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Tonometric assessment of jejunal mucosal nitric oxide formation in anaesthetized pigs

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ABSTRACT

Nitric oxide (NO) in the gut has attracted increasing interest as a regulatory factor for a wide variety of intestinal functions. This study was performed to evaluate some methodological aspects and jejunal sources for NO synthesis. Bench side evaluations and an animal model using chloralose-anaesthetized pigs were used. Immunohistochemistry was performed on samples from pig intestine and direct measurements of intestinal NO formation were performed using intraluminal tonometry. Tonometric measurements were quantitatively accurate and with high reproducibility. A substantial NO formation was assessed which was markedly inhibited by luminal administration of the non-selective NOS inhibitor L-NAME. Intravenous administration of L-NAME also reduced jejunal NO formation but to a lesser extent. Immunohistochemistry revealed staining for the inducible type of NOS in the mucosal surface epithelium whereas endothelial and neuronal subtypes were located in deeper layers of the jejunal wall. The study argues for that the source of jejunal NO production, as measured by intraluminal tonometry, is located in close proximity with the intestinal mucosa. The NOS in this compartment is predominantly of the inducible type.

Keywords animal, epithelium, immunohistochemistry, inducible, jejunal, measurement, nitric oxide synthase, nitric oxide.

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The radical nitric oxide (NO) has been ascribed important biological functions as being a messenger in the vasculature and in autonomic nerves, mediator of smooth muscle relaxation in general, as well as, a cytotoxic factor in host defence mechanisms (Ignarro *et al.* 1987, Palmer *et al.* 1987, Shikano *et al.* 1987, Gillespie & Sheng 1988, Hibbs *et al.* 1988, Li & Rand 1989, Boeckxstaens *et al.* 1990, Bredt *et al.* 1990). In the gut, NO has been demonstrated to act as a regulator of a multitude of functions, e.g. motility, mucosal barrier properties and epithelial transport. Intestinal NO is generated by three different pathways: the enzymatic, the non-enzymatic and the bacterial NO formation (Benjamin *et al.* 1994, Lundberg *et al.* 1994a, Salzman 1995). The former is of great biological importance, whereras the roles of the latter two remain speculative. Sources of enzymatic-NO production include enterocytes, endothelial cells, smooth muscle cells, intestinal neurones and leukocytes. Principally, NO is generated during degradation of L-arginine to citrulline, a reaction catalysed by specific enzymes, i.e. nitric oxide synthases (NOS). There exist three main isoforms of NOS: the endothelial type (eNOS) which is constitutive and mainly present in the endothelium of vessels; the neuronal or brain type (nNOS) which also is constitutive but located principally in nerves; and finally the inducible type (iNOS) which is expressed in certain cells, e.g. phagocytes, upon stimulation (for review see Nathan 1994, Forstermann *et al.* 1991). A growing number of reports show that the surface epithelium of the intestinal mucosa contains NOS of the inducible type (Hoffman *et al.* 1997, Holm 1999), and particularly so, during inflammatory states (Boughton-Smith *et al.* 1993, Tepperman *et al.* 1993, Whittle *et al.* 1995, Kimura *et al.* 1997, Kolios *et al.* 1998, Lamarque *et al.*

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1998). The role for intestinal mucosal NO formation is obscure. During physiological conditions, NO participates in the regulation of epithelial electrolyte transport and maintenance of a selective epithelial permeability (Kubes 1992, 1993, Kanwar *et al.* 1994). On the other hand, during pathological conditions following, e.g. sepsis, NO formation is up-regulated to cytotoxic levels which may distort the mucosal lining (Tepperman *et al.* 1993, Miller *et al.* 1993, Sorrells *et al.* 1996, Witthoft *et al.* 1998). Much of the present knowledge concerning NO-mediated effects in the gut are based on indirect observations by pharmacological intervention using NOS inhibitors. There is a need for direct determination of local NO formation which can be put in relation to functional variables. We recently proposed that tonometrical assessment of NO in the jejunal lumen may be a useful predictor of gastrointestinal hypo-perfusion associated with critical illness (Åneman *et al.* 1998). The present paper evaluates some methodological aspects and, by use of NOS inhibitors and immunohistochemistry, the jejunal source for NO found by the tonometric approach was investigated.

