

## L-Arginine, nitric oxide, and intestinal secretion: studies in rat jejunum in vivo

F H Mourad, L J D O'Donnell, E A Andre, C P Bearcroft, R A Owen, M L Clark, M J G Farthing

### Abstract

**Background**—L-Arginine has been shown to induce fluid secretion in human jejunum. Nitric oxide, a derivative of L-arginine is thought to have an important role as an intestinal secretagogue.

**Aim**—To determine the effect of L-arginine and the nitric oxide synthase inhibitor, nitro L-arginine methyl ester (L-NAME), on fluid and electrolyte movement in rat jejunum.

**Methods**—A 25 cm segment of rat jejunum was perfused in situ with iso-osmotic solutions containing either (1) saline, (2) D-arginine 20, (3) L-arginine 20, (4) L-NAME 0.1, 1, or 20 mmol/l, or (5) a combination of L-arginine 20 and L-NAME 0.1, 1, or 20 mmol/l. In further groups the effect of a subcutaneous injection of L-NAME 100 mg/kg was examined in rats pretreated with either D- or L-arginine 500 mg/kg.

**Results**—L-Arginine, unlike D-arginine, induced fluid secretion despite being better absorbed (mean  $-7.3$  v  $17.0$   $\mu\text{l}/\text{min}/\text{g}$ ;  $p < 0.01$ ). L-NAME at 0.1 mmol/l had no effect on basal fluid movement but reversed L-arginine induced secretion ( $7.8$ ;  $p < 0.05$ ). L-NAME at 1 and 20 mmol/l induced fluid secretion ( $-15.4$  and  $-28.4$ , respectively), which was enhanced by the addition of L-arginine ( $-30.0$  and  $-41.0$ , respectively; both  $p < 0.05$ ). A subcutaneous injection of L-NAME resulted in marked fluid secretion ( $-39.9$ ) and histological evidence of intestinal ischaemia. These changes were attenuated or reversed by pretreatment with subcutaneous L- but not D-arginine.

**Conclusions**—L-Arginine induces intestinal fluid secretion through production of nitric oxide. There is a delicate balance between the effect of nitric oxide as a secretagogue and its effect on maintaining blood flow and thus preventing intestinal ischaemia.

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Keywords: arginine, nitric oxide, L-NAME, intestinal transport, bowel ischaemia.

Actively transported sugars and most amino acids enhance small intestinal absorption of water and electrolytes.<sup>1-4</sup> However, L-arginine (L-Arg), unlike other amino acids, has been shown by us and others to induce water

secretion when perfused in human jejunum.<sup>1-5</sup> A satisfactory explanation has never been put forward to explain this phenomenon, though it has been suggested that L-Arg induced secretion by a local effect that could be inhibited by the calmodulin antagonist chlorpromazine.<sup>5</sup>

Over the past few years L-Arg has been found to be the precursor of the free radical nitric oxide (NO), which has an important role as a mediator of neural, cardiovascular, and gastrointestinal function.<sup>6-8</sup> Nitric oxide is derived from the guanidino terminal of L-Arg by the action of the stereospecific enzyme nitric oxide synthase (NOS) which, in the gut, is present in the myenteric plexus<sup>9,10</sup> and submucosal arterioles and venules.<sup>11,12</sup> In addition, it has been shown that NO could be produced by enterocytes through both the constitutive and the inducible NOS.<sup>13</sup> In vitro studies have unveiled a role for NO as a secretagogue in the ileum<sup>14,15</sup> and colon,<sup>16-18</sup> and in vivo studies have suggested a possible role for NO in the laxative action of castor oil,<sup>19</sup> magnesium sulphate,<sup>20</sup> and in the pathophysiology of secretion induced by *Escherichia coli* heat stable enterotoxin.<sup>15</sup> In addition, NO is a vasodilator and its continuous endogenous production is important in maintaining gastric microcirculation and mucosal integrity.<sup>21,22</sup> However, the effect of endogenous NO on small intestinal water absorption/secretion and mucosal integrity is unknown. The aim of this study was to examine (a) the pathophysiology of L-Arg induced jejunal secretion and its relation with NO production, and (b) the structural and functional effects of NOS inhibition in rat jejunum by examining the water and electrolyte movement and the histological changes in the jejunum after administration of the NOS inhibitor nitro L-arginine methyl ester (L-NAME).

