# Excitatory role of nitric oxide on 2-deoxy-D-glucose-induced gastric motility in rats

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**ABSTRACT**

Previous studies have shown that 2-deoxy-D-glucose (2-DG) increases gastric motility via the vagus nerve, but the underlying mechanism remains elusive. Since nitric oxide (NO) is involved in gastric motility, a possible interplay between 2-DG and NO can be suggested. In the present study, Wistar rats (250–350 g) of both sexes were intravenously injected with 2-DG (200 $mg \cdot kg^{-1}$), and the effects of the intravenous injection of the nitric oxide synthase (NOS) inhibitors, nitro-L-arginine methyl ester (L-NAME, 10 $mg \cdot kg^{-1}$) and $N^{\omega}$-nitro-L-arginine (L-NNA, 10 $mg \cdot kg^{-1}$), were investigated. Animals were anaesthetized and cannulated for intravenous drug injections while the left vagal nerve was electrically stimulated (0.1–10 Hz, 0.5 ms duration, 12 V, for 60 s), and intragastric pressure and motility changes were monitored using a latex gastric balloon. 2-DG increased the mean intragastric pressure (baseline: 5.2 ± 0.3 cm $H_2O$; after 2-DG: 14.4 ± 1.5 cm $H_2O$; $P$ = 0.0156) and significantly increased the motility index, while NOS inhibitors significantly attenuated the motility index (2-DG: 3634.6 ± 1135.8; L-NAME: 1326 ± 490.8, $P$ = 0.0156). However, pretreatment with NOS inhibitors significantly augmented the gastric responses to peripheral electrical vagal stimulation. These results suggest that NO plays an excitatory role in the gastric responsiveness to 2-DG, which may take place in the central nervous system.

**Key words:** Nitric oxide, 2-deoxy-D-glucose, gastric motility, intragastric pressure, motility index, L-NAME, L-NNA

## 1. Introduction

Gastric motility is controlled by the enteric system and the central nervous system, in which nitric oxide (NO) serves as a neuromodulator that acts as an intercellular messenger (Garthwaite, Charles, & Chess-Williams, 1988; Hashitani, Garcia-Londoño, Hirst, & Edwards, 2005; Moncada & Higgs, 1995).

The primary components involved in the central control of gastric motility are the nucleus of the solitary track (NTS), which is the sensory centre, and the dorsal motor nucleus (DMN) of the brain stem, which is the primary motor output centre along with the nucleus ambiguous (NA). These three nuclei also make several other connections with different neural areas that may have an effect on gastric motility.

Immunohistochemical studies have shown the presence of nitric oxide synthase (NOS) in the DMN, vagal afferents, and intrinsic neurons of the NTS (Lin, Cassell, Sandra, & Talman, 1998; Zheng, Rogers, & Travagli, 1999). NOS serves as a smooth muscle relaxant for the enteric nervous system, and NOS inhibitors have been shown to improve the basal contraction of gastric smooth muscle strips *in vitro* (Meile, Glatzle, Habermann, Kreis, & Zittel, 2006). NO, released from nonadrenergic-noncholinergic nerve fibres contained in the vagal nerve, mediates relaxation of the gastrointestinal smooth muscle (Boeckxstaens et al., 1991; Desai, Sessa, & Vane, 1991; Lefebvre, Hasrat, & Gobert, 1992).

2-Deoxy-L-glucose (2-DG) is an anti-metabolic glucose analogue that is transported across the blood–brain barrier into the brain where it is phosphorylated to form 2-deoxy-D-glucose-6-phosphate (2DG6P), but is not metabolized further down in the glycolytic pathway. 2DG6P then inhibits glucose-6-phosphate isomerase, thereby blocking glycolysis and the oxidative metabolism of glucose (Horton, Meldrum, & Bachelard, 1973). This results in intracellular glucoprivation while simultaneously

increasing the blood glucose level (Elman, Sokoloff, M. Adler, Weisenfeld, & Breier, 1999). In addition to its hyperglycaemic effects, 2-DG profoundly decreases body temperature, which is significantly correlated with changes in hypothalamic blood flow (Elman et al., 1999; Hervias, Lasheras, & Aguirre, 2000). 2-DG also stimulates gastric acid secretion and gastric motility via central mechanisms (Colin-Jones & Himsworth, 1970; Muramatsu, Chaki, Arai, & Aihara, 1990), which can be blocked by atropine or by vagotomy (Colin-Jones & Himsworth, 1970; Tsukamoto et al., 1967), suggesting a pivotal role of cholinergic mechanisms. Interestingly, high doses of 2-DG repress gastric motility, and Cato et al. (Cato, Flanagan, Verbalis, & Stricker, 1990) proposed that this effect could be mediated by oxytocin through hypothalamic-vagal projections. Since both NO and 2-DG have profound effects on the gastrointestinal system, it is highly likely that a significant interaction between them exists. Thus, we aimed to investigate this possibility using NOS inhibitors, $N^{\omega}$-nitro-L-arginine (L-NNA) and $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME), in anaesthetized rats that were surgically prepared for monitoring their gastric motility via intragastric balloon.

## 2. Materials and methods

### *a. Ethical approval*

This project was approved by the Institutional Experimental Animal Care and Use Ethics Committee of Hacettepe University (Approval Number: 2006/6-7) before the commencement of any intervention. Therefore, the Guiding Principles for the Care and Use of Laboratory Animals along with the related Turkish by-laws were strictly adhered to during the execution of all the procedures described within this manuscript.

