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The role of the L-arginine-nitric oxide pathway in relaxation of the opossum lower oesophageal sphincter

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1 The role of the L-arginine-nitric oxide pathway in lower oesophageal sphincter (LOS) relaxation and oesophageal peristalsis was investigated.

2 Twenty four adult opossums were anaesthetized and the right vagus nerve was isolated in the neck and sectioned. Electrical stimulation, applied to the peripheral end of the nerve, resulted in a frequency-dependent relaxation of the LOS, and peristaltic and non-peristaltic contractions in the oesophageal body.

3 N^{ω}-nitro-L-arginine (L-NNA, 10^{-8}–10^{-5} mol kg^{-1}), an inhibitor of the L-arginine-nitric oxide pathway, inhibited LOS relaxation in a dose-dependent manner, but did not affect resting LOS pressure. At the highest dose of L-NNA no relaxation of the LOS was elicited in response to vagal stimulation. The effect of L-NNA, (10^{-5} mol kg^{-1}) was fully reversed by infusion of 10^{-4} mol kg^{-1} L-arginine. Peristaltic velocity and amplitude of contractions in the oesophageal body were unaffected by L-NNA.

4 Infusion of sodium nitroprusside reduced LOS pressure to zero, and the drug was equally potent in control animals ($-\log ED_{50}$: 8.1 ± 0.2 mol kg^{-1}) and in animals pretreated with L-NNA ($-\log ED_{50}$: 8.2 ± 0.3 mol kg^{-1}). This suggests that the effect of L-NNA was not directly on guanylate cyclase.

5 A significant elevation of blood pressure was recorded after administration of L-NNA (10^{-5} mol kg^{-1}).

6 It is suggested that the L-arginine-nitric oxide pathway plays an important functional role for relaxation of the LOS, but not for oesophageal peristalsis. Whether the active substance is nitric oxide or a related nitroso-compound remains to be settled.

Keywords: Non-adrenergic non-cholinergic; nitric oxide; NANC nerves; L-arginine metabolism; oesophageal peristalsis; nitroprusside, N^{ω}-nitro-L-arginine

Introduction

Resting tone in the lower oesophageal sphincter (LOS) is independent of neuronal activity and thus 'myogenic' in nature (Goyal & Rattan, 1976). The sphincter relaxes during swallowing, and this relaxation is caused by an activation of non-adrenergic, non-cholinergic (NANC) nerves (Goyal & Rattan, 1975). The transmitter substance of these NANC nerves has not yet been identified but recent *in vitro* studies have suggested that nitric oxide (NO), or a related product of the L-arginine-NO pathway, is involved in the mediation of NANC inhibition in a number of tissues, including the LOS (Gillespie *et al.*, 1989; Li & Rand, 1989; Bult *et al.*, 1990; Gibson *et al.*, 1990; Tucker *et al.*, 1990; Tøttrup *et al.*, 1991). NO is synthesized from L-arginine (Palmer *et al.*, 1988), and this process is inhibited by a number of L-arginine analogues with a substituted guanidino group (Rees *et al.*, 1989a; Moore *et al.*, 1990). Most of the reports so far published concern the effects of L-arginine analogues on the responses to transmural field stimulation in isolated muscle preparations from different NANC innervated organs. To what extent the findings in isolated tissues are valid for *in vivo* organ function remains to be settled. The aim of the present study was to investigate the role of the L-arginine-NO pathway in motor function of the distal oesophageal body and the LOS, *in vivo*.

