The inhibitory role of nitric oxide in the control of porcine and human sphincter of Oddi activity

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Abstract

Background—The possible role of nitric oxide in the regulation of the sphincter of Oddi is not known in species with a resistor–like sphincter of Oddi such as humans and pigs.

Methods—Sphincter of Oddi perfusion manometry and simultaneous electromyography (EMG) were recorded transduodenally in eight anaesthetised pigs. Acetylcholine (4 µg/kg) was given intravenously, with or without sodium nitroprusside (10–100 µg/kg), an exogenous nitric oxide donor. For in vitro studies the sphincter was removed from the eight pigs and from six patients undergoing pancreatecoduodenectomy, cut into rings, and the amplitude of contraction was measured in an ex vivo bath. Each ring was stimulated with acetylcholine (100 µM) and KCl (125 mM). The stimulation was repeated after incubation with L-NAME (a stereospecific competitive inhibitor of nitric oxide synthase), with L-NAME plus L-arginine (a substrate for nitric oxide synthase), and with sodium nitroprusside. The sphincter rings were then submerged in liquid nitrogen and stored. Immunohistochemical analysis was used to localise nitric oxide synthase in the pig and human sphincter specimens.

Results—In vivo EMG revealed 2–3 phasic bursts per minute with the basal pressure variation 6–40 mm Hg. Acetylcholine induced a large electrical burst and the pressure increased by (mean [SE]) 20 (10) mm Hg (p <0.01) for 17 (4) seconds. After sodium nitroprusside (10 µg/kg) acetylcholine did not induce pressure changes and electrical activity was almost abolished. In vitro, L-NAME increased the KCl induced sphincter contraction in both pig and human specimens (p<0.01). In human, but not in pig, specimens L-NAME increased the amplitude of acetylcholine induced contraction (p<0.01). L-Arginine partly reversed the effect of L-NAME. The sphincter rings were then incubated with a polyurethane catheter (22G/0.9×100 mm) used for intra-arterial drug administration and blood pressure monitoring.

The sphincter of Oddi (SO) regulates bile flow into the duodenum by behaving either as a pump (category I) or as a passive resistor (category II), depending upon the species of animal.3–7 The physiological control of the SO is complex and includes both neural and humoral factors. In recent years much attention has been focused on the role of nitric oxide (NO), a potent smooth muscle relaxant produced by enteric nerves in the regulation of gastrointestinal smooth muscle function. NO is known to cause relaxation of the SO in category I species (with a pump-like sphincter) such as guinea pig, opossum, Australian possum, rabbit, and prairie dog.3–7 However, the effects of NO on the SO in category II species (resister-like sphincter) such as pig, cat, and humans is less well studied. Preliminary evidence suggests a physiological role for this neurotransmitter in the regulation of SO motility in these species too. NO synthase (NOS), the enzyme generating NO, has been localised in cat and human SO.4–10 In addition, exogenous topical application on the papilla of Vater or systemic administration of NO donors have been observed to decrease human SO motility10–11 during endoscopy.

The aims of these studies were to localise NOS activity in porcine and human SO and to evaluate a possible physiological role for NO in the control of SO function in these species.

Methods

IN VIVO STUDIES (PIG)

Preparation

Eight female pigs weighing 25–28 kg were used for animal studies. They were anaesthetised with controlled 1–2% efrane inhalation anaesthesia alone with ketamine (10 mg/kg im) and thiopentone (10 mg/kg iv) induction. The duodenum was opened through a midline laparotomy and the gall bladder was drained to avoid pressure changes inside the biliary tree. The pancreaticoduodenal artery was cannulated with a polyurethane catheter (22 G/0.9×100 mm) used for intra-arterial drug administration and blood pressure monitoring.
The signal from the pressure transducer was recorded by a personal computer.

**SO manometry**
The SO was cannulated through the duodenum with a custom-made perfusion manometry catheter (outer diameter 2 mm, three side holes with 120° interval between holes) connected to a microcapillary infusion system (Abbott Laboratories, Salt Lake City, Utah, USA). Pressure changes were recorded by a pressure transducer (AME Ab, Norway) connected to the personal computer. The high pressure (SO) zone was localised using a slow pull-back technique.

