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Patients with achalasia lack nitric oxide synthase in the gastro-oesophageal junction

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Abstract. The abnormal function of the lower oesophageal sphincter in achalasia is likely to be due to impaired nonadrenergic, noncholinergic (NANC) inhibitory input. Since recent studies in animals suggest that nitric oxide (NO) is implicated physiologically in the inhibitory responses of the lower oesophageal sphincter, we have investigated whether the synthesis of NO is altered in the gastro-oesophageal junction of patients with achalasia. NO synthase activity was investigated in samples of tissue from the gastro-oesophageal junction obtained during surgery in eight patients with typical achalasia and six non-achalasic controls who underwent oesophagectomy for reasons other than sphincter dysfunction. The NO synthase activity was determined by the transformation of ^{14}C-L-arginine into ^{14}C-L-citrulline in tissue homogenates. In addition, immunohistochemical staining of the tissues was performed using a polyclonal antibody raised against a peptide sequence of rat brain NO synthase. Furthermore, the relaxant response to an exogenous NO donor (sodium nitroprusside, SNP) was measured *in vitro* in muscle strips obtained from two patients with achalasia and in two non-achalasic controls. NO synthase activity was detected in each of the samples obtained from six control patients ($0{\cdot}59 \pm 0{\cdot}21$ pmol mg^{-1} min^{-1}; mean ± SE). By contrast, none of the samples obtained from the eight patients with achalasia had any detectable NO synthase activity. Immunohistochemical studies confirmed the presence of NO synthase in the myenteric plexus of the gastro-oesophageal junction of control patients and its absence in achalasia. SNP relaxed muscle strips precontracted with bethanechol in both control samples and those from patients with achalasia. We suggest that the absence of NO synthase in the myenteric plexus of the gastro-oesophageal junction explains the impaired function of the lower oesophageal sphincter in achalasia.

Keywords. Achalasia, gastro-oesophageal junction, lower oesophageal sphincter relaxation, nitric oxide, nitric oxide synthase, nonadrenergic, noncholinergic innervation.

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Introduction

Achalasia is a primary oesophageal motor disorder characterized by elevated resting pressure of the lower oesophageal sphincter, failure of the sphincter to relax completely, and non-peristaltic contractions of the oesophageal smooth muscle [1]. The aetiology of this condition is not clear, and although there is considerable evidence that the lower oesophageal sphincter dysfunction is due to impairment of nonadrenergic, noncholinergic (NANC) neurotransmission [2,3], the precise nature of this defect has not been elucidated.

Recent studies in animals suggest that under physiological conditions NO, the product of the L-arginine: NO pathway [4], is involved in the mediation of the NANC inhibitory response of the lower oesophageal sphincter [5–8]. Therefore, the absence of NO at the gastro-oesophageal junction could explain an impaired relaxant activity of the lower oesophageal sphincter. In view of this, we decided to determine whether there is a defect in the L-arginine: NO pathway in the gastro-oesophageal junction of patients with achalasia. For this, we investigated the presence of an NO synthase and its localization by immunohistochemistry in surgical specimens obtained from the gastro-oesophageal junction of patients with achalasia and of non-achalasic surgical controls.

Methods

Patients

Full-thickness muscle specimens of the gastro-oesophageal junction were obtained during surgery from eight patients with achalasia and from six non-achalasic patients who underwent surgical procedures in the oesophagogastric area. All patients with achalasia (four women and four men; mean age: 45 years; range: 26–86 years) fulfilled clinical, endoscopic and manometric criteria for the diagnosis of the disease [1] (Table 1). Non-achalasic patients (three women and three men; mean age: 66 years; range: 41–86 years) were operated on because of cancer in the gastric antrum ($n = 2$; total gastrectomy), cancer in the upper or middle part of the oesophagus ($n = 2$), oesophageal peptic stricture ($n = 1$) and giant hiatal hernia ($n = 1$). In none of the cancer cases was the gastro-oesophageal

Table 1. Clinical and manometric data of patients with achalasia

Patient no.	Sex	Age (years)	Symptoms duration (mo)	Mean LES pressure (mmHg)	LES relaxation to swallowing (%)	Mean oesophageal waves amplitude (mmHg)	Simultaneous oesophageal waves (%)
1	M	26	52	48	12	8	100
2	M	42	19	70	18	20	100
3	F	86	48	37	n.m.	15	100
4	M	31	264	30	0	24	100
5	F	43	24	34	50	28	100
6	M	38	10	42	30	22	100
7	F	34	11	62	20	10	100
8	F	57	120	55	0	10	100

n.m. = not measured.

junction infiltrated by the tumour. In cases of oesophageal cancer, functional behaviour of the gastro-oesophageal junction was verified manometrically as normal prior to surgery, i.e. mean lower oesophageal sphincter pressure lower than 35 mmHg and greater than 90% relaxation in response to wet deglution. This study was approved by the Institutional Review Board of the Hospital General Vall d'Hebron.

