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Mechanisms mediating N^G-nitro-L-arginine methyl ester-induced hypophagia in mice

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Abstract

N^G-Nitro-L-arginine methyl ester (50 mg/kg s.c.), an inhibitor of nitric oxide (NO) synthase, has been reported to increase brain serotonin (5-hydroxytryptamine, 5-HT) metabolism and induce hypophagia. Conversely, enhanced NO synthase activity is found to be accompanied by a decrease in 5-HT level. This negative correlation between NO and 5-HT in the regulation of food intake was further studied in mice. 5-HT depletion by *p*-chlorophenylalanine (250 mg/kg i.p., twice daily for 2 days) failed to antagonize the hypophagic effect of N^G-nitro-L-arginine methyl ester. Similarly, treatment with the NO synthesis precursor, L-arginine (1000 mg/kg s.c.), did not reverse the anorexia induced by fenfluramine (10 mg/kg s.c.), a 5-HT releaser/uptake inhibitor. Pretreatment with (−)-pindolol, methysergide and ritanserin had no effect on the hypophagic action of N^G-nitro-L-arginine methyl ester, suggesting the lack of involvement of 5-HT_1 and 5-HT_2 receptors. The selective neuronal NO synthase inhibitor, 7-nitroindazole (12.5–50.0 mg/kg i.p.), however, did not exhibit any hypophagic effect whilst N^G-nitro-L-arginine methyl ester increased gastric retention, which may subsequently induce satiety. Moreover, the hypophagic effect of N^G-nitro-L-arginine methyl ester, which was unassociated with changes in water intake and malaise induction, was also unattenuated by cholecystokinin (CCK) receptor antagonists, devazepide (10 mg/kg i.p.) and PD 135,158 ([1*S*-[1α,2β[*S**(*S**)],4α]]-4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]carbonyl]amino]propyl]amino]-1-phenylethyl] amino]-4-oxo-butanoic acid *N*-methyl-D-glucamine salt; 1 mg/kg i.p.). Furthermore, a decrease in exploratory activity and diminished preference for favorable sensory cues were observed in N^G-nitro-L-arginine methyl ester-treated animals. These results suggest that mechanisms other than 5-HT are possibly involved in mediating the hypophagic effect of N^G-nitro-L-arginine methyl ester treatment in mice.

Keywords: Nitric oxide (NO); N^G-Nitro-L-arginine methyl ester; 5-HT (5-hydroxytryptamine, serotonin); Anorexia; Gastric emptying; Food intake

1. Introduction

Nitric oxide (NO) is an ubiquitous free radical that performs the role of an intercellular messenger (Moncada et al., 1991). It exhibits diverse physiological effects which include mediation of endothelium-dependent vascular relaxation and modulation of peripheral nonadrenergic-noncholinergic (NANC) neurotransmission (Sanders and Ward, 1992; Rand, 1992). Several physiological effects of NO on central nervous system (CNS)-mediated functions have been reported. N^G-Nitro-L-arginine methyl ester, an inhibitor of nitric oxide (NO) synthase, exhibited opioid-independent antinociceptive activity in the formalin test (Moore et al., 1991) and also blocked intrathecal *N*-methyl-D-aspartate (NMDA)-induced thermal hyperalgesia (Malmberg and Yaskh, 1993). N^G-Nitro-L-arginine methyl ester and N^G-nitro-L-arginine diminished naloxone-precipitated withdrawal signs in morphine-dependent mice (Cappendijk et al., 1993). L-Arginine, the substrate for NO synthesis, elicited antidipsogenic effects that were reversed by N^G-nitro-L-arginine methyl ester (Calapai et al., 1992). Food intake was also decreased by N^G-nitro-L-arginine methyl ester and N^G-nitro-L-arginine treatments in mice (Morley and Flood, 1991) and rats (Squadrito et al., 1993).

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The central serotonergic system may be involved in N^G-nitro-L-arginine-induced anorexia. Fasted animals exhibited an increase in NO synthase activity in association with a reduction in 5-HT levels (Squadrito et al., 1994a). N^G-Nitro-L-arginine increased serotonin metabolism and the anorectic effect could be attenuated by pretreatment with metergoline, a non-selective 5-HT receptor antagonist (Squadrito et al., 1994b). These results therefore suggested that neuronal NO has a modulatory effect on serotonin receptors which are involved in feeding regulation. Enhancement of serotonergic neurotransmission reliably suppressed feeding and this involved mediation by multiple 5-HT receptor subtypes. Agonists acting on post-synaptic 5-HT_{1B} and 5-HT_{2C} receptors (Kennett et al., 1986; Kennett and Curzon, 1991) induced hypophagia whereas activation of pre-synaptic 5-HT_{1A} autoreceptors increased feeding by reducing serotonin release (Dourish et al., 1986). The characterization of the functional involvement of these receptor subtypes is therefore essential in the understanding of the NO–5-HT interaction related to hypophagia. In this study, attempts were made to confirm the presence of a correlation between NO and 5-HT in the control of feeding and to clarify the 5-HT receptor subtypes involved in mediating such an interaction. Since it has been demonstrated by other researchers that N^G-nitro-L-arginine methyl ester can inhibit feeding induced by centrally administered neuropeptide Y (Morley and Flood, 1992), other orexigenic agents like 2-deoxy-D-glucose and diazepam were therefore tested in order to assess the robustness of its anorectic action. In addition, the possible involvement of a peripheral mechanism such as gastric distension and its consequent promotion of satiety was also investigated in light of a recent finding which indicates delayed gastric emptying of a non-nutrient solution following N^G-nitro-L-arginine methyl ester treatment (Plourde et al., 1994).

