EXPERIMENTAL STUDY

Contrasting effects of nitric oxide on food intake and GH secretion stimulated by a GH-releasing peptide

Antonello E Rigamonti, Silvano G Cella, Guido M Cavallera, Romano Deghenghi¹, Vittorio Locatelli, Nicolaos Pitsikas² and Eugenio E Müller

Department of Medical Pharmacology, University of Milan, Milan, Italy, ¹Europeptides, Argenteuil, France and ²Boehringer Ingelheim Italia SpA, Milan, Italy

(Correspondence should be addressed to E E Müller, Department of Medical Pharmacology, University of Milan, via Vanvitelli 32 20129 Milan, Italy; Email: eugenio.muller@unimi.it)

Abstract

Objective: Among the many actions of nitric oxide (NO) are those on endocrine and feeding behaviour. Based on NO involvement in the GH-releasing effect of the GH-releasing peptides (GHRPs) and the reported orexigenic activity of these compounds, we sought to evaluate the effect of the combined administration of a long-acting NO donor, molsidomine, and the newly synthesized GHRP EP92632 on food intake and GH secretion in rats. Moreover, to verify the specificity of a potential NO involvement, we evaluated whether or not the effects of GHRPs were abolished by a pre-treatment with an inhibitor of NO synthase, N-nitro-arginine-methyl-ester (NAME).

Methods: In the food intake experiments, adult Sprague–Dawley male rats underwent acute administration of: (1) EP92632 (160 µg/kg, s.c.); (2) molsidomine (100 mg/kg, i.p.); (3) EP92632+molsidomine; (4) L-NAME (40 and 60 mg/kg, i.p.); (5) EP92632+L-NAME (60 mg/kg, i.p.); (6) EP92632+molsidomine+L-NAME (60 mg/kg, i.p.); and (7) 0.9% saline (0.1 ml/kg, i.p.). After treatments, the cumulative food intake in the 6 post-treatment hours was carefully evaluated. In the neuroendocrine experiments, rats were given the same compounds according to the above reported schedule, except for the use of one dose of NAME (60 mg/kg, i.p.) and a lower EP92632 dose (80 µg/kg, s.c.), and were sampled via atrial cannula.

Results: EP92632 significantly stimulated food intake, an effect which was further enhanced by molsidomine, though the latter did not elicit *per se* any orexigenic effect. L-NAME given alone significantly decreased food intake and abolished the orexigenic effect of the GHRP and the enhancing effect of molsidomine. Plasma GH levels increased significantly following administration of EP92632 but, in contrast to the food intake experiments, molsidomine significantly inhibited both basal and EP92632-stimulated GH secretion; moreover, NAME had a biphasic effect on the EP92632-stimulated GH release: initially inhibitory and then, from 45 min on, stimulatory. NAME did not affect basal GH levels but, surprisingly, combined administration of molsidomine and NAME induced a striking inhibition of both basal and the peptide-stimulated GH release.

Conclusions: In summary, these data indicate that NO in the rat is physiologically involved in a stimulatory way in the GHRP-mediated effect on food intake, but exerts a dual action, probably stimulatory at hypothalamic and inhibitory at pituitary levels, on basal and GHRP-stimulated GH secretion.

European Journal of Endocrinology 144 155–162

Introduction

Nitric oxide (NO) is a highly reactive gas with many actions (1, 2). In the last few years, it has been reported that NO acts as neurotransmitter/neuromodulator also in the endocrine system (3–5), including the somatotrophic axis (6–8). In addition, many data suggest that NO exerts a physiological role in the regulation of food intake (9–14).

Three main isoforms of NO synthase (NOS), the enzyme responsible of the synthesis of NO from

L-arginine, have been characterized: the brain NOS (b-NOS) and the endothelial NOS (e-NOS), which are constitutive enzymes; and the macrophagic NOS, which is an inducible enzyme (i-NOS) (1, 15).

The prevalent localization of the b-NOS in different hypothalamic areas (16, 17), but also at pituitary level (3), raises the issue of the possible dual action of NO as neuroendocrine modulator at either site.

Concerning the somatotrophic axis, haemoglobin, which is known to strongly bind NO, or N-methyl-L-arginine, a NOS inhibitor, potentiated in a dose-related

manner the release of growth hormone (GH) stimulated by growth hormone-releasing hormone (GHRH) from rat pituitary cell cultures, without affecting the basal secretion of GH (18). Since, conversely, nitroprusside, which releases NO, suppressed this response, Kato's observations were compatible with an inhibitory role of NO on pituitary GH release. In contrast, other *in vivo* and *in vitro* experiments (7) have shown that NO, endogenously produced, exerts a facilitatory role on the pituitary in the GH secretion induced by GHRH or GH-releasing peptides (GHRPs). The latter are synthetic compounds which act on specific receptors, different from those of GHRH and of other GH secretagogues, but are extremely effective in stimulating somatotroph function in both animals and humans (19–27). It has also been reported that GHRH increases mRNA levels and the release of somatostatin (SS) in the rat periventricular nucleus via a NO-mediated mechanism (6).

