ELSEVIER

European Journal of Pharmacology 311 (1996) 15–19

ejp

Short communication

Inhibition of feeding by a nitric oxide synthase inhibitor: effects of aging

John E. Morley [a,b,*], Vijaya B. Kumar [a,b], Michael B. Mattammal [a,b], Sue Farr [a,b], Patricia M.K. Morley [a,b], James F. Flood [a,b]

[a] *Geriatric Research, Education and Clinical Center, St. Louis VA Medical Center, St. Louis, MO, USA*
[b] *Division of Geriatric Medicine, St. Louis University Medical School, St. Louis, MO, USA*

Received 11 June 1996; revised 4 July 1996; accepted 9 July 1996

Abstract

Nitric oxide has been demonstrated to play a role in the modulation of food intake. With advancing age, there is a physiological decrease in food intake. The effect of the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) on food intake in C57BL/6Nnia mice aged 3, 12 and 24 months was studied. L-NAME was more effective at decreasing food intake in 12- and 24-month-old mice than in the 3-month-old mice. NO synthase levels in the hypothalamus were increased in 16- and 25-month-old mice compared to 6-month-old mice ($P < 0.01$). NO synthase mRNA increased in 16- compared to 6-month-old mice, but decreased in 25-month-old mice. Overall, these studies may suggest that nitric oxide may play an increasingly important role in the feeding drive with advancing age.

Keywords: Nitric oxide (NO); Anorexia; Food intake; Aging; Hypothalamus; Nitric oxide (NO) synthase

1. Introduction

With advancing age, there is a decrease in food intake in both humans and rodents (Anonymous, 1994; Peng et al., 1980; Wurtman et al., 1988). This physiological decrease in food intake has been termed the 'anorexia of aging' (Morley and Silver, 1988). Multiple neurotransmitters have been identified as playing a role in the regulation of food intake (Morley, 1980, 1987). Recently, a role of nitric oxide in the regulation of food intake has been identified (Morley and Flood, 1991). Nitric oxide is a biological messenger molecule which is synthesized in high levels in the brain and other mammalian tissues (Bredt et al., 1991). Inhibitors of nitric oxide (NO) synthase decrease food intake in rodents (Calignano et al., 1993; Morley et al., 1995a; Morley and Flood, 1992, 1994; Squadrito et al., 1993). An NO synthase inhibitor has been demonstrated to decrease body weight by 10% in genetically obese (ob/ob) mice (Morley and Flood, 1994). These genetically obese (ob/ob) mice have been demonstrated to have increased levels of NO synthase and its mRNA in the hypothalamus compared to their lean littermate (ob/c) controls (Morley et al., 1995b).

Previous studies in rodents have provided evidence that the anorexia of aging is due tc a decrease in the opioid feeding drive (Gosnell et al., 1983; Kavaliers and Hirst, 1985) and an increase in the satiating effect of the gastrointestinal peptide, cholecystokinin (Silver et al., 1988). Previously we have found that the nitric oxide donor, glyceryl trinitrite, can increase food intake in some older persons with early satiation (Morley, 1990). The purpose of this study was to determine whether nitric oxide plays a role in the anorexia of aging in rodents. These studies report on the effects of pharmacological antagonism of NO synthase on food intake in mice of different ages and the levels of NO synthase and its mRNA in this species.

2. Materials and methods

2.1. Subjects

C57BL/6Nnia mice aged 3 ($n = 30$), 12 ($n = 29$) and 24 ($n = 22$) months were obtained from Charles River Laboratories, Wilmington, MA, through the National Institute on Aging. Mice were housed in single cages with 12:12 h light-dark cycles with a room temperature between 20–26°C. Food and water was available ad libitum except where stated.

* Corresponding author. St. Louis University School of Medicine, 1402 S. Grand Blvd., Rm M238, St. Louis, MO 63104, USA. Tel.: 314-577-8462; fax: 314-771-8575.