### *b. Animals*

Wistar albino rats (250–300 g) were obtained from the Experimental Animals Breeding Unit of Refik Saydam Hygiene Centre of The Turkish Republic Ministry of Health in Ankara, and housed in the Laboratory Animal Husbandry Facility of the Department of Pharmacology, Faculty of Medicine, Hacettepe University until beginning the experiments. All rats were acclimatized for two weeks prior to the experiments and kept under environmentally controlled conditions at 21 ± 2 °C and 30–70% relative humidity with a 12-h dark/12-h light illumination sequence (the lights were on between 07.00 and 19.00 h) with *ad libitum* access to tap water (drinking bottle) and standard pellet dairy chow (Dokuz Tug Yem Sanayii, Ankara, Turkey).

***c. General procedures***

Animals were fasted for 14–18 h before the experiments and terminally anaesthetized with intraperitoneal injection of urethane (1.5 $g \cdot kg^{-1}$) as described in several previous gastric motility studies (Andrews & Scratcherd, 1980; Piché, Watanabe, & Hotta, 2014; E Quintana et al., 2001). The obtundation of the responses to painful stimuli was established by pinching the toe with surgical tweezers. Once the desired level of anaesthesia was reached, the trachea was cannulated to allow adequate ventilation, and the right external jugular vein was cannulated for the administration of drugs. The vagal nerve on both sides was exposed at the cervical region and isolated accordingly. During the experiments, the body temperatures were kept at 37.0 ± 0.1 °C using a rectal thermistor probe-controlled incandescent lamp (100 W) placed approximately 30 cm above the animals. All drugs were prepared daily, dissolved in non-pyrogenic sterile saline (0.9% NaCl), and warmed to body temperature (approximately 37 °C) before the injections.

***d. Surgical procedures***

Although gastric motility can be recorded using extraluminal strain gauges, these recordings will represent only a portion of the activity of the gastric walls (Krowicki, Sharkey, Serron, Nathan, & Hornby, 1997). Therefore, we used intragastric pressure recording for the determination of gastric motility. The procedures were carried out as previously described (E Quintana et al., 2001). In brief, after performing a laparotomy, an intraluminal latex balloon was inserted in the stomach through an incision in the fore-fundus at 2 mm above the fundus–corpus junction and held in place with a ligature. The balloon and catheter system were connected to a pressure transducer, and the intragastric pressure was registered online by a multi-channel recorder (MP35 Biopac Data Recording System, CA, USA). After reaching an intragastric pressure of 4.5–5.5 cm $H_2O$ by filling the balloon with 1.5–2 ml non-pyrogenic sterile saline (0.9% NaCl), the animals were allowed to stabilize for 30 min.

### *e. Experimental procedures*

Validation studies were performed before initiating the experiments to optimize the experimental conditions. Firstly, the duration of the effect of 2-DG on the gastric motility was assessed by injecting pure 2-DG in two rats. In addition, solvent (0.9% NaCl control infusions were performed to eliminate the effect of the solvent from the effect of the three target solutions (2-DG, L-NAME, L-NNA).

In the first group of experiments, 2-DG (n = 14, 200 $mg \cdot kg^{-1}$) was administered with a bolus intravenous (iv) injection, and intragastric pressure was monitored for 45 min. To analyse the role of NO, the rats were allocated into two groups and they were administered 10 $mg \cdot kg^{-1}$ (iv) of either L-NAME (n = 7) or L-NNA (n = 7) before the intragastric pressure was monitored for an additional 15 min. All experimental data are expressed as the motility index, which was calculated as the area under the curve for

a 10-min period once the responses stabilized, and the mean intragastric pressure at the same period was chosen to register the motility index.

To investigate whether the interaction between 2-DG and NO takes place at the periphery or in the central nervous system, a second group of experiments was performed using bilateral vagotomized animals. After cutting the left vagal nerve, the peripheral end was placed over bipolar silver electrodes and stimulated for 1 min at 12 V with a 0.5-ms duration at different frequencies (0.1–10 Hz). These animals were also allocated into two groups to receive L-NAME (n = 7) or L-NNA (n = 6) at a dose of 10 $mg \cdot kg^{-1}$ (iv). After obtaining the control responses and allowing for a 15-min equilibration period, the vagal stimulation was repeated using the same parameters. All data are expressed as the mean intragastric pressure during the stimulation periods, and intragastric pressure changes were calculated as the difference from the basal levels of responses.

At the end of the experiments, the animals were exsanguinated by cutting the carotid artery.

### *f. Statistical analyses*

All values are reported as the arithmetic mean ± standard error of the mean (SEM) of replicate experiments, and the Wilcoxon signed rank test or two-way analysis of variance for repeated measures was used as appropriate to analyse the data. Because of the high variability of the data, sufficient power to detect statistical significance would require a large number of animals in each group. As an alternative, the signed rank test was used to keep the animal number as low as possible. Differences were considered statistically significant when $P < 0.05$.

### *g. Drugs and reagents*

2-DG, L-NNA, and L-NAME were obtained from Sigma, (ABD), and urethane was obtained from Aarhus (Denmark). All drugs were dissolved in non-pyrogenic sterile saline (0.9% NaCl w/v in distilled water).

