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## Reduction of the nociceptive response to gastric distension by nitrate ingestion in rats

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### SUMMARY

**Background:** Dietary nitrates are known to produce nitric oxide in the stomach, which may influence gastric function.

**Aim:** To investigate whether nitrate ingestion modifies gastric sensitivity to distension through a mechanism involving nitric oxide production.

**Methods:** Nociception, associated with gastric distension ranging from 10 to 40 mmHg, was assessed in anaesthetized rats by the amplitude of cardiovascular depressor responses. Gastric volume corresponding to each distension was recorded. The following intragastric administrations (1 mL) were performed before distension: water (control), KNO<sub>3</sub>, NaNO<sub>3</sub>, KCl, NaCl (all at 0.1 mmol/kg), standard food (0.5 g), sodium nitroprusside, a nitric oxide donor (5 mg/kg), and haemoglobin, a nitric oxide scavenger (150 mg/kg) given either with water or KNO<sub>3</sub>.

**Results:** In controls, the fall in blood pressure increased from  $7.8 \pm 2.0$  to  $31.6 \pm 2.7$  mmHg at distending pressures from 10 to 40 mmHg, respectively. KNO<sub>3</sub> significantly reduced the amplitude of blood pressure response for the highest distending pressures (35 and 40 mmHg), while KCl induced a reduction in blood pressure response at all gastric pressures. NaNO<sub>3</sub> and NaCl did not induce significant changes in distension-induced depressor responses. Administration of 0.5 g of standard food or sodium nitroprusside reproduced the effect of KNO<sub>3</sub>, which was reversed by haemoglobin. None of the compounds modified the gastric pressure–volume relationship, except KNO<sub>3</sub>, which increased gastric volume for the lowest distending pressures, and haemoglobin, which reduced the volume for the highest pressure.

**Conclusions:** Ingestion of potassium nitrate reduces the sensitivity to gastric distension, through a mechanism involving nitric oxide.

### INTRODUCTION

Dietary nitrate, mainly derived from green and leafy vegetables, is known to produce nitric oxide (NO) in the stomach. Nitrate is absorbed from the stomach and proximal small intestine into the plasma and then concentrated in saliva.<sup>1</sup> Nitrate in saliva is rapidly reduced to nitrite by anaerobic bacteria and then to NO

under the acidic conditions of the stomach.<sup>2</sup> In humans, ingestion of small amounts of nitrate (i.e. 2 mmol, which corresponds to the amount normally found in a lettuce<sup>3</sup>) generates high concentrations of NO in the stomach.<sup>4</sup>

Numerous actions have been proposed for gastric NO. It has been found to protect gastric mucosa from damage induced by topically applied ethanol.<sup>5</sup> This effect can be mediated by the increase of mucosal blood flow induced by NO.<sup>6</sup> Mucus secretion is considered as a primary level of mucosal defence against acid in the stomach and NO has been found to stimulate mucus

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release<sup>7</sup> and to inhibit gastric acid secretion through suppression of histamine release from enterochromaffin-like cells.<sup>8</sup> Moreover, NO is considered as a neurotransmitter of the nonadrenergic noncholinergic innervation responsible for gastric relaxation<sup>9</sup> and has been found involved in the vagally mediated gastric receptive relaxation.<sup>10</sup>

NO has also been found implied in the pathophysiology of pain, but its role in pain transmission remains controversial. Several reports indicate an involvement of NO in some models of hyperalgesia,<sup>11</sup> while others indicate an antinociceptive action of NO.<sup>12</sup> On the other hand, one of the main factors found in idiopathic dyspepsia in humans is hypersensitivity to gastric distension, pain thresholds being decreased in comparison with healthy controls.<sup>13</sup> Consequently, according to the gastric production of NO from dietary nitrate and the role of NO in gastric physiology and in pain transmission, the aim of this study was to determine whether nitrate can modify pain induced by gastric distension in rats. Nociceptive responses to gastric distension were assessed in anaesthetized rats by the amplitude of the reflex cardiovascular depressor response, which is considered to be indicative of nociception.<sup>14</sup> Despite the increase in blood pressure observed in awake animals, the decrease in blood pressure induced by visceral distension has been validated to assess pain in anaesthetized animals.<sup>15–17</sup>

## MATERIALS AND METHODS

### *Animals*

Male Wistar rats (Harlan, Gannat, France) weighing between 300 and 350 g at the time of the experiments were housed under controlled lighting with standard diet (UAR 03, Epinay-sur-Orge) and water provided *ad libitum*. All protocols were approved by the local Institutional Animal Care and Use Committee.