### Methods

#### *In vivo perfusion model*

Male adult Wistar rats (180-220 g body weight) were fasted for 18 hours with free access to water. The rats were anaesthetised by intraperitoneal injection of sodium pentobarbitone (60 mg/kg) and maintained throughout the experiment by interval intraperitoneal injections (15-30 mg/kg) as necessary. The abdomen was opened through a midline incision and a 25 cm segment of jejunum

Digestive Diseases Research Centre, Medical College of St Bartholomew's Hospital, Charterhouse Square, London  
 F H Mourad  
 L J D O'Donnell  
 E A Andre  
 C P Bearcroft  
 R A Owen  
 M L Clark  
 M J G Farthing

Correspondence to: Professor M J G Farthing, Digestive Diseases Research Centre, St Bartholomew's and the Royal London School of Medicine and Dentistry, Turner Street, London E1 2AD.

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TABLE I Composition and osmolality of the different solutions perfused into rat jejunum

	L-Arg (mmol/l)	L-NAME (mmol/l)	Na (mmol/l)	Cl (mmol/l)	Osmolality (mOsmol/kg)
Saline			158.2	156.5	300
L-Arg	20		139.9	159.6	300
D-Arg	20		139.5	160	300
L-Arg+L-NAME 0.1	20	0.1	140	158	300
L-Arg+L-NAME 1	20	1	140	159	300
L-Arg+L-NAME 20	20	20	128	150.8	300
L-NAME 0.1		0.1	156	158	300
L-NAME 1		1	154.5	159	300
L-NAME 20		20	136.4	157	300

starting 5 cm distal to the ligament of Treitz was isolated between two cannulae and the abdomen closed.<sup>23</sup> The isolated segment was perfused in situ with the test solution at a rate of 0.5 ml/min for 30 minutes to reach steady state and then three effluent collections of 10 minutes each were obtained for assessment of water and electrolyte movement. All solutions used were rendered iso-osmotic (300 mOsmol/kg) by addition of NaCl, adjusted to pH 7.2 by the addition of 1 N HCl or 1 N NaOH, and contained 4  $\mu$ Ci/l [<sup>14</sup>C]polyethylene glycol 4000 as a non-absorbable volume marker. Animals were kept at 37°C using a heat pad. At the end of the experiments, urine and blood samples were taken and the rats were killed by an overdose of pentobarbitone; the perfused intestinal segment was removed, rinsed, blotted, and desiccated in an oven at 100°C to obtain dry weight. The samples of effluent were analysed immediately or kept frozen at -20°C and analysed within two weeks. Steady state condition was shown by less than 5% variation in water movement between consecutive 10 minute collections, and the values were accepted only if recovery of radioactive PEG fell between 95 and 105%<sup>23</sup> and no radioactivity was detected in urine and blood samples. At the end of the experiments the gross appearance of the intestine was assessed by an independent observer for the presence of ischaemia and then jejunal sections were taken for histological examination by light microscopy. Tissue sections were coded and examined 'blind' by a histopathologist.

#### *Effect of NO precursor, L-Arg, on water and electrolyte transport in rat jejunum*

In three groups of animals the intestinal segment was perfused with either iso-osmotic saline or a solution containing L-Arg (20 mmol/l) or its inactive enantiomer D-Arg at the same concentration. Effluent radioactivity, sodium chloride, and Arg were measured and net water, electrolyte, and Arg movement calculated accordingly.

In parallel groups of animals L-Arg or D-Arg in doses of 100 and 500 mg/kg in 0.3 ml saline was given subcutaneously and 35 minutes later jejunal perfusion was performed with iso-osmotic saline.

#### *Effect of L-NAME on water and electrolyte transport in rat jejunum*

Intestinal perfusion was performed in six groups of animals with solutions containing the

NOS inhibitor, L-NAME at concentrations of 0.1, 1, or 20 mmol/l with or without L-Arg 20 mmol/l (Table I). The solutions were perfused for 30 minutes to reach steady state and then three collections of the effluent of 10 minutes each were collected.