## 3. Results

During the validation experiments, the effect of 2-DG was sustained for over 1 h. This finding is aligned with the literature (Colin-Jones &Himsworth, 1970; E Quintana et al., 2001) and the duration was sufficiently long for the procedures planned for this study, since the recording time was limited to 1 h. Experiments conducted to examine the solvent's effect revealed no significant variations.

### *a. Effects of NOS blockers on gastric motility induced by 2-DG*

After the stabilization period, urethane-anaesthetized rats exhibited a mean intragastric pressure of 5.0 ± 0.4 cm $H_2O$ (n = 27), which was significantly increased following 2-DG (200 mg $kg^{-1}$ iv, n = 14) administration to 14.4 ± 1.5 cm $H_2O$, $P < 0.05$) (Figures 1–2).

2-DG also significantly augmented both the frequency and the amplitude of the gastric contractions and thus increased the gastric motility index from the basal level of 218.8 ± 64.0 cm $H_2O$ to 2783.4 ± 767.4 cm $H_2O$ (n = 14; $P < 0.05$) (Figure 3). This effect was completely abolished by the vagotomy or by atropine administration (1 mg·$kg^{-1}$, iv) (data not shown).

L-NAME decreased the 2-DG-induced mean intragastric pressure from 12.8 ± 1 cm $H_2O$ to 9.7 ± 1.2 cm $H_2O$ (n = 7, $P < 0.05$) and the gastric motility index from 3634.6 ± 1135.8 cm $H_2O$ to 1326 ± 490.8 cm $H_2O$ (n = 7, $P < 0.05$). Similarly, L-NNA decreased

the 2-DG-induced mean intragastric pressure from 16 ± 2.7 cm $H_2O$)to 9.5 ± 1.5 cm $H_2O$ (n = 7, $P < 0.05$) and the gastric motility index from 1932 ± 742.8 cm $H_2O$ to 683.6 ± 144.4 cm $H_2O$ ((n = 7, $P < 0.05$). In summary, the inhibition of NOS either by L-NAME or by L-NNA caused a significant decrease in the 2-DG-induced augmentation of the frequency and amplitude of gastric contractions, which resulted in a significant decrease of the intragastric pressure and gastric motility index.

***b. Effects of NOS blockers on gastric motility induced by electrical vagal stimulation.***

Figure 4 shows the gastric responses to electrical stimulation (12 V, 0.5 ms, 1 min) before and after the administration of NOS inhibitors. The electrical stimulation of the vagal nerve increased the mean intragastric pressure in a frequency-dependent manner (n = 13, stabilization period 5 ± 0.1 cm $H_2O$, 1 Hz 6 ± 0.2 cm $H_2O$, 10 Hz 7.6 ± 0.4 cm $H_2O$, $P < 0.0001$). This effect was also represented as changes in the intragastric pressure (Figures 5 and 6).

L-NAME increased the mean intragastric pressure response to 1 Hz, 3 Hz, and 10 Hz stimulation from 6.1 ± 0.2 cm $H_2O$ to 7.7 ± 0.5 cm $H_2O$, 7.2 ± 0.4 cm $H_2O$ to 10.4 ± 0.6 cm $H_2O$, and 7.7 ± 0.5 cm $H_2O$ to 14.8 ± 1 cm $H_2O$, respectively (n = 7, $P < 0.0001$). L-NAME also increased the intragastric pressure changes (i.e., 3 Hz: 4.8 ± 0.9 cm $H_2O$ to 9.3 ± 1.3 cm $H_2O$, n = 7, $P < 0.05$).

Similarly, L-NNA increased the mean intragastric pressure response to 1 Hz, 3 Hz, and 10 Hz stimulation from 5.2 ± 0.8 cm $H_2O$ to 5.1± 1.1 cm $H_2O$, 6.2 ± 0.6 cm $H_2O$ to 8.9 ± 1.3 cm $H_2O$, and 7.9 ± 0.8 cm $H_2O$ to 14.5 ± 1.3 cm $H_2O$, respectively (n = 6, $P < 0.05$). L-NNA also increased the change in intragastric pressure (i.e., 3 Hz: 4.8 ± 0.8 cm $H_2O$ to 8.4 ± 1.3 cm $H_2O$, n = 6, $P < 0.05$).

Thus, in contrast to the 2-DG experiments, NOS inhibition augmented the responsiveness to electrical stimulation, which was also frequency-dependent.

## 4. Discussion

Although their separate profound effects on the gastrointestinal system have been well established, a possible interaction between 2-DG and NO on gastric motility has not been investigated thoroughly. Using NOS inhibitors, the present study confirmed a significant interplay between these two factors. Overall, our results confirm previous findings that 2-DG increases the intragastric pressure and gastric motility, which requires an intact vagal pathway (Andrews & Scratcherd, 1980; Cato et al., 1990; E Quintana et al., 2001; Tsukamoto et al., 1967). Moreover, This study further expands our understanding of these effects, by demonstrating the existence of an interaction between 2-DG and NO, which presumably occurs in the central nervous system.

### *Interplay between NO and 2-DG*

In the first group of experiments, we showed a profound stimulatory property of NO in modulating the responsiveness to 2-DG; this finding is in contrast to the existing undisputed faith in the direct inhibitory properties of NO on gastric motility. Since this phenomenon was blocked by L-NAME, which is a non-specific NOS inhibitor with additional anti-muscarinic properties (Buxton et al., 1993), it is possible that inhibition of the 2-DG-induced motility by L-NAME is due to anti-muscarinic blockade. However, our results with L-NNA, another non-specific inhibitor of NOS lacking in a muscarinic blockade effect, confirmed that a profound and significant stimulatory interaction between NO and 2-DG does exist, which is specific to the inhibition of NOS.