### *Surgical procedure*

Animals were deprived of food the night prior to experimentation, and had free access to water. They were anaesthetized with pentobarbitone sodium (Nembutal, SANOFI, Libourne, France, 60 mg/kg, *s.c.*). Anaesthesia was maintained by re-administration of pentobarbitone sodium (15 mg · h/kg). Anaesthetized animals were maintained at 35 °C with a heat pad.

The trachea was cannulated to prevent airway obstruction. A second cannula was introduced into the thoracic aorta via the left common carotid artery for constant retrograde intra-arterial infusion of saline and further administration of anaesthetic. The rate of infusion was 40 µL/min. Systemic blood pressure was recorded from a side-arm of the carotid cannula by means of a Statham pressure transducer (P231ID) connected to a polygraph (R411 Dynograph, Beckman, Palo Alto, USA).

For gastric distension experiments, a midline incision was made along the abdomen under the diaphragm to expose the stomach. A balloon was placed into the stomach according to a previously described technique.<sup>18, 19</sup> The balloon was 5.0–5.5 cm long and made from a latex condom. It was sutured with silk to a Tygon R3606 flexible plastic tubing (1.0 mm inner diameter, 1.8 mm outer diameter). The portion of tubing inserted into the condom was repeatedly perforated to permit an easy air transfer. The volume of the balloon was greater than that of the stomach so that the balloon itself offered no resistance to inflation during the distension procedure. The balloon was inserted into the stomach through the upper part of the rumen (nonglandular part of the fundus in the rat), at 1 cm from the gastro-oesophageal junction. The tubing was exteriorized through the abdominal incision, and abdominal muscles and skin were sutured.

Isobaric distensions of the stomach were performed by connecting the balloon to a computerized barostat<sup>20</sup> and gastric volume was continuously monitored on a channel of the Beckman polygraph.

Intragastric administrations were made through a PE10 catheter coupled to the distending tubing and exteriorized in the same manner.

### *Experimental procedure*

The basal arterial pressure was recorded for 5 min before intragastric administration and then for 30 min before the first gastric distension. Isobaric gastric distensions were performed step by step with intermittent deflations of the balloon. Each distension step lasted 20 s and was followed by a 5-min deflation period. The first distension was performed at a pressure of 10 mmHg and an increment of 5 mmHg was added at each following step, until a maximal pressure of 40 mmHg was reached. Five minutes after the last distension, sensitivity to somatic pain was investigated

by quantifying the reflex depressor response to the pinching of abdominal skin with forceps during 10 s.

Nine groups of eight rats were used. They received, respectively: distilled water,  $\text{KNO}_3$ ,  $\text{NaNO}_3$ , KCl, NaCl, chow mixture, haemoglobin followed by distilled water, haemoglobin followed by  $\text{KNO}_3$ , sodium nitroprusside.

$\text{KNO}_3$ ,  $\text{NaNO}_3$ , KCl and NaCl (Merck, Darmstadt, Germany) dissolved in distilled water were administered intragastrically at the concentration of 0.1 mmol/kg. This dose corresponds to about three times the dose of  $\text{KNO}_3$ , expressed in mmole per kg of body weight, which induced a large production of gastric NO in humans.<sup>4</sup> However, taking into account the relative larger size of the stomach in rats compared with humans, this dose is similar to that given to humans when expressed per unit of gastric volume. Chow mixture was prepared with 0.5 g of standard food finely ground and humidified with 1 mL of distilled water. Sodium nitroprusside and haemoglobin (Sigma, St Louis, Missouri, USA) solutions were freshly prepared in 1 mL distilled water administered intragastrically at doses of 5 and 150 mg/kg, respectively, slowly enough to avoid mechanical stimulation of the gastric wall.

