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Influence of the selective neuronal NO synthase inhibitor ARL 17477 on nitrergic neurotransmission in porcine stomach

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Abstract

Selective neuronal NOS (nNOS) inhibitors have been developed for possible application in cerebral ischemia and neurodegenerative disorders. To investigate the degree of interference with peripheral nNOS, the influence of the selective nNOS inhibitor ARL 17477 was studied on electrically induced nitrergic relaxations in pig gastric fundus strips and on gastric fundic compliance in conscious pig. Circular muscle strips of porcine gastric fundus were electrically stimulated (10 s trains at 4 Hz, 0.1 ms and 40 V). ARL 17477 inhibited the electrically induced relaxations in a concentration-dependent way (3×10^{-6} M–10^{-4} M). The inhibitory effect of ARL 17477 developed more progressively than that of N^G-nitro-L-arginine methyl ester (L-NAME; 3×10^{-4} M). In conscious pigs, instrumented with a fundic cannula, L-NAME (20 mg/kg i.v.) significantly increased mean arterial blood pressure and decreased fundic compliance in the fasted state (71±13 ml/mm Hg versus 185±37 ml/mm Hg after saline; $P<0.05$). ARL 17477 (3 mg/kg, i.v.) did not influence blood pressure but influenced gastric fundic volume–pressure curves in a similar way as L-NAME. Plasma concentration analysis of ARL 17477 indicated a half-life of less than 30 min in pig. ARL 17477 thus inhibits the effect of nitrergic neurons in the pig gastric fundus in vitro, leading to inhibited gastric compliance in the conscious pig. The study indicates that selective nNOS inhibitors, applied for cerebral disorders, might also interfere with neuronal nitrergic regulation of gastrointestinal motility.

Keywords: Nitrergic neurotransmission; Fundic compliance; Neuronal nitric oxide synthase; ARL 17477; (Pig)

1. Introduction

Nitric oxide (NO) has been established as a non-adrenergic non-cholinergic (NANC) neurotransmitter in the gastrointestinal tract, inducing smooth muscle relaxation (Brookes, 1993). NO is synthetized from L-arginine by a family of isoenzymes, i.e. the constitutive neuronal NO synthase (nNOS) and endothelial NO synthase (eNOS), and the inducible NO synthase (iNOS). Both constitutive enzymes, nNOS and eNOS are present in the gastrointestinal tract under physiological conditions, nNOS being confined to neural elements and eNOS being expressed by endothelial cells (Fischer et al., 1999). In porcine gastric fundus circular and longitudinal smooth muscle strips, we have shown before the presence of nitrergic neuronal cell bodies and fibers; NANC relaxations induced by short trains of electrical field stimulation were largely nitrergic (Lefebvre et al., 1995). The nitrergic neurons in the proximal stomach have been implicated as important mediators of the reflex gastric relaxation to accommodate food (Desai et al., 1991; Tack et al., 2002). By use of an inflatable air bag, inserted in the proximal segment of the stomach via a chronic gastric cannula, and connected to an electronic barostat, we observed recently in conscious pigs that the non-selective NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) decreased fundic compliance during incremental pressure increases in the intragastric bag (Lefebvre et al., 2005).

L-NAME is a non-selective NOS inhibitor. With regard to potential therapeutic applications, great interest for NOS inhibitors selective for one of the NOS isoforms, especially iNOS and nNOS, has developed (Mayer and Andrew, 1998).

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Selective iNOS inhibitors might be useful in the treatment of septic shock and chronic inflammation (Hobbs et al., 1999). nNOS has been implicated in cerebral ischemia-induced cell damage (Huang et al., 1994) and neurodegenerative disorders (Dawson and Dawson, 1996); nNOS-selective inhibitors might be useful in these conditions. ARL 17477 is a selective nNOS inhibitor (Reif et al., 2000; Fedorov et al., 2004) that was shown to have neuroprotective effects in animal models of cerebral ischemia (Zhang et al., 1996; Tseng et al., 1999; O'Neill et al., 2000). One of the possible problems upon therapeutic use of nNOS-selective inhibitors are their peripheral side effects by interference with peripheral nNOS-mediated functions (Moore and Handy, 1997). We therefore compared the influence of ARL 17477 with that of L-NAME in pig stomach, in vitro on electrically induced nitrergic relaxations and in vivo on fundic compliance.