Interestingly, our results can be taken as expository evidence to explain the unexpected findings of a previous report (Meile et al., 2006), which showed that the inhibition of NOS by L-NMMA decreased gastric motor activity. Although their study focused on the effects of NOS inhibition, neither their nor our findings can be explained by a direct effect of NO on gastric smooth muscle cells (Boeckxstaens et al., 1991; Desai et al., 1991; Lefebvre et al., 1992), and therefore the possibility of an indirect neurogenic effect should also be considered.

The interplay between NO and 2-DG was investigated rather indirectly using control groups in a previous report that focused on the effects of endotoxin on gastric motility, and found no significant interaction (E Quintana et al., 2001). The discrepancy between our results from these previous findings can be attributed to the utilization of paired statistical tests in the former but an unpaired t-test in the latter for the analysis of gastric motility data, which by its nature exhibits a rather wide variation. By contrast, Hasebe and colleagues (Hasebe, Horie, Noji, Watanabe, & Yano, 2005) demonstrated that L-NNA decreased 2-DG-induced gastric acid secretion in a dose-dependent manner, which can be taken as a supportive evidence for our present findings.

***Where does the interaction take place?***

Our findings suggested that the interaction between NOS and 2-DG might take place in the central nervous system, because the NOS inhibitors L-NAME and L-NNA blocked 2-DG-induced gastric motility but augmented the responsiveness to the peripheral electrical stimulation of the vagus nerve in a frequency-dependent manner, which is in line with previous studies (Hryhorenko, Woskowska, & Fox-Threlkeld, 1994; Takahashi & Owyang, 1995). Since NOS inhibition blocks only the 2-DG-induced gastric motility, there might be a gastric motility control pathway in the central nervous system in which

NO acts as an excitatory neurotransmitter/neuromodulator. NO is generally considered as an inhibitory neuromodulator, although some studies have shown that NO may actually have excitatory properties (Travagli & Gillis, 1994; Wang, Paton, & Kasparov, 2007). Nevertheless, from our perspective, NO also appears to form part of an inhibition circuit.

Since the NTS, DMN, and NA regulate gastric motor activity, it appears feasible to suggest that the NO–2-DG interaction takes place in this pathway.

A recent study showed that stimulation of different parts of the DMN results in a decrease or an increase in gastric motor activities, but these results were NO-independent (Cruz, Murphy, Sahibzada, Verbalis, & Gillis, 2007). By contrast, Barrachina and her group (Elsa Quintana, Hernández, Moran, Esplugues, & Barrachina, 2005) reported that lipopolysaccharide injection to the dorsal vagal nucleus increased nNOS transcription and decreased the 2-DG-induced intragastric pressure changes. Moreover, Hornby and her group (Krowicki et al., 1997) showed that while L-NAME increased, L-arginine microinjection into the DMN significantly decreased the intragastric pressure. By taking our findings and these previous reports into consideration, we can speculate that the DMN is likely not the site of the NO–2-DG interaction to affect gastric motility. However, Travagli and Gillis (Travagli & Gillis, 1994) reported that L-arginine significantly increased the firing rate of the dorsal motor complex *in vitro*. Therefore, we cannot completely exclude the possibility that the DMN might be the site of this interaction.

Another recent study showed that NO has inhibitory effects on the NA (Sun, Zhao, & Ai, 2012). However, to the best of our knowledge, there has been no study directly investigating the effect of 2-DG on the NA.

Kasparov and his group showed that NO within the NST could produce excitatory post-synaptic potentials, while higher concentrations were required to directly engage GABAergic inhibition (Wang et al., 2007). This report could be the key to understanding the discrepancy between our results and those of Cato et al. (Cato et al., 1990), who showed that 500 $mg \cdot kg^{-1}$ 2-DG decreased the gastric motility, while lower doses caused an increase in motility.

Other than the DMN and NA, the nucleus tractus solitarii is the last primary site to investigate a possible NO–2-DG interaction. In fact, a very recent study showed that 2-DG inhibits NST and activates DMN neurons, and these effects were dependent on intact astrocytes. Therefore, the authors suggested that ATP might be responsible for the observed inhibitory interactions (Hermann, Viard, & Rogers, 2014). Given that we established a similar interaction between NO and 2-DG in the present study, we can now suggest that the inhibitory mediator secreted between the NST and DMN could actually be NO.

Although our study provides clues into the central neuroexcitatory effects of NO, it might only form a part of a larger inhibitory circuit that regulates gastric motility. The present study has shown that there is a profound stimulatory interaction between NO and 2-DG on gastric motor activity, which possibly takes place in the central nervous system. This hypothesis could be more strongly supported in future studies using central injections. Therefore, additional studies, especially those designed to investigate the interaction between NO and 2-DG, are needed to validate this phenomenon and to determine the precise site of this interaction.

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*Author contributions:* AM Sevgili: designed the research study, performed the research, analysed the data, and wrote the paper. ZD Balkanci: designed the research study, contributed essential reagents and tools, and edited the manuscript. A Erdem: contributed essential reagents and tools and edited the manuscript.