#### Data analysis

The depressor response was quantified by measuring the maximum fall in blood pressure observed after the nociceptive stimulus, which was expressed as the absolute change in blood pressure in mmHg. Values were expressed as mean  $\pm$  S.E.M. Statistical analysis was performed by either one-way ANOVA followed by Student's *t*-test adapted for unpaired values or repeated measure ANOVA. Differences were considered significant for  $P < 0.05$ .

## RESULTS

### Blood pressure

In control conditions obtained after intragastric administration of 1 mL of distilled water, the fall in mean arterial pressure increased linearly from  $7.8 \pm 2.0$  to  $31.6 \pm 2.7$  mmHg at distending pressures ranging from 10 to 40 mmHg. After intragastric administration of  $\text{KNO}_3$  (0.1 mmol/kg), the amplitude of arterial pressure falls was not significantly modified in comparison with controls for distending pressures from 10 to 30 mmHg, but the amplitude of blood pressure responses for

distending pressures of 35 and 40 mmHg were significantly reduced ( $P < 0.05$ ) (Figure 1). The depressor response was not significantly modified ( $P > 0.05$ ) by intragastric administration of  $\text{NaNO}_3$  at the same dose (0.1 mmol/kg), whatever the distending pressure, despite a tendency to a decreased amplitude for the distending pressures of 35 and 40 mmHg. After KCl administration (0.1 mmol/kg), the amplitude of the depressor response was lower than that of controls at all distending pressures and the curves were found significantly different by repeated measure ANOVA ( $P < 0.05$ ). NaCl (0.1 mmol/kg) did not induce any significant difference from controls, whatever the distending pressure.

Intragastric administration of 0.5 g of chow mixture induced a depressor response following a curve very similar to that observed with  $\text{KNO}_3$  (Figure 1), falls in blood pressure being significantly lower ( $P < 0.05$ ) than those observed in controls for the gastric distending pressures of 35 and 40 mmHg.

Haemoglobin, a NO scavenger (150 mg/kg) administered alone into the stomach did not modify the depressor responses in comparison with controls, whatever the gastric distending pressure. However, the decrease in the amplitude of the depressor response induced by  $\text{KNO}_3$  at the distending pressure of 35 and 40 mmHg was suppressed by a previous administration of haemoglobin (Figure 1). Intragastric administration of sodium nitroprusside, a NO donor (5 mg/kg), significantly decreased ( $P < 0.05$ ) the depressor response for the highest distending pressure (30–40 mmHg).

The control fall in blood pressure induced by pinching the abdominal skin was  $18.7 \pm 2.1$  mmHg and was not significantly modified ( $P > 0.05$ ) by intragastric administrations of  $\text{KNO}_3$ ,  $\text{NaNO}_3$ , KCl, NaCl, chow, sodium nitroprusside or haemoglobin.

### Gastric volume

In control studies, the gastric volume increased from  $0.7 \pm 0.3$  mL to  $5.5 \pm 0.4$  mL for the distending pressures of 10 and 40 mmHg. Intragastric administration of  $\text{KNO}_3$  increased gastric volume, in comparison with controls, at all distending pressures, but a significant difference ( $P < 0.05$ ) was reached only for the lowest distending pressures of 10 and 15 mmHg (Figure 2). This effect of  $\text{KNO}_3$  was abolished by a previous administration of haemoglobin. No significant change in gastric volume was observed after administration of