These observations recently prompted us to study the role of the NOergic system in the control of GH secretion in the dog, using as a tool Hexarelin, a potent GHRP analogue (28, 29). The main findings of these studies were that erithrityl tetranitrate, a liposoluble NO-donor compound, strikingly enhanced GH secretion stimulated by Hexarelin in both young and old dogs. Pre-treatment with N-nitro-L-arginine-methyl-ester (L-NAME), a NOS inhibitor, abolished GH release induced by the peptide in young dogs but, paradoxically, enhanced that of old dogs. N-nitro-D-arginine-methyl-ester (D-NAME), the inactive stereoisomer, did not affect GH response to Hexarelin in either young or old dogs (30).

Concerning NO involvement in behavioural aspects, some authors have reported that NOS inhibition induces in the mouse a reduction of food intake under basal conditions or when stimulated by neuropeptide Y (NPY), a potent orexigenic peptide (10, 12), and a similar effect was present in obese Zucker rats treated with NOS antagonists (31). Interestingly, fasting, in turn, increases in the rat the activity of b-NOS in the hypothalamus (32), as it occurs for orexigenic neuropeptides (33).

On the basis of NO involvement in the GH-releasing effect of GHRPs (see above) and reports that some GHRP molecules have an orexigenic activity (34–37), the present study was aimed at evaluating in the rat the GH-releasing and orexigenic effects of the combined administration of molsidomine, a long-acting NO-donor, and EP92632, an analogue of Hexarelin, endowed with GH-releasing and orexigenic actions (A E Rigamonti, unpublished results). In addition, the study sought to assess whether pre-treatment with L-NAME counteracted the neuroendocrine and orexigenic effects of the GHRPs.

Materials and methods

Fifty Sprague–Dawley male rats (200–250 g body weight) were used. Rats were provided with dry food (Standard Diet, Charles River, Calco, Italy) and water,

and allowed to feed *ad libitum*. They were in a 12 h light: 12 h darkness regimen, with light on at 0700 h.

Food intake experiments

Two days before testing, rats were placed singularly in a clear Plexiglas cage. According to a cross-over experimental design, rats were treated with (1) 0.9% physiological saline (1 ml/kg; i.p.; –60 min)+EP92632 (Tyr-Hexarelin; Europeptides, Argenteuil, France; 160 µg/kg, s.c.; 0 min); (2) molsidomine (Sigma-Aldrich, Milan, Italy; 100 mg/kg, i.p.; –60 min)+saline (1 ml/kg, i.p.; 0 min); (3) EP92632+molsidomine; (4) NAME (L-NAME; Sigma-Aldrich; 40 and 60 mg/kg, i.p.; –60 min)+saline (1 ml/kg; i.p.; 0 min); (5) NAME (60 mg/kg, i.p.; –60 min)+EP92632; (6) NAME+molsidomine (in combination at time –60 min)+saline; (7) NAME+molsidomine+EP92632; (8) saline+saline. Doses of NAME used in these experiments, selected according to the studies of Tena-Sempere *et al.* (7), did not affect blood pressure in the rat (Dr M Bernareggi, personal communication).

Each rat was injected six or seven times maximally, with an interval between two subsequent injections of at least 48 h. Each rat received at least one saline injection.

Starting at 2100 h, rats were provided a pre-measured amount of food. To ensure that the rats were satiated, they were given a freshly prepared diet consisting of standard pellets. After administration of the above compounds, the cumulative food intake was carefully evaluated every hour for 6 h, according to a standard procedure (36).

Neuroendocrine experiments

Groups of six rats were treated with the same compounds according to the schedule used in the food intake experiments, the only exception being the use of one dose of NAME (60 mg/kg, i.p.) and a lower EP92632 dose (80 µg/kg, s.c.). In these experiments, a lower dose of the peptide was chosen because with the dose used in food intake experiments a maximal GH release is elicited (data not shown).

A polyethylene cannula was inserted into the right atrium of rats anaesthetized with ketamine (0.05 ml/100 g, i.p.) and xylazine (0.1 ml/100 g, i.p.) to allow unstressed samplings. At times 0, 10, 15, 30, 45, 60 and 90 min 300 µl of blood were drawn from the cannula and were replaced by isovolumetric amounts of physiological saline.