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## Figures

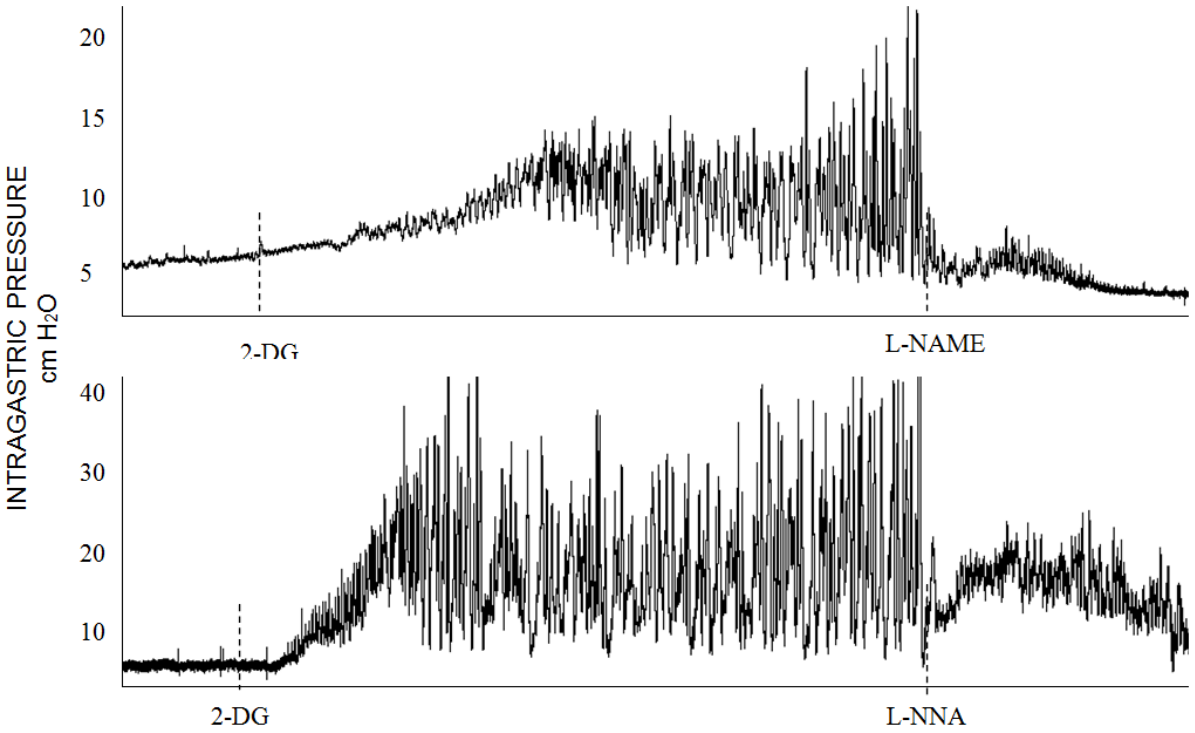
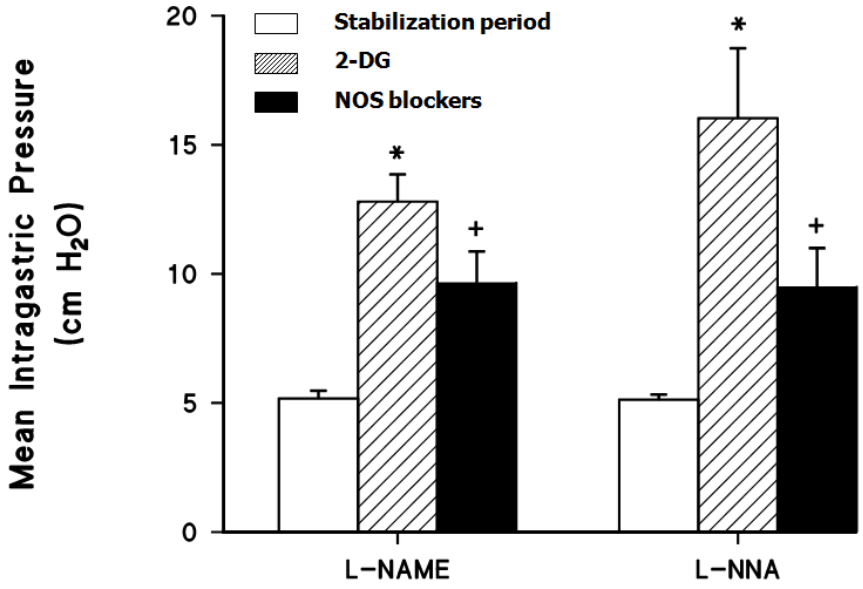