Methods

Twenty four North American opossums (*Didelphis virginiana*, 1100–2650 g) were anaesthetized with intraperitoneal pentobarbitone (35 mg kg^{-1}). Twelve animals had been given atropine (30 μg kg^{-1}, i.v.) as premedication to reduce shortening of the oesophagus during peristalsis and to avoid bradycardia in relation to manipulation and stimulation of the vagus nerve. Since atropine previously has been shown to affect the contractile pattern of the oesophageal body to vagal stimulation (Dodds *et al.*, 1978) data from these animals were not included in the calculations of peristaltic velocity and amplitude of contractions. On the other hand, atropine inhibits the shortening of the longitudinal muscle of the oesophagus during vagal stimulation (Dodds *et al.*, 1978) thereby preventing movement of the oesophagus in relation to the catheter, but leaves LOS relaxation unaffected, at least in the dose used for the present experiments (Goyal & Rattan, 1975). To avoid aspiration of saliva and saline, a rubber tube was inserted in the trachea, but the animals were kept on spontaneous respiration. The right vagus nerve was isolated in the neck, and sectioned. Electrical stimulation, applied to the peripheral end of the nerve, was delivered in trains of impulses, separated by 1 min (1 s train duration, 0.4 ms impulse duration, 20–50 V, varying frequency). Oesophageal pressures were monitored by a 4 channel, perfused catheter assembly. Each channel had a side hole for pressure recording, and the distal hole was covered with a 2 cm long 'cuff' for continuous measurement of LOS pressure (Gustavsson & Tucker, 1988). The three proximal side holes were located 1, 3 and 5 cm above the cuff, respectively. The catheter was constantly perfused (0.2 ml min^{-1}) with bubble-free saline by a low compliance, pneumohydraulic system and pressures were registered on an Elema Siemens Mingograph by external transducers (Elema Siemens 746, Sweden). The LOS was located by a slow pull-through technique, and the cuff was placed to yield the highest possible resting sphincter pressure. In addition, the following criteria were used to ensure optimal placement of the catheter: (1) a distinct relaxation of the LOS should be recorded in response to vagal stimulation, (2) within 20 s after cessation of vagal stimulation, LOS pressure should have returned to pre-stimulus level. All pressures mentioned are end-expiratory and given with the fundic pressure as reference. Peristaltic velocity was calculated by measuring the time interval between the major upstroke of the peristaltic contraction at the different measuring sites in the oesophageal body. Arterial blood pressure was continuously monitored in 6 animals by a catheter inserted in the femoral artery. All drugs were dissolved in

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0.9% saline, and given as intravenous bolus injections (1 ml kg^{-1}) over 20 s in cumulatively increasing doses through a cannula inserted in the external jugular vein or in the cephalic vein. Responses were compared by a paired or an unpaired Student's t test, where appropriate.

Determination of ED_{50}-values

The dose producing half-maximal effect (ED_{50}) was assessed by linear interpolation on the semilogarithmic dose-response curve and was expressed as $-\log ED_{50}$.

Drugs

Atropine (Danish Pharmacy Labs); sodium pentobarbitone (Danish Pharmacy Labs); N^{ω}-nitro-D-arginine (D-NNA, Serva), L-arginine, N^{ω}-nitro-L-arginine (L-NNA) and sodium nitroprusside were obtained from Sigma Chemical Co.

Results

Basal performance

Resting LOS pressure was 31 ± 3 mmHg above fundic pressure (Table 1). No spontaneous contractile activity was observed in the oesophageal body except when the animals swallowed spontaneously or when electrical stimulation was applied to the vagus nerve. Vagal stimulation resulted in peristaltic or simultaneous contractions in the oesophageal body and relaxation of the LOS. The latter was frequency-dependent and reached 80% at 10 Hz (Figure 1). The onset of the LOS relaxation began 1–3 s after cessation of vagal stimulation, while contraction at the recording site 3 cm above the LOS began 2–4 s after cessation of the stimulus. The response of the oesophageal body to vagal stimulation varied. In 6 out of 12 animals not given atropine, peristaltic contractions with a propagation velocity <6 cm s^{-1} were constantly elicited. In the remaining 6 animals the velocity of propagation was much faster, and occasionally simultaneous contractions were evoked. All measurements of peristaltic velocity and amplitude were made in animals exhibiting the first-named type of contractions, since these most closely resemble swallowing-induced peristalsis (Dodds *et al.*, 1978).

