osaka, Japan) was then passed through the ileal segment, which was then transferred to an organ bath during constant perfusion with oxygenated fluorocarbon solution (FC-43, Green Cross, Osaka, Japan). After adequate perfusion was established, the oral and aboral ends of the loop were cut and gently flushed with warm Krebs-bicarbonate buffer in an aboral direction to remove any fecal residue. A micromanometric assembly was then passed through the ileal segment, which was then transferred to an organ bath for equilibration. Arterial perfusion of fluorocarbon at a rate of 1 ml/min continued for the duration of the experiment (9). Animals were then killed painlessly with an anesthetic overdose.

Pressures were recorded using a pneumohydraulic apparatus with a six-channel micromanometric assembly with an outside diameter of 2 mm (Dentsleeve, Wayville, South Australia) (9). The assembly was perfused at 0.04 ml/min/channel with degassed distilled water. The aboral end was left open to permit free drainage of intraluminal perfusate. Pressure data were acquired and analyzed using purpose-designed software (MAD3, Charles Malbert, Institut National de la Recherche Agronomique, Rennes, France) on a Macintosh computer.

**Drugs.** The following drugs were added to the arterial perfusate to determine their effect on motility: 0.1 μM TTX (*n* = 4), 100 μM hexamethonium bromide (*n* = 4), 1 μM atropine (*n* = 6), 30 μM pirenzepine (*n* = 6), and 3 μM methoctamine (*n* = 6). All drugs were freshly prepared with 0.9% saline solution. 1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP; 3 μM, *n* = 6) was initially dissolved in DMSO and then diluted using saline to the required concentration. A segment of the irradiated ileum was used as a control to assess any effects DMSO might have on motility over a similar time course. No changes in motility were observed. Concentrations of drugs were chosen as maximal based on previous studies with only one drug investigated per ileal segment. The effective dose in this preparation was determined in pilot studies by ascending cumulative doses within the limits of selectivity for specific receptors (17).

**Protocol.** In each experiment, there was an equilibration period during which the ileal segment was allowed to equilibrate in 37°C carbogenated Krebs-bicarbonate buffer for 15 min before data were recorded. Predrug baseline manometric pressures were then collected for 15 min before a further 15 min recording of intra-arterial drug infusion. This was followed by a 15-min drug washout period, during which motility patterns were observed to return toward control, although this was not routinely measured due to persistence of some drug effects such as with TTX.

**Data analysis.** Pressure waves were scored if their amplitudes were >5 mmHg. The amplitude (mmHg) and rate (numbers/min/channel) of all pressure waves were calculated. As in previous studies, pressure waves with amplitudes of >20 mmHg and durations longer than 6 s were scored as HALD pressure waves (9). The rate (numbers/min/channel), amplitude (mmHg), and duration (s) of each HALD pressure wave were determined (Fig. 1).

All data are means ± SE. The effects of all drugs were compared with predrug baseline recordings (postequilibration) in each intestinal loop using a paired Students *t*-test. A *P* value of <0.05 was considered significant in all analyses.

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**Fig. 1.** Example of a 6-channel micromanometric pressure recording from an isolated arterially perfused segment of the irradiated small intestine. Numerous high-amplitude, long-duration (HALD) pressure waves are shown. Criteria used for HALD pressure wave classification are illustrated in the inset.
RESULTS

Irradiated rats lost weight (55.4 ± 3.8 g) over the 4 days postirradiation, which was associated with a reduction in food and water intake and a decrease in urine and fecal output. Diarrhea was produced in response to radiation-induced intestinal injury (9, 14).

Blockade of neuronal sodium channels. TTX infusion virtually abolished all HALD pressure waves in ileal segments from irradiated rats compared with predrug baseline periods (*P < 0.01, Fig. 2 and Fig. 3, i). The rate of LASD pressure waves was significantly increased with TTX infusion (*P < 0.04) while, concurrently, the amplitudes of LASD pressure waves were significantly decreased during TTX infusion (*P < 0.04; Fig. 4, i).

Antagonism of nicotinic cholinergic receptors. An intrarterial hexamethonium infusion significantly reduced the occurrence of HALD pressure waves compared with predrug baseline data (*P < 0.03; Fig. 3, ii). No effects on HALD amplitude or duration were observed. Hexamethonium had no

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Fig. 2. Multichannel micromanometric pressure tracings showing examples of the effects of arterially infused TTX on irradiated small intestinal motility. The arrow indicates the beginning of the TTX infusion. HALD pressure waves were rapidly abolished, with a significant increase observed in the low-amplitude, short-duration (LASD) pressure waves (inset).