**Figure 1**

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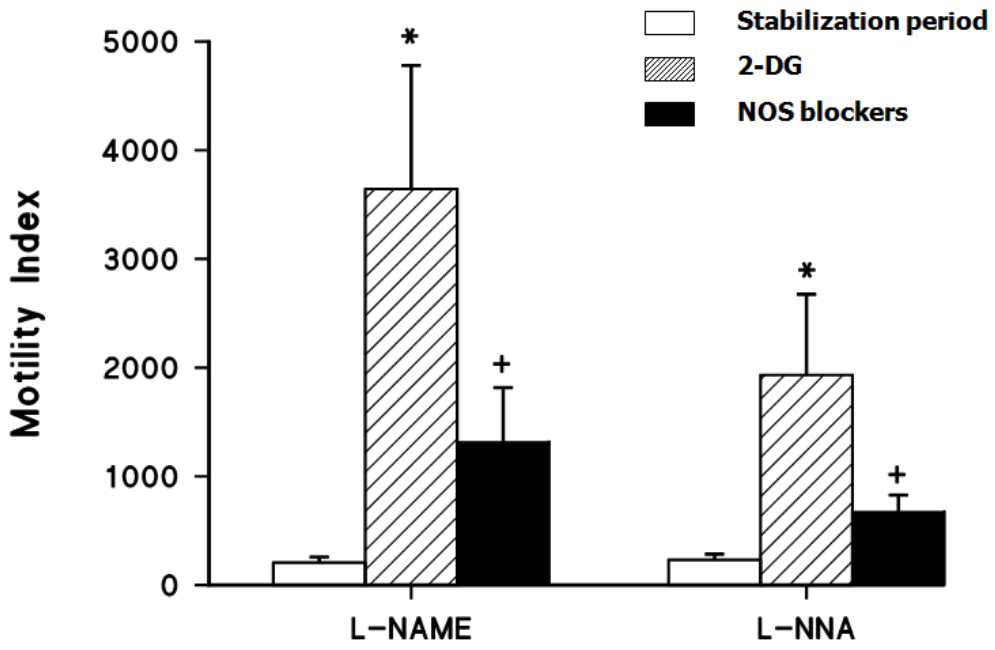


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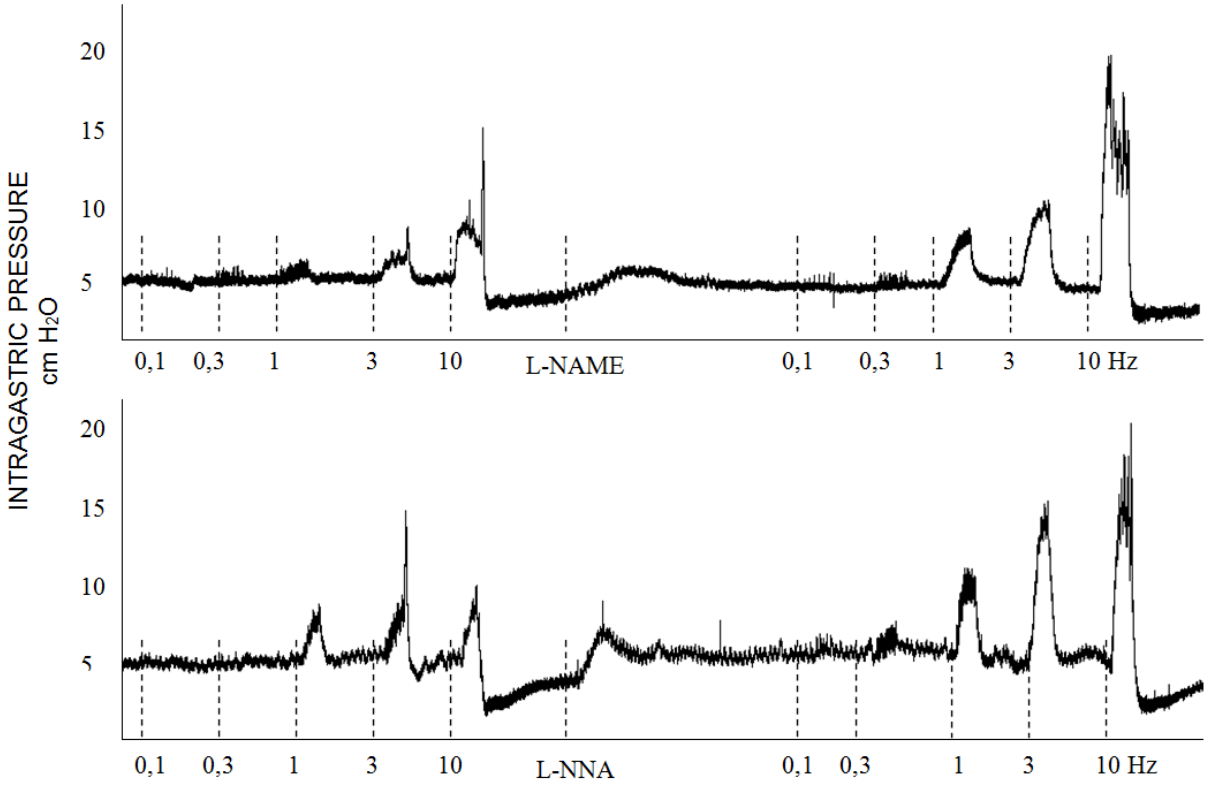