**SO electromyography**
Simultaneous electromyographic (EMG) registration was performed using two flexible Teflon coated silver wires (Medwire, New York, NY, USA) inserted with a small injection needle into the SO smooth muscle on opposite sides. The needle was removed and a separate grounding electrode was placed on the skin of the abdomen. After amplification the signal from the electrodes was recorded by the same personal computer as the blood pressure and SO manometry signals. The software used was ASYST (Asyst Software Technologies, New York, USA).

**Drug testing**
After an initial stabilisation period of 30 minutes acetylcholine chloride (Sigma Chemical Co, St Louis, Missouri, USA) was administered intra-arterially in a dose of 4 μg/kg (this dose had been previously determined by us to result in submaximal SO contraction). After a 10 minute washout period to allow for normalisation of SO pressure and EMG activity, sodium nitroprusside (E Merck AG, Darmstadt, Germany) was injected intra-arterially (10 or 100 µg/kg) followed by acetylcholine as previously administered.

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_In vitro drug testing (pig and human)_
After a stabilisation period of 30 minutes the SO rings were stimulated by 100 μM exogenous acetylcholine or 125 mM potassium chloride (KCl), a potent direct smooth muscle stimulant. In preliminary studies using five pig and two human SO preparations it was determined that these concentrations elicited maximal SO contractions which were reproducible in successive stimulations. After each stimulant the rings were rinsed twice for five minutes each with fresh incubation medium; 10 μM sodium nitroprusside (an exogenous NO donor) or 0.1 mM N-nitro-L-arginine methyl ester (L-NAME) (Sigma), a stereospecific inhibitor of NO synthase, were then added to the medium and the stimulation was repeated after 20 minutes of incubation. In order to compete with the effects of L-NAME a 10-fold concentration (1 mM) of L-arginine (Sigma), a substrate for NO synthase, was further added to the rings, which had been previously incubated with L-NAME, and the stimulation was repeated again after 20 minutes of incubation. The force of contractions was compared with the maximum contraction force after adding KCl or acetylcholine at the beginning of the experiment (taken as 100%).

**Localisation studies (pig and human)**
After the above pharmacological studies a rod was pierced through the SO rings and they were submersed in liquid nitrogen and stored and shipped in dry ice to the University of Wisconsin for histological studies. The specimens were thawed on the day of fixation for about 40 minutes and pinned down flat and fixed in 4% picroformaldehyde for four hours at 4°C. The tissue was then washed repeatedly (at least six times) for 24 hours and placed into PBS containing 15% sucrose for 24 hours and, using OCT (Tissue Tek, Miles Laboratories, La Jolla, California, USA), were cryosectioned at 10 µm onto gelatine coated slides. Several such sections from each specimen were taken onto the slides. At least five slides were evaluated for each specimen.

Protein gene product (PGP) 9.5 was used as a general neuronal marker. We have previously characterised PGP 9.5 in various human and animal systems and found it to be the most reliable general neuronal marker which also works very well under these experimental conditions. Polyclonal rabbit primary antisera against NO synthase (Transduction Laboratories, Lexington, Kentucky, USA; dilution 1 in 1000) was used to assess the nitrinergic components of the enteral nervous system. We have previously tested this NO synthase antibody against the original polyclonal antibody (gift of Snyder and Dawson, Johns Hopkins University) and found it to be of comparable staining characteristics in the human and opossum gastrointestinal tract. The primary antibodies were incubated for two hours at room tempera-
ture and, after thorough rinsing (six times, 10 minutes each), they were incubated with goat anti-rabbit conjugated to fluorescein isothiocyanate (Cappel, Durham, North Carolina, USA) at 1 in 60 for one hour at room temperature. All immunostaining was carried out with a “Cadenza” automated immunostainer (Shandon-Lipshaw Inc, Pittsburgh, Pennsylvania, USA) to assure uniformity of staining. Sites of antigenic binding were visualised under epifluorescence with an Olympus BHT-2 research microscope (Olympus Inc, Tokyo, Japan) and areas were photographed by attached photographic equipment. Appropriate positive, omission, and pre-incubation controls were also performed.

At least two slides per subject containing several sections each were rinsed in PBS and incubated for 15 minutes at 37°C in a mixture of nicotinamide adenine dinucleotide phosphate (NADPH) (1 mg/ml), nitroblue tetrazolium (0.4 mg/ml) (both from Sigma), and 0.5% Triton X-100 in 0.1 M Tris buffer, pH 7.6, as previously described. The sites of NADPH-diaphorase indicative of NO synthase stained brilliantly blue. Sections were then cover slipped in carbonate buffered glycerol and qualitative evaluations of the staining in the SO muscle layer, neurons, mucosa, and submucosa were undertaken (CS).