Fig. 3. Group data for the numbers of HALD pressure waves per minute per channel (HALDS/min/ch) before and during drug infusions. Significant decreases in the rate of HALD pressure waves were observed for drug infusions (A–F). Atropine (1 μM; C) and methoctramine (3 μM; E) infusion virtually abolished all HALD pressure waves.
effect on either the rate or amplitude of LASD pressure waves in loops from irradiated animals (Fig. 4, ii). Antagonism of muscarinic cholinergic receptors. There was a significant reduction in the occurrence of HALD pressure waves following the infusion with atropine compared with predrgu baseline data ($P < 0.01$; Fig. 3, iii) with no effect on amplitude or duration. Atropine did not affect the rate but significantly reduced the amplitude of LASD pressure waves ($P < 0.05$; Fig. 4, iii).
**MUSCARINIC RECEPTORS IN RADIATION-INDUCED DYSMOTILITY**

Muscarinic receptor antagonism: pirenzepine ($M_1$), methoctramine ($M_2$), and 4-DAMP ($M_3$). Pirenzepine infusion significantly reduced the occurrence of HALD pressure waves compared with baseline frequencies ($P < 0.03$; Fig. 3,iv). Pirenzepine also significantly reduced the rate ($P < 0.02$) and amplitude of LASD pressure waves ($P < 0.005$; Fig. 4,iv).

Methoctramine virtually abolished HALD pressure waves ($P < 0.001$; Fig. 3,v). However, it also reduced both the rate ($P < 0.0003$) and amplitude ($P < 0.003$) of LASD pressure waves to the extent that many contractions did not reach the amplitude (>5 mmHg) required for classification (Fig. 4,v).

4-DAMP significantly reduced the number of HALD pressure waves ($P < 0.03$; Figs. 3,vi, and (Fig. 5). The rate of LASD pressure waves was unaffected by 4-DAMP, but a small reduction in the amplitude of LASD pressure waves was observed ($P < 0.05$; Fig. 4,vi).

Pirenzepine, methoctramine, and 4-DAMP had no significant effect on HALD amplitude or duration compared with predrug data.

**DISCUSSION**

The aim of this study was to identify potential therapeutic agents to normalize pathological patterns of intestinal motility without significant disruption of the physiological patterns that underlie normal digestive function. We have demonstrated that the HALD pressure waves previously identified in radiation enteritis (9) are neurally mediated and rely on cholinergic transmission, in contrast to low-amplitude, regular intestinal pressure waves.

In addition, these results indicate a pivotal role for muscarinic M1 receptors in the mediation of HALD pressure waves and suggest these receptors as potential therapeutic targets in motility disturbances such as those involved in radiation enteritis.

Our previous description of the duration and amplitude of HALD pressure waves suggested they were likely to have a neural etiology (9). The abolition of HALD activity with TTX confirms that neural mechanisms are responsible for their generation. In contrast, non-HALD pressure waves were slightly augmented in their rate of occurrence by TTX, suggesting they arise in response to myogenic mechanisms under tonic neural inhibition. HALD amplitude, however, was reduced by TTX, suggesting that they are augmented by release of excitatory transmitters onto smooth muscle. Although nerves are generally believed to be resistant to radiation-induced injury, evidence suggests that there are ultrastructural changes that may have a role in mediating abnormal motility (12), and these most likely would parallel changes in gene expression leading to altered function.

To further define the mechanisms underlying this radiation-induced dysmotility, we examined the effects of nicotinic and muscarinic blockade on HALD and non-HALD pressure wave activity. These data indicate that both nicotinic and muscarinic pathways are important in HALD pressure wave activity.

Hexamethonium abolished HALDs but had no effect on non-HALD pressure waves, suggesting that synaptic activation within the enteric nervous system is important in activating the motor pathways that underlie HALD production. The pathogenesis of non-HALD pressure waves is unclear, but the small increase following TTX, and the lack of response to hexamethonium, suggests that these are locally mediated and do not require inputs from enteric neural pathways.

The response to atropine indicated that muscarinic mechanisms were important in the control of radiation-induced dysmotility. Muscarinic M1 receptors are known to be selectively localized on neural pathways, in particular, on myenteric neurons (5), where they mediate excitation of inhibitory and excitatory motorneurons and interneurons (20, 21). In contrast, M2 and M3 receptors are preferentially localized in longitudinal and circular smooth muscle layers, where they mediate contractile effects of ACh (6), although the two subtypes may be expressed in different proportions depending on the region of the gastrointestinal tract (1).