PII S0014-2999(96)00546-8

2.2. Food intake experiments

All experiments were done between 0800 and 0900. Animals were food deprived 18 h before the experiments were conducted. Following injection of drug or saline, food was reintroduced into the cage and the food intake over the next 60 min was quantitated. Animals were given a minimum of 72 h for recovery between experiments. N^G-Nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma Biochemical, St. Louis, MO, and administered subcutaneously in a volume of 0.5 ml of saline. All mice received all doses in a randomized design.

2.3. Hypothalamic dissection

Mice were food deprived 18–20 h before killing. C57BL/6 of different ages mice were killed alternately. Mice were killed by decapitation and brains were rapidly removed from the skull and whole hypothalamus dissected en bloc. The brain was placed on its ventral surface and vertical slices were made between the optic chiasm anteriorly and the mammillary bodies posteriorly. Parasagittal cuts were made through the perihypothalamic sulcus and a horizontal cut made below the anterior commissure. Hypothalami were divided into half, one half being utilized for the synthase assay and one for its mRNA.

2.4. Nitric oxide synthase assay

NO synthase was measured by determining the production of [^{3}H]citrulline from [^{3}H]L-arginine.

$$[^3H]\text{L-arginine} \xrightarrow{\text{NO synthase}} \text{NO} + [^3H]\text{L-citrulline}$$

One equivalent of arginine yields one equivalent of NO and one equivalent of citrulline.

2.4.1. Enzyme preparation from tissues

Tissue (20–50 mg) was homogenized in 10 vols. of (w/v) 0.32 M sucrose/10 mM Hepes/1 mM dithiothreitol (pH 7.4), using 20 up and down strokes of a teflon-glass homogenizer (800 rpm). The nuclear material was removed by centrifugation at $500 \times g$ for 10 min. The supernatant S_1 was removed and centrifuged at 1000 rpm to get a crude pellet. The pellet was resuspended in the homogenizing buffer (200–300 μl, 10 up and down strokes of a teflon homogenizer). The suspension was then centrifuged at $100\,000 \times g$ for 30 min to obtain the soluble fraction. Protein in the soluble fraction was determined by the method of Bradford (1976).

2.4.2. Incubation

To a mixture of 10 μM [^{3}H]L-arginine (43.5 Ci/mmol, Amersham), 1.0 mM NADPH, 0.1 mM dithiothreitol, 100 μM H_4biopetrin, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, and calmodulin (10 μg/ml) in 400 μl of Tris-HCl (0.1 M buffer, pH 7.4) was added 100 μl of supernatant prepared above. The reaction mixture was incubated for 30 min, at 37°C. The reaction was terminated by adding 40 μl of 20% $HClO_4$. The mixture was cooled on ice and centrifuged at 4000 rpm. A 100 μl aliquot of the supernatant was injected onto a high pressure liquid chromatography, equipped with a Zorbax 300 SCX silica column (4.6 mm × 25 cm) (sulfonate). The eluting solvent was: 0.1 M KH_2PO_4 (arginine, R_t = 10 min; citrulline, R_t = 4.5 min). The flow rate was 1.0 ml/min. 1.0 ml fractions were collected (ISCO, Retriever-2) and radioactivity in the fractions was determined using liquid scintillation spectrometry. Blanks consisted of 100 μl of buffer incubated 30 min.

2.5. RNase protection assay

The RNase protection assay to measure the mRNA levels was carried out by a modification of procedures described earlier (Melton et al., 1984). A 196 bp polymerase chain reaction product which extends from amino acid 420 to 485 of rat brain NO synthase was made (Xie et al., 1992). For generating the polymerase chain reaction product, rat brain NO synthase cDNA provided by David Bredt, Johns Hopkins University, Baltimore, MD, was used (Bredt et al., 1991). The reverse and forward primers used in the polymerase chain reaction fraction were 5′-CAGTGGTCAAGCTG CAG and 5′-TAGCCCGCGTAGCG GAT respectively. T_7 and T_3 promoter sequences were attached to the forward and reverse side of the polymerase chain reaction fragment. The amplified region is between the cAMP dependent phosphorylation and Ca^{2+} calmodulin binding sides of NO synthase. This region showed 77–80% homology with mouse macrophage (Bredt et al., 1991) and human endothelial (Marsden et al., 1992) NO synthase at both nucleotide and protein level.