Effects of L-NNA, D-NNA and L-arginine

Administration of L-NNA resulted in a dose-dependent inhibition of LOS relaxation (Figures 1 and 2). The maximal effect at each dose of L-NNA was seen after approximately 10 min. At 10^{-5} mol kg^{-1}, the LOS failed to relax in response to vagal stimulation at any frequency studied (Figure 1). Resting LOS pressure did not change after infusion of L-NNA (Table 1). The effect of L-NNA (10^{-5} mol kg^{-1}) was fully reversed by infusion of L-arginine, 10^{-4} mol kg^{-1} (Figures 1 and 2). This reversal by L-arginine was maximal after 10–15 min. In animals not given L-arginine, the effect of L-NNA persisted to the end of the study period (at least 30 min after last injection of L-NNA). D-NNA had no influence on LOS relaxation induced by vagal stimulation (Figure 1). Peristaltic velocity was 3.6 ± 0.6 cm s^{-1} in the oesophageal body when a frequency of 10 Hz was used for vagal stimulation. This parameter and the amplitude of contractions were unchanged after infusion of L-NNA (Table 1).

Table 1 Effects of N^{ω}-nitro-L-arginine (L-NNA 10^{-5} mol kg^{-1}) on blood pressure, lower oesophageal sphincter (LOS) pressure and oesophageal peristalsis

	Control	*After L-NNA*	P
Systolic pressure ($n = 6$)	107 ± 12	127 ± 11	<0.05
Diastolic pressure ($n = 6$)	70 ± 5	85 ± 6	<0.05
LOS pressure ($n = 12$ and 7)	31 ± 3	30 ± 3	0.83
Oesophageal peristalsis			
Velocity (cm s^{-1}) ($n = 6$)	3.7 ± 0.6	4.0 ± 0.6	0.81
Pressure amplitude ($n = 6$)	76 ± 7	77 ± 6	0.87

Pressures are given in mmHg. Values are means with s.e.mean indicated. A paired Student's t test was used for statistical comparisons. P represents probability.

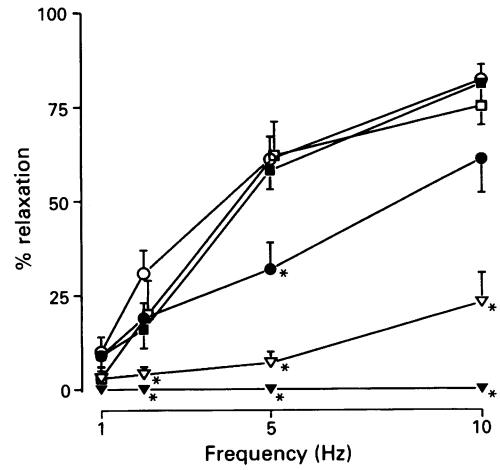

Figure 1 The influence of N^{ω}-nitro-L-arginine (L-NNA) on vagally-induced relaxation of the lower oesophageal sphincter (LOS). Vagal stimulation (1 s trains of impulses, 0.4 ms impulse duration) produced a frequency-dependent relaxation of the LOS (control: ○). L-NNA dose-dependently (*$P < 0.01$) inhibited LOS relaxation (●: 10^{-7} mol kg^{-1}; ▽: 10^{-6} mol kg^{-1}; ▼: 10^{-5} mol kg^{-1}). The effect of L-NNA, 10^{-5} mol kg^{-1} was reversed by infusion of L-arginine, 10^{-4} mol kg^{-1} (□). D-NNA, 10^{-5} mol kg^{-1} (■), had no influence on the relaxations induced by vagal stimulation. All drugs were given intravenously as bolus injections over 20 s. Each point is mean of 7–10 observations with s.e.mean shown by vertical bars.

