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Effects of intracerebroventricular leptin administration on food intake, body weight gain and diencephalic nitric oxide synthase activity in the mouse

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1 Intracranial administration of leptin reduces both food intake and body weight gain in the mouse. Inhibitors of nitric oxide (NO) synthase produce similar effects.

2 To investigate the role of the brain L-arginine/NO pathway in mediating this effect of leptin, we have evaluated food intake and body weight gain after daily (5 days) intracerebroventricular (i.c.v.) administration of leptin (0.5–2 μg) alone or in association with L-arginine (10 μg). Moreover, we measured diencephalic nitric oxide synthase (NOS) activity after a single i.c.v. leptin (0.25–2 μg) injection and after consecutive doses of leptin (0.25–2 μg) over 5 days. The time course of the effect of leptin on NOS activity was also evaluated.

3 I.c.v. injected leptin (1 and 2 μg) significantly and dose-dependently reduced food intake and body weight gain with respect to vehicle (food intake: 5.97 ± 0.16 g 24 h^{-1} and 4.27 ± 0.18 g 24 h^{-1}, respectively, vs 8.05 ± 0.34 g 24 h^{-1}, $P<0.001$, $n=6$ for each group; body weight gain: $-10.7 \pm 0.46\%$ and $-15.2 \pm 0.65\%$, respectively, vs $5.14 \pm 0.38\%$, $P<0.001$, $n=6$ for each group). This effect was antagonized by L-arginine (food intake: 7.90 ± 0.37 g 24 h; body weight gain: $5.11 \pm 0.31\%$, $n=6$). Diencephalic NOS activity was significantly reduced by the highest doses of leptin with respect to vehicle (vehicle: 0.90 ± 0.04 nmol citrulline min^{-1} g^{-1} tissue; leptin 1 μg: 0.62 ± 0.03 nmol citrulline min^{-1} g^{-1} tissue, $P<0.001$; leptin 2 μg: 0.44 ± 0.03 nmol citrulline min^{-1} g^{-1} tissue, $P<0.001$, $n=6$ for each group). Similar results were obtained in animals treated with daily consecutive doses of leptin. The inhibitory effect appeared rapidly (within 30 min) and was long lasting (up to 12 h).

4 Our results suggest that the brain L-arginine/NO pathway may be involved in the central effect of leptin on feeding behaviour and body weight gain in mice.

Keywords: Leptin; nitric oxide; L-arginine; food intake; body weight gain; brain; obesity

Introduction

The recent cloning of the mouse and human *obese* genes and the characterization of its protein product, leptin (Zhang *et al*., 1994), has been an important breakthrough for the understanding of obesity pathophysiology. Leptin is a hormone produced exclusively by the adipocyte that conveys to the brain information on the size of energy stores and activates hypothalamic centres regulating energy intake and expenditure (Flier, 1997). This protein also regulates feeding behaviour by reducing food consumption (Pelleymounter *et al*., 1995; Tritos & Mantzoros, 1997). Leptin produces its effects on food intake mainly by the reduction of neuropeptide Y (NPY) levels, and intracerebroventricular (i.c.v.) leptin administration results in a more potent response compared with the response to systemic leptin administration, suggesting that the central nervous system (CNS) is a major site of its action (Schwartz *et al*., 1996). Moreover, leptin seems to affect several neuroendocrine mechanisms and the hypothalamic-pituitary axis (Flier, 1997). On the other hand, the specific brain pathways engaged by the hormone are still largely unknown.

Over the last few years, another molecule, nitric oxide (NO), has been shown to be involved in regulating ingestive behaviour (Calapai *et al*., 1992; Morley & Flood, 1991). More exactly, administration of inhibitors of nitric oxide synthase (NOS) activity causes anorectic effects and reduces body weight gain in both lean and obese rats (Squadrito *et al*., 1993). It has also recently been observed that nitric oxide overproduction is reduced by leptin in cultured islets isolated from obese Zucker diabetic fatty rats (Wang *et al*., 1998). Moreover, both leptin and NO seem to exert their effects by hypothalamic action (Calapai *et al*., 1994; Elmquist *et al*., 1997).