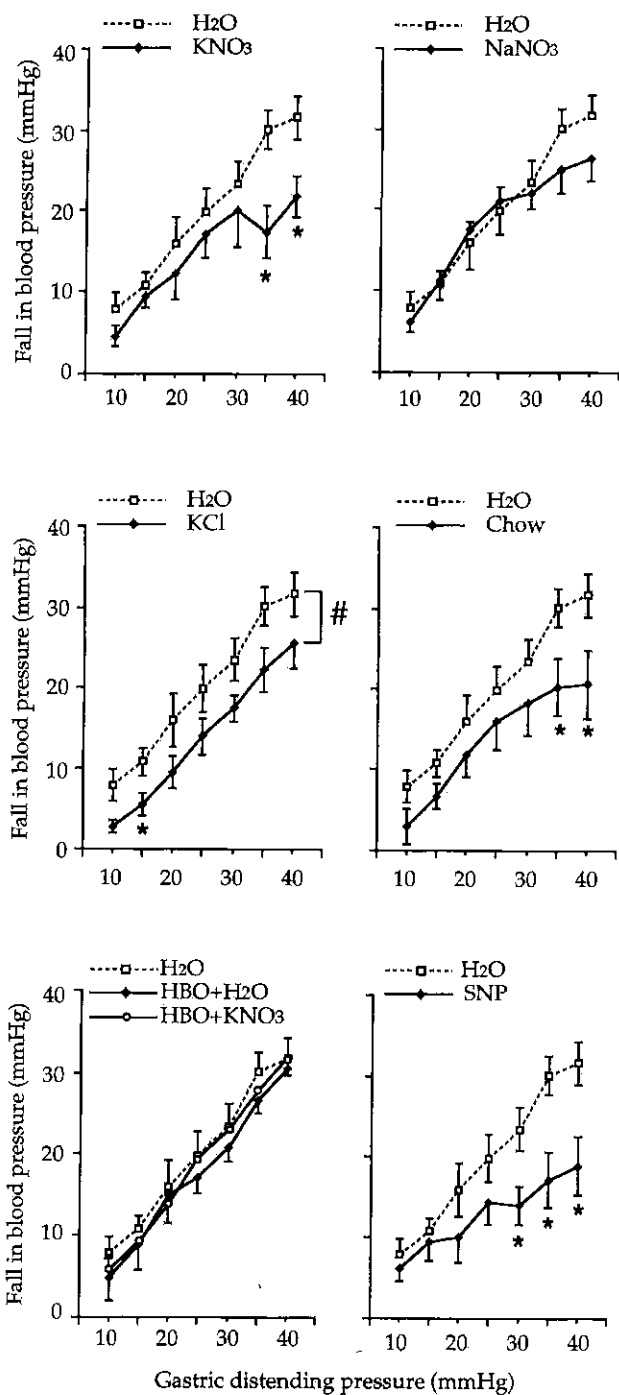


Figure 1. Effect of different compounds given intragastrically on the fall in blood pressure induced by gastric distension. The amounts were 0.1 mmol/kg of  $\text{KNO}_3$ ,  $\text{NaNO}_3$  and  $\text{KCl}$ , 0.5 g of chow, 5 mg/kg of haemoglobin (HBO), and 150 mg/kg of sodium nitroprusside (SNP). Control values ( $\text{H}_2\text{O}$ , 1 mL) correspond to a unique experiment. \* $P < 0.05$  vs.  $\text{H}_2\text{O}$  at the same distending pressure, # $P < 0.05$  vs.  $\text{H}_2\text{O}$  curve ( $n = 8$ ).