To study the effect of systemic L-NAME, three groups of animals were injected subcutaneously with either L-Arg or D-Arg 500 mg/kg in 0.3 ml saline, or 0.3 ml saline alone, followed after 15 minutes by subcutaneous L-NAME 100 mg/kg in 0.3 ml saline. After a further 20 minutes jejunal perfusion with an iso-osmotic saline solution was performed.

#### *Analytical methods*

[<sup>14</sup>C]PEG concentrations in the effluent were measured in triplicate by liquid scintillation spectroscopy in LKB Wallac Ultra-beta 1210 scintillation counter. Sodium and potassium concentrations were determined by flame photometry (Instrument Laboratories 943), and chloride concentration by Corning 925 chloride analyser (Corning, UK). Solution osmolality was analysed using the vapour pressure technique with a Wescor 5500 osmometer. L-Arg and D-Arg concentrations in the effluent were measured by the colorimetric method of Sakagushi as previously described.<sup>24, 25</sup> In brief, 0.5 ml of sample solution and 0.5 ml of 20% potassium hydroxide were mixed in a test tube colled in an ice-water bath. Acetic anhydride (0.075 ml) was added and the tube shaken vigorously in the ice. One hour later 0.5 ml of 0.01% 1-naphthol in 10% potassium hydroxide solution was added followed by 0.2 ml of 0.06% hypobromite solution. This was followed by 0.5 ml of alcohol and one hour later a second addition of 0.2 ml of hypobromite solution was made. The optical density was measured at 520 nm and compared with that of known concentrations of Arg. The net water, electrolyte, and Arg movement was calculated and expressed respectively in  $\mu$ l/min/g and  $\mu$ mol/min/g of dry intestinal weight. Positive values denote absorption and negative values denote secretion.

#### *Histology*

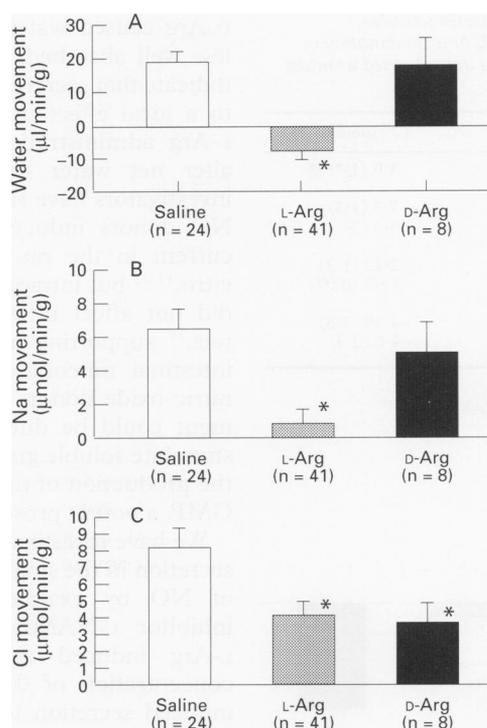
Jejunal sections were stained with haematoxylin and eosin and histology was evaluated by a pathologist unaware of the experimental protocol from which the tissue originated.

#### *Materials*

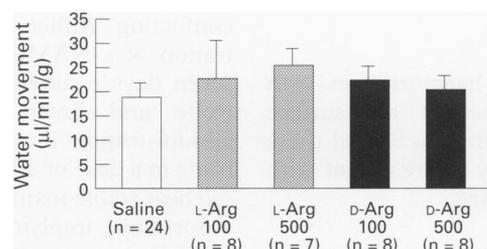
L-Arg, D-Arg, and L-NAME were obtained from Sigma Chemical Company, UK. Radio-labelled polyethylene glycol ([<sup>14</sup>C]PEG 4000) was obtained from Amersham International and all other chemicals were supplied by British Drug House (BDH Chemicals).

