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Effects of long-term laxative treatment on neuropeptides in rat mesenteric vessels and caecum

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Abstract—In order to investigate the toxic effects of long-term treatment with anthraquinone laxatives, rats were fed either chocolate alone, or chocolate adulterated with senna or danthron (1,8-dihydroxyanthraquinone) for 5 months. Mesenteric blood vessels and the outer muscle layers of the caecum, together with the myenteric plexus, were examined using ultrastructural, histochemical, immunohistochemical and immunoassay techniques. There was no ultrastructural evidence of degeneration in either the mesenteric vessels or the caecum. In the mesenteric vessels, levels of neuropeptide Y were significantly reduced in the danthron-fed rats, but levels of substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) were unaffected by all treatments. In the caecum, VIP-, SP- and CGRP-immunoreactivity and catecholamine-fluorescence were unchanged by the laxative treatments.

Laxatives have been used in enormous quantities for the relief of constipation, a distressing complaint affecting all age groups. In 1989, 1·5% of all National Health Service prescriptions were for laxatives. Ingestion of the anthracene laxatives has been particularly widespread, even though their precise mode of action is unknown (Smith 1968; de Witte & Lemli 1988). The specific influence of these laxatives on the large intestine, their low oral toxicity and lack of side effects with controlled dosage have determined their popularity of use (Godding 1976); however, these drugs are often self-administered and subject to much misuse (Nelemans 1976). Excessive, long-term dosage of such purgatives in man can lead to the development of a cathartic colon with complete loss of motor and functional activity, sometimes necessitating colectomy (Reimann et al 1980). Loss of intrinsic innervation, myenteric neuronal damage and atrophy of smooth muscle coats have been reported (Smith 1968, 1972).

Sennosides A and B are naturally occurring anthraquinones that are catabolized by the microorganisms in the colon to their aglycones, rhein and rhein-9-anthrone. These compounds act on epithelial cells and submucosal neurones, producing net secretion of electrolytes and water, and induce synthesis of prostaglandins (Leng-Peschlow 1986; Beubler & Kollar 1988; Claus et al 1988). There have been conflicting reports in the literature on animal studies of the effects of long-term administration of anthraquinones on neurones of the myenteric plexus (Smith 1968; Dufour & Gendre 1984, 1988; Kiernan & Heinicke 1989; Heinicke & Kiernan 1990).

In the present study, we have used ultrastructural, histochemical, immunohistochemical and immunoassay techniques to investigate the toxic effects of long-term treatment of rats with either sennosides or the synthetic anthraquinone, 1,8-dihydroxyanthraquinone (danthron), on mesenteric vessels and the muscle coat of the caecum containing the myenteric plexus. We have investigated these tissues because, in the rat, sennosides are largely catabolized to their active compounds by the bacterial flora in the caecum and there is evidence that senna and its metabolites may affect mesenteric vessels, where the active metabolites of senna accumulate (de Witte & Lemli 1988), to cause sensory neuropathy of the resistance vessels (Ralevic et al 1990).

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Materials and methods

Animals and treatment. Female Sprague-Dawley rats, 208–265 g, were used in this study. There were nine rats in each of the four study groups: controls were untreated; chocolate-fed animals were given 5 g of commercial milk chocolate every day; senna-fed animals were given 5 g of chocolate adulterated with ground senna pods per day, thus receiving a daily dose of sennoside B of 3·8 mg (17–20 mg kg^{-1}); and danthron-treated animals were given 5 g of chocolate adulterated with 5 mg danthron (18–24 mg kg^{-1}). All groups had free access to water and food. The treated animals were individually housed and ate the chocolate avidly. Animals were maintained on their respective diets for 23–25 weeks until they were killed by stunning and exsanguination. By 1–2 weeks, the stools of the senna and danthron groups of rats were soft and pale but by 3–4 weeks they appeared to be normal, i.e. they were hard and dark. There were no significant differences in weight gain amongst the four groups.

Immunoassay. The caecum and a section of mesentery (a section of vein and artery) were dissected out and frozen in liquid nitrogen for storage. The samples were later thawed, the outer muscle layers containing the myenteric plexus were dissected from the whole thickness of the caecum and weighed, and the mesenteric vessels were cleaned of connective tissue and fat and the length of vessels measured. Peptides were extracted from the tissues by boiling in 0·5 M acetic acid for 10 min, followed by homogenization, centrifugation and lyophilization of the supernatant. The tissue contents of calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) in the mesentery, and VIP and SP in the caecum, were measured using an inhibition enzyme-linked immunosorbent assay (ELISA) as previously described (Belai et al 1988), and expressed as pmol (g caecal tissue)$^{-1}$ and pmol (cm length of mesenteric vessel)$^{-1}$.

Immunohistochemistry Segments of caecum were dissected out, cut open lengthwise and stretched out onto strips of Sylgard silicone rubber with the mucosal side downward. The tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 h at 4°C. The longitudinal and circular muscle layers, together with the myenteric plexus, were peeled away from the rest of the caecum and whole mounts of tissue were processed for immunohistochemical detection of SP, CGRP and VIP, as previously described (Belai et al 1988).