MATERIALS AND METHODS

Thirty-six landrace breed pigs of both sexes (mean weight 32 kg, range 28–36 kg) were used in the study, which was approved by the Committee for Ethical Review of Animal Experiments at the Göteborg University. All animals were fasted overnight with free access to water.

Anaesthesia

Following induction with ketamine (Ketalar™, Parke Davis, Solna, Sweden) intramuscularly (bolus, 30 mg kg^{-1} body weight) and α-chloralose (Merck, Darmstadt, Germany) intravenously (bolus, 100 mg kg^{-1} body weight), anaesthesia was maintained by an i.v. infusion of (α-chloralose (pH 7.40) at 25 mg h^{-1} kg^{-1} body weight. Each animal was tracheotomized and mechanically ventilated (Servo 900, Siemens, Stockholm, Sweden) with air to maintain normocapnia and arterial pH around 7.4. Two heating blankets kept core body temperature at +38–39 °C. Isotonic Ringer's acetated solution with 2.5% glucose was infused to maintain normovolaemia as gauged by central venous pressure and pulmonary arterial capillary wedge pressure (data not shown).

Surgical preparation

Catheters (PE240) were positioned in the left internal jugular vein, as well as in the left femoral artery and vein to monitor blood pressures and to provide vascular access for blood sampling and infusions. A midline laparotomy was performed and an ultrasonic transit time flowmeter probe (inner diameter 16 mm, Transonic Systems, Ithaca, NY) was placed around the portal vein to measure mesenteric blood flow. A tonometer (TRIP™ NGS catheter, Tonometrics Division, Instrumentarium Corporation, Helsinki, Finland) was positioned together with an infusion/drainage catheter (PE120) in the jejunal lumen, 50 cm aboral to the ligament of Treitz via a short anti-mesenteric jejunotomy and secured with a purse-string suture.

Nitric oxide measurements

The gas permeable silastic balloon of the tonometer was inflated with 5 mL of room air. Equilibration between NO in the intrajejunal atmosphere and the air of the inflated balloon was allowed during 10 min. The equilibrated gas in the balloon was then transferred into a gas-tight syringe and immediately injected into the sample line of a CLD700AL chemiluminescence analyser (Eco Physics, Dürnten, Switzerland) to determine the amount of NO in the sample by computing the area under the curve using the trapezoidal method for numerical integration (Power Macintosh 6100/66, Apple Computer, Cupertino, CA and LabView, National Instruments, Austin, TX). This value was then divided with the measuring-time in the chemiluminescence analyser (0.45 s) to calculate the concentration (parts per billion, ppb).

Nitric oxide synthase immunohistochemistry

Whole thickness specimens of the jejunum were obtained from six control animals from the area where the incision was made for tube insertions. The specimens were fixed in 4% (w/v) *p*-formaldehyde in phosphate-buffered saline (pH 7.4) for 60 min in 4 °C. The tissue was then frozen in liquid nitrogen and embedded in OCT compound (Sakura Finetek USA, Torrance, CA, USA). Eight to 10 μm sections were cut from the frozen tissue and mounted on slides. The slides were first pre-incubated with a 5% non-fat milk solution and then incubated with primary antibodies against different isoforms of NOS overnight at 4 °C. Polyclonal antibodies against iNOS, eNOS and nNOS were raised in rabbits (Affinity BioReagents, Boulder, CO, USA) and used at 1:800, 1:500 and 1:800, respectively. As control, sections were incubated overnight with phosphate-buffered saline instead of the primary antibody. After washing, the slides were incubated with biotin-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Burmingham, AL, USA) and FITC-conjugated Neutralite™ avidin

(Southern Biotechnology Associates, Burmingham, AL, USA). Immunofluorescence of coded sections were scored arbitrarily with regard to localization using a Nikon Microphot FXA with a fluorescent unit (Nikon Corporation, Tokyo, Japan) by an observer blinded to the treatments.

Drugs

L-NAME (N^G-nitro-L-arginine methyl ester), D-NAME, L-arginine and D-arginine were purchased from Sigma Chemical (St Louis, MO) and the NO donor sodium nitroprusside from Merck, Darmstadt, Germany. All compounds were freshly dissolved in saline and either infused intravenously or administered intraluminally close to the tonometer balloon.