#### *Statistics*

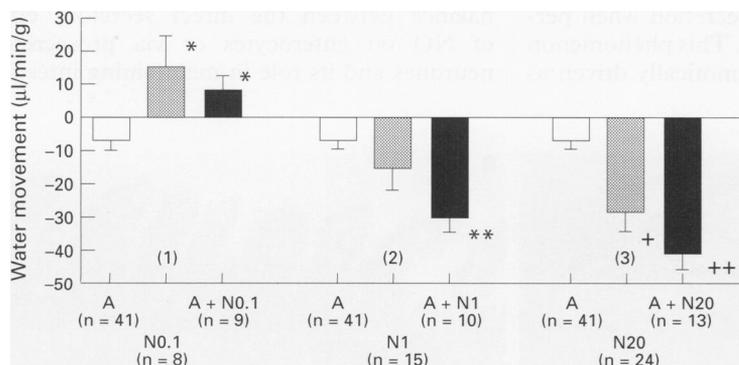
Results are expressed as mean (SEM) in each group of animals studied. Analysis of variance (ANOVA) was used for multiple comparisons



**Figure 1:** Water (A), sodium (B) and chloride (C) movement in rat jejunum perfused with either saline, L-Arg 20 mmol/l, or D-Arg 20 mmol/l. Values are expressed as mean (SEM). Positive values denote absorption and negative values denote secretion. (A) ANOVA  $p < 0.001$ ; \* $p < 0.00001$  compared with saline and  $p < 0.01$  compared with D-Arg. (B) ANOVA  $p < 0.01$ ; \* $p < 0.001$  compared with saline and  $p < 0.05$  compared with D-Arg. (C) ANOVA  $p < 0.05$ ; \* $p < 0.05$  compared with saline.



**Figure 2:** Water movement in rat jejunum perfused with iso-osmotic saline solution after a subcutaneous injection of L-Arg or D-Arg at a dose of 100 and 500 mg/kg. Values are expressed as mean and SEM. Positive values denote absorption and negative values denote secretion. ANOVA NS.



**Figure 3:** Water movement in rat jejunum after perfusion with solutions containing L-Arg, L-NAME, or L-Arg L-NAME. Values are expressed as mean (SEM). Positive values denote absorption and negative values denote secretion. (1) ANOVA  $p < 0.05$ ; \* $p < 0.01$  compared with A. (2) ANOVA  $p < 0.01$ ; \*\* $p < 0.004$  compared with A and  $p < 0.05$  compared with N1. (3) ANOVA  $p < 0.01$ ; + $p < 0.003$  and ++ $p < 0.0001$  compared with A, and ++ $p < 0.05$  compared with N20. (A=L-Arg 20 mmol/l; N0.1, N1, N20=L-NAME at 0.1, 1, and 20 mmol/l respectively).

and an unpaired Student's *t* test for individual comparisons.

## Results

### Effect of the NO precursor, L-Arg on water and electrolyte transport

Perfusion of rat jejunum with an iso-osmotic saline solution (Fig 1A) resulted in a mean (SEM) net water absorption of 18.3 (3.6)  $\mu\text{l}/\text{min}/\text{g}$  dry intestinal weight, whereas 20 mmol/l of L-Arg caused water secretion ( $-7.3$  (2.6);  $p < 0.00001$  compared with saline). This phenomenon was not observed with D-Arg where net water absorption (17.0 (9.0)) was not different from the saline control, but significantly different from the L-Arg ( $p < 0.05$ ) (Fig 1A). Likewise, sodium and chloride absorption were greater with saline solution than with L-Arg containing solution (Figs 1B and 1C). Paradoxically, D-Arg resulted in a decrease in chloride absorption than saline (Fig 1C). Absorption of L-Arg was greater than its D-enantiomer (9.8 (2.0)  $\mu\text{mol}/\text{min}/\text{g}$  v 2.2 (0.5);  $p < 0.01$ ).

Subcutaneous administration of L-Arg or D-Arg in a dose of 100 or 500 mg/kg had no effect on water (Fig 2) and electrolyte movement (data not shown). All histological sections obtained after either perfusion with Arg or following subcutaneous administration of Arg were normal.

### Effect of NOS inhibition with L-NAME

The NOS inhibitor L-NAME perfused intraluminally in a concentration of 0.1 mmol/l had no effect on water and electrolyte movement, but in higher doses of 1 and 20 mmol/l it led to water secretion (Fig 3) and to a marked decrease or reversal of electrolyte absorption (Table II).