2. Material and methods

2.1. Drugs used

Atropine sulphate, guanethidine sulphate, N^G-nitro-L-arginine methyl ester (L-NAME), prostaglandin $F_{2\alpha}$ and sodium nitroprusside were purchased from Sigma Chemicals (St. Louis, MO, USA) and ARL 17477 (*N*-[4-(2-{[(3-chlorophenyl) methyl]amino}ethyl)phenyl]-2-thiophenecarboximidamide dihydrochloride) was a gift from AstraZeneca (Wilmington, USA). All drugs were dissolved in deionized water for the in vitro experiments. A saturated NO solution was obtained as described by Kelm and Schrader (1990) by bubbling argon gas and then NO gas through 3 consecutive in-line connected gas-tight vials, the first 2 containing KOH solutions, the latter deionized water. For the in vivo experiments, L-NAME (20 mg/kg) and ARL 17477 (3 mg/kg) were dissolved in saline (5 ml in toto).

2.2. In vitro evaluation of the inhibitory effect of ARL 17477

Stomachs were obtained from healthy 6-month-old male castrated pigs, slaughtered at a local abattoir according to Belgium regulations and transported to the laboratory in ice-chilled physiological salt solution. The mucosa was removed from the ventral part of the gastric fundus and 8 muscle strips (15×3 mm) were cut in the direction of the circular muscle layer. Strips were used immediately after preparation and suspended between 2 platinum plate electrodes under a load of 2 g in 10 ml organ baths, containing physiological salt solution, maintained at 37° C and gassed with a mixture of 95% O_2 and 5% CO_2. The physiological salt solution had the following composition (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 124.1, HCO_3^- 25, $H_2PO_4^-$ 1.2 and glucose 11.5. It always contained 10^{-6} M atropine and 4×10^{-6} M guanethidine to inhibit cholinergic and noradrenergic responses. Tension was recorded auxotonically (Grass force displacement transducer FT03 connected in series with a 1 g/cm spring) on a Graphtec Linearcorder FWR3701 or a Kipp & Zonen BD112 recorder. Electrical field stimulation was performed by means of a Grass S44 or S88 stimulator with a constant voltage unit. The tissues were equilibrated for 1.5 h with rinsing every 15 min.

After the equilibration period, tone was raised by administration of 3×10^{-7} M prostaglandin $F_{2\alpha}$. Once a stable contraction was obtained, relaxation was induced by addition of 10^{-5} M sodium nitroprusside. The tissues were then rinsed every 10 min for 1 h. Prostaglandin $F_{2\alpha}$ (3×10^{-7} M) was then administered again and once a stable contraction occurred, 10^{-5} M NO was added inducing a short-lasting relaxation. After 5 min, the tissues were then electrically stimulated 29 times at 5 min intervals with 10 s trains at 4 Hz, 0.1 ms and 40 V. This induced short-lasting relaxations. Immediately after the fifth electrical stimulation, L-NAME (3×10^{-4} M), or ARL 17477 (3×10^{-7} M to 10^{-4} M) was administered in 7 parallel tissues, so that 5 electrically induced responses were obtained before (predrug) and 24 responses were obtained after (postdrug) the addition of drug. In the 8th tissue, nothing was added (time control). Five minutes after the last electrical stimulation, NO (10^{-5} M) was administered again.

2.3. In vivo experiments

2.3.1. Experimental protocol

Experiments were performed according to French regulation on animal experiments under the official agreement A35-622 and 01894.

Three female Large White Pigs (39.8±0.8 kg) were used for fundic compliance recordings. Each animal was studied 6 times and at least 48 h separated consecutive experiments performed on the same animal. Ten hours fasting was always achieved prior the onset of each experimental session. Either saline (5 ml), L-NAME (20 mg/kg) or ARL 17477 (3 mg/kg), both dissolved in 5 ml saline, was administered i.v. 90 min, 30 min and 90 min, respectively, before the beginning of the fundic compliance measurements. Due to large expected intra-animal variability, the experiments were duplicated for each experimental condition.

In sessions with ARL 17477, blood samples were collected in heparinized tubes 15 and 45 min after its administration. Plasma was immediately separated (4000 rpm, 10 min) and stored at −20 °C for later analysis. Plasma concentrations of ARL 17477 were determined by high performance liquid chromatography-mass spectrometry (HPLC-MS) after extraction with ethyl acetate.