Statistics

All results are presented as means and standard errors of the mean (SEM). Statistical analysis (StatView 4.5, Abacus Concepts, Berkley, CA) was made by one-way or two-way analysis of variance (ANOVA) as appropriate followed by Bonferroni's post-hoc test. Calibration experiments were evaluated by linear regression, and the significance was established using Spearman's correlation coefficient. A P-value <0.05 was considered statistically significant (Altman 1997).

RESULTS

Bench side tests

Bench side calibrations of the tonometer were performed to allow for quantitative measurements. Incremental amounts of sodium nitrite (Chromatography standard, Merck, Darmstadt, Germany) were added to a 0.1 M potassium iodide (Riedel-de Haën, Seelze, Germany) in 0.1 M sulphuric acid to yield known concentrations of NO. The tonometric catheter was submerged into the solution and the NO concentration measured as described above. The equilibration of NO in the tonometric balloon was rapid with more than 90% of the NO concentration in the solution recovered after 5 min (Fig. 1). No significant increase in the recovered NO concentration was observed at 10 or 15 min and a 10-min equlibration period was used throughout the study. The linear correlation between NO recovered tonometrically and the concentration of the calibration solution was good ($r = 0.93$) (Fig. 2). The inter-sample coefficient of variation was 9% when 10 identical determination procedures were performed (data not shown). Furthermore, the detection limit for the tonometry balloon was in the magnitude of 50 ppb and the resolution was in the magnitude of 10%.

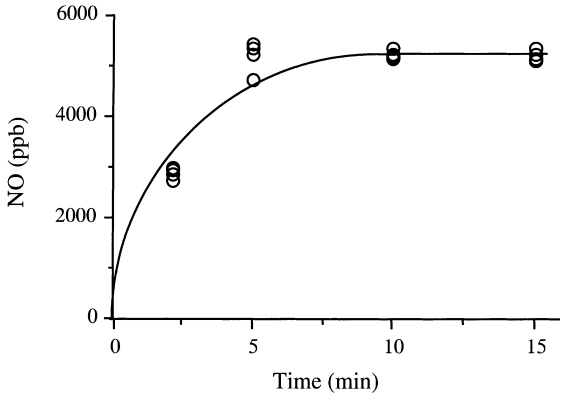

Figure 1 The *in vitro* recovery of NO by use of the tonometric technique. More than 90% of the maximal measured NO concentration was recovered during 5 min of equilibration. No additional increase in measured NO concentration was observed following 10 min of equilibration.

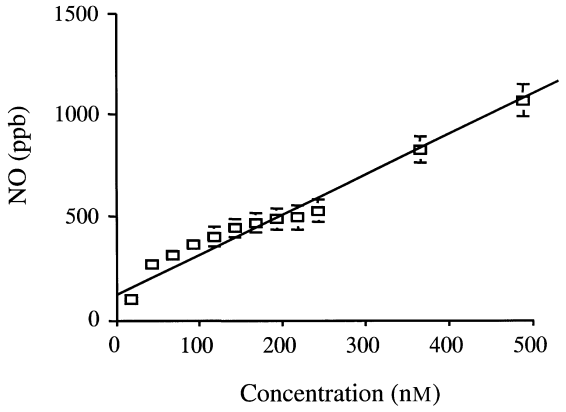

Figure 2 The *in vitro* recovery of NO by use of the tonometric technique. Data represent experiments using four different tonometric catheters submerged in a solution of potassium iodide in sulphuric acid and sodium nitrite in various concentrations. Results are shown as mean ± SEM.