The polymerase chain reaction mixture contained 60 mM Tris-HCl pH 8.8, 6 mM $MgCl_2$, 1 mM mercaptoethanol, 16 mM ammonium sulfate, 50 pm of each primer and 1 mM of each deoxyribonucleotides. The sample was denatured at 94°C for 5 min and then subjected to 34 cycles of polymerase chain reaction of 1 min each at 55°C, 72°C and 94°C. The product was gel purified and subcloned into a TA cloning vector (PCR II) obtained from Invitrogen (San Diego, CA).

For the synthesis of complimentary RNA, the probe was excised from TA cloning vector using 10 U of *Eco*RI/μg of plasmid DNA. The excised fragment was gel purified and ^{32}P-labeled complimentary RNA was prepared in a total volume of 20 μl. The reaction mixture contained 1 μg of the probe, 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 10 mM dithiothreitol, 0.5 mM each of ATP, UTP and GTP, 12.5 U of RNase inhibitor, 50 μCi (800 Ci/mmol) α-^{32}P-CTP and 10 U of T_7 polymerase. The reaction was performed at 37°C for 1 h and the product was electrophoresed on a 5% polyacrylamide gel in TBE (50 mM Tris, 50 mM boric

acid and 1 mM EDTA pH 8.0). Noncomplimentary ^{3}H-labeled RNA was prepared under identical conditions using T_3 polymerase. Known concentrations of this RNA were mixed with yeast RNA hybridized with complementary RNA and subjected to hybridization to estimate the efficacy of hybridization. 1–2 μg of rat brain RNA was hybridized to 20×10^3 cpm of the complementary RNA in a total volume of 50 μl containing 400 mM NaCl, 100 mM EDTA, 40 mM Pipes, 40% deionized formamide at 55°C for 40 h. Following hybridization, the mixture was diluted to 300 μl with water containing 10 μg RNase A and 17 U of RNase T_1 and incubation was continued at 30°C for 60 min. Then the contents were phenol-chloroform extracted, dissolved in 90% formamide, 20 mM EDTA and 0.3% bromophenol blue and subjected to gel electrophoresis in 6% gel containing 30% urea. The cpm in each band was estimated by scanning the gel with the Ambis-System. From the specific activity of the CTP used, the amount of probe hybridized was calculated by assuming equal distribution of the four nucleotides by the following formula:

pg of specific mRNA
= pmol of CTP hybridized × 4
× efficiency of hybridization × 340.

2.6. Statistics

All data is expressed as mean ± standard error of the mean. Statistically significant differences were determined by a one or two-way analysis of variance followed by Dunnet's *t*-test or Tukey *t*-test where appropriate.

3. Results

The results of the effects of L-NAME on food intake in food deprived mice are shown in Fig. 1. Both age ($F(2,306) = 25.4$, $P < 0.001$) and dose ($F(3,306) = 29.1$, $P < 0.001$), but not the interaction between them, was significant. In 5-month-old mice only the highest dose of L-NAME suppressed food intake. In the two older groups, all three doses of L-NAME decreased food intake, suggesting increased sensitivity of middle aged and older mice to pharmacological antagonism of NO synthase.

