Regulation of gall bladder motility by the arginine-nitric oxide pathway in guinea pigs

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Abstract

Nitric oxide (NO) synthesised from L-arginine is an intercellular messenger in various biological actions including endothelial dependent relaxation and inhibition of platelet aggregation. This study explored the role of the L-arginine-NO pathway in the regulation of gall bladder motility. Intraluminal gall bladder pressure was recorded in anaesthetised guinea pigs in response to cholecystokinin or bethanechol before and after treatment with specific NO synthase inhibitors (N^G -nitro-L-arginine, N^G -nitro-L-arginine methyl ester, or N^G -monomethyl-L-arginine), or with an NO donor (sodium nitroprusside). Baseline gall bladder pressure significantly increased after treatment with the NO synthase inhibitors. Responses to cholecystokinin (0.025–1.25 nmol/kg) were significantly enhanced after treatment with N^G -nitro-L-arginine methyl ester and lasted two to threefold longer than in control experiments. The effect of the inhibitor both on resting pressure and on cholecystokinin induced changes was reversed by L-arginine but not by D-arginine. Pre-treatment with the inhibitors also induced a significant enhancement of the response to bethanechol. On the other hand, sodium nitroprusside abolished the response to low dose cholecystokinin and reduced the response to a high dose by about 80%. In vitro experiments with isolated gall bladder strips showed a significant enhancement of the contractile response to cholecystokinin or bethanechol after preincubation with the NO synthase inhibitor. Calcium dependent activity of NO synthase was detected in fresh homogenates from gall bladder tissue and incubation with endotoxin induced considerable calcium independent activity. These findings support the existence of a key L-arginine-nitric oxide pathway regulating gall bladder contraction.

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An enzymatic pathway that synthesises nitric oxide (NO) from the terminal guanidino nitrogen of L-arginine was originally shown in vascular endothelial cells.¹ This was followed by the finding that the L-arginine-NO pathway is a widespread tissue system involved in the regulation of cell function and communication.² The L-arginine-NO system exerts various biological actions including vascular smooth muscle relaxation³ and inhibition of platelet aggregation.⁴ Recent experimental data suggest that NO acts as a neurotransmitter in non-adrenergic non-

cholinergic pathways of the gastrointestinal tract.⁵ It is now well established that NO exerts its actions through stimulation of a cytosolic guanylate cyclase with a consequent rise in cyclic guanosine monophosphate (cGMP) concentrations.^{3,6}

In gall bladder smooth muscle, accumulation of intracellular cGMP and muscle contraction are inversely related events.⁷ Cholecystokinin (CCK) is the main physiological agent that induces gall bladder contraction.^{8,9} Thus cGMP has been shown to relax gall bladder smooth muscle and antagonise the action of CCK.⁷ On the other hand, non-adrenergic non-cholinergic nerves conduct inhibitory signals that induce gall bladder relaxation.¹⁰ These data suggest that NO may be involved in some of these processes but the potential role of the L-arginine-NO pathway in gall bladder physiology has not been explored. Thus we designed the present experiments to investigate the effect of NO synthase inhibition on gall bladder muscular activity in vivo and in vitro. We also investigated the presence of the enzyme able to convert L-arginine to NO in tissue homogenates from guinea pig gall bladder.

Materials and methods

ANIMALS

Male albino guinea pigs weighing 350–450 g (Charles River, France) were used. They were maintained in a temperature controlled environment provided with a 12:12 hour light:dark cycle and given regular guinea pig food and tap water ad libitum.

IN VIVO STUDIES

Intraluminal gall bladder pressure was measured in vivo by the method described by Poston *et al* for guinea pigs.¹¹ After an overnight fast, the animals were anaesthetised with 2 g/kg intraperitoneal urethane (Fluka, Bucks, Switzerland). A tracheostomy was performed and a catheter contained heparinised (10 U/ml) saline was inserted into the right carotid artery for continuous monitoring of arterial blood pressure. A second catheter was inserted into the internal jugular vein to give drugs intravenously. After a midline abdominal incision, a catheter (internal diameter 0.89 mm) was inserted through the gall bladder fundus into the lumen and tied in position to prevent any leakage. The contents of the gall bladder were emptied and 2 ml normal saline injected. Thereafter, saline was constantly infused at a rate of 0.1 ml/min through the gall bladder catheter with an ASID Bonz PP infusion

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pump (Germany). A side channel from the infusion catheter was connected to a pressure transducer (model 1280C, Hewlett-Packard) on line with a recording system. This infusion rate was needed to maintain a constant baseline pressure within the open biliary system. Thus gall bladder muscle contractions could be recorded as changes in intraluminal gall bladder pressure.