Blood was centrifuged and plasma samples stored at –20 °C until assessment of rat GH (rGH) by RIA assay.

Plasma rGH concentrations

Plasma rGH concentrations were determined by a double antibody RIA. Highly purified amounts of this

hormone as standard and for iodination and the related antibody were kindly provided by Dr A F Parlow (Pituitary Hormones and Antisera Center, Torrance, CA, USA). The sensitivity of this assay was 0.39 ng/ml; intra-assay variability was 5%. To avoid possible interassay variation, all samples of a given experiment were assayed in a single RIA.

Statistical analysis

Food intake was expressed as a cumulative value of the food ingested at the test meal after administration of the compounds until 6 h. Plasma rGH concentrations were evaluated either as absolute mean values (ng/ml) $\pm$ S.E.M. or as area under the GH response curve (AUC_{0–90 min}: ng/ml per min), calculated by the trapezoid method.

Statistical comparisons of the mean value were performed by Bonferroni test, preceded by ANOVA; $P < 0.05$ was taken to be statistically significant.

Results

Food intake experiments

EP92632 significantly stimulated food intake (3.4 ± 0.6 vs 2.6 ± 0.7 g, $P < 0.01$), an effect particularly prominent in the first 2 h. This effect was further enhanced by molsidomine (6.4 ± 0.6 g, $P < 0.01$), though the latter did not elicit *per se* any orexigenic effect (Fig. 1a). NAME given alone (40 and 60 mg/kg, i.p.) dose-dependently decreased food intake (2.0 ± 0.8 and 1.0 ± 0.6 g respectively, $P < 0.01$ vs saline+saline) (Fig. 1b); moreover, at the dose of 60 mg/kg, i.p. it abolished the orexigenic effect of the GHRP and the enhancing effect of molsidomine (1.9 ± 0.4 and 2.1 ± 0.4 g, $P < 0.01$ vs saline+saline) (Fig. 1c and d). Administration of NAME in the molsidomine-treated rats further reduced basal food intake (1.1 ± 0.6 , $P < 0.01$ vs saline+saline) (Fig. 1d).

Neuroendocrine experiments

Plasma GH levels were increased significantly by administration of EP92632 (AUC_{rGH 0–90}: 18617.9 ± 2267.8 ng/ml per min vs 10267.1 ± 1445.6 ng/ml per min, $P < 0.01$) (Table 1). The GH-releasing effect of the peptide was evident at each post-injection period ($P < 0.01$) (Fig. 2). In contrast to the pattern of the food-intake experiments, molsidomine significantly inhibited both basal and EP92632-stimulated GH secretion at 10, 15 and 30 min and at 30 min respectively ($P < 0.01$ vs saline+saline) (Fig. 2). These results were confirmed by the AUC data only when related to the peptide-stimulated GH release (AUC_{rGH 0–90}: 12378.3 ± 1643.0 ng/ml per min vs 18617.9 ± 2267.8 ng/ml per min; $P < 0.01$) (Table 1).

Table 1 Areas under curves of plasma rGH concentrations following different treatments.

Treatment	AUC _{rGH 0–90 min} (ng/ml per min)
Saline+saline	10267.1 ± 1445.6
Molsidomine+saline	10590.6 ± 2159.2
NAME+saline	11540.3 ± 1421.7
NAME/molsidomine+saline	4307.8 ± 688.1^a
Saline+EP92632	18617.9 ± 2267.8^a
Molsidomine+EP92632	12378.3 ± 1643.0^b
NAME+EP92632	17533.2 ± 1655.1^a
NAME/molsidomine+EP92632	14032.3 ± 1828.3^b

^a $P < 0.01$ vs saline+saline.

^b $P < 0.01$ vs saline+EP92632.

NAME, paradoxically, had a biphasic effect. Initially it inhibited the EP92632 GH-releasing effect at 10, 15 and 30 min ($P < 0.01$); then a marked elevation of GH release over values present in rats given GHRP alone was present from 45 min onwards (at 60 min; $P < 0.01$) (Fig. 3). The dual effects of NAME cancelled each other when differences in the AUC vs the EP92632 alone treated group were evaluated (AUC_{rGH 0–90}: 17533.2 ± 1655.1 ng/ml per min vs 18617.9 ± 2267.8 ng/ml per min; $P = \text{NS}$) (Table 1). NAME did not affect basal GH levels (AUC_{rGH 0–90}: 11540.3 ± 1421.7 ng/ml per min; $P = \text{NS}$ vs saline+saline) (Table 1).