Table 1
Levels of nitric oxide synthase and its messenger RNA in mice of different ages ([a] $P < 0.01$; [b] $P < 0.05$)

Age months	NO synthase nmol/mg/min	NO synthase mRNA pg NO synthase mRNA/μg total RNA
6	0.0526 ± 0.0019	34.0 ± 3.7
14	0.0783 ± 0.0068 [a]	45.9 ± 3.2
25	0.0700 ± 0.0045 [a]	16.6 ± 6.1 [b]

$F(2,12) = 6.39$, $P < 0.01$ $F(2,6) = 10.53$, $P < 0.01$

The levels of NO synthase and its mRNA are shown in Table 1. NO synthase activity was increased in 13- and 25-month-old mice compared to 6-month-old mice ($F(2,12) = 6.39$, $P < 0.01$). NO synthase mRNA increased at 14 months and then decreased at 25 months.

4. Discussion

The studies reported here demonstrate an increased sensitivity to pharmacological antagonism of NO synthase in middle aged and older mice. NO synthase levels were higher in the middle aged and older mice than the younger mice. mRNA levels of NO synthase were decreased in the oldest animals, demonstrating a dichotomy between mRNA and enzyme levels with aging.

Human epidemiological studies have found that there is a linear decrease in total energy intake in persons greater than 20 years of age over the lifespan (Anonymous, 1994). Wurtman et al. (1988) in a laboratory situation found a reduction of food intake in healthy old males and females compared to young controls. Older persons fail to demonstrate the hyperphagia seen in young persons after a period

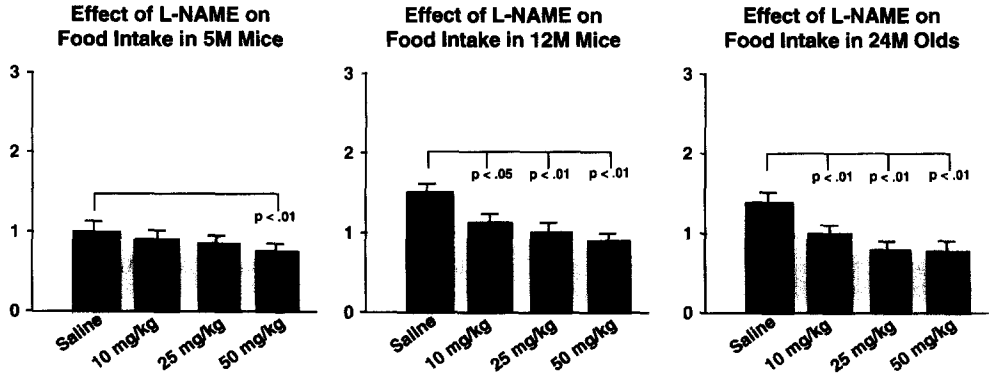

Fig. 1. Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on food intake in mice of different ages (age $F(2,306) = 25.4$, $P < 0.001$; dose $F(3,306) = 29.1$, $P < 0.001$), 5M = 5 months, 12M = 12 months and 24M = 24 months.

of underfeeding (Roberts et al., 1994). Clarkston et al. (1994) found that healthy older persons have a greater satiation response after being fed a standardized meal compared to younger persons. Rodent studies demonstrate a similar physiological decrease in food intake leading to weight loss in advanced age (Peng et al., 1980).

The causes of this physiological anorexia of aging have been investigated in detail in rodents. Endogenous opioids, in particular the dynorphin-κ-opioid system, have been demonstrated to play an important role in the regulation of food intake (Morley et al., 1983). In both rats (Gosnell et al., 1983) and mice (Kavaliers and Hirst, 1985) there is evidence that the anorexia of aging is, in part, due to a decrease in the opioid feeding drive. This appears to be predominantly due to a decrease in opioid receptors in the hypothalamus (Morley et al., 1990). In addition, studies in mice have demonstrated an increase in the satiating effect of the gastrointestinal hormone, cholecystokinin, with advancing age (Silver et al., 1988).