Quantification of NO synthase activity

Specimens of oesophagus were frozen in liquid nitrogen immediately after they were obtained in the operating room and were stored at −80°C until used. The enzymatic transformation of ^{14}C-L-arginine into ^{14}C-L-citrulline by homogenates of the specimens was determined according to the method described by Knowles *et al.* [9].

The incubation medium consisted of 50 mM potassium phosphate buffer (pH 7·2), 60 mM valine, 120 μM NADPH, 1·2 mM L-citrulline, 24 μM L-arginine, 150 000 dpm L-^{14}C-arginine, 1·2 mM $MgCl_2$ and 0·24 mM $CaCl_2$. Samples were incubated for 10 min at 37°C before the reaction was stopped by removal of substrate and dilution by the addition of H_2O: Dowex-50 W-resin mix. The presence of ^{14}C-citrulline was measured by liquid scintillation counting. The activity of the Ca^{2+}-dependent NO synthase was calculated as the difference between the citrulline generated in the absence and presence of 1 mM EGTA. Activity of the Ca^{2+}-independent enzyme was calculated as the difference between samples containing 1 mM EGTA and samples containing 1 mM EGTA plus 1 mM N^G-nitro-L-arginine methyl ester (L-NAME, Sigma, St Louis, MO, USA), an inhibitor of NO synthase.

Immunohistochemical staining of NO synthase

For immunohistochemistry, surgical specimens were divided into two parts; one of the samples was immediately frozen in liquid nitrogen and stored at −80°C, and the other fixed by immersion in 10% buffered formaldehyde. The immunohistochemical study was performed following the protocol of Springall *et al.* [10] using a polyclonal antibody (p-53) raised in rabbits at Wellcome Research Laboratories (Beckenham, UK) against a 21 amino acid sequence of the rat brain constitutive NO synthase. Peptide p-53 (LPLLLQANGNDPELFQIPPEL) corresponds to position 519–540 of the published sequence [11]. The antibody is able to recognize a similar protein in human central and peripheral nervous systems [12]. The characterization of the antibody has been published elsewhere [13]. Immunohistochemistry was performed in a Microprove Staining Station (Biomeda Corp., Foster City, CA, USA). Five μm sections of either frozen or formalin-fixed paraffin-embedded tissue from patients with achalasia and controls were obtained. Endogenous peroxidase was blocked by immersing slides in 0·03% hydrogen peroxide in methanol, and non-specific binding was prevented by incubation in Redusol® (Biomeda Corp., Foster City, CA, USA). The sections were incubated with NO synthase antiserum (p-53) at 1 : 2000 dilution for 9 min. After washing in PBS, sections were incubated with biotinylated anti-rabbit immunoglobulin link antibody and peroxidase-conjugated streptavidin (Universal LSAB kit/HRP, DAKO Corp., Carpinteria, CA, USA) for 4 min. Sections incubated with pre-immune serum provided negative controls. All the incubations were performed at 40°C. Diaminobenzidine was used

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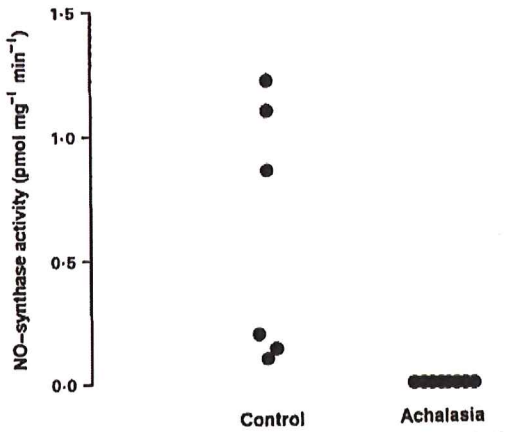

Figure 1. Individual values of nitric oxide (NO) synthase activity in the gastro-oesophageal junction of non-achalasic controls and patients with achalasia.