2. Materials and methods

2.1. Animals

Male ICR mice (30–35 g) were obtained from the Laboratory Animal Unit, University of Hong Kong. Animals were housed under a standard 12:12 light-dark cycle (lights on at 06.00 h). Room temperature and humidity were maintained at 22 ± 1°C and 60–70% respectively. Food (Ralston Purina chow 5001) and water were available ad libitum. All animals had at least one week to acclimate to the laboratory prior to the start of the experiment. Feeding studies were conducted between 10.00 and 14.00 h. Food intake was measured at intervals of 1, 2 and 4 h following drug injection. Mice were deprived of food, but not water, overnight for 24 h to establish a stable baseline intake. Food intake was subtracted from an initially preweighed quantity placed in the food hoppers to obtain the consumption at each time interval. In the case of fluid intake studies, the procedures were the same except that animals were allowed access to food pellets. All experiments were performed in the animal's home cage.

2.2. Drugs

L-Arginine hydrochloride (Sigma, St. Louis, MO, USA), N^G-nitro-L-arginine methyl ester hydrochloride (RBI, Natick, MA, USA), (±)-*p*-chlorophenylalanine (RBI), *S*-(±)-fenfluramine hydrochloride (RBI), PD 135,158 ([1*S*-[1α,2β[*S**(*S**)],4α]]-4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[(1,7,7-trimethylbicyclo[2.2.1]-hept-2-yl)oxy]carbonyl]amino]propyl]amino]-1-phenyl-ethyl] amino]-4-oxo-butanoic acid *N*-methyl-D-glucamine salt) (RBI) and 2-deoxy-D-glucose (Sigma) were dissolved in 0.9% NaCl. Methysergide maleate (RBI) and ritanserin (RBI) were dissolved in glacial acetic acid and made up to 1% with 0.9% NaCl. (−)-Pindolol (RBI) was dissolved in 0.1 M HCl in 0.9% NaCl. Devazepide (L-364,718, Merck) was dissolved in a vehicle of 20% dimethyl sulfoxide/80% propylene glycol. 7-Nitroindazole (Cookson, Southampton, UK) was prepared as a uniform suspension in olive oil. Diazepam (Valium, Roche) was diluted to its appropriate concentration with 0.9% NaCl. All drugs were injected in a volume of 1 ml/100 g and the controls were administered an equivalent volume of vehicle. *p*-Chlorophenylalanine, 7-nitroindazole, 2-deoxy-D-glucose, diazepam and devazepide were administered via the intraperitoneal route while the rest of the drugs were given subcutaneously. Pretreatment with antagonists and other drugs was given 30 min prior to N^G-nitro-L-arginine methyl ester. In 5-HT-depleted animals, N^G-nitro-L-arginine methyl ester was administered 18 h after the final injection of *p*-chlorophenylalanine. Doses of antagonists used in these experiments were selected according to those used in other studies (Khosla and Crawley, 1988; Rodgers and Shepherd, 1989; Hughes et al., 1990; Tokuyama et al., 1993) to achieve effective blockade of 5-HT and CCK receptors in mice. A moderate dose of (−)-pindolol was used in the present study since at high doses it may act as a partial 5-HT_{1A} agonist (Rodgers and Shepherd, 1989).

2.3. Experimental design and procedure

Experiment 1

To confirm the negative correlation between NO and 5-HT in feeding regulation, mice were pretreated with *p*-chlorophenylalanine (250 mg/kg twice daily for

2 days), a 5-HT synthesis inhibitor, and the anorectic effect of N^G-nitro-L-arginine methyl ester (50 mg/kg) was then tested 18 h after the final injection of *p*-chorophenylalanine. Alternatively, the anorectic effect induced by fenfluramine (10 mg/kg), a 5-HT releaser/uptake inhibitor, was examined following the stimulation of NO production by administration of its substrate, L-arginine (1000 mg/kg). The involvement of various 5-HT receptor subtypes was also studied using the following antagonists with different selectivities for 5-HT receptor sites: methysergide (5 mg/kg; 5-HT_{2A}, 5-HT_{2C} > 5-HT_{1A}), ritanserin (5 mg/kg; 5-HT_{2A}, 5-HT_{2C}) and (−)-pindolol (2 mg/kg; 5-HT_{1A}, 5-HT_{1B}). In order to confirm the involvement of a specific central action of N^G-nitro-L-arginine methyl ester in inducing hypophagia, animals were treated with 7-nitroindazole (12.5, 25.0, 50.0 mg/kg), a selective inhibitor of neuronal NO synthase.

Experiment 2

The inhibitory effect of N^G-nitro-L-arginine methyl ester on feeding was studied in the presence of two orexigenic agents: 2-deoxy-D-glucose (750 mg/kg) and diazepam (2.5 mg/kg). The different mechanisms underlying the hyperphagic action of these drugs served to test the robustness of the anorectic efficacy of N^G-nitro-L-arginine methyl ester.