Effect of sodium nitroprusside

Infusion of sodium nitroprusside, which was given either in untreated animals or when 10^{-5} mol kg^{-1} of L-NNA had abolished LOS responses to vagal stimulation, resulted in a dose-dependent and complete relaxation of LOS pressure ($-\log ED_{50}$: 8.1 ± 0.2 mol kg^{-1} and 8.2 ± 0.3 mol kg^{-1} in control- and L-NNA pretreated animals, respectively; $n = 4$ and 8). Arterial blood pressure was reduced to 15–35 mmHg (systolic) and 5–15 mmHg (diastolic) with the highest dose of sodium nitroprusside (10^{-5} mol kg^{-1}). A comparable

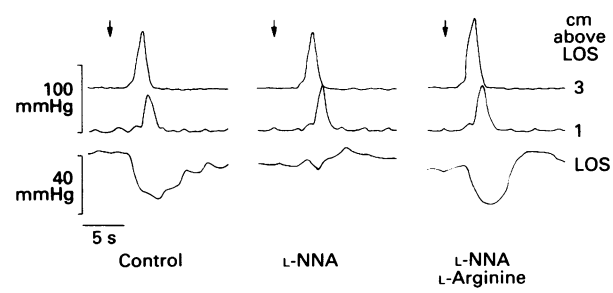

Figure 2 The effect of vagal stimulation on pressures in the oesophageal body and the lower oesophageal sphincter (LOS). The lower tracings represent the LOS and the two upper tracings represent recordings 1 and 3 cm above the LOS, respectively. All three responses were induced by stimulation of the right vagus (1 s train, 40 V, 0.4 ms impulse duration and 10 Hz). Arrows at the top indicate cessation of the stimulus train. The control response is shown on the left. The response in the middle, labelled L-NNA, represents the response recorded 10 min after infusion of N^{ω}-nitro-L-arginine (L-NNA, 10^{-5} mol kg^{-1}). The right response shows the reversing effect of L-arginine (10^{-4} mol kg^{-1}) on LOS relaxation in the same animal. Notice that the peristaltic contractions are unchanged.

reduction in blood pressure by bleeding has been shown to cause only a minor decrease in LOS pressure (Goyal & Rattan, 1980).

Discussion

The external muscle layers of the gastrointestinal tract receive a potent inhibitory innervation, which is thought to play a role in peristalsis and relaxation of sphincters. It is evident that the transmitter(s) mediating the inhibitory responses is neither acetylcholine nor noradrenaline, and it is therefore designated as NANC in character. Different substances, including vasoactive intestinal polypeptide (VIP) and adenosine triphosphate (ATP) have been proposed as participating in the NANC responses of several gut muscles, but significant data fail to support these candidates as principal mediators of NANC inhibition in the oesophagus (Daniel *et al.*, 1983; Torphy *et al.*, 1986). Recently, *in vitro* studies have shown that NO, or a related nitroso compound, might be involved in NANC inhibition of both gastrointestinal and non-gastrointestinal smooth muscle (Gillespie *et al.*, 1989; Li & Rand, 1989; Gibson *et al.*, 1990; Bult *et al.*, 1990; Tucker *et al.*, 1990; Tøttrup *et al.*, 1991). The evidence presented in these studies is based on the assumption that NO, or a related nitroso compound, is synthesized from L-arginine, and that the process is inhibited by L-arginine analogues with a substituted N-guanidino group. Of these analogues, L-NNA seems to be one of the most potent (Moore *et al.*, 1990).