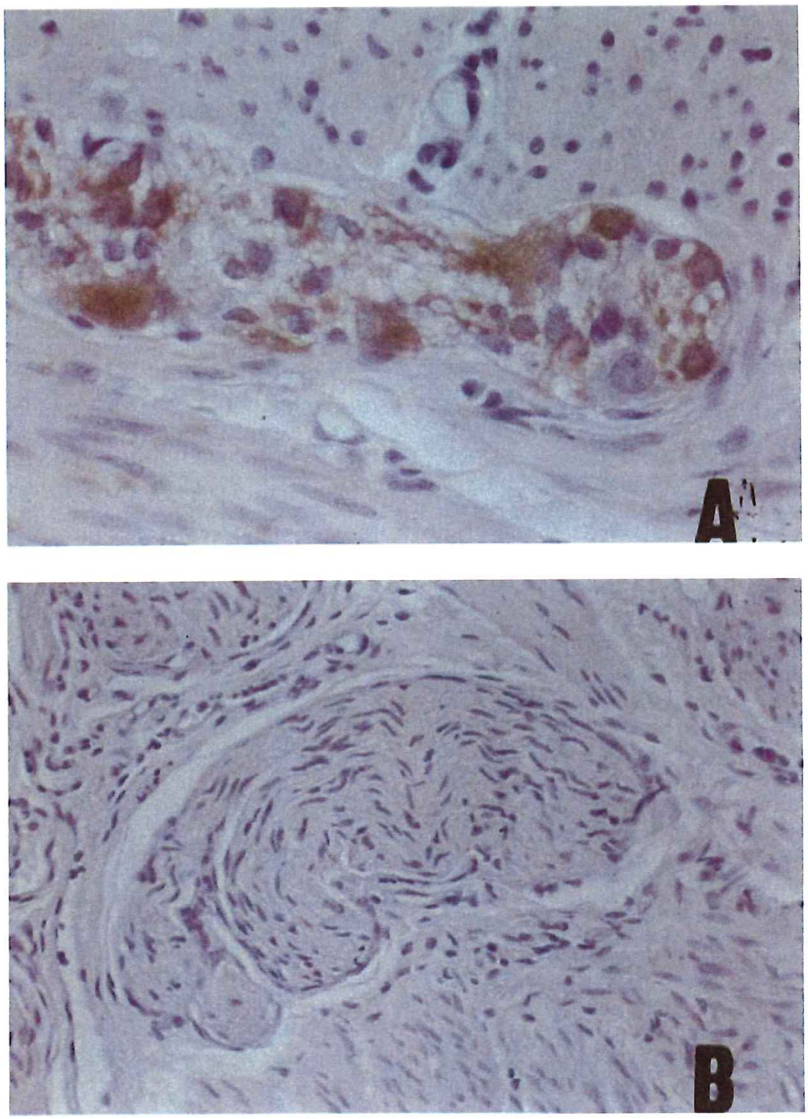

Figure 2. Immunostaining with antisera to nitric oxide (NO) synthase in gastro-oesophageal junction specimens. (A) Strong immunoreactivity in the myenteric plexus in a control subject. (B) Absence of immunoreactivity in a patient with achalasia. (Magnification A × 400; B × 250).

as the chromogen. Sections were counterstained with haematoxylin, dehydrated and mounted.

Pharmacological studies of the gastro-oesophageal junction with SNP in vitro

Muscle strips from the gastro-oesophageal junction, about 6 mm long and 3 mm wide, were obtained from two patients with achalasia and two non-achalasic control subjects. The strips were incubated in organ baths containing 20 ml of Krebs solution at 37°C, continuously bubbled with O_2:CO_2 (95%:5%). The resting strip tension was adjusted to 1 g and changes in tension were monitored by an isometric force transducer (Letica, Barcelona, Spain) and recorded by a polygraph connected on-line. Experiments were started after a 2 h stabilization period. The strips were then contracted with 2 $\mu g\ ml^{-1}$ of bethanechol (Roig Farma, Barcelona, Spain) and relaxed with 0·25 $\mu g\ ml^{-1}$ SNP (Merck, Darmstadt, Germany).

Results

Quantification of NO synthase activity

A constitutive, Ca^{2+}-dependent, NO synthase activity was detected in each of the six control samples (mean

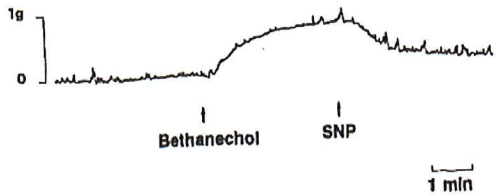

Figure 3. *In vitro* relaxant response induced by sodium nitroprusside (SNP) in a gastro-oesophageal junction smooth muscle strip precontracted with bethanechol. Example in patient with achalasia, typical of response seen in two patients with achalasia and two control subjects.

value ± SE = 0·59 ± 0·21 pmol citrulline mg^{-1} min^{-1}). By contrast, none of the eight achalasia samples showed any detectable NO synthase activity (lower limit of detection of assay 0·04 pmol mg^{-1} min^{-1}) (Fig. 1). The Ca^{2+}-independent NO synthase (inducible form) was not detected in any of the samples from either patients with achalasia or non-achalasic controls.