Experiment 3

The effect of N^G-nitro-L-arginine methyl ester on gastric emptying of a semi-solid diet was tested according to Samanin et al. (1991). Mice were first placed on a restricted feeding schedule for 1 week, during which time they were allowed daily access to 5 g of mash diet (50% ground Purina chow, 50% water by weight) for 30 min. Immediately afterwards, they were given free access to chow pellets for 4 h only. The latter was given to ensure an adequate daily caloric intake. After this 1-week restricted feeding schedule, the test was conducted by allowing the animals to consume the wet mash for 30 min, and N^G-nitro-L-arginine methyl ester (50 mg/kg) or saline (1 ml/100 g) was then injected and the animals were killed 2 or 4 h later. Conditioning and experiments were carried out in Perspex boxes (25 × 15 × 15 cm). Gastric contents collected in individual glass vials were weighed and dried to a constant weight. Food retained was taken as the difference between the amount eaten and the weight of the stomach content. Results, presented as percent food retention, are expressed in terms of both dry and wet weight. Since gastric distension may promote satiety, and cholecystokinin (CCK) has been proposed as a major satiety signal, the effects of devazepide (10 mg/kg), a CCK_A receptor antagonist, and PD 135,158 (1 mg/kg), a CCK_B receptor antagonist, on N^G-nitro-L-arginine methyl ester-induced anorexia were also investigated.

Experiment 4

To rule out any possible non-specific depressant effects of N^G-nitro-L-arginine methyl ester on ingestive behavior, the intake of water in previously 24-h liquid-deprived animals was assessed. In addition, the consumption of a sweet caloric drink (5% sucrose) was tested in 24-h water-deprived animals in order to evaluate their responses to favorable sensory cues. This served as a measure of the arousal and attention status of the animals following N^G-nitro-L-arginine methyl ester (12.5, 25.0, 50.0 mg/kg) treatment. The possibility of N^G-nitro-L-arginine methyl ester-induced anorexia being secondary to the induction of malaise was tested by using the two-bottle conditioned taste aversion paradigm. Mice were subjected to a restricted 1-h access to water daily for 1 week. On day 8, a novel tasting saccharin solution (1 g/l) was presented immediately following subcutaneous N^G-nitro-L-arginine methyl ester (50 mg/kg) injection. Control animals received injections of an equivalent volume (1 ml/100 g) of NaCl (0.6 nmol/kg) or LiCl (0.6 nmol/kg, positive control). The animals were allowed to drink water on days 9 and 10 according to the original conditioning schedule. The test was performed on day 11 and animals were allowed to choose between water and saccharin following NaCl, LiCl or N^G-nitro-L-arginine methyl ester administration.

Experiment 5

The effect of N^G-nitro-L-arginine methyl ester on locomotor activity was assessed in an open field arena (length 350 mm, width 245 mm, height 430 mm). The monitoring system (Watchman I, constructed by the Electronic Services Unit, University of Hong Kong) contains four open-field chambers and is interphased to an IBM PC/AT computer for data storage and calculations (Qiu et al., 1992). Immediately following N^G-nitro-L-arginine methyl ester (50 mg/kg) administration, animals were placed individually in separate monitoring chambers for 10 min to allow acclimatization to the surroundings. They were then monitored for 30 min, during which time the horizontal activities (mm/s) were calculated at 10-min intervals. Experiments were conducted between 10.00 and 12.00 h under illuminated conditions in a soundproofed room with regulated temperature and humidity.

2.4. Statistical analysis

The results are expressed as means ± S.E.M. Data were analysed by analysis of variance (ANOVA) followed by post-hoc comparisons using Fisher's least-significant difference (LSD) test. Comparison between

two related mean values was done by paired Student's t-test. Significance was set at $P < 0.05$ in all cases. Calculations were performed using SPSS 6.0 (Statistical Program for Social Sciences, SPSS, Chicago, IL, USA).

3. Results

3.1. NO–5-HT interaction and the effect of 5-HT antagonists on N^G-nitro-L-arginine methyl ester-induced hypophagia

As shown in Fig. 1, depletion of 5-HT with p-chlorophenylalanine was found not to affect N^G-nitro-L-arginine methyl ester-induced hypophagia. p-Chlorophenylalanine alone increased cumulative food intake at 4 h ($P < 0.05$). An increase in 5-HT availability with fenfluramine was associated with decreases ($P < 0.001$) in food intake at 1, 2 and 4 h. This effect was not reversed by L-arginine at a concentration reported to reverse N^G-nitro-L-arginine-induced hypophagia and to increase food intake. Instead, in the present study, L-arginine was shown to depress cumulative food intake at 2 and 4 h ($P < 0.05$). Antagonism of 5-HT_{1A} and 5-HT_{1B} receptors with (−)-pindolol failed to abolish the hypophagic effect of N^G-nitro-L-arginine methyl ester at 4 h (Fig. 2). (−)-Pindolol treatment itself depressed cumulative food intake at 1 and 2 h ($P < 0.01$ and $P < 0.05$). No reversal of the N^G-nitro-L-arginine