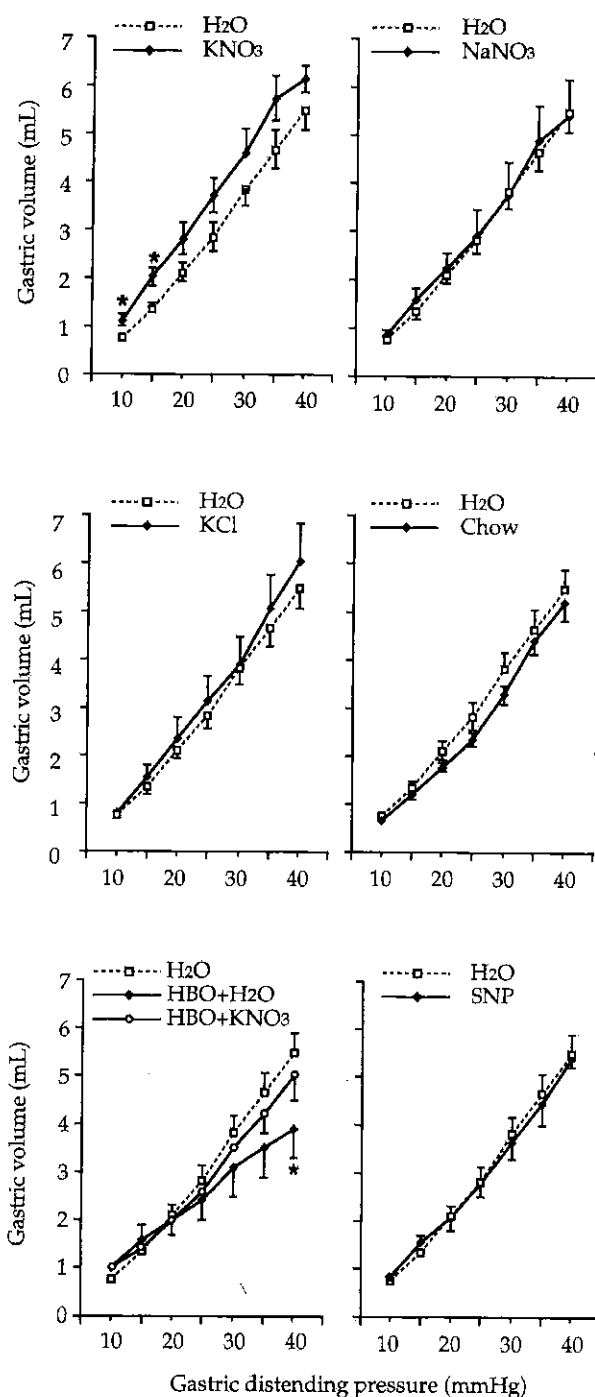


Figure 2. Gastric volumes corresponding to increasing gastric distensions after intragastric administration of different compounds. The amounts of the compounds were 0.1 mmol/kg of  $\text{KNO}_3$ ,  $\text{NaNO}_3$  and  $\text{KCl}$ , 0.5 g of chow, 5 mg/kg of haemoglobin (HBO), and 150 mg/kg of sodium nitroprusside (SNP). Control values ( $\text{H}_2\text{O}$ , 1 mL) correspond to a unique experiment. \* $P < 0.05$  vs.  $\text{H}_2\text{O}$  at the same distending pressure ( $n = 8$ ).

NaNO<sub>3</sub>, KCl, NaCl, chow and sodium nitroprusside, compared with controls. However, haemoglobin administered alone decreased gastric volumes for the highest distending pressures and the difference with controls was significant ( $P < 0.05$ ) for the gastric pressure of 40 mmHg (Figure 2).

## DISCUSSION

Our results indicate that ingestion of an amount of nitrate that can be found in food reduces gastric sensitivity to distension in rats. Pain induced by gastric distension was assessed by the amplitude of the decrease in arterial pressure according to a model widely used in nonhuman investigations of visceral nociception.<sup>14</sup> However, visceral distension is known to induce an increase in blood pressure in awake animals, while in pentobarbitone anaesthetized animals a reduction in blood pressure is observed. This fall in blood pressure has been validated to assess pain in anaesthetized animals. For example, in anaesthetized rats, the decrease in blood pressure induced by distension of the jejunum,<sup>15</sup> ileum<sup>16</sup> or renal pelvis<sup>17</sup> has been found to be blocked by morphine, an action reversed by naloxone.

The reduction of pain by KNO<sub>3</sub> observed for the highest pressure of gastric distension is very likely mediated by NO since it was reproduced by a NO donor, sodium nitroprusside, and abolished by a NO scavenger, haemoglobin. An antinociceptive action of NO is not surprising, whereas its role in pain transmission is very controversial. For example, NO has been found involved in inflammatory hyperalgesia<sup>11</sup> but it is also considered as a mediator of opiate analgesia.<sup>12</sup> However, the few studies performed in visceral or peritoneal pain indicate an antinociceptive action of NO.<sup>21–23</sup>