Assessment of jejunal nitirc oxide

Baseline jejunal NO concentrations ranged between 268 and 5559 ppb (median 2167 ppb, $n = 36$). Despite this large inter-animal variability, only small changes in jejunal luminal NO concentrations were observed within individual animals during 90 min at baseline conditions (Table 1). Intravenous administration of L-NAME (10 mg kg^{-1}, infused over a period of 10 min) was followed by reductions in luminal NO concentractions to 48% of baseline. However, L-NAME (0.3 mM, 20 mL) infused intraluminally close to the tonometric balloon almost completely abolished the occurrence of NO (Table 1). The effect of intraluminal L-NAME was not caused by quenching of NO in the saline vehicle for L-NAME, as equal amounts of saline alone did not change the NO concentration

Table 1 Effects of the NO synthase inhibitor L-NAME on jejunal NO

	% of baseline		
	30 min	60 min	90 min
Controls (n = 6)	99 ± 2	101 ± 5	98 ± 5
L-NAME 10 mg kg^{-1} i.v. (n = 6)	60 ± 13	48 ± 12	
L-NAME 0.3 mM intraluminally (n = 6)	10 ± 5	7 ± 4	
L-NAME 0.3 mM + L-Arg 3 mM intraluminally (n = 3)	43 ± 12	57 ± 13	
L-NAME 0.3 mM + D-Arg 3 mM intraluminally (n = 3)	10 ± 4	11 ± 2	

All values relate to baseline measurements. Results are shown as mean ± SEM.

($n = 3$ animals, data not shown). The specificity of NOS blockade was verified by simultaneous administration of L-arginine (3 mM, $n = 3$ animals) which counteracted the effect of L-NAME, whereas administration of the stereo-isomer D-arginine had no such action (3 mM, $n = 3$ animals) (Table 1). Intraluminal infusion of D-NAME (0.3 mM, 20 mL, $n = 3$ animals) or L-arginine alone (3 mM, 20 mL, $n = 3$ animals) did not result in any changes of measured NO levels (data not shown). Intraluminal instillation of the NO donor sodium nitroprusside resulted in increases in recorded NO ($n = 3$ animals) (Fig. 3). Intraluminal administration of L-NAME, L-arginine, D-arginine or sodium nitroprusside did not result in any significant changes to measured haemodynamic variables (arterial pressure and mesenteric perfusion as monitored by portal blood flow), whereas L-NAME intravenously was associated with increased mean arterial pressure (from 122 ± 9 to 170 ± 9 mmHg, $n = 6$ animals, $P < 0.05$) and decreased mesenteric blood flow (from 1040 ± 80 to 610 ± 60 mL min^{-1} $n = 6$ animals, $P < 0.05$).

Localization of NO synthase in porcine jejunum

Immunohistochemical analyses of specimens from six control animals demonstrated immunoreactivity for iNOS particularly in the surface epithelium of the villi (Fig. 4a). The eNOS immunoreactivity was, as expected, associated with blood vessel structures in the submucosa and muscularis (Fig. 4b). The nNOS immunoreactivity was mainly observed in the myenteric plexus and the circular smooth muscle, but also in the sub-mucosa in five out of the six animals (Fig. 4c). It was noted that villuos tissue was not labelled positively for nNOS or eNOS. Control specimens, treated similarly, but without the primary antibody, were dark.

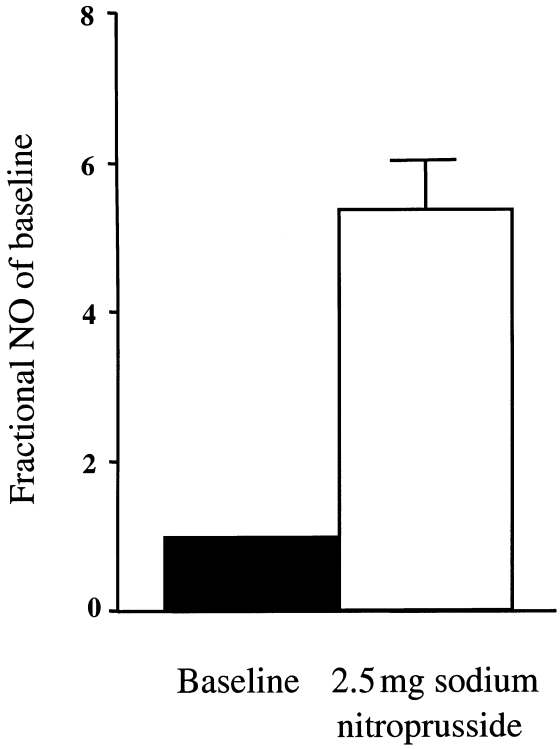

Figure 3 The fractional increase of recovered NO concentration ($n = 3$ animals) from a jejunal segment after intraluminal instillation of 2.5 mg sodium nitroprusside (20 mL).