Perfusion with combinations of L-NAME and L-Arg (Fig 3) showed that L-NAME at a concentration of 0.1 mmol/l reversed L-Arg induced secretion to absorption. At the higher concentrations of L-NAME (1 and 20 mmol/l), however, L-Arg induced secretion was enhanced rather than reversed (Fig 3).

All intestinal segments perfused with L-NAME at 1 and 20 mmol/l with or without L-Arg appeared grossly dusky and ischaemic but there were no obvious abnormalities on light microscopy.

A subcutaneous injection of L-NAME (100 mg/kg) 20 minutes before starting the jejunal perfusion caused severe intestinal secretion ( $-39.9$  (5.4)) (Fig 4). Pretreatment with L-Arg (500 mg/kg) subcutaneously, partially but significantly attenuated the secretory response. D-Arg had no effect on L-NAME induced intestinal secretion (Fig 4). Sodium and chloride movement paralleled that of water (Table II).

Intestinal segments perfused after L-NAME injection showed marked macroscopic changes; the intestine looked ischaemic with obvious areas of infarction. Microscopically, there were typical histological changes of ischaemia with

TABLE II Sodium and chloride movement in rat jejunum perfused with solutions containing either L-Arg 20 mmol/l, L-NAME 0.1, 1, or 20 mmol/l; or a combination of L-Arg 20 + L-NAME at the above different concentrations. Values are expressed as mean (SEM)

	Na ( $\mu\text{mol}/\text{min}/\text{g}$ )	Cl ( $\mu\text{mol}/\text{min}/\text{g}$ )
L-Arg	0.8 (1)*†§	3.9 (1)**‡¶
L-NAME 0.1 mmol/l	6 (1.2)	7.3 (1.3)
L-Arg+L-NAME 0.1 mmol/l	4.1 (0.8)	5.3 (1)
L-NAME 1 mmol/l	0.23 (2.8)††	-2.42 (1.2)
L-Arg+L-NAME 1 mmol/l	-5.7 (0.6)	-1.97 (0.07)
L-NAME 20 mmol/l	-1.36 (1.4)§§	-4.59 (1.8)
L-Arg+L-NAME 20 mmol/l	-4.96 (1.2)	-4.6 (2.3)

Comparison is made among groups. All ANOVA  $p < 0.05$ .

\* $p < 0.005$  and \*\* $p < 0.05$  compared with L-NAME 0.1 and L-Arg+L-NAME 0.1.

† $p < 0.0005$  and †† $p < 0.05$  compared with L-Arg+L-NAME 1.

‡ $p < 0.005$  compared with L-NAME 1 and L-Arg+L-NAME 1.

§ $p < 0.001$  and §§ $p < 0.05$  compared with L-Arg+L-NAME 20.

¶ $p < 0.003$  compared with L-NAME 20 and L-Arg+L-NAME 20.

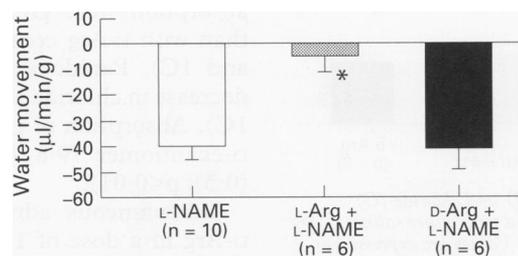


Figure 4: Water movement in rat jejunum perfused with an iso-osmotic saline solution after a subcutaneous injection of L-NAME 100 mg/kg alone, or after pretreatment with subcutaneous L-Arg or D-Arg 500 mg/kg. Values are expressed as mean (SEM). Positive values denote absorption and negative values denote secretion. ANOVA  $p < 0.01$ ; \* $p < 0.01$  compared with L-NAME and with D-Arg + L-NAME.

submucosal oedema and haemorrhages with early degenerative changes of the surface epithelium at the tip of villi (Fig 5). All these changes were prevented by pretreatment with L-Arg (Fig 5B) but not D-Arg.