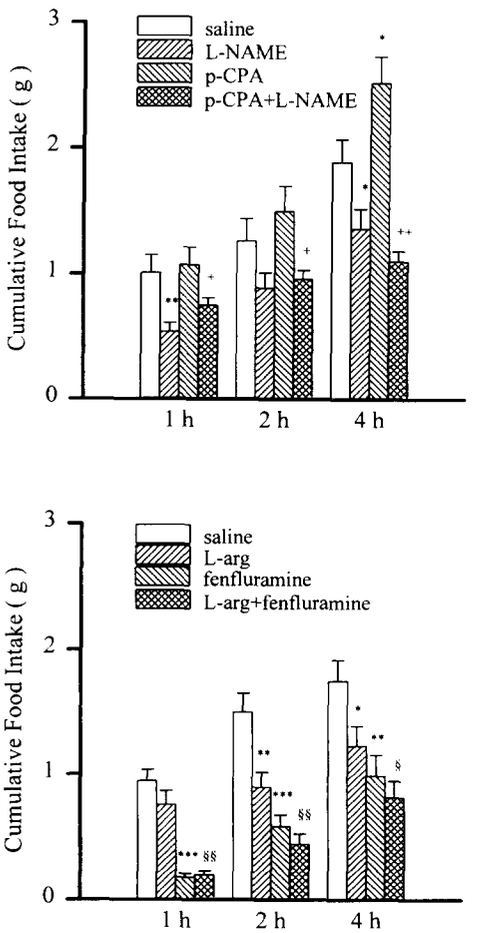

Fig. 1. Interaction of 5-HT and nitric oxide (NO) in the regulation of food intake in 24-h fasted mice. Doses of drugs used are: p-chlorophenylalanine (p-CPA, 250 mg/kg i.p.×2 for 2 days), L-arginine (L-arg, 1000 mg/kg s.c.), fenfluramine (10 mg/kg s.c.) and N^G-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg s.c.), Values shown are means ± S.E.M. ($n = 6$–8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. saline control; $^{+}P < 0.05$, $^{++}P < 0.001$ vs. drug-treated control; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs. L-arginine.

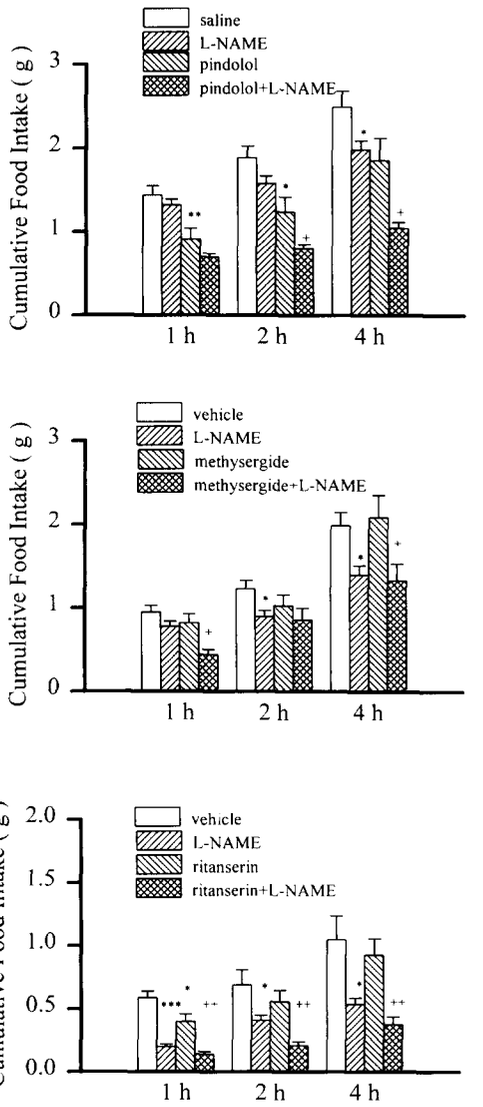

Fig. 2. Effect of 5-HT antagonists (−)-pindolol (2 mg/kg s.c.), methysergide (5 mg/kg s.c.) and ritanserin (5 mg/kg s.c.) on N^G-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg s.c.)-induced anorexia in 24-h fasted mice. Values shown are means ± S.E.M. ($n = 6$–8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. saline control; $^{+}P < 0.05$, $^{++}P < 0.01$ vs. drug-treated control.

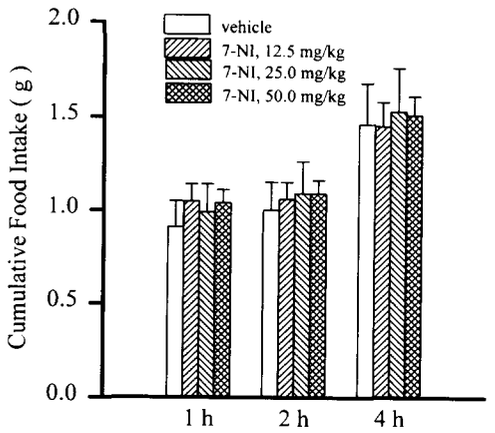

Fig. 3. Effect of 7-nitroindazole (7-NI, 12.5, 25.0 and 50 mg/kg i.p.) on N^G-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg s.c.)-induced anorexia in 24-h fasted mice. Values shown are means ± S.E.M. ($n = 8$).

methyl ester-induced hypophagic effect was observed with methysergide, a non-selective $5HT_1$ and $5HT_2$ antagonist, or with ritanserin treatment, a $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ antagonist (Fig. 2). Treatments with 7-nitroindazole, a specific central NO synthase inhibitor, also failed to reveal any difference in food intake compared to the vehicle-injected group (Fig. 3).