At the end of the series of experiments, the animals were killed with an overdose of pentobarbital sodium.

2.3.2. Surgical preparation

The pigs were pre-anesthetized with ketamine (5 mg/kg, i.m.). A surgical level of anesthesia was obtained with halothane (3–5% v/v) delivered by a mechanical ventilator (SAL 900, Siemens). Oxygen fraction (FiO_2) and tidal volume were adjusted so that spO_2 measured by pulse oxymetry (Ohmeda) was 98% or more and $spCO_2$ measured by IR capnometer (Amstrong) was less than 5%. A venous cannula was inserted into the marginal vein of the ear to infuse Ringer-lactate (500 ml/h) during surgery to prevent dehydration.

A T shaped silicon cannula (1 cm ID) was introduced in the fundus 10 cm distal from the lower esophageal sphincter and exteriorized in the left flank. A silicon catheter (ID = 0.76 mm) for the administration of the drugs was introduced into an external jugular vein and an arterial PVC catheter (ID = 1.02 mm) was inserted into the external carotid artery for monitoring mean arterial blood pressure (MABP). Those catheters were kept open by daily injection of heparinized saline (50 UI/ml).

Animals were allowed 1 week to recover from surgery before the start of the experiments.

2.3.3. Fundic compliance measurements

The volume–pressure ratio during fundic distension was obtained from an inflatable air bag inserted in the proximal segment of the stomach and maintained at a constant pressure using an electronic barostat (Synectics) according to the method already described (Samson et al., 1995; Whitehead et al., 1997). Briefly, a 2 l bag was inserted in the proximal segment of the stomach via the gastric cannula while the pig was standing in a sling frame. The bag was connected to the barostat by a double-lumen catheter allowing simultaneously inflation/deflation of the bag and pressure measurement within the bag. Once at minimal abdominal pressure, 2 mm Hg incremental pressure steps were performed until the pressure reached 24 mm Hg. Each of these steps lasted 200 s and the volume for each step was calculated as the median volume for the last 30 s of each step. The compliance was calculated as the larger slope of the pressure–volume curve (Whitehead et al., 1997).

2.3.4. Blood pressure recordings

The systolic and diastolic pressures were continuously measured, using an invasive blood pressure monitor (Kontron Medical 108 monitor), during the 33 min barostat observation. Mean arterial blood pressure was calculated as the sum of two-thirds of diastolic pressure and one-third of systolic pressure every 200 s during the compliance experiment. Mean arterial blood pressure values measured every 200 s during the 33 min compliance measurement were averaged over the 33 min recording period.

2.4. Data analysis

In the in vitro experiments, the mean relaxant response to electrical stimulation trains 3, 4 and 5 before drug administration was calculated. This mean response, as well as the response to the first administration of 10^{-5} M NO, was expressed as percentage of the sodium nitroprusside-induced relaxation at the beginning of the experiment. The responses induced by the 24 electrical stimulations postdrug were expressed as percentage of the mean electrically induced response predrug. The response to 10^{-5} M NO at the end of the experiment was expressed as percentage of the response to NO predrug.

Data were expressed as mean ± standard error of the mean (S.E.M.). Data from duplicate experiments were averaged for the 3 experimental conditions (saline, L-NAME, ARL 17477) in a particular pig. Comparison between treatments was achieved by repeated measures ANOVA and the *t*-test corrected for multiple comparisons (Bonferroni procedure). The significance threshold was 0.05 for all analyses.

3. Results

3.1. In vitro evaluation of the inhibitory effect of ARL 17477

Nitric oxide (10^{-5} M) and electrical field stimulation (EFS; 4 Hz, 0.1 ms, 40 V for 10 s) induced short-lasting relaxations in circular muscle strips of the pig gastric fundus (Fig. 1). In general, the amplitude of the response to NO was somewhat more pronounced than that to EFS, although the opposite also occurred in some tissues. In the series where 10^{-5} M ARL 17477 was administered, e.g., the relaxation by the first addition of NO and the mean response to predrug