In the light of these observations, we evaluated the effects of intracranial administration of leptin and L-arginine on food intake and body weight gain and of leptin on diencephalic neuronal NOS activity in mice.

Methods

Animals

Male, Swiss 8-week-old mice were used. The animals were housed at a constant temperature of 22 ± 2°C under a 12 h/12 h light-dark cycle (lights on at 06.00 h), with free access

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to Purina rat chow pellets and tap water, unless otherwise stated.

Intracerebroventricular administrations

For i.c.v. injections (left cerebral lateral ventricle coordinates: 1 mm posterior and 1 mm lateral to the bregma; depth 2.4 mm) mice were anaesthetized with intraperitoneal chloral hydrate (400 mg kg^{-1}) to produce full surgical anaesthesia, and the skull exposed. Injections (5 μl) were made at lambda with a 3.5 mm long, 27 gauge needle attached to a 10 μl Hamilton microsyringe (type 701N) and the wound sealed with epoxy resin. Injections of vehicle or drugs by this procedure led to a uniform distribution throughout the ventricular system within 10 min (De Sarro *et al*., 1992). Each group of animals was composed of six mice in all the experiments.

Food intake and body weight gain

Food intake was measured, corrected for spillage, by weighing the jars containing food (to the nearest 0.1 g) every 24 h at 10.00 h and data are expressed as g 24 h^{-1} mouse. Water intake was measured by chemical graduate burettes (to the nearest 0.1 ml). Mice were randomly assigned to eight groups, and their basal food consumption together with body weight gain (expressed as % of starting body weight) were obtained from measurements on the 7 days preceding the treatment (baseline period). Animals were successively treated i.c.v. for 5 days (treatment period) as follows: vehicle; leptin 0.5 μg; leptin 1 μg; leptin 2 μg; leptin 2 μg + L-arginine 10 μg; leptin 2 μg + D-arginine 10 μg; L-arginine 10 μg; D-arginine 10 μg.

Nitric oxide synthase activity

To measure neuronal diencephalic NOS activity the following experiments were performed. A group of mice was treated i.c.v. with different doses of leptin (0.25, 0.5, 1 or 2 μg mouse) or with vehicle (cerebrospinal fluid, CSF) and sacrificed 1 h after injection. Another group was treated i.c.v. with different doses of leptin (0.25, 0.5, 1 or 2 μg mouse) consecutively administered over 5 days. All the groups of animals were sacrificed under light anaesthesia with ether by decapitation. The brain was rapidly removed and diencephalon quickly dissected out and immediately frozen in liquid nitrogen.

Tissues were homogenized at 4°C in four volumes of HEPES buffer (20 mM pH 7.2) containing 320 mM sucrose, 1 mM DL-dithiothreitol, 10 μg ml^{-1} soybean trypsin inhibitor, 2 μg ml^{-1} aprotinin and 10 μg ml^{-1} leupeptin. The homogenates were centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatants, i.e. the cytosolic fractions containing NOS activity, were stored at −70°C until use. Protein concentration in the cytosolic fraction was measured spectrophotometrically according to Bradford using bovine serum albumin as standard (Bradford 1976).

NOS activity was evaluated by measuring the rate of conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline according to the method of Salter et al. (1991) and expressed as nmol citrulline min^{-1} g^{-1} tissue. Briefly, an aliquot of cytosolic fraction (100 mg of protein) was preincubated for 5 min at 37°C in 50 mM potassium phosphate buffer pH 7.2 containing 60 mM L-valine, 120 μM NADPH, 1.2 mM L-citrulline, 1.2 mM $MgCl_2$ and 0.24 mM $CaCl_2$ in the presence of drug or vehicle. Samples were then incubated for 10 min at 37°C with L-[U-^{14}C]arginine (150,000 d.p.m.) and 20 μM L-arginine. The reaction was stopped by the addition of 1.0 ml of a mixture of H_2O/Dowex-50 W 1 : 1 vv^{-1} (200–400, 8% cross-linked Na^+-form). The Na^+-form of Dowex-50 W was prepared by washing four times the H^+-form of the resin with 1 M NaOH and then with bi-distilled water until the pH was less than 7.5. The resin was settled by centrifugation (11,000 × *g* for 3 min) in a microfuge (Beckman, Microfuge 11) and an aliquot of the supernatant was taken for scintillation counting (4 ml Pico-Aqua; Packard 1500). The activity of Ca^{2+}/dependent NOS was determined from the difference between the [U-^{14}C]citrulline produced by control samples and samples containing 1 mM EGTA; the activity of the Ca^{2+}/independent enzyme was determined from the difference between the [U-^{14}C]citrulline produced by samples containing 1 mM EGTA and samples containing 1 mM EGTA plus 1 mM L-N^G monomethyl arginine (L-NMMA).