### Discussion

Amino acids are actively transported in the small intestine and in general enhance water and electrolyte absorption.<sup>1 3 4</sup> However, the dibasic amino acid L-Arg has been shown to induce water secretion in human jejunum.<sup>1 5</sup> Similarly, in our experiments we have shown that L-Arg induced water secretion when perfused in rat jejunum (Fig 1). This phenomenon is stereospecific and not osmotically driven as

D-Arg caused water absorption despite being less well absorbed. In addition, these studies indicate that secretion induced by L-Arg relates to a local effect in the intestine, as parental L-Arg administration in high dose failed to alter net water movement (Fig 2). Other investigators have shown that L-Arg and other NO donors induce changes in short circuit current in the rat and guinea pig ileum in vitro,<sup>14 15</sup> but intraperitoneal injection of L-Arg did not affect intestinal water movement in rats,<sup>19</sup> supporting a local effect of L-Arg on intestinal mucosa. The effect of L-Arg and nitric oxide donors on intestinal water movement could be due to the ability of NO to stimulate soluble guanylate cyclase resulting in the production of the second messenger cyclic GMP, a potent prosecretory agent.<sup>26</sup>

We have investigated whether L-Arg induces secretion in the small bowel through formation of NO by examining the effect of NOS inhibitor L-NAME given intraluminally on L-Arg induced secretion. L-NAME at a concentration of 0.1 mmol/l reversed L-Arg induced secretion to absorption, indicating a role for NO in this process. Surprisingly, however, L-NAME at a concentration of 1 and 20 mmol/l given alone caused secretion in the rat jejunum and enhanced rather than reversed water secretion induced by L-Arg, which may imply that these substances induce water secretion by different mechanisms. The effects of L-NAME at higher doses are more difficult to explain. The effect of L-NAME on intestinal water transport in previous studies has been conflicting. Miller *et al* found that administration of L-NAME orally to guinea pigs for seven days resulted in ileal water secretion.<sup>27</sup> Rolfe and Levin showed that L-NAME administration in rats by the intraperitoneal route in a dose of 40 mg/kg reversed *Escherichia coli* heat stable toxin (Sta) induced secretion to absorption, implying that NO is a secretagogue<sup>15</sup>; however Schirgi-Degen and Beubler found that intravenous L-NAME administration led to net fluid secretion in rat jejunum and enhanced Sta secretion, concluding that NO has a proabsorptive role in the intestine.<sup>28</sup> In these studies intestinal ischaemia and histology were not evaluated.

Our studies suggest that there is a fine balance between the direct secretory effect of NO on enterocytes or via prosecretory neurones and its role in maintaining intestinal

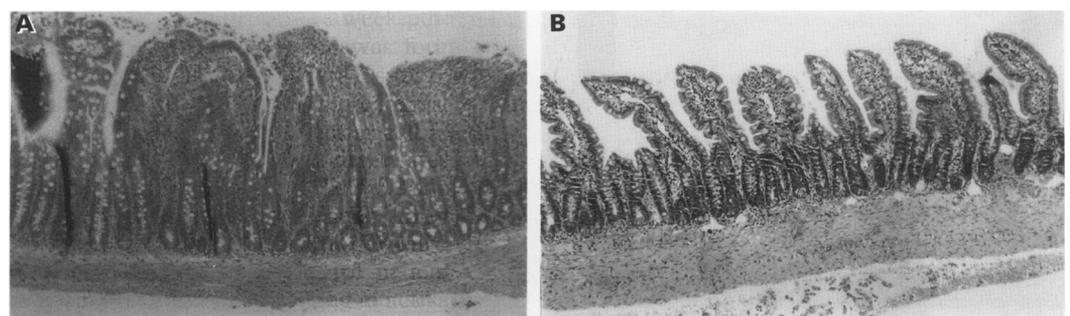


Figure 5: Histological sections from rat jejunum after a subcutaneous injection of L-NAME 100 mg/kg (A) showing submucosal oedema and haemorrhages with early degenerative changes at the tip of villi or (B) after pretreatment with subcutaneous L-Arg 500 mg/kg showing no histological abnormalities (original magnification  $\times 100$ ).