Fluorescence histochemistry. Segments of caecum were dissected out and stretched, as described above. The stretched tissues were immersed in 2% w/v glyoxylic acid solution in 0·1 M PBS, pH 7·4 for 1·5 h (Lindvall & Björklund 1974). They were then transferred onto clean slides, dried until translucent and placed in an oven at 80°C for 4 min. After the tissues were mounted, catecholamine-containing nerves were viewed under a Zeiss microscope fitted for epifluorescence with UV filters.

Ultrastructural examination. Transverse 1 mm slices of caecum and mesenteric artery were taken from control (n = 6), chocolate-fed (n = 7), danthron-fed (n = 6) and senna-fed (n = 7) rats.

Tissues were immersion-fixed in 1·5% glutaraldehyde, 1·5% freshly prepared paraformaldehyde in 0·1 M cacodylate buffer, post-fixed in 2% osmium tetroxide in 0·1 M cacodylate buffer and embedded in Araldite resin for conventional transmission electron miscroscopy. Ultra-thin sections were cut on an OMU2 ultramicrotome, stained with uranyl acetate and lead citrate and viewed in a Philips 300 electronmicroscope.

Statistical comparisons. The immunoassay data were expressed as mean ± s.e.m. and compared by analysis of variance and an unpaired Student's *t*-test. $P < 0.05$ was taken as being significant.

Results

Immunoassay data. In the mesenteric vessels and smooth muscle layers of the caecum containing the myenteric plexus, the levels of NPY, VIP, CGRP and SP were not significantly different in chocolate-fed rats compared with controls (Fig. 1). However, compared with the chocolate-fed rats (true controls), NPY levels were significantly reduced ($P < 0.05$) in the mesenteric vessels of the danthron-fed rats, although SP, CGRP and VIP levels were unaffected by the treatment.

There were no significant changes in the levels of VIP and SP in the tissue samples of caecum after laxative treatment compared with untreated and chocolate-fed controls (Table 1).

Immunohistochemical examination of innervation. Dense VIP- and CGRP-immunoreactive nerve fibres and occasional cell bodies were clearly visible in the myenteric plexus and circular muscle layer of all preparations. Fluorescent catecholamine-containing nerve fibres were most prominent around unstained ganglion cells and hardly visible in the muscle coats. There were no apparent differences in either the intensity or the density of fluorescent VIP-, CGRP- or SP-immunoreactive nerve fibres in the myenteric plexus and muscle coats of the caecum after any of the treatments. Catecholamine-fluorescence was also unaffected by chocolate, danthron or senna treatment.

Ultrastructural examination. Ultrastructural examination of sections of caecum and mesenteric artery from control and treated groups revealed no changes in tissue morphology. Examination of axon profiles and nerve cell bodies in the myenteric plexus of the caecum from the treated animals revealed that they were morphologically similar to those from the control animals. Similarly, axon profiles of the perivascular nerves of the mesenteric artery and endothelial cells lining the vessel showed no signs of damage. No ultrastructural changes were observed in smooth muscle cells from either tissue after treatment.

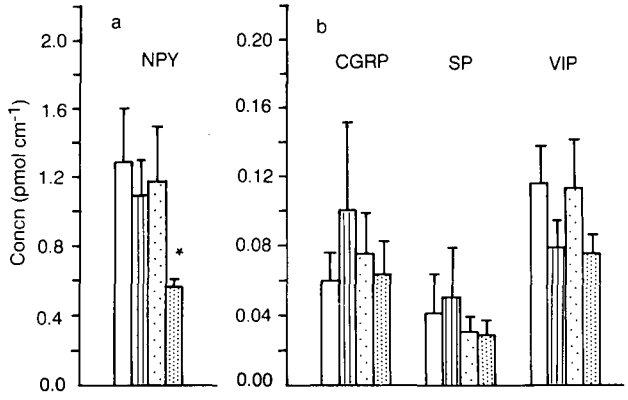

FIG. 1. Levels of (a) neuropeptide Y (NPY) and (b) calcitonin gene-related peptide (CGRP), substance P (SP) and vasoactive intestinal polypeptide (VIP) in mesenteric blood vessels from control (blank columns, n = 5), chocolate-fed (striped columns, n = 5), senna-fed (sparse dot columns, n = 6) and danthron-fed (dense dot columns, n = 6) rats. Data are expressed as pmol (cm length of mesenteric vessel)$^{-1}$. Levels of NPY were significantly reduced in the danthron-fed group compared with the chocolate-fed group. *$P < 0.05$.

Table 1. Levels of vasoactive intestinal polypeptide (VIP) and substance P (SP) in the outer muscle layers of the rat caecum containing the myenteric plexus.

