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The lipid peroxidation product 4-hydroxy-2,3-*trans*-1 nonenal decreases rat intestinal smooth muscle function in-vitro by alkylation of sulphydryl groups

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Abstract—The effects of the lipid peroxidation product 4-hydroxy-2,3-*trans*-1 nonenal (HNE) on intestinal smooth muscle function have been studied. Exposure of rat isolated small intestinal segments to HNE (0·1–0·5 mM) led to decreased muscarinic and β-adrenergic responses. The maximal response to the muscarinic agonist methacholine and its pEC50 decreased in a dose dependent manner. The response to the β-adrenoceptor agonist isoprenaline was affected in a similar manner, but at slightly higher concentrations of HNE. As HNE has been described to be sulphydryl-reactive these effects were compared with the effects of the sulphydryl-reactive agent *N*-ethylmaleimide (NEM). Incubation of intestinal segments with NEM had similar effects on pharmacological responses to methacholine, indicating that the effects of HNE like that of NEM are likely to be caused by alkylation of sulphydryl groups. Dithiothreitol, a compound which reduces oxidized sulphydryl groups, was unable to restore the effects of HNE or NEM, which suggests that the effects of HNE and NEM are irreversible.

Oxidative stress has been shown to alter smooth muscle function in the intestinal tract. Hydrogen peroxide (an important reactive oxygen metabolite which is formed after intestinal ischaemia and reperfusion or during inflammatory processes) or cumene hydroperoxide (a model compound which is used to induce lipid peroxidation) deteriorates receptor-dependent or -independent contraction of the smooth muscle (Van der Vliet et al 1989). The effect of cumene hydroperoxide has been shown to be related to the process of lipid peroxidation, since inhibitors of lipid peroxidation also prevent smooth muscle damage by cumene hydroperoxide (Van der Vliet et al 1990b). One major product of lipid peroxidation has been shown to be 4-hydroxy-2,3-*trans*-nonenal (HNE) (Benedetti et al 1980; Pryor & Porter 1990) and this compound has direct actions on smooth muscle (Van der Kraaij et al 1990) and is able to affect receptor responses (Leurs et al 1986; Haenen et al 1989). Therefore we have investigated the effects of HNE on receptor responses in the rat small intestinal smooth muscle.

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Materials and methods

Chemicals. Methacholine hydrochloride, (−)-isoprenaline hydrochloride, *N*-ethylmaleimide (NEM), dithiothreitol (DTT) and N^6-2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate (sodium salt) (dibutyryl-cAMP) were obtained from Sigma Chemical Co. (St Louis, USA). 4-Hydroxy-2,3-transnonenal (HNE) was synthesized in our laboratory as described earlier (Leurs et al 1986). All other chemicals were of reagent grade.

Pharmacological measurements. Male Wistar rats, 220–250 g (Harlan C.P.B., Zeist, The Netherlands), were killed by decapitation and the midpart of the small intestine was removed rapidly and rinsed with Krebs buffer. Segments of about 1·5 cm were mounted in 20 mL tissue baths and kept at 37°C in Krebs buffer which was continuously gassed with 95% O_2–5% CO_2 (pH = 7·4). After equilibration for 30 min concentration-response-curves for methacholine or isoprenaline were recorded by cumulative addition of the agonists (Van der Vliet et al 1989, 1990a). After construction of the control curve, the segments were exposed to various concentrations of HNE or NEM for 20 min, during which time the smooth muscle tension was recorded isotonically. After incubation with HNE or NEM and extensive washing (30 min, during which the buffer was refreshed three times), a second concentration-response-curve was measured. In some experiments 1 mM DDT was added after incubation of intestinal segments with HNE or NEM during the first 20 min of the washing period.

Calculation of the data and statistics. Activities of the agonists used are expressed as pEC50 (i.e. log EC50). Maximal responses of the small intestine to methacholine or (−)-isoprenaline after treatment with HNE or NEM were related to the maximal response before treatment. Time-matched control curves were recorded for statistical analysis of differences between treated and untreated segments, which were determined using Student's *t*-test. $P < 0.05$ was regarded as significant.

Results and discussion

At concentrations of 0·1–0·5 mM, HNE affects the basal motility of the small intestine, which is completely inhibited at higher

Table 1. Effects of HNE and NEM on rat intestinal smooth muscle contraction by methacholine.