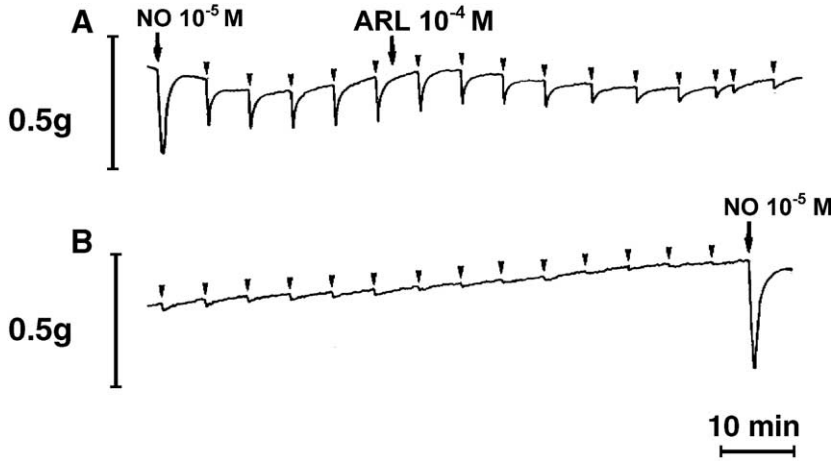

Fig. 1. Actual trace (panel B is the continuation of panel A) showing the relaxant response in a circular smooth muscle strip of the pig gastric fundus, to 10^{-5} M NO and to repetitive electrical field stimulation at 4 Hz, 0.1 ms, 40 V for 10 s before and after addition of 10^{-4} M ARL 17477. The tissue had been contracted by administration of 3×10^{-7} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