Combined pre-treatment of molsidomine plus NAME induced a striking inhibition of the peptide-stimulated GH release (at 10 and 30 min vs saline+EP92632; $P < 0.01$; AUC_{rGH 0–90}: 14032.3 ± 1828.3 ng/ml per min; $P < 0.01$ vs saline+EP92632) (Fig. 4; Table 1), which was greater than the inhibitory action on GH release exerted by molsidomine alone. Interestingly, co-administration of these compounds also reduced basal GH levels (10, 15, 30 and 45 min; $P < 0.01$ vs saline+saline; AUC_{rGH 0–90}: 4307.8 ± 688.1 ng/ml per min; $P < 0.01$ vs saline+saline) (Fig. 4; Table 1).

Discussion

To our knowledge there are no specific pharmacokinetic studies on the penetration of NAME and molsidomine through the blood–brain barrier (BBB). However, though inferentially, such a possibility is supported by many studies on the neuroendocrine effects of these compounds (38–43) and the lack of BBB in some brain areas, namely at the pituitary–medial–basal–hypothalamic level (44).

In this study in the rat, molsidomine enhanced the orexigenic effect of a GHRP whereas, conversely, NAME reduced both basal and GHRP-stimulated feeding behaviour. Unexpectedly, molsidomine (and NAME), administered either alone or together, inhibited basal and GHRP-induced GH release.

Figure 1 Cumulative food intake at 6 h in rats treated with: (a) 0.9% saline (0.1 ml/kg, i.p.), molsidomine (100 mg/kg, i.p.), EP92632 (160 µg/kg, s.c.) or molsidomine+EP92632, $*P<0.01$ vs saline; (b) 0.9% saline or L-NAME (40 and 60 mg/kg, i.p.), $*P<0.01$ vs saline; (c) 0.9% saline (0.1 ml/kg, i.p.), EP92632 (160 µg/kg, s.c.) or L-NAME (60 mg/kg, i.p.) + EP92632, $*P<0.01$ vs saline, $**P<0.01$ vs EP92632; (d) 0.9% saline (0.1 ml/kg, i.p.), NAME (60 mg/kg, i.p.)+molsidomine (100 mg/kg, i.p.)+saline, EP92632 (160 µg/kg, s.c.) or L-NAME+molsidomine+EP92632, $*P<0.01$ vs saline, $**P<0.01$ vs saline+EP92632. For this and following figures see text for details. Values are means $\pm$ S.E.M.

These puzzling findings may be compatible with a GHRP mechanism of action exerted at both hypothalamic and pituitary levels and with a dual action of NO, acting as neuroendocrine modulator at either site (45, 46).

In particular, to explain these findings, NO has to be viewed as an enhancer of the hypothalamic GHRP orexigenic and GH-releasing mechanisms but also, for GH secretion, as a compound endowed with an inhibitory action on the pituitary.

Figure 2 Profiles of plasma rGH concentrations in rats treated with 0.9% saline (0.1 ml/kg, i.p.), saline+molsidomine (100 mg/kg, i.p.), saline+EP92632 (160 µg/kg, s.c.) or molsidomine+EP92632. * $P < 0.01$ vs saline, ** $P < 0.01$ vs saline+EP92632. Values are means $\pm$ S.E.M.

Figure 4 Profiles of plasma rGH concentrations in rats treated with 0.9% saline (0.1 ml/kg, i.p.), NAME (60 mg/kg, i.p.)+molsidomine (100 mg/kg, i.p.)+saline, saline+EP92632 (160 µg/kg, s.c.) or L-NAME+molsidomine+EP92632. * $P < 0.01$ vs saline, ** $P < 0.01$ vs saline+EP92632. Values are means $\pm$ S.E.M.

This view is supported by *in vitro* studies showing that NOergic neurons play a permissive role in the medial basal hypothalamus to stimulate the release of many regulatory peptides, as corticotrophin-releasing hormone, luteinizing hormone releasing-hormone, GHRH, somatostatin and oxytocin (46). NO, however, has also been reported in some (18, 47), though not all

(7, 48), studies to inhibit GHRH-induced GH release from rat pituitary cell cultures and to affect basal and dopamine inhibition of prolactin secretion (5).

The results of this *in vivo* rat study, which showed an inhibitory action of either the NO donor or NOS antagonist on the GH response to GHRP, are in contrast with previous findings in young dogs, where erythritol tetranitrate, a NO donor, enhanced GH release stimulated by the GHRP peptide, Hexarelin, an effect which was abolished by NAME (30). The existence of species differences, and/or a different pharmacokinetic profile of molsidomine vs erythritol tetranitrate cannot be ruled out (49) as possible explanations of the discrepancy.