At least three slides each containing several sections from each animal and human were subjected to standard haematoxylin and eosin staining to confirm the presence of the entire sphincter with all the layers and to study its histological relation to the adjoining duodenal mucosa and the pancreatic tissue. Haematoxylin and eosin staining was also used to confirm the absence of any tumour infiltration in the sections studied in all human SO preparations.

**STATISTICAL ANALYSIS**

All results are expressed as mean (SE) unless otherwise stated. The paired Student’s t test (with logistic transformation in skewed distributions) was used to compare the groups and the significance was accepted at 0.05.

The study protocols were approved by the animal care committee of the University of Tampere, by the ethical committee of the University of Wisconsin, and by the human subject committee of the University of Wisconsin.

**Results**

**IN VIVO STUDIES (PIG)**

During the resting period EMG revealed 2–3 phasic 10–20 µV electrical bursts per minute and the SO baseline pressure varied between 6 and 40 mm Hg (mean (SE) 18 (13) mm Hg). Phasic contractions were occasionally seen synchronously with electrical bursts. However, they could not be reliably measured. The length of the high pressure zone of the SO varied from 4 mm to 6 mm as estimated by a pull-through technique. Acetylcholine induced a single large electrical burst (amplitude up to 100 µV) followed by phasic electric burst activity at a frequency of 5–10 per minute for 2–5 minutes. The initial electric burst was accompanied by an increase of 20 (10) mm Hg from basal SO pressure (p < 0.01) that was sustained for 17 (4) seconds (fig 1). No changes in arterial blood pressure were seen.

After pretreatment with sodium nitroprusside (10 µg/kg) acetylcholine induced only a few low amplitude electrical spikes without any detectable changes in the SO pressure. After pretreatment with 100 µg/kg sodium nitroprusside phasic electrical activity disappeared despite acetylcholine stimulation which resulted in only a slight decrease (1–2 mm Hg) in SO baseline pressure (fig 2). At the same time arterial blood pressure decreased by 30 (4) mm Hg (p<0.01). These effects of sodium nitroprusside lasted for 5–6 minutes.

**IN VITRO STUDIES (PIG AND HUMAN)**

All pig and human SO rings demonstrated significant contractions after incubation with KCl and acetylcholine, the former producing greater contractions than the latter (pig, 1397 (260) mg v 682 (120) mg, p<0.01; human, 689 (144) mg v 261 (61) mg, p<0.05, respectively).

When L-NAME was added to the medium and the stimulation was repeated with KCl the contractile force increased in all SO rings compared with KCl stimulation without L-NAME (figs 3 and 4). L-Arginine together with L-NAME resulted in a decreased contraction force after KCl stimulation in all pig SO rings, and tended to decrease contraction in the human SO rings compared with the contraction with L-NAME without L-arginine (figs 3 and 4).
When L-NAME was added to the medium and the stimulation was repeated with acetylcholine, the contractile force of the pig SO rings did not change compared with acetylcholine stimulation without L-NAME (fig 3). By contrast, the acetylcholine-induced SO contractile force in the human SO preparation was enhanced (fig 4). L-Arginine together with L-NAME resulted in decreased contraction after acetylcholine stimulation in pig SO rings and tended to decrease the SO contraction in human SO rings in comparison with the contraction with L-NAME without L-arginine (figs 3 and 4).

Exogenous sodium nitroprusside significantly decreased both KCl and acetylcholine-induced contractions in all pig and human SO rings compared with the stimulation without sodium nitroprusside (p<0.05 for KCl and p=0.01 for acetylcholine; figs 5 and 6).

LOCALISATION STUDIES (PIG AND HUMAN)
Haematoxylin and eosin staining
Staining of the SO with haematoxylin and eosin both in pig and human revealed that the mucosa was covered by one to three layers of
tall cuboidal epithelial cells with a well defined submucosa surrounded by radially arranged smooth muscle layers. The smooth muscle was 10–25 layers thick in the human SO preparation and 5–8 layers thick in the pig SO preparation. Unlike the rest of the gastrointestinal tract, several submucosal neurons were seen in the porcine SO preparation that stained positive for NOS antibody. The NOS staining in the human SO preparation closely paralleled those of the pig except for relatively greater numbers of fibres containing NOS in the muscle layer (fig 7B).