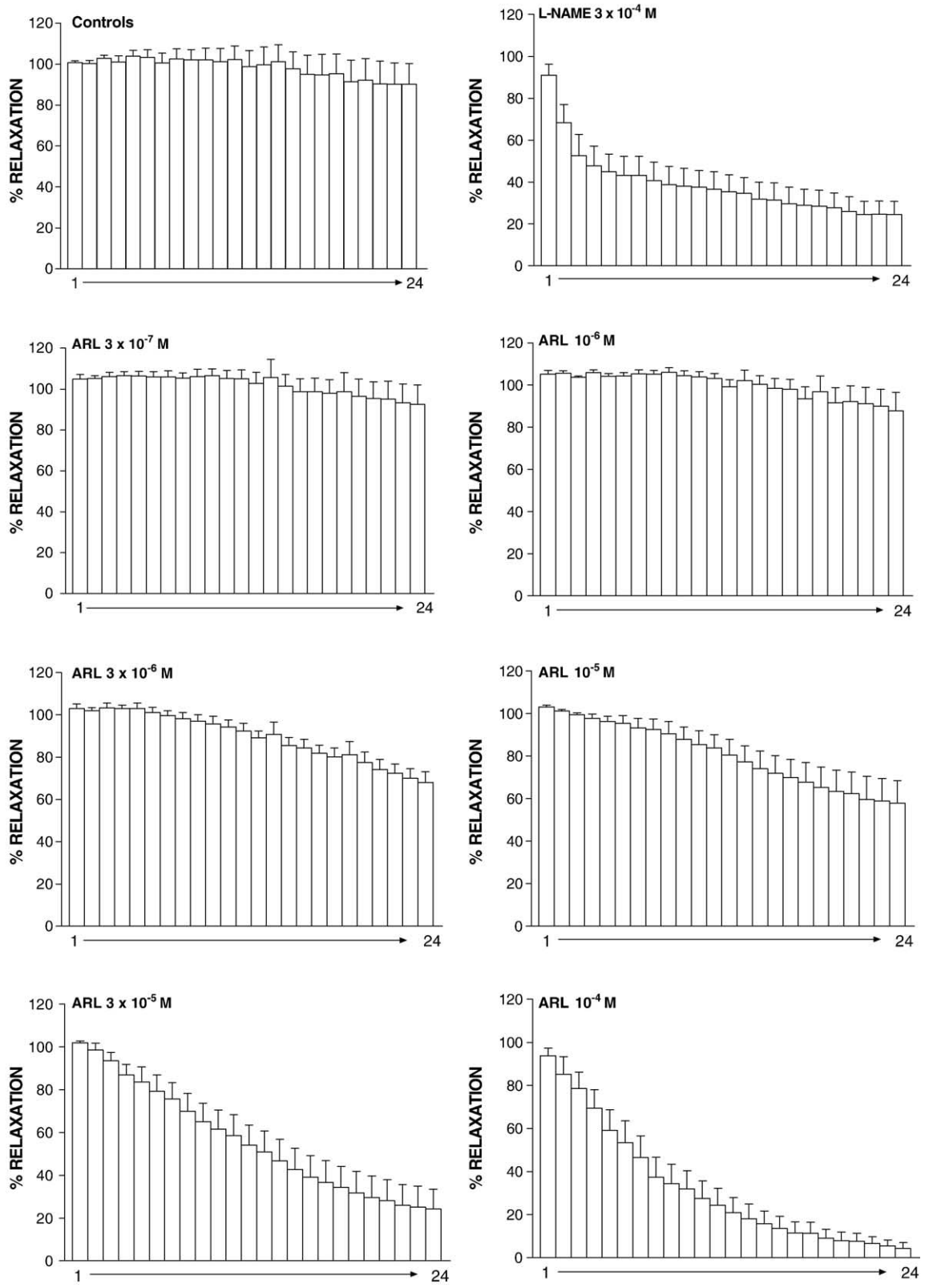

Fig. 2. Circular smooth muscle strips of the pig gastric fundus: mean relaxant responses to 24 electrical field stimulations (4 Hz, 0.1 ms, 40 V, 10 s) obtained in control conditions or after administration of 3×10^{-4} M L-NAME or 3×10^{-7} M–10^{-4} M ARL 17477. The responses were expressed as percentage of the mean response to the 3 electrical stimulations just before drug administration. Mean±S.E.M. from $n=6$–7.

stimulations 3 to 5 attained 58.7±11.3% and 44.4±4.0% (n=7) respectively of that induced by 10^{-5} M sodium nitroprusside, tested in the beginning of the experiment. In the control tissues, the response to EFS remained stable for 12 stimulations and then showed very moderate progressive decline (Fig. 2); the relaxation by the 24th postdrug EFS was 90.3±10.2% (n=7) of the EFS-induced reference predrug response. Also the response to NO was stable; the relaxation by the second administration of NO attained 94.8±12.3% (n=7) of the first response to NO.

As described previously (Dick and Lefebvre, 1997), 3×10^{-4} M L-NAME reduced the electrically induced relaxations (Fig. 2). The inhibitory effect was manifested quickly after its administration and tended to stabilize at the 7th EFS postdrug, although a very moderate progressive increase of the inhibitory effect could be observed till the end of the experiment. At low concentration, ARL 17477 (3×10^{-7} M and 10^{-6} M) had no influence on the EFS-induced relaxations as they were comparable to those in control tissues. From 3×10^{-6} M onwards, a concentration-dependent inhibitory effect of ARL 17477 was observed. Unlike L-NAME, the inhibitory effect of ARL 17477 did not stabilize after 6 stimulations but progressively increased with the increasing number of stimulations (Fig. 2). Neither L-NAME nor ARL 17477 influenced the NO-induced relaxations. Expressed as % of the first predrug response to NO, the relaxation to the second administration of NO was 83.6±9.4%, 90.4±6.4%, 99.0±9.5%, 119.1±8.2%, 117.2±9.1%, 110.0±8.7% and 86.2±8.4% in the presence of 3×10^{-4} M L-NAME and of the increasing concentrations of ARL 17477 (3×10^{-7} M to 10^{-4} M) respectively (n=6–7 per group).

3.2. In vivo experiments

3.2.1. Plasma concentrations of ARL 17477

The plasma concentrations at the 2 sessions with ARL 17477 in a particular pig were averaged to obtain a single value at 15 and 45 min after administration. The mean plasma concentrations of ARL 17477 at 15 and 45 min postdrug were 114.7±15.2 and 47.0±9.2 ng/ml (n=3).

3.2.2. Effect of the NOS inhibitors on arterial blood pressure

Mean arterial blood pressure (in mm Hg) over the 33 min recording period was significantly increased after administration of L-NAME: 86.0±3.6 after saline (n=3), 103.6±1.5 after L-NAME (n=3; P<0.01 versus saline, and versus ARL 17477) and 85.9±2.1 after ARL 17477 (n=3).