Drugs

Recombinant mouse leptin (Linco Research Inc., St. Charles, MO, U.S.A.) was dissolved in artificial CSF and administered i.c.v. at doses of 0.5, 1 and 2 μg per mouse. Control animals received i.c.v. an equal volume of CSF (vehicle). L-arginine and D-arginine hydrochloride were obtained by Sigma Chemical Company (St. Louis, MO, U.S.A.), L-NMMA was obtained from INALCO S.p.A. (Milano, Italy). L-[U-^{14}C]arginine hydrochloride (specific activity 304 mCi μmol^{-1}) was obtained from Amersham Ltd. (Buckinghamshire, U.K.).

Statistical analysis

All statistical procedures were performed using SPSS statistical software package release 6.1.3 (SPSS Inc., Chicago, IL, U.S.A.). Data analysis was performed using one way ANOVA with Scheffé *post hoc* test for multiple comparisons. Data are expressed as the means ± s.e.mean. Statistical significance was set at $P<0.05$.

Results

Food intake and body weight gain

Figure 1 shows the effects of i.c.v. administration of vehicle, leptin, leptin + L-arginine or leptin + D-arginine and L-arginine or D-arginine on food intake. During the baseline period no statistically significant difference was observed as regards food

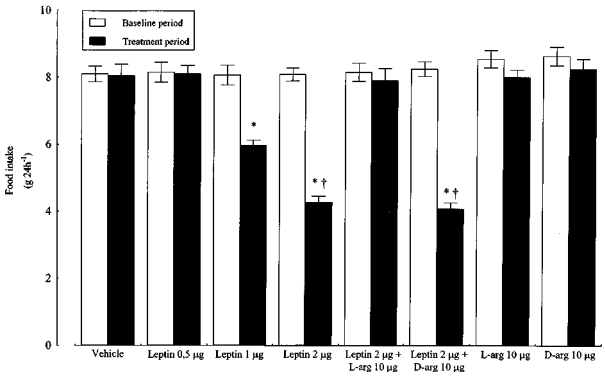

Figure 1 The effects of daily i.c.v. injection (5 days) of leptin (0.5–2 μg), leptin (2 μg) + L-arginine (10 μg) or leptin (2 μg) + D-arginine (10 μg) and L-arginine (10 μg) or D-arginine (10 μg) on food intake. Each column represents the mean ± s.e.mean for six animals. *$P<0.001$ vs vehicle, leptin (0.5 μg), leptin (2 μg) + L-arginine (10 μg), L-arginine (10 μg) and D-arginine (10 μg). †$P<0.01$ vs leptin (1 μg).

consumption among the groups studied. As previously reported (Pelleymounter *et al*., 1995), mice receiving leptin showed a significant reduction in food intake. No significant difference was observed among the groups studied with respect to water intake during the treatment period (Figure 2). Animals treated with both leptin and L-arginine showed an ingestive behaviour similar to that of the control group. Co-administration of leptin and D-arginine did not modify the anorectic effect of leptin. Finally, food intake was not significantly affected in the groups treated with L-arginine or D-arginine with respect to the baseline period (Figure 1).

Body weight gain of animals treated with leptin was reduced in a dose dependent manner in groups treated with the highest doses of leptin (1 and 2 μg). Simultaneous L-arginine, but not D-arginine administration, antagonized this effect. No significant modifications in body weight gain were found in the groups treated with L-arginine or D-arginine (Figure 3).