3.2. Effect of N^G-nitro-L-arginine methyl ester on 2-deoxy-D-glucose and diazepam hyperphagia

2-Deoxy-D-glucose increased cumulative food intake at 4 h, $P < 0.05$ (Fig. 4). This hyperphagic response was diminished in the presence of N^G-nitro-L-arginine methyl ester. Diazepam increased ($P < 0.05$) food intake at 2 h and the hyperphagia was also attenuated by N^G-nitro-L-arginine methyl ester treatment.

3.3. Effect of N^G-nitro-L-arginine methyl ester on gastric emptying

Gastric retention of a semi-solid mash diet was significantly higher ($P < 0.05$) in the N^G-nitro-L-arginine methyl ester-treated group when measured 2 h following food consumption Table 1). Higher retention was also observed in the N^G-nitro-L-arginine methyl ester-treated group when measured at 4 h, although this was not statistically significant ($P = 0.07$ and $P = 0.06$ in terms of percent dry and wet weights, respectively). As a consequence of this finding, the participation of CCK-mediated satiety occurring concomitantly with the gastric distension was also explored. Both devazepide (peripheral CCK_A receptor antagonist) and PD 135,158 (central CCK_B receptor antagonist) failed to antagonize the anorectic effect of N^G-nitro-L-arginine methyl ester on 1 and 4 h intake of a chow diet (Fig. 5).

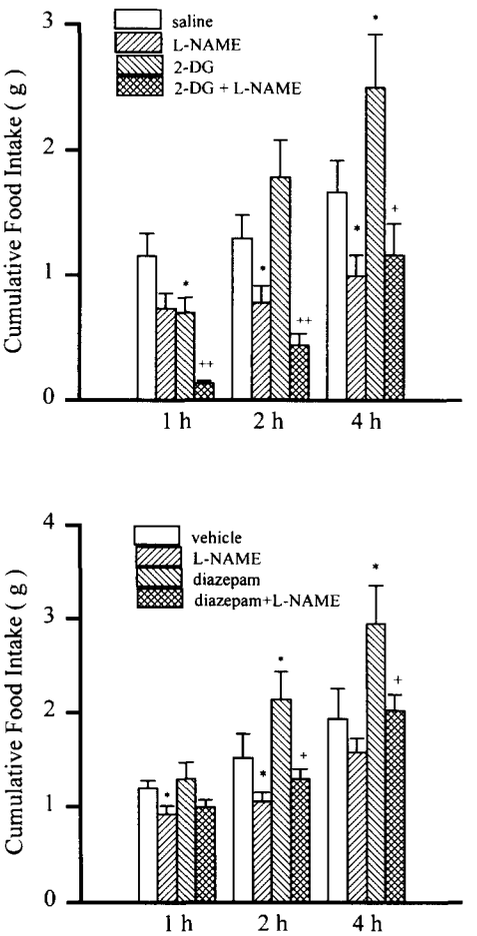

Fig. 4. Effect of orexigenic agents, 2-deoxy-D-glucose (2-DG, 750 mg/kg i.g.) and diazepam (2.5 mg/kg i.p.) on N^G-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg s.c.)-induced anorexia in 24-h fasted mice. Values shown are means ± S.E.M. ($n = 6$–8). * $P < 0.05$ vs. saline control; + $P < 0.05$, ++ $P < 0.01$ vs. drug-treated control.

3.4. Effect of N^G-nitro-L-arginine methyl ester on water consumption, arousal and malaise

Water intake was depressed ($P < 0.05$) in the N^G-nitro-L-arginine methyl ester-treated group during the first hour of rehydration following 24-h water deprivation (Table 2). Subsequent intakes were similar be-

Table 1
Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on gastric emptying in mice given a semi-solid mash diet after a 24-h fast

Treatment	n	Percentage food retention			
		Dry weight (g)		Wet weight (g)	
		2 h	4 h	2 h	4 h
Saline (1.0 ml/100 g)	7	20.8 ± 1.9	9.8 ± 1.7	31.2 ± 2.9	17.5 ± 2.0
L-NAME (50 mg/kg)	7	30.1 ± 3.5 [a]	16.4 ± 2.9	43.5 ± 3.6 [a]	25.7 ± 3.3

Values are means ± S.E.M. Significance from saline group, [a] $P < 0.05$, by Student's t-test.

Table 2
Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on fluid intake in 24-h water-deprived mice when given water or 5% sucrose

Treatment	n	Fluid consumption (ml/animal)		
		1 h	2 h	4 h
Water intake				
Saline (1 ml/100 g)	6	2.30±0.30	0.32±0.04	1.30±0.17
L-NAME (50 mg/kg)	6	1.50±0.22 [a]	0.25±0.04	1.20±0.18
5% Sucrose intake				
Saline (1 ml/100 g)	6	1.58±0.23	0.22±0.03	0.92±0.13
L-NAME (12.5 mg/kg)	6	1.32±0.21	0.13±0.02 [a]	0.75±0.13
L-NAME (25.0 mg/kg)	6	1.52±0.13	0.05±0.02 [b]	0.78±0.07
L-NAME (50.0 mg/kg)	6	0.77±0.12 [a]	0.00±0.00 [b]	0.20±0.03 [b]

Values are means±S.E.M. Significance from saline group, [a] $P < 0.05$, [b] $P < 0.001$, by Student's t-test or Fisher's LSD test following one-way ANOVA.