3.2.3. Effect of the NOS inhibitors on fundic compliance

After administration of L-NAME and ARL 17477, for equal pressure, intrabag volumes were lower than after administration of saline, reaching significance for L-NAME from 16 to 22 mm Hg and for ARL 17477 at 16 mm Hg (Fig. 3). As a consequence, compliance of the proximal stomach was significantly decreased after L-NAME (71±13 ml/mm Hg, n=3; P<0.05 versus saline, 185±37 ml/mm Hg, n=3). The compliance after ARL 17477 was similar to that obtained after L-NAME (73±17 ml/mm Hg, n=3) but was not significantly different from that in the saline session.

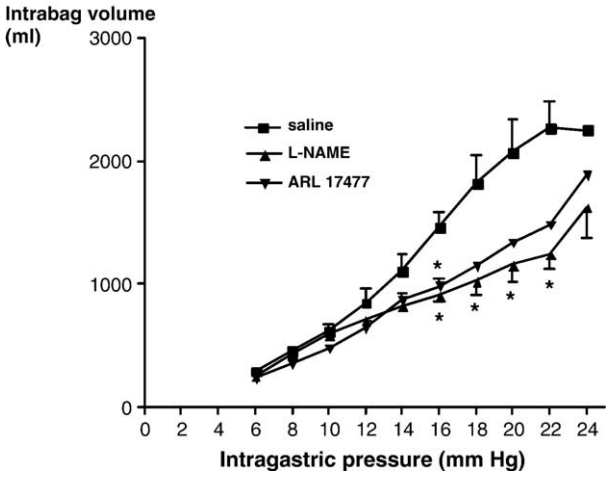

Fig. 3. Evolution of the relationship between the volume of the polyethylene bag and the intragastric pressure during stepwise inflation. Data correspond to mean±S.E.M. from n=3. *P<0.05, significantly different from saline.

4. Discussion

The present study shows that the nNOS-selective inhibitor ARL 17477 is able to interfere with gastrointestinal nitrergic effects. ARL 17477 was reported to be at least 100-fold selective for nNOS versus eNOS and iNOS; the IC_{50} values as assessed from inhibition of the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline were indeed 0.035, 3.5 and 5×10^{-6} M for nNOS from rat cerebellum, eNOS from human umbilical vein endothelial cells and iNOS from a murine macrophage cell line respectively (Reif et al., 2000). IC_{50} values determined with recombinant human nNOS and eNOS cell lines were 1 and 17×10^{-6} M respectively (O'Neill et al., 2000); this illustrates that IC_{50} values depend upon assay conditions but confirms a more than 10-fold selectivity of ARL 17477 for nNOS versus eNOS. A spectroscopic competition assay determining binding constants for ARL 17477 at the different NOS isoforms indicated that ARL 17477 binds most tightly to nNOS, and about 1 and 2 orders of magnitude more weakly to iNOS and eNOS respectively (Fedorov et al., 2004). In circular muscle strips of the pig gastric fundus, ARL 17477 had a concentration-dependent inhibitory effect on electrically induced NANC relaxations from 3×10^{-6} M onwards, reaching an inhibitory effect comparable to that of 3×10^{-4} M L-NAME at 3×10^{-5} M. We have previously shown by immunohistochemistry the presence of nitrergic neurons in the pig gastric fundus; functionally, the electrically induced NANC relaxations in circular muscle strips were fully neurogenic as they were abolished by tetrodotoxin, and largely nitrergic (Lefebvre et al., 1995). The reduction of the NANC relaxations by ARL 17477 thus illustrates that it interferes with nitrergic neurotransmission in the pig gastric fundus. The concentrations of ARL 17477 required to inhibit NANC relaxations in the pig gastric fundus are similar to those required to inhibit human recombinant eNOS in the above cited study (O'Neill et al., 2000). However,

concentrations obtained in an in vitro assay with isolated recombinant NOS cannot automatically be extrapolated to organ bath experiments with functional smooth muscle strips. Indeed, in the same study with recombinant NOS (O'Neill et al., 2000), the IC_{50} of the non-selective NOS inhibitor L-NAME versus nNOS and eNOS was 0.8 and 1.8×10^{-5} M respectively, while we showed previously that 1×10^{-4} M L-NAME is required to inhibit electrically induced NANC relaxations in the pig gastric fundus by 50% (Dick and Lefebvre, 1997). When taking this factor in account for ARL 17477, the concentrations needed to inhibit NANC relaxations in the pig gastric fundus overlap with those required to inhibit human recombinant nNOS in the study of O'Neill et al. (2000).