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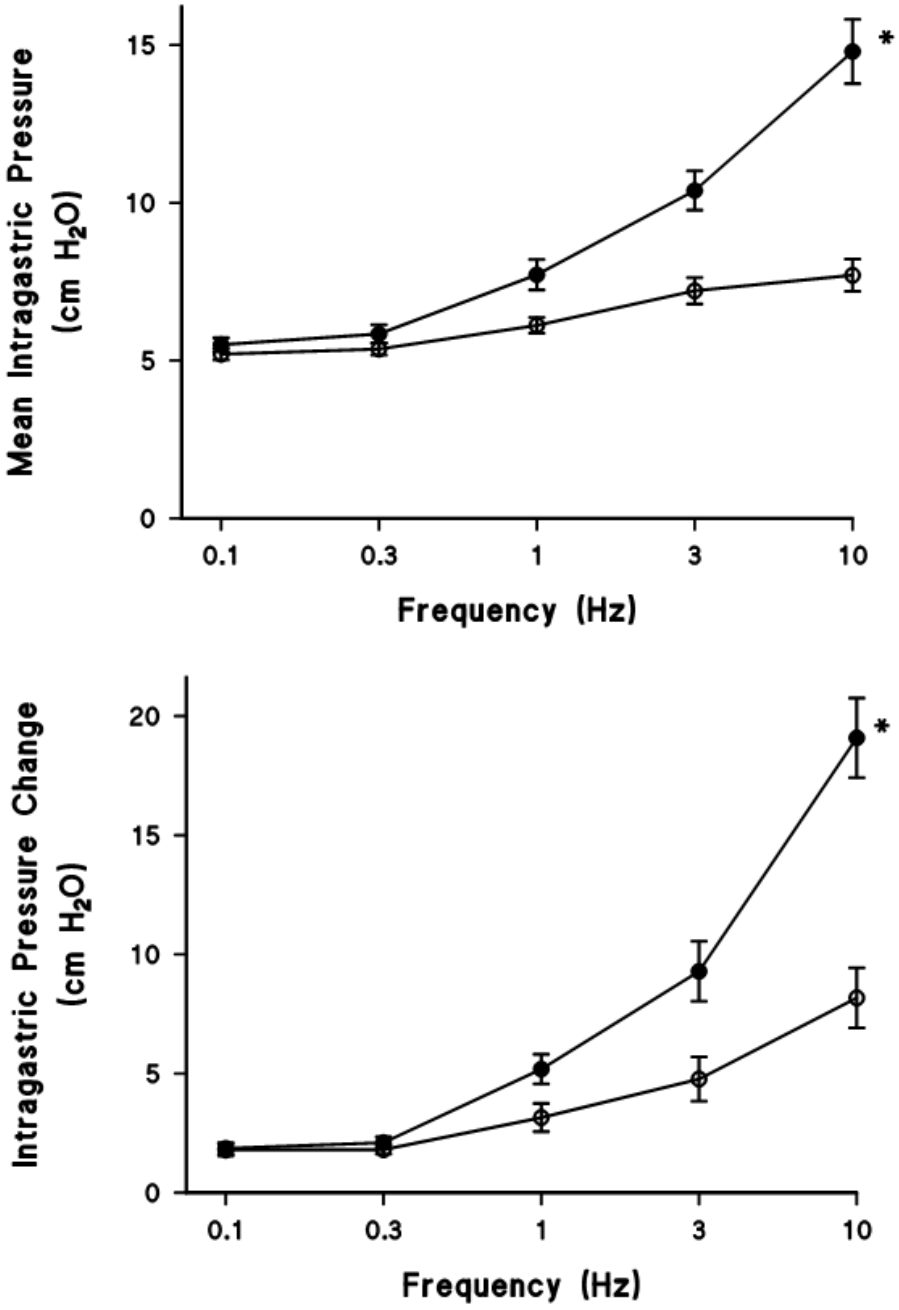
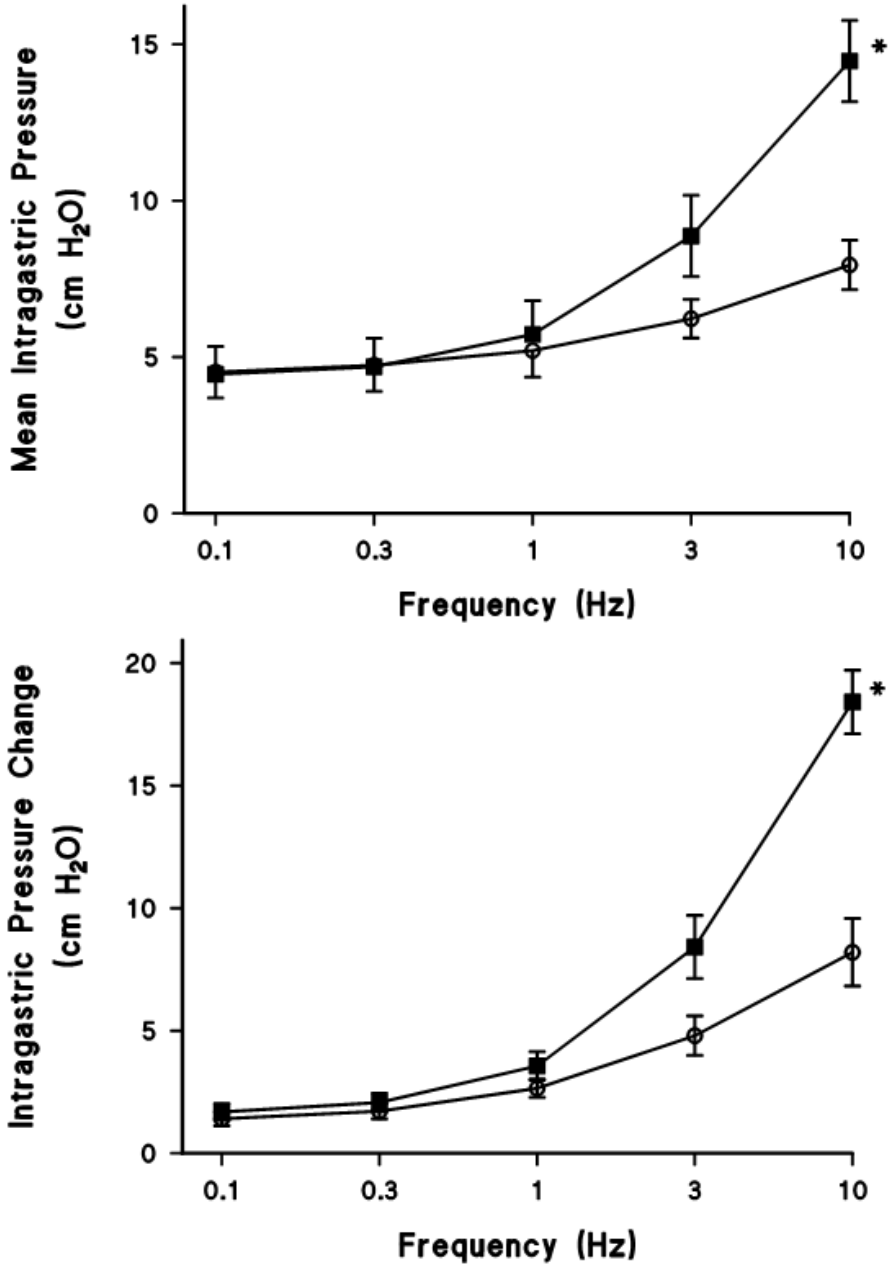