There is increasing evidence that nitric oxide plays a role in appetite regulation (Calignano et al., 1993; Morley and Flood, 1991, 1992; Squadrito et al., 1993) and elevations in NO synthase may play a role in the increased food intake seen in congenitally obese (ob/ob) mice (Morley and Flood, 1994; Morley et al., 1995a). In rats NO synthase has been demonstrated to be increased in the hypothalamus in food deprived animals (Squadrito et al., 1994). The studies reported here suggest a complex role for nitric oxide in the modulation of food intake with advancing age. The increased sensitivity to an NO synthase antagonist with advancing age suggests the possibility that there is an increased role for nitric oxide in the regulation of food intake in middle aged and older mice. Whether the effects of L-NAME on feeding behavior were due to a direct central nervous system effect or a peripheral effect, e.g. on the gastric fundus, could not be determined from this study.

The increase in hypothalamic NO synthase enzyme activity with age suggests that this may be an adaptive mechanism in an attempt to overcome the decline in the opioid feeding drive with aging. Alternatively, the increase in NO synthase could be secondary to a decrease in the ability of nitric oxide to stimulate intracellular cyclic GMP with advancing age. Further studies will be necessary to distinguish between these possibilities.

Overall these studies suggest a complex role for nitric oxide in the regulation of food intake with advancing age. The increase in NO synthase in the hypothalamus may be an adaptive reaction in response to the decrease in the endogenous opioid feeding drive with aging. Further studies will be necessary to determine if this is indeed the case. In particular, the use of other inhibitors of nitric oxide need to be utilized.

The dichotomy between NO synthase enzyme activity and mRNA with advancing age is similar to that seen with tyrosine hydroxylase and its mRNA (Pasinetti et al., 1992; Tumer et al., 1992). The data reported here suggest that aging is associated with increased post translational activity.

Acknowledgements

The authors gratefully acknowledge the editorial assistance of Carol McCleary. The illustrative work done by Connie Young and technical assistance of Terry Sasser and Matthew Walters are invaluable.

References

Anonymous, 1994, Food intake and NHANES III, MMWR 43, 116.

Bradford, M.M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding, Anal. Biochem. 72, 248.

Bredt, D.S., P.M. Hwang, C.E. Glatt, C. Lowenstein, R.R. Reed and S.H. Snyder, 1991, Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase, Nature 351, 714.

Calignano, A., P. Persico, F. Mancuso and L. Sorrentino, 1993, Endogenous nitric oxide modulates morphine-induced changes in locomotion and food intake in mice, Eur. J. Pharmacol. 231, 415.

Clarkston, W.K., M.M. Pantano, J.E. Morley, M. Horowitz, J.M. Littlefield and F.R. Burton, 1994, Evidence for the anorexia of aging: correlations with gastric emptying in healthy elderly vs. healthy young adults, J. Am. Geriatr. Soc. 42, SA1.

Gosnell, B.A., A.S. Levine and J.E. Morley, 1983, The effects of aging on opioid modulation of feeding in rats, Life Sci. 32, 2793.

Kavaliers, M. and M. Hirst, 1985, The influence of opiate agonists on day-night feeding rhythms in young and old mice, Brain Res. 326, 160.

Marsden, P.A., K.T. Schappert, H.S. Chen, M. Flowers, C.L. Sundell, J.N. Wilcox, S. Lamas and T. Michel, 1992, Molecular cloning and characterization of human endothelial nitric oxide synthase, FEBS Lett. 307, 287.

Melton, D.A., P.A. Kreig, M.R. Rebagliata, T. Maniatis, K. Zinn and M.R. Greem, 1984, Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter, Nucleic Acids Res. 12, 7035.

Morley, J.E., 1980, The neuroendocrine control of appetite: the role of endogenous opiates, cholecystokinin, TRH, gamma-amino-butyric-acid and the diazepam receptor, Life Sci. 27, 355.

Morley, J.E., 1987, Neuropeptide regulation of appetite and weight, Endocr. Rev. 8, 256.

Morley, J.E., 1990, Anorexia in older patients: its meaning and management, Geriatrics 45, 59.