In our study there was a non-specific plasma GH rise following saline administration, an effect likely due to the use of the xylazine-ketamine combination as an anaesthetic (50, 51). However, the uniform use of this anaesthesia in all experimental groups should have lessened this problem.

Regarding its effect on feeding behaviour, a wealth of information has been provided that NO acts centrally. For instance, alterations in the feeding state of rodents result in changes in NOS levels in the hypothalamus (52); the ob/ob mouse has elevated hypothalamic levels of NOS mRNA; administration of a NOS antagonist induces a marked decrease in food intake, and weight loss (11, 13).

The view proposed above does not rule out the possibility that two distinct NOergic pathways exist in the hypothalamus, respectively modulating the neuro-endocrine and orexigenic actions of GHRPs. In this context, it is noteworthy that in the rat the orexigenic

Figure 3 Profiles of plasma rGH concentrations in rats treated with 0.9% saline (0.1 ml/kg, i.p.) or saline+L-NAME (60 mg/kg, i.p.), saline+EP92632 (160 µg/kg, s.c.) or NAME+EP92632. * $P < 0.01$ vs saline, ** $P < 0.01$ vs saline+EP92632. Values are means $\pm$ S.E.M.

activity of some GHRPs appears to be divorced from the GH-releasing effect (36), pointing to the existence of different receptor or sub-receptor systems for GHRPs (53).

In accordance with the model of a dual action of NO on the hypothalamus and the pituitary, in our study, pre-treatment with molsidomine enhanced the GHRP-induced orexia for the exclusive stimulatory effect of NO at hypothalamic level, whereas molsidomine's decrease of the GHRP-induced GH release was probably due to the prevalent inhibition exerted by NO function impinging on somatotroph cells (pituitary 'closed gate'). In this context, the stimulatory effect of NO, due to molsidomine's action on the hypothalamic component of the GHRP machinery for GH release (26, 27), would have been masked by a downstream NO-mediated pituitary blockade.

In our study, pre-treatment with NAME, as expected, abolished GHRP-stimulated feeding behaviour for deprivation of NOergic function. This may also apply to NAME for the seemingly paradoxical reduction of the GH release induced by GHRP. In fact, although under these circumstances, pituitary NOS inhibition should have prompted release of GH from somatotrophs after the GHRP challenge – and, presumably, of other GH secretagogues – (pituitary 'opened gate'), abolishment of intra-hypothalamic NO function would suppress the hypothalamic machinery underlying the GHRP-induced GH release. The dual effect induced by NAME pre-treatment at hypothalamic and pituitary level would also be manifested by the delayed increase of GH response to GHRP, when the inhibitory hypothalamic effect of NAME subsides (54, 55) and the hypothalamic GHRP pathway returns to be active.

Combined NAME and molsidomine pre-treatment strikingly decreased both baseline food intake and plasma GH concentrations. These effects are presently beyond any sound interpretation. However, due to the extent of the reduction in the two parameters, they cannot be an artefact.

The consistency of our experimental results rests on the specificity of NAME and molsidomine effects on the hormonal and behaviour parameters. That the effects of NAME were specific and unrelated to the drug's vasoconstrictive activity is supported by its ability to abolish the GHRH-induced GH release also *in vitro* (7) and its failure to block GHRH-stimulated GH release also when given simultaneously with GHRH, despite its immediate vasoconstrictive effects (7). Finally, NAME at the doses used did not affect blood pressure (see Materials and methods), and, in addition, vasoconstriction, as that present in spontaneously hypertensive rats, is associated with an increased rather than a decreased responsiveness to GHRH (56). Concerning feeding, the ability of NAME to inhibit this behaviour also in obese Zucker rats, an experimental model of obesity with hypertension (57), and the ineffectiveness of molsidomine to affect basal food intake in our study

suggest the independence of the effects of these compounds from potential cardiovascular effects.

In summary, reports that leptin and NPY, two powerful modulators of feeding behaviour, alter hypothalamic NOS expression (9) and our findings that the orexigenic effect of GHRPs is modulated by NO strengthen the view that the latter plays an important role in the control of feeding in rats (this study) and dogs (30, 37). However, inconsistencies still remain which hinder proper evaluation of NO involvement in GH regulation, and also, partly, in the control of feeding, too. They need to be clarified before GHRP/NO donor compounds, endowed with strong orexigenic (and neuroendocrine?) actions, may be developed.

Acknowledgements

This work was partially supported by CNR Strategic Project 'Biology of aging and its consequences on Public Health System', Rome, Italy.