In our experiments, nitrate was administered directly into the stomach to the anaesthetized rat. The paradigm in which nitrate is absorbed from the stomach into the plasma and then concentrated in the saliva, where bacteria of the tongue reduce nitrate into nitrite,<sup>1, 4</sup> cannot be applied to our experimental conditions since no deglutition of saliva occurs during anaesthesia. However, nitrate-reducing flora has been identified in the rat stomach<sup>24</sup> and, moreover, the gastric mucosa has been found to be able to reduce nitrate into nitrite even in germ-free rats.<sup>25</sup> Since nitrite is transformed in nitrous acid and nitrogen oxides in the acidic conditions of the stomach,<sup>2</sup> a mediation by NO of the effects of

intra-gastric nitrate observed in the present study is highly possible.

The antinociceptive action of KNO<sub>3</sub> was not reproduced by NaNO<sub>3</sub>, despite the tendency observed for the highest distending pressures. This difference between KNO<sub>3</sub> and NaNO<sub>3</sub> cannot be explained by differences in ionic characteristics of the two salts, which would lead to different amounts of NO produced. However, it has been shown in several experimental models that low doses of potassium are able to stimulate NO release from different tissues<sup>26, 27</sup>. Thus, we can speculate that the antinociceptive action of KNO<sub>3</sub> depends both upon NO produced via a reduction from nitrate and upon the release of endogenous NO stimulated by potassium ions. The low antinociceptive effect of NaNO<sub>3</sub>, which did not reach statistical significance, would involve only exogenous production of NO.

We found an antinociceptive action of KCl, but the distending pressure–depressor response curve was different from that obtained with KNO<sub>3</sub> (Figure 1). The antinociceptive properties of KCl and KNO<sub>3</sub> likely involve different mechanisms. Several reports indicate an analgesic action of the potassium ion. For example, potassium-containing dentifrices have been found to reduce the pain of dentine hypersensitivity evoked by tactile stimulation or cold air blasts.<sup>28, 29</sup> However, the mechanism of action of potassium, which has been found to decrease action potential conduction in A $\delta$ - and C-fibers of rat spinal nerves,<sup>30</sup> remains unclear. We have also shown that introduction of standard food into the stomach reduced the sensitivity of this organ to distension. The relationships between food intake and analgesia are well documented but data are often controversial and mainly concern nonvisceral pain. For example, food deprivation is known to induce analgesia<sup>31</sup> while sucrose, and more generally palatable substances, also produces analgesia.<sup>32</sup> Concerning visceral pain, extra-physiologic hyperglycemia has been found to either decrease<sup>33</sup> or increase<sup>34</sup> rectal sensitivity to distension, while a 600-kCal meal decreased rectal sensitivity.<sup>35</sup> In our study, we show for the first time that very small amounts of food in the stomach are able to reduce the sensitivity to distension: the distending pressure–depressor response curves after either KNO<sub>3</sub> or standard food administration are very similar, but we cannot conclude that the effect of food depends upon its content in nitrate or potassium.

NO is considered as a mediator of nonadrenergic noncholinergic-mediated relaxation of the gastric



fundus<sup>36</sup> and NO donors have been found to mimic the gastric relaxation observed after vagal stimulation.<sup>37</sup> In our study, KNO<sub>3</sub> induced an increase in gastric volume under distension. This effect is very likely mediated by NO since it was abolished by the NO scavenger haemoglobin and not reproduced by KCl. However, the NO donor sodium nitroprusside did not induce any relaxation of the stomach. We can speculate that NO production from the amount of sodium nitroprusside we introduced in the stomach was too small to induce muscular relaxation. In addition, visceral pain induced by gastric distension was more sensitive to NO than smooth muscle relaxation, since sodium nitroprusside reduced the amplitude of the depressor response to distension. However, we can speculate that the antinociceptive sites of action of NO are very likely the sensory afferents of the gastric wall, but other sites of the reflex leading to a hypotensive response, from the gastric wall to the cardiovascular system, cannot be definitively excluded. Finally, our results also show for the first time that the NO scavenger haemoglobin reduces gastric volume for the highest distension pressure, suggesting a permanent tonic relaxant action of NO on the gastric wall.

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