	VIP (pmol (g tissue)$^{-1}$)	SP (pmol (g tissue)$^{-1}$)
Control	178·4 ± 46·4 (6)	48·1 ± 17·0 (6)
Chocolate-fed	166·4 ± 57·5 (6)	67·9 ± 37·8 (6)
Senna-fed	162·6 ± 52·5 (6)	24·0 ± 8·3 (6)
Danthron-fed	271·9 ± 66·3 (6)	64·4 ± 23·3 (6)

(n) = number of animals.

Discussion

This study shows that there is no evidence for neuropathy of myenteric neurones of the rat caecum after 5 months of ingestion of purgative doses of either senna or danthron. This concurs with reports on the rat colon (Kiernan & Heinicke 1989; Heinicke & Kiernan 1990) which show that there is no evidence of toxic destruction of any identifiable population of neurones after 4 months of ingestion of purgative doses of either senna or danthron. Morphological and histological studies of the rodent colon have suggested that enteric neuropathy reported after chronic administration of syrup of senna (Smith 1968) may be due to the action of free anthraquinones which are present in the syrup rather than the sennosides because sennosides A and B have no effect on intestinal and jejunal myenteric neurones whereas danthron causes axonal damage (Dufour & Gendre 1984, 1988). This has been disputed, however, by Heinicke & Kiernan (1990), who found no changes in either the total number of neurones, or neuropeptide immunoreactivity, in the intestinal myenteric plexus after chronic danthron treatment. Our findings in the caecum also show resistance to danthron-induced damage.

Nevertheless, it does seem that the mesenteric vessels may be more susceptible to the effect of long-term anthraquinone intake, maybe because of the accumulation of rhein-anthrone in these tissues. Whilst there were no ultrastructural abnormalities in these vessels after laxative treatment, the NPY content was significantly reduced in the danthron-treated animals. NPY and ATP are co-released with noradrenaline from sympathetic nerves innervating rat mesenteric vessels (Sjöblom-Widfeldt 1990). NPY is also contained in enteric neurones, some of which project to mesenteric vessels (Furness et al 1983). The functional significance of the reported change in NPY content is unknown; while pressor responses elicited by perivascular nerve stimulation are unaffected by long-term ingestion of danthron (Ralevic et al 1990), any changes in the predominant pre- and post-junctional neuromodulatory roles of NPY (Westfall et al 1987) have not been investigated. NPY is a potent inhibitor of intestinal fluid and epithelial ion transport in the gastrointestinal tract (Saria & Beubler 1985; Friel et al 1986; Cox & Cuthbert 1988). The action of anthraquinone derivatives is to induce colonic secretions, hence, reduced NPY levels in the mesentery may reflect changes in the levels of NPY in enteric neurones, which project to these vessels, in response to this stimulus.

Although there are already indications of a disturbance of sensory neurotransmission in the mesenteric vascular bed in the same model of long-term ingestion of anthraquinones (Ralevic et al 1990), there were no changes in the levels of the neuropeptides involved in sensory neurotransmission, namely, SP and CGRP, nor was there any ultrastructural evidence for neuropathy. The sensory neurotoxin, capsaicin, when applied topically, stimulates release of SP and CGRP from sensory nerve fibres (Maggi & Meli 1988) and causes relaxation of the preconstricted rat mesenteric vascular bed, largely due to stimulation of sensory nerve endings (Manzini & Perretti 1988). A repeated application of capsaicin to the mesenteric bed from senna- and danthron-fed rats causes a smaller inhibition of pressor responses compared with the control group, suggestive of a limited source of inhibitory neurotransmitter after laxative treatment (Ralevic et al 1990). Thus it seems that chronic laxative administration affects the mechanisms of neurotransmission rather than causes neurodegeneration.

Despite the lack of toxic effects of anthraquinones on the morphology and content of neuropeptides in neurones of the myenteric plexus and nerves that innervate the outer smooth muscle layers of the caecum, there is preliminary evidence that there is an impairment of the non-adrenergic, non-cholinergic (purinergic) component of neuromuscular transmission in the caecum after long-term administration of danthron (C. Hoyle, unpublished observations). Whether the sensory dysfunction detected in mesenteric blood vessels after chronic anthraquinone ingestion is also related to the ATP-component of neurogenic inhibition is unknown.

Although caution should be used in relating the effects of long-term laxative ingestion in rodents to the effects of chronic laxative abuse in man, these studies indicate that anthracene laxatives may not be as neurotoxic in the intestine as was originally thought (Smith 1973; Reimann & Schmidt 1982). Some of the changes reported in man should perhaps be attributed to motility disorders that may have initiated the need for laxative ingestion. For example, disturbances in the normal neural content of VIP and 5-hydroxytryptamine in the bowel wall in idiopathic chronic constipation in man have been reported which may contribute to the functional changes seen in this disorder (Koch et al 1988; Milner et al 1990; Lincoln et al 1990).

In summary, apart from a reduction in NPY in mesenteric vessels, no gross morphological or immunohistochemical abnormalities were observed following chronic laxative treatment with anthraquinones. This does not exclude the possibility, however, that changes in the physiology of neurotransmission occur; indeed, some have already been observed.

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