References

- Moncada S & Higgs EA. Endogenous nitric oxide: physiology, pathology and clinical relevance. *European Journal of Clinical Investigation* 1991 **21** 361–374.
- Moncada S. Nitric oxide gas: mediator, modulator, and pathophysiological entity. *Journal of Laboratory and Clinical Medicine* 1992 **120** 187–191.
- Ceccatelli S, Hulting AL, Zhang X, Gustafsson L, Villar M & Hökfelt T. Nitric oxide synthase in the rat anterior pituitary gland and the role of nitric oxide in regulation of luteinizing hormone secretion. *PNAS* 1993 **90** 11292–11296.
- Chiodera P, Volpi R & Coiro V. Inhibitory control of nitric oxide on the arginine-vasopressin and oxytocin response to hypoglycaemia in normal men. *Neuroreport* 1994 **5** 1822–1824.
- Duvilanski BH, Zambruno C, Seilicovich A, Pisera D, Lasaga M, Diaz MC *et al.* Role of nitric oxide in control of prolactin release by the adenohypophysis. *PNAS* 1995 **92** 170–174.
- Aguila MC. Growth hormone-releasing factor increases somatostatin release and mRNA levels in the rat periventricular nucleus via nitric oxide by activation of guanylate cyclase. *PNAS* 1994 **91** 782–786.
- Tena-Sempere M, Pinilla L, Gonzalez D & Aguilar E. Involvement of endogenous nitric oxide in the control of pituitary responsiveness to different elicitors of growth hormone release in prepubertal rats. *Neuroendocrinology* 1996 **64** 146–152.
- Volpi R, Chiodera P, Caffarri G, Vescovi PP, Capretti L, Gatti C *et al.* Influence of nitric oxide on hypoglycemia- or angiotensin II-stimulated ACTH and GH secretion in normal men. *Neuropeptides* 1996 **30** 528–532.
- Morley JE, Alshaher MM, Farr SA, Flood JF & Kumar VB. Leptin and neuropeptide Y (NPY) modulate nitric oxide synthase: further evidence for a role of nitric oxide in feeding. *Peptides* 1999 **20** 595–600.
- Morley JE & Flood JF. Competitive antagonism of nitric oxide synthase causes weight loss in mice. *Life Sciences* 1992 **51** 1285–1289.
- Morley JE & Flood JF. Effect of competitive antagonism of NO synthase on weight and food intake in obese and diabetic mice. *American Journal of Physiology* 1994 **266** R164–R168.
- Morley JE & Flood JF. Evidence that nitric oxide modulates food intake in mice. *Life Sciences* 1991 **49** 707–711.
- Morley JE, Kumar VB, Mattammal M & Villareal DT. Measurement of nitric oxide synthase and its mRNA in genetically obese (ob/ob) mice. *Life Sciences* 1995 **57** 1327–1331.