Table 3
Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on saccharin intake in a two-bottle conditioned taste aversion (CTA) paradigm.

Treatment	n	Water	Saccharin
Saline (0.6 nmol/kg)	7	2.22±0.36	1.23±0.19
LiCl (0.6 nmol/kg)	7	2.37±0.27	0.46±0.10 [a]
L-NAME (50 mg/kg)	6	2.55±0.45	1.18±0.21

Values are means±S.E.M. Significance from saline group, [a] $P < 0.001$, by Fisher's LSD test following one-way ANOVA.

tween the two groups. Consumption of a sweet solution (5% sucrose) was however consistently decreased by N^G-nitro-L-arginine methyl ester (50 mg/kg) up to 4 h ($P < 0.001$) in similarly water-deprived animals. The likelihood of N^G-nitro-L-arginine methyl ester treatment being associated with aversive consequences was also tested. Pairing of a novel-tasting saccharin solution with LiCl, which is known to induce malaise, reduced saccharin consumption upon subsequent LiCl treatment ($P < 0.01$) (Table 3). Pairing of N^G-nitro-L-arginine methyl ester with saccharin and its subsequent introduction did not diminish saccharin consumption when compared to that of saline controls (Table 3).

3.5. Effect of N^G-nitro-L-arginine methyl ester on locomotor activity

Exploratory activity in an open field was time-dependently decreased in N^G-nitro-L-arginine methyl ester-treated animals (ANOVA, $F(2,45) = 5.2$, $P < 0.01$). The average speed of locomotion was also decreased ($P < 0.05$) in the second and third 10-min intervals when compared to that of the control group (Table 4).

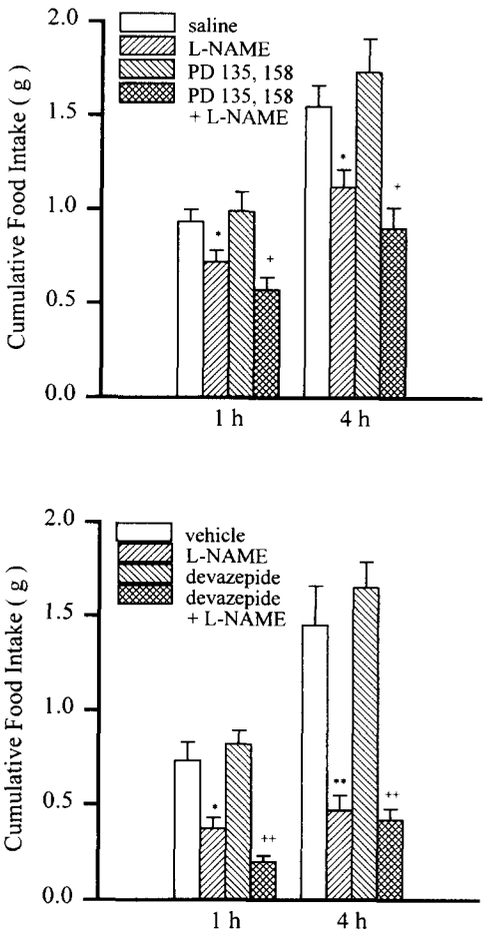

Fig. 5. Effect of central (PD 135,158, 1 mg/kg s.c.) and peripheral (devazepide, 10 mg/kg i.p.) CCK receptor antagonists on N^G-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg s.c.)-induced anorexia in 24-h fasted mice. Values shown are means±S.E.M. ($n = 6$). * $P < 0.05$, ** $P < 0.01$ vs. saline control; + $P < 0.01$, ++ $P < 0.001$ vs. drug-treated control.

4. Discussion

The present results do not support the involvement of 5-HT mechanisms in mediating the effect of NO on

Table 4
Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on locomotor activity of mice in an open field arena

Treatment	n	Average speed (mm/s)			Composite mean speed (mm/s)
		0–10 min	10–20 min	20–30 min	
Saline	16	21.7 ± 1.6	17.9 ± 1.6	17.6 ± 1.2	19.5 ± 1.2
L-NAME (50 mg/kg)	16	19.3 ± 2.2	12.4 ± 1.8 [a,b]	10.8 ± 2.3 [a,b]	14.2 ± 1.9 [a]

Values are means ± S.E.M. Significance from saline group, [a] $P < 0.05$, by Student's t-test. Significance from 0–10 min, [b] $P < 0.05$, by Fisher's LSD test following one-way ANOVA.