Treatment	pEC50	Max. effect (%)	n
Control	6·7±0·2	98±13	6
[HNE] (mM)			
0·1	6·7±0·2	88±10	6
0·2	6·3±0·2*	52±23*	7
0·2+1 mM DTT	6·1±0·2*	55±15*	3
0·5	5·9±0·4*	10±2**	4
[NEM] (μM)			
20	6·6±0·2	73±14*	4
50	6·4±0·3	27±14*	5
50+1 mM DTT	6·1±0·3*	39±31*	3

Rat intestinal segments were pretreated with various concentrations of 4-hydroxy-2,3-transnonenal (HNE) or *N*-ethylmaleimide (NEM) for 20 min and subsequently washed for 30 min. Dithiothreitol (DTT) was added immediately during the first 20 min of washing. After treatment, concentration-response curves were recorded for methacholine, which was added cumulatively. Control experiments were run simultaneously without addition of HNE or NEM. The maximal response was expressed relative to the maximal response to methacholine before incubation with HNE or NEM. Mean values±s.d. of n measurements are shown. * $P<0·01$ compared with corresponding control. ** $P<0·001$ compared with corresponding control.

concentrations. Additionally, HNE induces a slow relaxation of the intestinal smooth muscle. These effects correlate with the effects of the lipid peroxidation-inducing agent cumene hydroperoxide on the intestinal smooth muscle, as cumene hydroperoxide also reduces the basal motility (Van der Vliet et al 1990b). Van der Kraaij et al (1990) also described a correlation between the effects of cumene hydroperoxide and HNE, both of which induce coronary vasodilatation. This might indicate that the effects of cumene hydroperoxide are evoked via production of HNE. The concentrations of HNE used in this study may seem rather high, but there is evidence that HNE can reach concentrations of up to 10 mM in the membrane (Benedetti et al 1984), which implies that the concentration used here is within physiological or pathological situations.

Incubation of intestinal segments with HNE also decreases the muscarinic response to methacholine in a concentration-dependent manner (Table 1). Both the maximal response to methacholine and the pEC50 are decreased. The effect of HNE on muscarinic contraction of intestinal smooth muscle is different from the effect described earlier of induction by the well-known inducer of lipid peroxidation, cumene hydroperoxide, which has been also found to reduce the muscarinic contraction. However, at relatively low concentrations of cumene hydroperoxide only the pEC50 of methacholine is decreased without an effect on the maximal contraction (Van der Vliet et al 1990b). The major effect of the lipid peroxidation product HNE is a reduction of the maximal contraction by methacholine, with a relatively small effect on the pEC50. It can therefore be argued that HNE cannot be held fully responsible for the effect of cumene hydroperoxide on the muscarinic response of the intestinal smooth muscle. At higher concentrations cumene hydroperoxide also affects the maximal muscarinic contraction (unpublished results), which might be attributed to the production of HNE.

Relaxation of the intestinal smooth muscle by the β-adrenoceptor agonist isoprenaline is also affected after treatment with HNE; in this case the maximal response to isoprenaline as well as the pEC50 was decreased (Table 2). Moreover, exposure to 0·5 mM HNE leads to a decreased maximal response to isoprenaline relatively to the response induced by 0·5 mM dibutyryl-cAMP

Table 2. Effect of HNE on β-adrenoceptor-mediated relaxation of rat intestinal longitudinal smooth muscle.

[HNE] (mM)	pEC50	Max. effect (%)	n
Control	7·1±0·2	74±13	6
0·1	7·2±0·2	77±8	4
0·2	6·9±0·3	68±16	4
0·5	6·7±0·1*	43±10*	3

Rat intestinal segments were pretreated for 20 min with various concentrations of 4-hydroxy-2,3-transnonenal (HNE), and subsequently washed for 30 min. The response to isoprenaline was recorded by cumulative addition of the agonist, and was related to the response to 0·5 mM dibutyryl-cAMP, which was added with the highest isoprenaline concentration. Mean values and s.d. and the number of measurements (n) are given. * $P<0·05$ compared with control.

(Table 2), which implies that treatment with HNE modulates the β-adrenoceptor-mediated formation of cAMP.

Since HNE has been described to be a sulphydryl-reactive agent (Van Kuijk et al 1986) we compared the effects of HNE with the effects of a well known sulphydryl-reagent NEM. NEM has a similar direct action on the intestinal smooth muscle tone to that of HNE, but at lower concentrations (not shown). NEM (when used in similar concentrations to HNE) completely abolishes muscarinic contraction, which implies that NEM is more harmful to the smooth muscle contractile system compared with HNE. At lower concentrations NEM has a similar effect compared with HNE, namely a decrease in maximal muscarinic response. The pEC50 could not be shown to decrease significantly due to large deviations in the pEC50 after treatment with NEM. If HNE and NEM act by a similar mechanism this would indicate that HNE is weaker than NEM as a sulphydryl-reagent in this system. We studied the possible protective effect of DTT against HNE- or NEM-induced damage. DTT is able to reduce oxidized sulphydryl groups (Jones et al 1983), but is shown to be unable to restore HNE- or NEM-induced damage (Table 1). The similarity between the effects of HNE and NEM suggests that HNE as well as NEM alters the smooth muscle function by irreversible alkylation of thiol groups.