DISCUSSION

The present study evaluates a novel approach to assess intestinal NO formation. An air-filled, gas-permeable balloon was positioned into the jejunal lumen in close proximity with the intestinal mucosa and the air was then allowed to equilibrate with the intestinal atmosphere. The contents of the balloon were analysed with regard to NO by use of a commercially available chemiluminescence analyser which exclusively measures NO without interference from related oxides of nitrogen metabolites (Brien *et al.* 1996). This tonometric approach to jejunal NO formation was evaluated bench side demonstrating high precision with small variations upon repeated determinations of equal samples. Similar procedures have previously been published (Åneman *et al.* 1996, 1998, Herulf *et al.* 1998). When used *in vivo*, it was found that jejunal NO varied considerably between animals, but that repeated samples within an individual during baseline conditions were rather constant. Local administration of a NO-donating compound increased the recorded NO levels. Furthermore, basal endogenous NO occurrence in the jejunal lumen was more readily inhibited by an intraluminal than by a systemic route of administration of the NOS blocker L-NAME. This finding suggests that formation of NO, as detected by a tonometer placed in

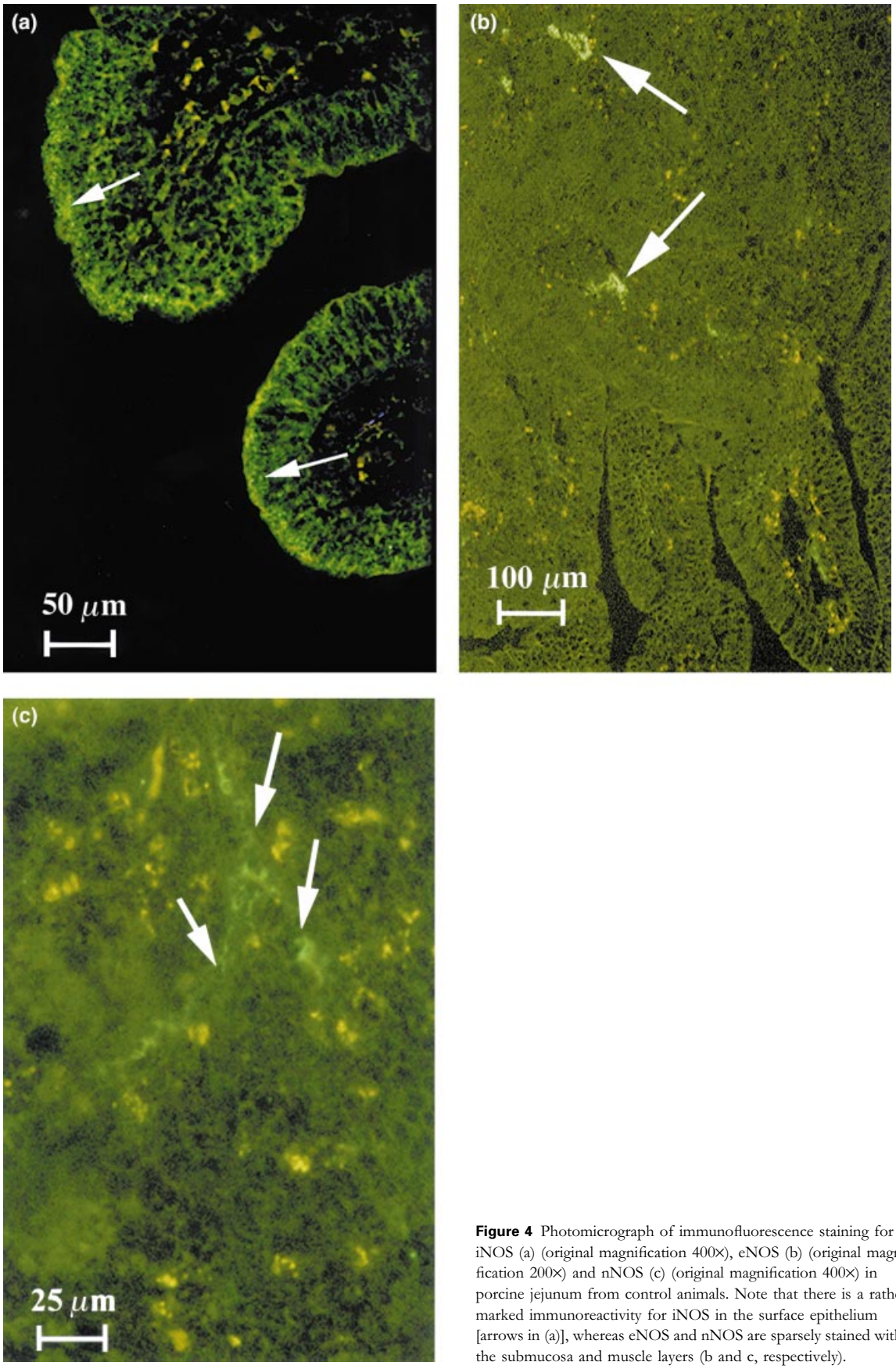

Figure 4 Photomicrograph of immunofluorescence staining for iNOS (a) (original magnification 400×), eNOS (b) (original magnification 200×) and nNOS (c) (original magnification 400×) in porcine jejunum from control animals. Note that there is a rather marked immunoreactivity for iNOS in the surface epithelium [arrows in (a)], whereas eNOS and nNOS are sparsely stained within the submucosa and muscle layers (b and c, respectively).

the lumen, is related to NOS activity in close proximity to the luminal compartment, probably in the cells of the epithelial lining. The lack of effects on arterial pressure and mesenteric blood flow by intraluminal administration of L-NAME argues against systemic absorption of the NOS blocker and further underlines the local nature of the NOS inhibitory response. Moreover, these results confirm similar conclusions made out of experiments in rats using local or systemic administration of NOS inhibitors on duodenal mucosal alkaline secretion (Holm *et al.* 1997). However, in another study on anaesthetized pigs, Holm *et al.* (1998) found that duodenal mucosal NO formation does not decrease subsequent to a NOS blocker administered intraluminally. This contrasts to the present study where jejunal NO formation was virtually abolished by luminal administration of the NOS inhibitor L-NAME. These diverging results may be because of regional differences regarding intestinal NO synthesis, different methodological approach (luminal perfusion vs. tonometry) and/or unknown dose–response relationships.