- 14 Morley JE, Kumar VB, Mattammal MB, Farr S, Morley PM & Flood JE. Inhibition of feeding by a nitric oxide synthase inhibitor: effects of aging. *European Journal of Pharmacology* 1996 **311** 15–19.
- 15 Knowles RG. Nitric oxide biochemistry. *Biochemical Society Transactions* 1997 **25** 895–901.
- 16 Bredt DS. Targeting nitric oxide to its targets. *Proceedings of the Society for Experimental Biology and Medicine* 1996 **211** 41–48.
- 17 Bhat G, Mahesh VB, Aguan K & Brann DW. Evidence that brain nitric oxide synthase is the major nitric oxide synthase isoform in the hypothalamus of the adult female rat and that nitric oxide potentially regulates hypothalamic cGMP levels. *Neuroendocrinology* 1996 **64** 93–102.
- 18 Kato M. Involvement of nitric oxide in growth hormone (GH)-releasing hormone-induced GH secretion in rat pituitary cells. *Endocrinology* 1992 **131** 2133–2138.
- 19 Micic D, Casabiell X, Gualillo O, Pombo M, Dieguez C & Casanueva FF. Growth hormone secretagogues: the clinical future. *Hormone Research* 1999 **51** 29–33.
- 20 Deghenghi R, Boutignon F, Luoni M, Grilli R, Guidi M & Locatelli V. Small peptides as potent releasers of growth hormone. *Journal of Pediatric Endocrinology and Metabolism* 1995 **8** 311–313.
- 21 Gertz BJ, Barrett JS, Eisenhandler R, Krupa DA, Wittreich JM, Seibold JR *et al.* Growth hormone response in man to L-692 429, a novel nonpeptide mimic of growth hormone-releasing peptide-6. *Journal of Clinical Endocrinology and Metabolism* 1993 **77** 1393–1397.
- 22 Ghigo E, Arvat E, Muccioli G & Camanni F. Growth hormone-releasing peptides. *European Journal of Endocrinology* 1997 **136** 445–460.
- 23 Howard AD, Feighner SD, Cully DF, Arena JP, Liberators PA, Rosenblum CI *et al.* A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 1996 **273** 974–977.
- 24 Jacks T, Hickey G, Judith F, Taylor J, Chen H, Krupa D *et al.* Effects of acute and repeated intravenous administration of L-692 585, a novel non-peptidyl growth hormone secretagogue, on plasma growth hormone, IGF-I, ACTH, cortisol, prolactin, insulin, and thyroxine levels in beagles. *Journal of Endocrinology* 1994 **143** 399–406.
- 25 Muccioli G, Ghe C, Ghigo MC, Papotti M, Arvat E, Boghen MF *et al.* Specific receptors for synthetic GH secretagogues in the human brain and pituitary gland. *Journal of Endocrinology* 1998 **157** 99–106.
- 26 Müller EE, Locatelli V & Cocchi D. Neuroendocrine control of growth hormone secretion. *Physiological Reviews* 1999 **79** 511–607.
- 27 Locatelli V & Torsello A. Growth hormone secretagogues: focus on the growth hormone-releasing peptides. *Pharmacological Research* 1997 **36** 415–423.
- 28 Cella SG, Locatelli V, Poratelli M, De Gennaro Colonna V, Imbimbo BP, Deghenghi R *et al.* Hexarelin, a potent GHRP analogue: interactions with GHRH and clonidine in young and aged dogs. *Peptides* 1995 **16** 81–86.
- 29 Torsello A, Grilli R, Luoni M, Guidi M, Ghigo MC, Wehrenberg WB *et al.* Mechanism of action of Hexarelin I. Growth hormone-releasing activity in the rat. *European Journal of Endocrinology* 1996 **135** 481–488.
- 30 Rigamonti AE, Cella SG, Marazzi N & Müller EE. The nitric oxide (NO) modulation of the GH-releasing activity of Hexarelin in young and old dogs. *Metabolism* 1999 **48** 176–182.
- 31 Squadrito F, Calapai G, Cucinotta D, Altavilla D, Zingarelli B, Ioculano M *et al.* Anorectic activity of NG-nitro-L-arginine, an inhibitor of brain nitric oxide synthase, in obese Zucker rats. *European Journal of Pharmacology* 1993 **230** 125–128.
- 32 Squadrito F, Calapai G, Altavilla D, Cucinotta D, Zingarelli B, Campo GM *et al.* Food deprivation increases brain nitric oxide synthase and depresses brain serotonin levels in rats. *Neuropharmacology* 1994 **33** 83–86.
- 33 Calapai G, Corica F, Allegra A, Corsonello A, Sautebin L, De Gregorio T *et al.* Effects of intracerebroventricular leptin administration on food intake, body weight gain and diencephalic nitric oxide synthase activity in the mouse. *British Journal of Pharmacology* 1998 **125** 798–802.
- 34 Locke W, Kirgis HD, Bowers CY & Abdoh AA. Intracerebroventricular growth hormone-releasing peptide-6 stimulates eating without affecting plasma growth hormone responses in rats. *Life Sciences* 1995 **56** 1347–1352.
- 35 Okada K, Ishii S, Minami S, Sugihara H, Shibasaki T & Wakabayashi I. Intracerebroventricular administration of the growth hormone-releasing peptide KP-102 increases food intake in free-feeding rats. *Endocrinology* 1996 **137** 5155–5158.
- 36 Torsello A, Luoni M, Schweiger F, Grilli R, Guidi M, Bresciani E *et al.* Novel hexarelin analogs stimulate feeding in the rat though a mechanism not involving growth hormone release. *European Journal of Pharmacology* 1998 **360** 123–129.
- 37 Rigamonti AE, Cella SG, Marazzi N & Müller EE. Six-week treatment with Hexarelin in young dogs: evaluation of the GH responsiveness to acute Hexarelin or GHRH administration, and of the orexigenic effect of Hexarelin. *European Journal of Endocrinology* 1999 **141** 313–320.
- 38 Gonzalez D & Aguilar E. *In vitro*, nitric oxide (NO) stimulates LH secretion and partially prevents the inhibitory effect of dopamine on PRL release. *Journal of Endocrinological Investigation* 1999 **22** 772–780.
- 39 Bugajski J, Gadek-Michalska A, Glod R, Borycz J & Bugajski AJ. Blockade of nitric oxide formation impairs adrenergic-induced ACTH and corticosterone secretion. *Journal of Physiology and Pharmacology* 1999 **50** 327–334.
- 40 Pinilla L, Tena-Sempere M, Gonzalez D & Aguilar E. The role of nitric oxide in the control of basal and LHRH-stimulated LH secretion. *Journal of Endocrinological Investigation* 1999 **22** 340–348.
- 41 Fisker S, Nielsen S, Ebdrup L, Bech JN, Christiansen JS, Pedersen EB *et al.* The role of nitric oxide in L-arginine-stimulated growth hormone release. *Journal of Endocrinological Investigation* 1999 **22** 89–93.
- 42 Maccario M, Oleandri SE, Procopio M, Grottoli S, Avogadri E, Camanni F *et al.* Comparison among the effects of arginine, a nitric oxide precursor, isosorbide dinitrate and molsidomine, two nitric oxide donors, on hormonal secretions and blood pressure in man. *Journal of Endocrinological Investigation* 1997 **20** 488–492.
- 43 Korbonsits M, Trainer PJ, Fanciulli G, Oliva O, Pala A, Dettori A *et al.* L-arginine is unlikely to exert neuroendocrine effects in humans via the generation of nitric oxide. *European Journal of Endocrinology* 1996 **135** 543–547.
- 44 Broadwell RD, Balin BJ, Salzman M & Kaplan RS. Brain–blood barrier? Yes and no. *PNAS* 1983 **80** 7352–7356.
- 45 Nelson RJ, Kriegsfeld LJ, Dawson VL & Dawson TM. Effects of nitric oxide on neuroendocrine function and behavior. *Frontiers in Neuroendocrinology* 1997 **18** 463–491.
- 46 McCann SM. The nitric oxide hypothesis of brain aging. *Experimental Gerontology* 1997 **32** 431–440.
- 47 Tsumori M, Murakami Y, Koshimura K & Kato Y. Endogenous nitric oxide inhibits growth hormone secretion through cyclic guanosine monophosphate-dependent mechanisms in GH3 cells. *Endocrine Journal* 1999 **46** 779–785.
- 48 Pinilla L, Tena-Sempere M & Aguilar E. Nitric oxide stimulates growth hormone secretion *in vitro* through a calcium- and cyclic guanosine monophosphate-independent mechanism. *Hormone Research* 1999 **51** 242–247.
- 49 Gonzalez MC & Llorente E. Methylene blue inhibits stimulatory effect of sodium nitroprusside but not of 3-morpholino sydnominine on prolactin secretion in freely moving male rats. *Brain Research Bulletin* 1998 **46** 229–231.
- 50 Kasuya E, Hodate K, Matsumoto M, Sakaguchi M, Hashizume T & Kanematsu S. The effects of xylazine on plasma concentrations of growth hormone, insulin-like growth factor-I, glucose and insulin in calves. *Endocrine Journal* 1996 **43** 145–149.