It has been shown previously that HNE decreases the number of β-adrenoceptors in rat lung membranes at concentrations used in this study (Leurs et al 1986). This might imply that treatment of the intestine with HNE may also lead to a decreased number of intestinal β-adrenoceptors, and will thus induce a diminished receptor mediated response. In heart tissue HNE also affects the β-adrenoceptor function but is unlikely to do so at the level of β-adrenoceptors (Haenen et al 1989). It has been established that all components of the β-adrenoceptor system contain essential sulphydryl groups (Stadel & Lefkowitz 1979). Although the β-adrenoceptors on rat intestinal smooth muscle are different from those on airway or heart muscular tissue (Van der Vliet et al 1990a), we suggest that HNE will reduce the β-adrenergic relaxation of intestinal smooth muscle by a direct action on the receptor system, probably by reduction of sulphydryl groups.

It is unlikely that HNE selectively diminishes the muscarinic contraction of intestinal smooth muscle at the receptor level, since, after exposure to HNE, intestinal segments did not further contract on increasing the potassium level maximally contracted with methacholine (not shown). This indicates that non-receptor-mediated contraction is also decreased after incubation with HNE. In this respect the effects of HNE can be compared with the effects of cumene hydroperoxide, which also diminishes receptor as well as non-receptor-mediated contraction of the intestinal smooth muscle (Van der Vliet et al 1989). The

lipophilicity of HNE suggests that the effect of HNE might be limited to the membrane, and it is therefore likely that membrane-associated enzymes are mainly affected by HNE. Other membrane associated enzymes can be targets for sulphydryl-reactive agents. It is well established that Ca^{2+}-ATPases are vulnerable to oxidative stress, and which are shown to be inactivated by modification of sulphydryl groups (Thor et al 1985; Kaneko et al 1989). This will lead to a disturbance of the Ca^{2+} homeostasis, which affects a variety of cellular functions and might also cause a diminished smooth muscle contractility.

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The effect of a 5-HT_3-antagonist on the ileal brake mechanism in the rat

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Abstract—Studies have been carried out on 7 male adult rats to investigate how the action of the selective 5-HT_3 receptor antagonist, granisetron, influences gastrointestinal transit under control conditions and when it is delayed by ileal infusion of lipid. Stomach to caecum transit time (SCTT) was measured using environmental hydrogen analysis. Subcutaneous administration of granisetron (BRL 43694, 40 $\mu g\ kg^{-1}$) significantly delayed the passage of the head of the baked bean meal through the stomach and the small intestine under control conditions ($P < 0.05$). The same compound, however, significantly reversed the delay in SCTT induced by ileal infusion of lipid ($P < 0.001$). These apparently paradoxical results may be rationalized by postulating inhibition of receptors on afferent nerves initiating reflexes that both accelerate and delay transit.

A number of observations suggest that 5-hydroxytryptamine (5-HT) may play an important role in mediating the responses to chemical and mechanical stimulation of the intestinal wall. Over 90% of the total 5-HT in the body is contained in enterochromaffin cells of the gastrointestinal mucosa in close association with afferent nerve terminals (Andrews & Hawthorn 1988). Blockade of 5-HT_3 receptors has been shown to reduce the emetic responses to cisplatin and radiation (Andrews et al 1988), decrease the sensitivity of the rectum to distension in patients with the irritable bowel syndrome (Prior & Read 1990) and reverse the delay in gastric emptying induced by the presence of lipid in the proximal small intestine (Gamse 1989). These observations have led to the suggestion that 5-HT_3 receptors may be present on afferent neurones. Stimulation of 5-HT_3 receptors with 2-methyl-5-HT leads to the release of substance P, acetylcholine and noradrenaline from enteric neurones (Richardson et al 1985). The aim of our studies was to determine how inhibition of 5-HT_3 receptors with granisetron would influence gastrointestinal transit both under control conditions and when it is delayed by the presence of lipid in the ileum.

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Materials and methods

Animals. Experiments were carried out on a total of 7 adult male albino rats, 250–300 g, from Sheffield Field Laboratories. The