feeding. Neither a simultaneous decrease in 5-HT and NO nor a parallel increase in them had any effect on the feeding response under each condition. The hypophagia elicited by N^G-nitro-L-arginine methyl ester was unchanged in spite of a depletion in 5-HT level caused by *p*-chlorophenylalanine. Similarly, the increase in 5-HT level induced by fenfluramine produced a marked anorexia that was not altered by L-arginine. In previous studies, the same dose of L-arginine has been reported to reverse N^G-nitro-L-arginine methyl ester-induced hypophagia but was by itself devoid of any influence on food intake (Morley and Flood, 1992; Squadrito et al., 1994a). A significant decrease in food intake was however observed with L-arginine alone in this study. Since the dose of L-arginine used can saturate nitric oxide synthase (Nathan, 1992), it is possible that the synthesis of NO is maintained at a maximal level under conditions of a constant supply of cofactors. The decrease in food intake by L-arginine as observed in this study could thus be considered as a non-specific occurrence unrelated to the underlying experimental objective of increasing NO synthesis. In this regard, it might be more appropriate to consider L-arginine pretreatment followed by fenfluramine as a condition of increased 5-HT level unassociated with decreases in NO level. The use of NO releasers such as *S*-nitroso-*N*-acetylpenicillamine or 3-morpholinosydnonimine in place of L-arginine was excluded due to possible non-specific effects on feeding secondary to their potent hypotensive actions.

As an alternative approach to investigate the 5-HT involvement in N^G-nitro-L-arginine methyl ester-induced hypophagia, antagonists at different 5-HT receptor sites were used. Previous study has shown that 5-HT_1 but not 5-HT_2 receptor antagonists are capable of reversing the anorectic action of N^G-nitro-L-arginine (Squadrito et al., 1993). The results in this study however failed to confirm the involvement of 5-HT_{1A} and 5-HT_{1B} receptors since (−)-pindolol pretreatment did not attenuate the effect of N^G-nitro-L-arginine methyl ester. Methysergide and ritanserin were similarly shown to be ineffective, suggesting the lack of involvement of 5-HT_{2A} and 5-HT_{2C} receptors. The heightened response to N^G-nitro-L-arginine methyl ester elicited by (−)-pindolol may be due to additivity of drug effects. A slight depressant action of (−)-pindolol on food intake was also observed by Kennett et al. (1986). 5-HT_{1A} inhibition-induced hypophagia was demonstrated with the selective 5-HT_{1A} antagonist, (*S*)-UH-301 (Moreau et al., 1992). These data, taken together, do not support the role of increased 5-HT metabolism in mediating the anorectic action of N^G-nitro-L-arginine methyl ester as suggested previously (Squadrito et al., 1994a,b). The discrepancy cannot be explained by mere differences in the animal model (rat and mice) and drug treatment (N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester) used. An alternative suggestion is that NO may act through multiple mechanisms (central and peripheral) to control feeding. This would allow the expression of the hypophagic action of N^G-nitro-L-arginine methyl ester independently of 5-HT pathways, as found in the present study.

N^G-Nitro-L-arginine methyl ester-induced anorexia is reported to be mediated centrally. This suggestion was supported by results showing an increase in 5-HT metabolism in discrete brain regions (cortex, diencephalon and medulla pons) following N^G-nitro-L-arginine administration peripherally (Squadrito et al., 1994b). Direct intracerebroventricular injection of N^G-nitro-L-arginine also reduced food intake while the same dose administered intravenously was ineffective (Squadrito et al., 1993). In this study, no hypophagic action was observed following administration of 7-nitroindazole. The specificity of 7-nitroindazole for neuronal NO synthase has been well characterized, both in vivo and in vitro. It has the same potency as N^G-nitro-L-arginine methyl ester but has no accompanying cardiovascular effects (Babbedge et al., 1993; Moore et al., 1993). Absorption following administration is rapid, as evidenced by maximal neuronal NO synthase inhibition within 18–30 min following i.p. injection in oil medium (Moore et al., 1993). Its selectivity for neuronal NO synthase in vivo is inferred from studies showing antinociceptive activity and reversal of brain focal infarction when the compound was applied by the peritoneal route (Moore et al., 1993; Yoshida et al., 1994). Inhibition of NO synthase activity in gastric tissues is also absent (Babbedge et al., 1993). Thus, it is rather unlikely that a central site of action is involved in N^G-nitro-L-arginine methyl ester-induced anorexia.

In order to evaluate the anorectic potency of N^G-nitro-L-arginine methyl ester in models other than food deprivation-induced eating, 2-deoxy-D-glucose- and diazepam-induced hyperphagia models were also used. 2-Deoxy-D-glucose induces glucoprivation, which leads to an increase in feeding. Diazepam increases feeding most likely through an enhancement of GABAergic transmission (Montgomery, 1991). The 2-deoxy-D-glucose-and diazepam-induced hyperphagia were both attenuated by N^G-nitro-L-arginine methyl ester. These results, together with the efficacy of N^G-nitro-L-arginine methyl ester in blocking the orexigenic action of neuropeptide Y (Morley and Flood, 1992) suggest that N^G-nitro-L-arginine methyl ester may be a potential therapeutic tool in the management of obesity.