As mentioned, the sensitivity to local jejunal NOS inhibition and NO donation indicates that the tonometric method probably mainly reflects mucosal NO formation. Immunohistological studies have previously demonstrated the presence of NOS in intestinal neurones and epithelial cells of intestinal mucosa (Brown *et al.* 1992). Also, these results were confirmed in the present study in the pig, showing distinct immunoreactivity for the inducible isoform of NOS (iNOS) in the surface epithelium of the jejunal mucosa, whereas, immunoreactivity for constitutive isoforms (eNOS and nNOS) were situated in deeper layers. Taken together, data suggest that luminal NO, as measured by the tonometric method, probably mainly emerge from the luminal lining of the mucosa. However, it cannot be excluded that cells in the intestinal interstitium, such as fibroblasts, neutrophils and smooth muscle cells may have contributed to the NO detected intraluminally.

As reported in the present study, the epithelial NOS isoform appears to be of the inducible type, a finding that has recently been reported (Hoffman *et al.* 1997, Holm 1999). The role for epithelial NO formation is, however, obscure. A low rate of iNOS expression in the surface epithelium during physiological conditions may be involved in the regulation of epithelial electrolyte transport and the maintenance of normal selective mucosal permeability (Kubes 1992, 1993, Tepperman *et al.* 1993, Sorrells *et al.* 1996, Izzo *et al.* 1998). Intestinal epithelial NO formation is dramatically up-regulated during inflammatory conditions (Lundberg *et al.* 1994b, Rachmilewitz *et al.* 1995) and recent data show that expression of epithelial iNOS increases in response to proinflammatory cytokins, luminal aggressors like acidity and endotoxin (Boughton-Smith *et al.* 1993, Whittle *et al.* 1995, Holm 1999). The NO formation by the intestinal epithelial cells may thus constitute a part of the first line of host defence against luminal pathogens. Nitric oxide tonometry can prove to be very helpful in future elucidation of such a host defence system.

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