Mean Intragastric Pressure
(cm $H_2O$)
15
10
5
0
0.1
0.3
1
3
10
Frequency (Hz)
*
Intragastric Pressure Change
(cm $H_2O$)
20
15
10
5
0
0.1
0.3
1
3
10
Frequency (Hz)
*

**Figure 5**


Mean Intragastric Pressure
(cm $H_2O$)
15
10
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*
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3
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Frequency (Hz)
Intragastric Pressure Change
(cm $H_2O$)
20
15
10
5
0
*
0.1
0.3
1
3
10
Frequency (Hz)

**Figure 6**

## Figure captions

**Figure 1. Interplay of 2-DG and NOS blockers on gastric motility.** Original demonstrative recordings showing the effects of iv administration of NOS blockers (10 mg·kg$^{-1}$ L-NAME, 10 mg·kg$^{-1}$ L-NNA) on intragastric pressure induced by 2-DG (200 mg·kg$^{-1}$).

**Figure 2. Mean intragastric pressure changes.** 2-DG increased the mean intragastric pressure in both groups, and L-NAME ($P$ = 0.0156) and L-NNA ($P$ = 0.0156) administration significantly impaired this effect. Vertical bars indicate the standard error of the mean from seven experiments. * indicates the statistical significance between the stabilization period and 2-DG measurements, + indicates the statistical significance between 2-DG measurements and NOS blocker measurements.

**Figure 3. Effects of 2-DG and NOS blockers on the motility index.** 2-DG increased the motility index in both groups, and L-NAME ($P$ = 0.0156) and L-NNA ($P$ = 0.0156) administration significantly impaired this effect. Vertical bars indicate the standard error of the mean of seven experiments. * indicates the statistical significance between the stabilization period and 2-DG measurements, + indicates the statistical significance between 2-DG measurements and NOS blocker measurements.

**Figure 4. Effect of NOS blockers on electrically stimulated gastric motility.** Original recordings showing the effects of intravenous administration of nitric oxide synthase blockers (10 mg·kg$^{-1}$ L-NAME, 10 mg·kg$^{-1}$ L-NNA) on frequency-dependent intragastric pressure changes.

**Figure 5. Effect of L-NAME on electrically stimulated gastric motility**. Upper panel represents mean intragastric pressure values of rats observed before (control; open circles) and after (solid circles) L-NAME (10 mg·kg$^{-1}$, iv) administration in response to electrical

vagus nerve stimulations (12 V, 0.5 ms, 1 min) with different frequencies. Values are mean ± standard error of the mean of seven experiments. Two-way analysis of variance for repeated measures applied to “Control” versus “L-NAME” curves indicated a significant difference ($P < 0.0001$). Lower panel represents intragastric pressure values of rats observed before (control; open circles) and after (solid circles) L-NAME (10 $mg \cdot kg^{-1}$, iv) administration in response to electrical vagus nerve stimulations (12 V, 0.5 ms, 1 min) with different frequencies. Values are mean ± standard error of the mean of seven experiments. Two-way analysis of variance for repeated measures applied to “Control” versus “L-NAME” curves indicated a significant difference ($P = 0.0008$).

**Figure 6. Effect of L-NNA on electrically stimulated gastric motility.** Upper panel represents mean intragastric pressure values of rats observed before (control; open circles) and after (solid squares) L-NNA (10 $mg \cdot kg^{-1}$, iv) administration in response to electrical vagus nerve stimulations (12 V, 0.5 ms, 1 min) with different frequencies. Values are mean ± standard error of the mean of seven experiments. Two-way analysis of variance for repeated measures applied to “Control” versus “L-NNA” curves indicated a significant difference ($P = 0.0277$). Lower panel represents intragastric pressure values of rats observed before (control; open circles) and after (solid squares) L-NNA (10 $mg \cdot kg^{-1}$, iv) administration in response to electrical vagus nerve stimulations (12 V, 0.5 ms, 1 min) with different frequencies. Values are mean ± standard error of the mean of seven experiments. Two-way analysis of variance for repeated measures applied to “Control” versus “L-NNA” curves indicated a significant difference ($P = 0.0035$).