Morley, J.E. and J.F. Flood, 1991, Evidence that nitric oxide modulates food intake in mice, Life Sci. 49, 707.

Morley, J.E. and J.F. Flood, 1992, Competitive antagonism of nitric oxide synthetase causes weight loss in mice, Life Sci. 51, 1285.

Morley, J.E. and J.F. Flood, 1994, Effect of competitive antagonism of NO synthetase on weight and food intake in obese and diabetic mice, Am. J. Physiol. 266, R164.

Morley, J.E. and A.J. Silver, 1988, Anorexia in the elderly, Neurobiol. Aging 9, 9.

Morley, J.E., A.S. Levine, G.K. Yim and M.T. Lowy, 1983, Opioid modulation of appetite, Neurosci. Biobehav. Rev. 7, 281.

Morley, J.E., J.F. Flood and A.J. Silver, 1990, Opioid peptides and aging, Ann. N.Y. Acad. Sci. 579, 123.

Morley, J.E., S.A. Farr, M.D. Suarez and J.F. Flood, 1995a, Nitric oxide

synthase inhibition and food intake: effects on motivation to eat and in female mice, Pharmacol. Biochem. Behav. 50, 369.

Morley, J.E., V.B. Kumar, M. Mattammal and D.T. Villareal, 1995b, Measurement of nitric oxide synthase and its mRNA in genetically obese (ob/ob) mice, Life Sci. 57, 1327.

Pasinetti, G.M., H.H. Osterburg, A.B. Kelly, S. Kohama, D.G. Morgan, J.F. Reinhard, Jr., R.H. Stellwagen and C.E. Finch, 1992, Slow changes of tyrosine hydroxylase gene expression in dopaminergic brain neurons after neurotoxin lesioning: a model for neuron aging, Brain Res. (Mol. Brain Res.) 13, 63.

Peng, M.T., M.J. Jiang and H.K. Hsu, 1980, Changes in running-wheel activity, eating and drinking and their day/night distributions throughout the life span of the rat, J. Gerontol. 35, 339.

Roberts, S.B., P. Fuss, M.B. Heyman, W.J. Evans, R. Tsay, H. Rasmussen, M. Fiatarone, J. Cortiella, G.E. Dallal and V.R. Young, 1994, Control of food intake in older men, J. Am. Med. Assoc. 272, 1601.

Silver, A.J., J.F. Flood and J.E. Morley, 1988, Effect of gastrointestinal peptides on ingestion in old and young mice, Peptides 9, 221.

Squadrito, F., G. Calapai, D. Cucinotta, D. Altavilla, B. Zingarelli, M. Ioculano, G. Uma, A. Sardella, G.M. Campo and A.P. Caputi, 1993, Anorectic activity of N^G-nitro-L-arginine, an inhibitor of brain nitric oxide synthase, in obese Zucker rats, Eur. J. Pharmacol. 230, 125.

Squadrito, F., G. Calapai, D. Altavilla, D. Cucinotta, B. Zingarelli, G.M. Campo, V. Arcoraci, L. Sautebin, G. Mazzaglia and A.P. Caputi, 1994, Food deprivation increases brain nitric oxide synthase and depresses brain serotonin levels in rats, Neuropharmacology 33, 83.

Turner, N., C. Hale, J. Lawler and R. Strong, 1992, Modulation of tyrosine hydroxylase gene expression in the rat adrenal gland by exercise: effects of age, Brain Res. (Mol. Brain Res.) 14, 51.

Wurtman, J.J., H. Lieberman, R. Tsay, T. Nader and B. Chew, 1988, Calorie and nutrient intakes of elderly and young subjects measured under identical conditions, J. Gerontol. 43, B174.

Xie, Q.W., H.J. Cho, J. Calaycay, R.A. Mumford, K.M. Swiderek, T.D. Lee, A. Ding, T. Troso and C. Nathan, 1992, Cloning and characterization of inducible nitric oxide synthase from mouse macrophages, Science 256, 225.