The present finding that vagally-induced relaxation of the LOS was abolished by intravenous administration of L-NNA implies a physiological role of the L-arginine-NO pathway in LOS relaxation. The reversal by L-arginine and the lack of effect of the D-isomer (D-NNA) support this view. The hypothesis is further strengthened by earlier reports that guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels increase during NANC stimulation of the LOS (Torphy *et al.*, 1986), since NO and other nitrogen-containing compounds are potent stimulators of guanylate cyclase (Arnold *et al.*, 1977; Rapoport & Murad, 1983; Boulanger *et al.*, 1990). Sodium nitroprusside, another activator of guanylate cyclase, relaxed the LOS completely when vagally-induced relaxation was absent due to the influence of L-NNA. Moreover, the sensitivity to sodium nitroprusside was identical whether or not the animals had been given L-NNA indicating that the inhibitory influence of L-NNA was not directly on the guanylate cyclase. During 'resting' conditions, NO does not seem to be released in an amount to affect the LOS, since L-NNA had no effects on resting LOS pressure. By contrast, a significant rise in arterial blood pressure was recorded after infusion of L-NNA (Table 1) supporting results of an earlier investigation (Rees *et al.*, 1989b).

NANC inhibition of the circular muscle layer of the oesophageal body has been shown to precede the peristaltic wave of contraction (Rattan *et al.*, 1983; Sugarbaker *et al.*, 1984; Paterson, 1989), and gradients in the latency from cessation of the inhibition to the onset of the contraction have been suggested to play a role in the mechanism of peristalsis (Weisbrodt & Christensen, 1972; Christensen *et al.*, 1979; Paterson, 1989). The inhibitory NANC transmitter of the oesophageal body is probably a product of the L-arginine-NO pathway, since the inhibitory junction potential evoked by stimulation of these nerves is abolished by L-NNA (E.E. Daniel, personal communication), and the prominent 'off' contraction elicited in circular oesophageal muscle strips by transmural field stimulation disappears after L-NNA (Knudsen & Tøttrup, unpublished observations). The short trains used for vagal stimulation in the present study are optimal for relaxation of the lower oesophageal sphincter, but less well suited for studies of oesophageal peristalsis since both contractions with a normal propagation velocity as well as contractions propagating much faster may be elicited (Dodds *et al.*, 1978). In animals showing only the first-named type of contractions, L-NNA did not affect either velocity of propagation or amplitude of contractions. This suggests that, at least under experimental conditions, the inhibitory innervation is not essential for the basic pattern of oesophageal peristalsis. A 'myogenic' mechanism may therefore exist for determination of propagation (Bartlet, 1973; Sarna *et al.*, 1977; Helm *et al.*, 1989), although this mechanism may allow contractions to propagate in both directions when the nerves are blocked.

Two crucial questions that cannot be addressed from the present findings are: (1) is NO the active agent, and (2) is NO, or the closely related compound, a transmitter in the classical sense? Vascular endothelial cells liberate a relaxing factor (endothelium-derived relaxing factor, EDRF) in response to certain stimuli, and several lines of evidence have suggested that EDRF is identical to NO (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). Certain pharmacological differences between EDRF and NO (Shikano *et al.*, 1987) have been demonstrated, and recent experiments have shown a closer similarity between EDRF and a nitrosothiol (Myers *et al.*, 1990; Wei & Kontos, 1990). A linkage to another molecule would clearly be an advantage in the nervous system, since storage of NO is unlikely, due to its instability after contact with oxygen and the superoxide anion (Gryglewski *et al.*, 1986). This leads directly to the question of the compound as a neurotransmitter. The enzyme required for conversion of L-arginine to NO (and citrulline) has recently been demonstrated by immunohistochemical staining in the brain, in autonomic nerves and in the myenteric plexus (Bredt *et al.*, 1990) favouring a transmitter role of NO or the closely related compound. If this is true, we anticipate that the substance released upon activation is either not stored as other neurotransmitters, or is different from NO. The cellular location of the process, however, might also be extraneuronal.

In conclusion, the present findings show that L-NNA is an efficient inhibitor of the NANC-mediated LOS relaxation in intact opossums. This indicates an important role of the L-arginine-NO pathway in LOS motor function. Propagated contractions in the oesophageal body may be generated when the L-arginine-NO pathway is blocked. Further characterization of this mediator system in the LOS, and elsewhere, may be of major clinical importance.

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