As it has been suggested that muscarinic receptors ($M_1$) localized to enteric ganglia may have a role in motility following inflammation (22), additional experiments were undertaken to examine the effects of more selective muscarinic receptor blockade of neural pathways controlling gastrointestinal motility. The reduction in HALD pressure wave activity following pirenzepine infusion suggested that $M_1$ receptors may play a role during radiation-induced injury, but the concomitant reduction in low-amplitude pressure waves indicated that this effect was not selective for the pathophysiological pattern.

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**Figure 5.** 4-DAMP arterial infusion (as indicated by the arrow) produced loss of both HALD and LASD pressure waves.
The abolition of HALD pressure waves by methoctramine indicates that M₂ receptors are important in HALD mediation. The exact localization of these receptors is unresolved, as both prejunglational and postjunglational M₂ receptors have been reported to be involved in the ascending reflex contraction in the rat ileum (17). However, similar to findings with M₁ receptors, the effects of methoctramine were not selective for HALD motility. The fact that methoctramine reduced motility to a greater extent than atropine would suggest that some of the additional M₁ blockade by atropine was along inhibitory neural pathways to the smooth muscle, thus removing both inhibition and excitation. We would expect a pure M₂ effect to be more restricted to excitatory pathways.

Interestingly, our results indicated that while M₂ receptor antagonism reduces both HALD and LASD pressure waves, M₃ receptor antagonism is more selective for HALD pressure waves. As both subtypes are expressed on rat small intestinal smooth muscle (16, 26, 28), there are two possible explanations for the observed effects. The first is that M₂ receptors are positioned to respond to physiological levels of ACh release at the interstitial cells of Cajal (ICCs) that form the neuromuscular junction (7), whereas M₃ receptors may be located elsewhere on smooth muscle or ICCs and respond only to supra-physiological levels of ACh release, such as may occur during prolonged activation of excitatory motorneurons. A second possible explanation would be that there are specialized junctions between a subpopulation of excitatory motorneurons and ICCs that encode the program for HALD pressure waves. Considering the occurrence (albeit rare) of HALD in the normal state, we consider the first explanation to be more likely, as this would not require the development of a special class of motorneurons for a relatively infrequent motor pattern. Interestingly, M₃ receptors have been implicated in the giant migrating contractions (GMCs) recorded in the normal rat colon in vivo (18), further indicating that they are physiologically activated.

The precise triggering mechanisms for HALDs is unknown. Peristaltic pressure waves similar to HALDs are seen during distension at 10 ml/h (3) and increased by higher distension rates (2). It is thus possible that after irradiation, there is an exaggerated response to the infused perfusate in loops, although this requires direct assessment.

Although nonselective muscarinic antagonists such as atropine provide some symptomatic benefit during acute radiation enteritis, our data suggest that a more specific muscarinic blockade, possibly with novel M₁ receptor antagonists, may have more specific effects without anticholinergic side effects. Furthermore, the virtual abolishment of all HALD and non-HALD pressure waves in response to methoctramine infusion suggests that M₂ receptors could also be considered in the treatment of radiation enteritis. Another group of targets that are beyond the scope of the present study are tachykinin receptors. These play important roles, particularly in diseases of the gut (13), and may give rise to specific motor patterns (4). Their role in mediation of dysmotility in radiation enteritis remains to be elucidated. Notwithstanding, the selective effect of the M₁ receptor antagonist that we have identified should provide a stimulus for further preclinical and clinical studies of the therapeutic potential of this class of drugs.

As we have recorded HALD pressure waves in isolated tissue, we cannot directly ascribe a physiological correlate to them. In the dog, high-amplitude, lumen-occlusive contractions, which migrate rapidly in aboral GMCs or oral directions retrograde giant contractions, allows rapid transport of intestinal contents along the gut tube (23). We hypothesize that the HALD pressure waves seen in our preparation may represent an in vitro correlate of these motor events. GMCs have a far longer duration than ordinary intestinal contractions and propagate far more rapidly than lower-amplitude contractions (23), characteristics similar to those of HALD pressure waves, which have durations five to six times that of low-amplitude waves and also migrate rapidly along the intestinal segment. In addition, GMCs are neurally mediated and sensitive to hexamethonium (27), features in common with HALD pressure waves. However, further studies are required to examine this potential relationship.

In conclusion, we have shown that in vitro dysmotility (HALDs) associated with radiation enteritis is neurally mediated and involves both muscarinic and nicotinic pathways. Both M₁ (pirenzepine) and M₂ (methoctramine) receptor blockade reduced the occurrence of HALDs but also inhibited normal low-amplitude pressure activity. In contrast, selective M₃ receptor blockade (4-DAMP) reduced HALD activity with minimal effects on normal motor function, suggesting a potential role for M₃ receptor antagonists in the control of dysmotility associated with acute radiation enteritis.

GRANTS
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REFERENCES