blood flow. Nitric oxide could be involved in intestinal secretion either by acting directly on the epithelium or indirectly stimulating neuronal reflexes<sup>15</sup> or stimulating the release of other agents from enteric nerves that can stimulate secretion. In addition, oxyradicals have been shown to stimulate intestinal electrolyte transport in rabbit and rat intestine,<sup>29, 30</sup> and therefore NO and other nitrogen oxides could stimulate secretion by the nature of their free radical structure. L-NAME is absorbed from the small intestine and Gardiner *et al* have found a 25% decrease in mesenteric blood flow in rats drinking a solution containing 0.1 mg/ml L-NAME.<sup>31</sup> We would expect the highest concentration and the total amount of L-NAME administered in our experiments would cause a significant fall in mesenteric blood flow. With perfusions of L-NAME at 1 and 20 mmol/l the intestine was clearly ischaemic, though not severely enough to cause histological changes. This may be due to the fact that histological changes usually appear within 30 minutes to one hour after total circulatory arrest to the intestine,<sup>32, 33</sup> whereas in the present experiments there was probably a decrease but not complete interruption of intestinal blood flow. It is well known that vasoconstrictor agents like vasopressin cause water and electrolyte secretion in the small intestine.<sup>34</sup> In addition Robinson *et al* have shown that intestinal ischaemia causes net water and electrolyte secretion secondary to a profound inhibition of water and sodium absorption by villus enterocytes, whereas the intact crypt cells continue to secrete sodium chloride and water.<sup>35, 36</sup> The concentration of L-NAME required to reverse L-Arg induced secretion in our study was low compared with previous studies, where the dose of L-Arg able to counteract the L-NAME effect can be threefold to 100-fold higher than that of the NOS inhibitor depending on tissues and species.<sup>37</sup> This could be explained by the fact that L-NAME may be better absorbed than L-Arg, or the concentration of NO needed to activate secretion either directly or through neuronal reflexes is much lower than that required for vasodilatation. Although L-NAME has been found to have an antimuscarinic effect,<sup>38</sup> it is unlikely that it has an important role in our experiments as the antimuscarinic effect is independent of NO production and thus it would not be antagonised by the NO precursor L-Arg.

Systemic administration of L-NAME led to severe intestinal secretion associated with macroscopic and microscopic evidence of intestinal ischaemia with some areas of infarction. This is further evidence of the importance of NO in maintaining mesenteric circulation and thus intestinal mucosal integrity. Both intestinal ischaemia and intestinal secretion were markedly attenuated by pretreatment with L-Arg but not D-Arg, confirming the specificity of the effect. Our results are in accordance with those of Schirgi-Degen and Beubler,<sup>28</sup> in that L-NAME given parenterally induced secretion in rat jejunum; we disagree, however, with their conclusion that NO is

an absorbagogue as the L-NAME induced secretion in their experiments is most likely due to decreased blood flow.

Nitric oxide exerts its biological effects locally either as a paracrine mediator or as a neurotransmitter. We propose that within the intestine, inhibition of NO release in the vicinity of the vasculature causes ischaemia and consequent intestinal secretion, and that increased NO production in the vicinity of enterocytes also promotes intestinal secretion. The secretion induced by L-Arg perfusion is due to increased NO production in the vicinity of enterocytes. The secretion produced by subcutaneous administration of L-NAME is due to intestinal ischaemia. Secretion induced by perfusion with L-Arg is reversed by perfusion in combination with 0.1 mmol/l L-NAME owing to inhibition of NO production in the vicinity of the enterocytes. However, perfusion with higher concentrations of L-NAME paradoxically enhances L-Arg induced secretion by affecting the vasculature and producing ischaemia and consequent secretion. Thus there is a delicate balance between the effect of NO and inhibitors of NO on enterocyte and vascular function.

In summary, L-Arg induces water and electrolyte secretion in the rat small intestine through an NO mediated mechanism due either to a direct effect on enterocytes or through neuronal activation. Intraluminal and systemic NOS inhibition leads to severe intestinal secretion by decreasing mesenteric blood flow resulting in intestinal ischaemia. There seems to be a fine balance between the prosecretory effect of NO on enterocytes or myenteric neurones and its role in maintaining blood flow and thus preventing intestinal ischaemia.

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