- 51 Hampshire J & Altszuler N. Clonidine or xylazine as provocative tests for growth hormone secretion in the dog. *American Journal of Veterinary Research* 1981 **42** 1073–1076.
- 52 Ueta Y, Levy A, Chowdrey HS & Lightman SL. Inhibition of hypothalamic nitric oxide synthase gene expression in the rat paraventricular nucleus by food deprivation is independent of serotonin depletion. *Journal of Neuroendocrinology* 1995 **7** 861–865.
- 53 Smith RG, Palyha OC, Feighner SD, Tan CP, McKee KK, Hreniuk DL *et al.* Growth hormone releasing substances: types and their receptors. *Hormone Research* 1999 **51** 1–8.
- 54 Avontuur JA, Buijk SL & Bruining HA. Distribution and metabolism of N(G)-nitro-L-arginine methyl ester in patients with septic shock. *European Journal of Clinical Pharmacology* 1998 **54** 627–631.
- 55 Pfeiffer S, Leopold E, Schmidt K, Brunner F & Mayer B. Inhibition of nitric oxide synthesis by NG-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, NG-nitro-L-arginine. *British Journal of Pharmacology* 1996 **118** 1433–1440.
- 56 Aguilar E, Rodriguez Padilla ML, Bellido C, Tena-Sempere M & Pinilla L. Changes in follicle-stimulating hormone secretion in spontaneously hypertensive rats. *Neuroendocrinology* 1991 **56** 85–93.
- 57 Kim JH, Snider T, Abel M & Zemel MB. Hypertension in young, healthy Zucker obese rats is not responsive to reduced salt intake. *Journal of Nutrition* 1994 **124** 713–716.

Received 7 August 2000

Accepted 19 October 2000