The protocol consisted of constructing dose-response curves of intraluminal gall bladder pressure in response to cholecystokinin octapeptide (CCK-8, Sigma, St Louis, Mo) at doses ranging from 0.025 to 1.25 nmol/kg or to bethanechol (Roig Farma, Barcelona, Spain) at doses ranging from 12.5 to 50 µg/kg, before and after inhibition of the NO synthase with the arginine analogues N^G-monomethyl-L-arginine (L-NMMA, 100 mg/kg, Wellcome Research Laboratories, Beckenham, Kent, UK), N^G-nitro-L-arginine (L-NA, 10 mg/kg, Sigma), or N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg, Sigma).¹² Resting gall bladder pressure was the mean pressure recorded over a five minute period immediately before an intravenous bolus of either CCK-8 or bethanechol, and the response was defined as the maximal change in intraluminal pressure recorded during the first five minute period after the bolus. Responses to CCK-8 or bethanechol were recorded 10 minutes after pretreatment with the NO synthase inhibitors or with saline (all drugs were administered as an intravenous bolus dissolved in 0.9% NaCl). In some experiments, the specific substrate of NO synthase, L-arginine (300 mg/kg, Sigma), was given to reverse the effects of the inhibitors, whereas the enantiomer D-arginine (300 mg/kg, Sigma) was used as the inactive control compound. Other experiments were performed on animals pretreated with sodium nitroprusside (2 mg/kg, Merck, Darmstadt, Germany), an exogenous non-enzymatic donor of NO,¹³ to assess the effects of NO on gall bladder responses to CCK-8. Finally, to discriminate the effect on gall bladder pressure of changes in systemic blood pressure induced by the NO synthase inhibitors, the vasopressor agent phenylephrine (10 µg/kg/min, Roig Farma) was infused intravenously into anaesthetised guinea pigs and changes in both systemic and intraluminal gall bladder pressure were measured.

IN VITRO STUDIES

Longitudinal gall bladder strips were taken from male albino guinea pigs that had been fasted overnight. The strips were placed in organ baths containing 20 ml of Krebs solution at 37°C continuously bubbled with O₂:CO₂ (95%:5%). Resting strip tension was adjusted to 1 g and changes were monitored by an isometric force transducer (Letica, Barcelona, Spain) and recorded by a polygraph connected on line. Experiments were started after a two hour stabilisation period. Tension responses to CCK-8 (25 ng/ml) and bethanechol (2 µg/ml) were tested in control experiments and after addition of L-NAME (27 µg/ml) to the incubation medium.

DETERMINATION OF NO SYNTHASE ACTIVITY

The enzymatic transformation of L-arginine to L-citrulline was determined in tissue homogenates with the method described by Knowles *et al.*¹⁴ Aliquots of tissue homogenate (20 µl) were added to 10 ml plastic tubes containing 100 µl of a buffer consisting of 50 mM potassium phosphate pH 7.2, 60 mM L-valine, 120 µM NADPH, 1.2 mM L-citrulline, 24 µM L-arginine, L-[U-¹⁴C]-arginine (150 000 dpm), 1.2 mM MgCl₂, and 0.24 mM CaCl₂. Samples were incubated for 10 minutes at 37°C before the reaction was stopped by removal of substrate and dilution by addition of 1.5 ml of 1:1 (vol:vol) H₂O:Dowex-50W (200–400, 8% cross linked, Na⁺-form). Five ml of H₂O were added to the resin incubated mix, this was left to settle for 10 minutes, and 4 ml of supernatant was removed and examined for the presence of ¹⁴C-citrulline by liquid scintillation counting. The activity of the calcium dependent NO synthase was found from the difference between the ¹⁴C-citrulline produced from control samples and samples containing 1 mM ethylene glycol tetra-acetic acid (EGTA); the activity of the calcium independent enzyme was determined from the difference between samples containing 1mM EGTA and samples containing 1 mM EGTA and 1 mM L-NAME. A further series of enzyme activities were measured after incubation of gall bladder tissue in the presence of lipopolysaccharide from *S typhosa* (Sigma) at a concentration of 10 µg/ml.