In most areas the NADPH-diaphorase histochemical reaction paralleled the NOS staining patterns in both human and pig SO preparations. However, this technique did not stain the plexi of submucosal nerve fibres previously seen with NOS staining.

Discussion

The physiological regulation of SO function has been widely studied. During the past few years one of the main questions has been the possible role of NO in this process, particularly in species such as man, pig and cat where the SO supposedly acts as a passive resistor or gate against bile flow. NOS has previously been localised in cat and human SO and exogenous NO donors have been observed to decrease human SO motility during endoscopic manometry.8–11 In this study we have evaluated the role of NO in the regulation of pig and human SO. Pig SO was chosen for comparison in this study because its physiological nature and peptidergic innervation resembles that of humans.14–16 To our knowledge NO has not been studied in porcine SO regulation.

Our experiments have shown the presence of NOS in the nerve endings in both human and porcine SO. An intrinsic role for this enzyme is suggested by the enhancement of stimulated contractions by L-NAME (an inhibitor of endogenous NOS) and by the fact that this effect of L-NAME can be reversed by L-arginine, an endogenous substrate for NOS. Further, the use of an NO donor (nitroprusside) effectively antagonised the stimulation induced by acetylcholine. To our knowledge these in vitro experiments have not previously been performed in either human or pig preparations. Taken together, these findings strongly suggest that NO released by enteric neurons in the SO is an intrinsic inhibitor of muscle contractility in category II species. Although this is similar to the effect described in category I species (with a pump-like sphincter),17,18 the net physiological result may be quite the opposite. Thus, inhibition of SO motility may decrease bile flow if the SO behaves like a pump, and increase flow if the SO acts predominantly as a resistor. This assumption is supported by a recent finding that L-NAME, an inhibitor of NOS, impairs bile flow in the cat.17

In vivo monitoring of SO electrical activity has not previously been extensively studied.18
In anaesthetised pigs the SO demonstrated 2–3 phasic electrical bursts per minute. However, although some simultaneous fluctuations in the baseline values were observed, phasic contractions of the SO were difficult to detect during the resting period. One explanation for this is that the pig SO is very short and continuous monitoring at the high pressure zone is difficult without interference by respiratory movements. Acetylcholine, a potent excitatory agent in SO regulation, induced a remarkable increase in SO pressure at all doses tested, as expected. This contraction occurred simultaneously with a huge electrical burst in the EMG. This coupling of electrical and contractile activity has been observed in opssum but not, to our knowledge, in category II (resistor-like) SO. When sodium nitroprusside, an exogenous NO donor, was given in pharmacological doses, the basal SO pressure did not change but the phasic electrical burst activity disappeared in the EMG and the stimulatory effect of acetylcholine was abolished, both in EMG and SO pressure. Our studies therefore indicate that SO electrical activity correlates well with both excitatory and inhibitory effects on SO pressure, suggesting a potential role for EMG in the functional investigations of SO.

The enhancement of acetylcholine induced contractions was more striking in porcine SO than in the human organ. This may be due to weak muscarine receptor antagonism by L-NAME, as has been reported. The different effects of L-NAME on acetylcholine stimulation of SO in the two species may therefore result from possible differences in the expression of muscarinic receptors.

Haematoxylin and eosin staining of the SO in pigs and humans showed a similar architecture in the two species. Immunohistochemical staining for the general neuronal marker PGP 9.5 showed the presence of a rich network of fibres at the mucosa and abundant nerve fibres within the muscle. Several ganglia containing neuronal cell bodies similar to those seen elsewhere in the gastrointestinal tract were also noted. Immunohistochemical analysis revealed rich staining for NOS and NADPH-diaphorase in a similar distribution in both porcine and human SO preparations. NOS has previously been localised in resistor-like sphincters in cat and human SO, but not in porcine SO, and at pump-like sphincters in at least the rabbit and Australian possum.

In conclusion, both in pig and human SO there is endogenous NOS activity and innervation. Furthermore, we have provided evidence that NO may play an inhibitory role in intrinsic regulation in human SO. The physiological similarities between porcine and human SO that we have observed, along with the previously reported similarities in SO innervations, support the use of the pig in experimental models of SO function. Finally, our data suggest that monitoring of SO electrical activity may be a valid method of assessing the physiology of the SO.

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