In an attempt to find out the possible mechanism(s) responsible for mediating the effects of N^G-nitro-L-arginine methyl ester on feeding, attention was paid to other peripheral factors such as gastric distention and satiety induction. NO acting as a NANC neurotransmitter has been shown to mediate gastric accommodation (Lefebvre et al., 1992) and to decrease intestinal

motility (Calignano et al., 1992) in vivo. This NANC-mediated relaxation of gastrointestinal smooth muscle has been shown to be abolished by N^G-nitro-L-arginine methyl ester which also initiates phasic intestinal contractions (Calignano et al., 1992) and disruption of gastric cyclic motor activity (Sarna et al., 1992). Spontaneous tonic and phasic contractions of the pylorus are also increased by N^G-nitro-L-arginine methyl ester (Allescher et al., 1992). Since increased pyloric resistance impedes antral emptying into the duodenum, the outcome would be a promotion of gastric stasis and distension, which could eventually lead to satiety. In this study, N^G-nitro-L-arginine methyl ester treatment was associated with significantly greater food retention (50% increase). This is in agreement with the findings of Plourde et al. (1994), who have demonstrated a slow gastric emptying of a non-nutrient solution following N^G-nitro-L-arginine methyl ester administration. Two additional studies also provide strong and convincing evidence in support of the pylorus being a major target of N^G-nitro-L-arginine methyl ester. In human infantile pyloric stenosis, enlargement of the pyloric musculature and a defect in its relaxation causes gastric outlet obstruction. Immunohistochemical studies have pointed to a lack of NO synthase activity within the enteric nerve fibers of the hypertrophied pylorus (Vanderwinden et al., 1992). In a mutant mouse strain deficient in the expression of neuronal NO synthase, similar manifestations of pyloric stenosis have again been observed (Huang et al., 1993). Thus, pyloric stenosis may account for the delayed gastric emptying observed in N^G-nitro-L-arginine methyl ester-treated animals.

Following an increase in intragastric pressure as a result of delayed emptying, it is likely that some mechanisms mediating satiety are invoked to reduce feeding. CCK is known to be a potent satiety signal (McHugh and Moran, 1986) and hence the effect of blockade of CCK receptors on N^G-nitro-L-arginine methyl ester-induced anorexia was investigated. Our results showed that neither devazepide nor PD 135,158 was capable of antagonizing the anorexia elicited by N^G-nitro-L-arginine methyl ester, therefore implying the lack of involvement of both peripheral and central CCK mechanisms. Furthermore, the lack of discrimination between a caloric and non-caloric meal in the expression of the anorectic action of N^G-nitro-L-arginine methyl ester also suggested that the effect was not dependent on the release of a nutrient-sensitive intestinal factor which acts on the pyloric or gastric musculature to delay emptying. The satiety effect produced by gastric distention may be related to the activation of vagal afferents sensitive to increases in gastric muscular tone (Flanagan et al., 1989).

As some anorectic agents are known to reduce food intake through the production of malaise, the possible aversive consequence of N^G-nitro-L-arginine methyl ester treatment was therefore studied. Results showed that N^G-nitro-L-arginine methyl ester did not reduce saccharin intake in a two-bottle conditioned taste aversion (CTA) paradigm. This suggests that the production of sickness or aversive consequences is unlikely to be the reason for the reduction in food intake.

The reduction in food intake induced by N^G-nitro-L-arginine methyl ester is also unlikely to be related to a non-specific depressant effect on ingestive behavior. Water consumption in animals motivated to drink by prior 24-h liquid deprivation was unaffected by N^G-nitro-L-arginine methyl ester treatment, except during the first hour. The intake of a sweet solution under similar conditions was however consistently depressed by N^G-nitro-L-arginine methyl ester. This provides some suggestion for a deficit in the arousal and attention status of the animal upon presentation of a favorable stimulus (5% sucrose). Additional support for this contention is that the exploratory activity of food-deprived animals in an open field was also time-dependently reduced by N^G-nitro-L-arginine methyl ester treatment. This sedative property of N^G-nitro-L-arginine methyl ester on locomotor activity and exploratory behaviour has been also observed by Moore et al. (1991) under non-fasted condition, with the exception that it was recorded only at higher doses (600 mg/kg). It is possible that the sedative action of N^G-nitro-L-arginine methyl ester may combine with the gastric distention factor to promote a reduction in food intake, at least during the first hour of feeding. This hypothesis is supported by the fact that both water and food consumption were reduced in the first hour whereas only food consumption remained depressed up to 4 h. In the latter circumstance, the action of gastric distention may predominate. Elaborate behavioral assessment involving simultaneous monitoring of locomotor activity and feeding pattern may provide evidence for the presence of such an interaction.

The dose of N^G-nitro-L-arginine methyl ester (50 mg/kg) used in this study has been shown to cause a 30–40% increase in blood pressure in mice by Moore et al. (1991). This hypertensive effect of N^G-nitro-L-arginine methyl ester may contribute to its hypophagic action. However, it has been shown that the increase in blood pressure cannot be overcome by blockade of the autonomic nervous and renin-angiotensin system (Wang and Pang, 1991). In this regard, there is a dearth of procedures available for antagonizing the hypertensive component of the action of N^G-nitro-L-arginine methyl ester. In spite of this, it has been demonstrated by other workers (Knardahl, 1986; Niewiadowska and Lukaszewska, 1988; Shido and Nagasaka, 1989) that both feeding motivation and locomotor activity are enhanced in spontaneously hypertensive rats (SHR). These characteristics are seemingly in contrast to that

exhibited by N^G-nitro-L-arginine methyl ester-treated animals. Therefore, the involvement of hypertension in N^G-nitro-L-arginine methyl ester-induced hypophagia is unlikely.

In conclusion, this study suggests that peripherally mediated mechanisms working through the induction of gastric distension and its consequent promotion of satiety may be involved in the hypophagic effects of N^G-nitro-L-arginine methyl ester.

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