STATISTICAL ANALYSIS

Results are expressed as mean (SEM). In the in vivo studies, differences between means were tested by analysis of variance for multiple comparisons and by Student's *t* test for unpaired data for single comparisons. In the in vitro experiments, a Student's *t* test for paired data was used for comparing changes before and after incubation with L-NAME.

Results

Basal intraluminal gall bladder pressure was stable and averaged 8 (1) mm Hg under our in vivo experimental conditions. The NO synthase inhibitors L-NAME, L-NMMA, or L-NA significantly increased baseline gall bladder pressure (Fig 1). The effect of these inhibitors was evident within a few minutes and lasted for at least one hour. Administration of L-arginine, a substrate of NO synthase, reversed the effect of the inhibitors; however, the enantiomer D-arginine was ineffective.

Figure 2 shows the effect of inhibition of NO synthase on the response of the gall bladder to CCK-8. Changes in intraluminal gall bladder pressure were dose-dependent and occurred 10 to 20 seconds after CCK-8 was given. Interestingly, pretreatment with L-NAME resulted in a shift to the left of the dose-response curve, as CCK-8 induced higher pressure responses in animals given L-NAME than in saline controls. At every CCK-8 dose tested, differences between animals treated with L-NAME and with saline were significant. The effect of NO synthase inhibition was not only indicated by an increase

Figure 1: Effect of nitric oxide (NO) synthase inhibition on resting intraluminal gall bladder pressure. Gall bladder pressure increased after 10 mg/kg L-NAME, 100 mg/kg L-NMMA, or 10 mg/kg L-NA given intravenously. The effect of L-NA was reversed by L-arginine (L-ARG) (300 mg/kg) but not by D-arginine (D-ARG). Each bar represents the mean (SEM) of five separate experiments (* $p < 0.05$ v baseline). L-NA = N^G -nitro-L-arginine; L-NAME = N^G -nitro-L-arginine methylester; L-NMMA = N^G -monomethyl-L-arginine.

in the maximal response induced by CCK-8 but also by a prolonged duration of the response. Figure 3 shows that the duration of changes in gall bladder pressure induced by CCK-8 increased in relation to the dose of CCK-8 given. The higher the dose, the longer the response to CCK-8. Pretreatment with L-NAME significantly prolonged the response to CCK-8 at all doses studied.

The effect of L-NAME on changes in intraluminal gall bladder pressure induced by CCK was reversed by a simultaneous dose of L-arginine. Figure 4 shows that the effect of L-NAME on the response of the gall bladder to CCK-8 was not found in animals receiving L-arginine. The specific substrate of the NO synthase was able to reverse the effect of the inhibitor.

In a second series of experiments, the cholinergic agonist bethanechol was used as a cholecystokinetic agent. Bethanechol produced a dose dependent increase in intraluminal gall bladder pressure in animals treated with saline (Fig 5). Again, as noted for CCK-8, pretreatment with L-NAME greatly increased the responses of the gall bladder to bethanechol.

Figure 2: Effect of NO synthase inhibition with L-NAME (10 mg/kg) on gall bladder pressure changes induced by CCK-8. Each point is the mean (SEM) of five experiments (* $p < 0.05$ and ** $p < 0.01$ v saline). CCK = cholecystokinin; other abbreviations as for Fig 1.

Figure 3: Effect of NO synthase inhibition with L-NAME (10 mg/kg) on the duration of the gall bladder response to CCK-8. Each point is the mean (SEM) of five experiments (* $p < 0.05$ and ** $p < 0.01$ v saline). Abbreviations as for Figs 1 and 2.

Differences were significant throughout the dose range studied by comparison with the saline group. In these experiments, the duration of changes in intraluminal gall bladder pressure was not modified by the inhibitor.