Immunohistochemical staining of NO synthase

Immunohistochemical studies confirmed the presence of NO synthase in the gastro-oesophageal junction of control specimens and its absence in the gastro-oesophageal junction of specimens from patients with achalasia. In myenteric plexuses of control subjects, immunoreactivity to NO synthase was found in neuronal cytoplasm and fibres (Fig. 2A) and in intramuscular fibres. The same intensity of immunostaining was observed in frozen and in formalin-fixed paraffin-embedded tissue but was completely absent from sections incubated with pre-immune serum (negative controls). The myenteric plexuses from patients with achalasia were devoid of ganglion cells, and the nerve fibres did not exhibit immunoreactivity (Fig. 2B).

Response of the gastro-oesophageal junction to SNP

The relaxant response to 0·25 μg ml^{-1} SNP in smooth muscle strips precontracted with bethanechol from patients with achalasia was similar to that observed in strips from control subjects. This concentration of SNP caused a > 80% reduction in the tension generated by bethanecol in each of the tissue samples tested (Fig. 3).

Discussion

The pathogenic mechanism of achalasia has, until now, remained unknown. Absent or incomplete relaxation of the lower oesophageal sphincter in response to swallowing [14] and a paradoxical contractile response of the sphincter to cholecystokinin-octapeptide [2] indicate impairment of postganglionic inhibitory nerves. Several recent studies suggest that the incomplete relaxation of the sphincter in achalasia may be due to the absence or functional impairment of NANC inhibitory nerves in the presence of postganglionic cholinergic excitatory nerves which are functionally intact [3,15]. However, the precise nature of the NANC transmitter which may be lacking in achalasia is not clear. Vasoactive intestinal polypeptide (VIP) is absent in specimens from the gastro-oesophageal junction in achalasia [16]; however, VIP does not appear to be the principal mediator of NANC inhibition in the oesophagus [6,17]. Recent *in vitro* and *in vivo* animal studies have suggested that NO plays an important functional role in the relaxation of the lower oesophageal sphincter [5–8]. Thus neurogenic relaxation of this tissue in the opossum [5,6], dog [7] and guinea pig [8] was blocked by inhibitors of the generation of NO, while reversal of this effect was obtained with L-arginine [6,7]. These findings are in agreement with previous reports indicating that guanosine 3′-5′-cyclic monophosphate (cGMP) increases during NANC stimulation of the lower oesophageal sphincter [18], since NO is a potent stimulator of guanylate cyclase [19,20].

We have now demonstrated biochemically and immunologically the absence of NO synthase in the gastro-oesophageal junction of patients with achalasia. In contrast, specimens of gastro-oesophageal junction from control subjects clearly showed activity of the NO-generating enzyme. Two general types of NO synthase have been identified, a Ca^{2+}-independent inducible and a Ca^{2+}-dependent constitutive enzyme [4]. The latter type has previously been identified in NANC nerves of animals [19] and our present study demonstrates a similar type of enzyme involved in NANC nerves of animals [21] and our present study chemical staining in control gastro-oesophageal junction specimens showed that NO synthase was located in the myenteric plexus. This observation is consistent with the neurotransmitter role of NO previously postulated for the brain, autonomic nerves and gut myenteric plexus [10,22,23].

Nitrates, which act by donating NO [24], have been shown to relax the human lower oesophageal sphincter in normal subjects and in patients with achalasia [25]. In our *in vitro* preparations a single dose of the NO donor SNP relaxed oesophageal muscle strips precontracted with bethanechol from both patients with achalasia and control subjects, suggesting that the soluble guanylate cyclase, the receptor for NO, is unaffected in this condition. However, it remains to be established whether the lack of generation of endogenous NO results in a hypersensitivity to nitrovasodilators in the gastro-oesophageal junction of these patients.

The evidence accumulated since the discovery of the L-arginine: NO pathway indicates its relevance in a variety of biological functions [4]. These include NANC neurotransmission in the vasculature and in organs of the genitourinary tract as well as in organs of the gastrointestinal system, including the lower oesophageal sphincter [6], stomach [26], gallbladder [27],

ileum [28] and colon [29]. Our present results, together with the finding that NO synthase may be absent in human pyloric tissue in infantile hypertrophic pyloric stenosis [30], are beginning to unravel the pathophysiological significance of defects in the L-arginine: NO pathway.

In summary, we have demonstrated biochemically and by immunohistochemistry the absence of NO synthase in the myenteric plexus of the gastro-oesophageal junction in patients with achalasia. We postulate that the lack of this key enzyme, associated with impaired local production of NO, is responsible for the defective lower oesophageal sphincter function in this condition. Although achalasia is a motility disorder supposedly confined to the oesophagus, motor abnormalities of the stomach [31,32], small bowel [33] and sphincter of Oddi [34] have been described in some patients and it is reasonable to assume that the same defect may account for these abnormalities.

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