A dose of 3 mg/kg ARL 17477 was selected for use in the in vivo part of our study as this dose in the rat significantly reduced cortical NOS activity without influencing cerebral blood flow, while a dose of 10 mg/kg i.v. reduced the latter suggesting that inhibition of eNOS started to develop (Zhang et al., 1996). Our in vitro experiments showed that the time course for ARL 17477-induced inhibition is very slow compared to the effect of L-NAME that stabilized after 30 min. This was the rationale for administering ARL 17477 90 min before the start of the barostat test instead of 30 min for L-NAME. ARL 17477 had a similar effect to that of L-NAME on the gastric fundus volume–pressure curves yielding smaller volumes for a given pressure. The value for gastric compliance after administration of ARL 17477 (73±17 ml/mm Hg) was very close to that in the session with L-NAME (71±13 ml/mm Hg), although it did not reach a significant difference from the value in the saline session (185±37 ml/mm Hg); the latter might be due to the limited number of experiments in this in vivo series. Also the pharmacokinetic characteristics of ARL 17477 in the pig might have played a role. With its MW of 442.84, the plasma concentrations at 15 and 45 min after administration correspond to 2.59×10^{-7} M and 1.06×10^{-7} M. These concentrations are smaller than those required to inhibit peripheral nNOS in the in vitro study, although we should realize that plasma concentrations are only indicative of tissue concentrations. In a pharmacokinetic study in rats, brain concentrations of ARL 17477 were more than 10-fold higher than plasma concentrations after i.v. bolus injection (O'Neill et al., 2000). Still, the plasma concentrations in the pig were more than halved within 30 min, suggesting that ARL 17477 has a very short half-life in the pig. Assuming a half-life of 30 min, the quantity injected i.v. in the body will have decreased to 1/8 at 90 min after administration and 1/16 at 120 min when the compliance experiment comes to its end. This might contribute to the observation that the effect of ARL 17477 tended to be somewhat less pronounced than that of L-NAME, but it is clear that even in the experimental conditions used ARL 17477 is able to interfere with gastric compliance. The interference with the relaxant capacity of the stomach might disturb gastric accommodation upon food intake and alter gastric emptying. As impaired gastric accommodation has indeed been proposed as a pathophysiological mechanism in a subgroup of functional dyspepsia patients (Tack et al., 1998), this might lead to dyspeptic symptoms.

It has been reported that 10 mg/kg L-NAME i.v. did not change mean arterial blood pressure in the anesthetized pig (Van Gelderen et al., 1993) but the dose of 20 mg/kg i.v. in this study clearly increased mean arterial blood pressure in the conscious pig; this is related to inhibition of vascular eNOS. ARL 17477 (3 mg/kg i.v.) did not influence arterial blood pressure in the conscious pig in this study, corresponding to reports in the rat (1, 3, 10 mg/kg i.v. in bolus; Zhang et al., 1996) and in the dog (1.5 mg/kg i.v. infusion for 30 min; Tseng et al., 1999). However, in another study in the rat, 3 mg/kg i.v. of ARL 17477 significantly increased arterial blood pressure (Harukuni et al., 1999), and very recently, ARL 17477 (5 mg/kg i.v.) was shown to increase arterial blood pressure in anesthetized pigs (Zhang et al., 2005). This does not necessarily indicate non-selective inhibition of eNOS as administration of antisense oligodeoxynucleotides to nNOS into the rat nucleus tractus solitarii increased blood pressure (Maeda et al., 1999) suggesting a possible role for central nNOS in the control of blood pressure. In line with these results, microinjection of ARL 17477 into the rat nucleus tractus solitarii increased arterial blood pressure and suppressed baroreflex responses, suggesting that nNOS has a role in transmission of baroreflex signals through the nucleus tractus solitarii (Talman and Dragon, 2004).

In conclusion, the nNOS-selective inhibitor ARL 17477 is able to inhibit the effects of the nitrergic neurons in the pig gastric fundus in vitro, which translates to inhibition of gastric compliance in the conscious pig. When nNOS-selective inhibitors would be applied for cerebral disorders, it can thus be expected that they will also interfere with neuronal nitrergic regulation of gastrointestinal motility.

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