Figure 6 shows the effect of sodium nitroprusside on gall bladder contraction induced by CCK. Sodium nitroprusside is able to act as a donor of NO. Note that the effect of sodium nitroprusside was the opposite of that found with the NO synthase inhibitors and its administration greatly reduced the response of the gall bladder to CCK-8. Responses to low dose CCK-8 were completely abolished whereas responses to a high dose were reduced by about 80%.

Systemic arterial blood pressure in anaesthetised guinea pigs averaged 40 (2) mm Hg. The NO synthase inhibitors L-NA and L-NAME produced a comparable increase in blood pressure that persisted for at least one hour. When L-arginine was given after the inhibitors, blood pressure returned to resting values within five minutes. By contrast, D-arginine did not reverse the pressor effects induced by the NO synthase inhibitors. Administration of L-arginine alone did not alter the resting blood pressure in untreated animals. Intravenous infusion of phenylephrine produced a sustained increase in

Figure 4: Reversal by L-arginine (300 mg/kg) of the effect induced by L-NAME (10 mg/kg) on the gall bladder pressure response to CCK-8. The dose of CCK-8 used was 0.025 nmol/kg. Each bar represents the mean (SEM) of at least five experiments (* $p < 0.05$ v CCK-8). Abbreviations as for Figs 1 and 2.

Figure 5: Effect of L-NAME (10 mg/kg) on gall bladder pressure response to bethanechol. Each point represents the mean (SEM) of five experiments (**p* < 0.01 *v* saline). Abbreviations as for Fig 1.

blood pressure, but phenylephrine did not affect either basal gall bladder pressure or the response to CCK-8 (Table I).

Table II shows the effect of L-NAME on in vitro gall bladder strip contraction induced either by CCK-8 or bethanechol. Both agents greatly increased the isometric tension generated by the muscle strips. In the presence of L-NAME, responses to CCK-8 or bethanechol were significantly enhanced.

Considerable activity of calcium dependent NO synthase was detected in whole homogenates from guinea pig gall bladder (7.9 (1.5) pmol/h/mg of protein), indicating the presence of constitutive NO synthase in normal gall bladder tissue. Calcium independent activity was not detected under these experimental conditions. After incubation of fresh tissue with 10 μg/ml endotoxin for five hours, however, a high rate of calcium independent activity was found (30.0 (5.7) pmol/h/mg of protein). These results suggest the existence of an isoenzyme that is inducible by endotoxin, as described in other tissue preparations.¹⁴

Discussion

Data presented here indicate that (a) resting gall bladder pressure and contractions induced by both CCK-8 and bethanechol are enhanced by inhibition of the NO synthase (shown by our in vivo and in vitro experiments); (b) these effects are specifically reversed by the NO synthase

Figure 6: Effect of sodium nitroprusside (SNP) (2 mg/kg) on gall bladder pressure response to CCK-8. Each point represents the mean (SEM) of five experiments (**p* < 0.01 *v* saline). Abbreviations as for Fig 2.

TABLE I Lack of correlation between changes in systemic arterial pressure and intraluminal gall bladder pressure

	MAP mm Hg	IGP mm Hg
Basal	40 (2)	8 (1)
Phenylephrine (10 μg/kg: min)	61 (1)*	7 (2)
L-NA (10 mg/kg)	68 (4)*	16 (3)†
CCK-8 (0.025 nmol/kg)	40 (4)	13 (2)†
L-NAME (10 mg/kg) plus CCK-8	78 (2)*	25 (5)‡
Phenylephrine (10 μg/kg: min) plus CCK-8	61 (1)*	14 (2)†

**p* < 0.01 *v* basal and *v* CCK-8; †*p* < 0.05 *v* basal. No difference *v* CCK-8; ‡*p* < 0.01 *v* basal and *p* < 0.05 *v* CCK-8; MAP = mean arterial pressure (mean (SEM) of five experiments); IGP = intraluminal gall bladder pressure; L-NA = N^G-nitro-L-arginine; CCK = cholecystokinin; L-NAME = N^G-nitro-L-arginine methylester.

substrate L-arginine; (c) sodium nitroprusside, an exogenous donor of NO,¹³ counteracts the effect of CCK-8 on gall bladder pressure; and (d) a constitutive calcium dependent NO synthase can be detected in normal gall bladder tissue. Taken together, these findings suggest that local generation of NO participates in the regulation of gall bladder motility in guinea pigs.

Generation of NO was first described in the vascular endothelium in response to acetylcholine or other vasorelaxants.¹² It is well known that the inhibition of NO synthesis with L-arginine analogues induces a sustained vasoconstriction and causes an increase in systemic arterial blood pressure.¹² In our study, changes in intraluminal gall bladder pressure after treatment with the L-arginine analogues were associated with the known vasomotor effects induced by NO synthase inhibition. It is unlikely, however, that vasoconstriction accounted for the results found in our experiments as phenylephrine was able to increase systemic arterial blood pressure to a similar extent without changing intraluminal gall bladder pressure. Also, the experiments performed in vitro clearly show that the effects of inhibition of NO synthase are independent of blood flow.

Changes in intraluminal gall bladder pressure may reflect changes in the intrinsic tone of its musculoelastic wall or in the resistance of the sphincter of Oddi to bile flow.^{15,16} Our findings suggest that synthesis of NO from L-arginine may play an important part in regulating gall bladder tone, as the in vitro experiments showed a significant increment in muscular tension after inhibition of NO synthase.¹⁷ We have also found, by a different method, that NO synthase inhibition increases resistance to bile flow by an action on the intrinsic muscular activity of the sphincter of Oddi. Nevertheless, the experiments presented in our paper point out the role of the L-arginine-NO pathway among the mechanisms that modulate the compliance of the gall bladder wall. The presence of constitutive NO synthase in normal gall bladder tissue together with the

TABLE II Effect of N^G-nitro-L-arginine methylester (L-NAME) on in vitro gall bladder strip contraction†

	Control	L-NAME (27 μg/ml)
CCK-8 (25 ng/ml)	750 (80)	900 (80)*
Bethanechol (2 μg/ml)	620 (170)	930 (170)*

**p* < 0.05 *v* control; †Results are expressed as mg of isometric tension and represent changes from baseline (mean (SEM) of three experiments); CCK = cholecystokinin.

effect of the inhibitors on resting gall bladder pressure suggest that the L-arginine-NO pathway is permanently active for normal gall bladder physiology.

The gall bladder is a reservoir that empties at a slow pace. In humans, for instance, gall bladder emptying elicited by CCK is a slow steady process that generally requires 30–60 minutes to reach its maximum. The most important determinant of gall bladder emptying is contraction of gall bladder smooth muscle, although the resistance to bile flow at the cystic duct and sphincter of Oddi may also modulate the process.^{15,16} As found in our experiments, inhibition of NO synthesis may enhance the contraction of the smooth muscle of the gall bladder and the tonic response to CCK-8 can be prolonged, favouring the delivery of bile. Non-adrenergic non-cholinergic nerves conduct inhibitory signals that modulate gall bladder contraction.¹⁰ As NO has been shown to act as a neurotransmitter of non-adrenergic non-cholinergic signals in the gastrointestinal tract, it is plausible that inhibition of NO synthase results in a blockade of the non-adrenergic non-cholinergic pathways. The absence of the neural modulation of the contraction would enhance the response to CCK-8 and bethanechol as found in our experiments.

Additional support for the role of NO in gall bladder motility is provided by the results from the experiment with sodium nitroprusside. It is known that sodium nitroprusside releases NO through a spontaneous reaction independently of the enzymatic pathway that uses L-arginine as substrate.¹³ We found that sodium nitroprusside given before CCK produced a shift in the pressure dose-response curve of the gall bladder that was opposite to that produced by the NO synthase inhibitors. The reduced responsiveness of gall bladder muscle to CCK by such an exogenous source of NO can probably be explained by the accumulation of cGMP in the gall bladder smooth muscle induced by NO.^{3,6,7}

Finally, our data show that endotoxin is able to induce a calcium independent NO synthase in guinea pig gall bladder tissue. According to our findings, the inducible enzyme may display an activity four to five times higher than that of the constitutive enzyme. The extraordinary generation of NO induced by endotoxin could explain the absence of motor activity that usually occurs in association with gall bladder infection.

In summary, the L-arginine-NO pathway is present in guinea pig bladder tissue. This pathway generates NO and plays a significant part in the regulation of resting gall bladder tone and in gall bladder emptying.

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