Nitric oxide synthase activity

Measurement of diencephalic NOS activity revealed only the presence of calcium-dependent constitutive NOS activity. Mice treated with a single dose or consecutive doses of leptin over 5 days showed a significant and dose-dependent reduction in neuronal NOS activity which was apparent at a dose of 1 μg (Figure 4). The time course of the *ex vivo* NOS inhibitory effect of leptin revealed a long lasting effect, since inhibition of neuronal NOS activity was still apparent 12 h after a single leptin i.c.v. injection. In mice treated with leptin, NOS activity returned to values similar to vehicle groups within 24 h (Figure 5).

Discussion

It has been observed that leptin may exert its central action mainly through a decreased NPY gene expression in hypothalamic arcuate nucleus. Neuropeptide Y also acts on the hypothalamus, reducing energy expenditure and potently stimulating food intake (Schwartz *et al*., 1996). However, leptin administration in NPY knockout ($-/-$) mice decreases food intake, body weight and fat mass, and these mice, after 48 h of food deprivation, show a body weight gain and a feeding behaviour similar to that observed in control ($+/+$) (Erickson *et al*., 1996), thus suggesting that NPY is not the only neurotransmitter mediating leptin effects on food intake. For this reason, we investigated the possibility that nitric oxide, another neurotransmitter capable of controlling food intake (Squadrito *et al*., 1994), is involved in brain leptin effects.

Nitric oxide acts in the central nervous system as a messenger molecule to mediate the increased cyclic GMP

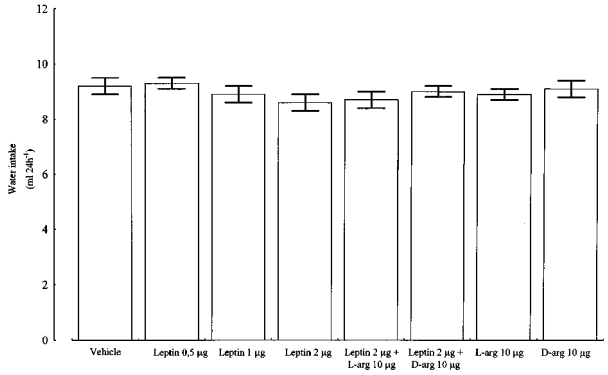

Figure 2 The effects of daily i.c.v. injection (5 days) of leptin (0.5–2 μg), leptin (2 μg) + L-arginine (10 μg) or leptin (2 μg) + D-arginine (10 μg) and L-arginine (10 μg) or D-arginine (10 μg) on water intake. Each column represents the mean ± s.e.mean for six animals.

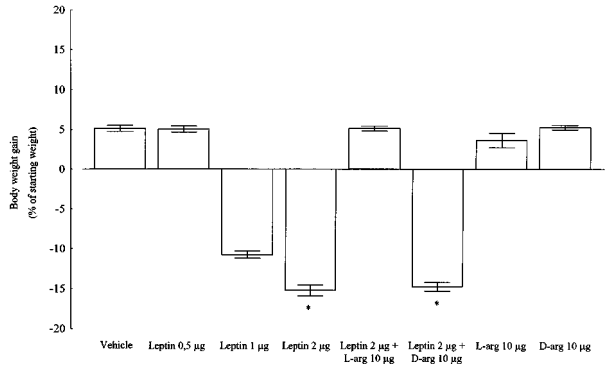

Figure 3 The effect of daily i.c.v. injection (5 days) of leptin (0.5–2 μg), leptin (2 μg) + L-arginine (10 μg) or leptin (2 μg) + D-arginine (10 μg) and L-arginine (10 μg) or D-arginine (10 μg) on body weight gain. Each column represents the mean ± s.e.mean for six animals. $*P<0.001$ vs vehicle, leptin (0.5 μg), leptin (1 μg), leptin (2 μg) + L-arg (10 μg), L-arg (10 μg) and D-arg (10 μg).

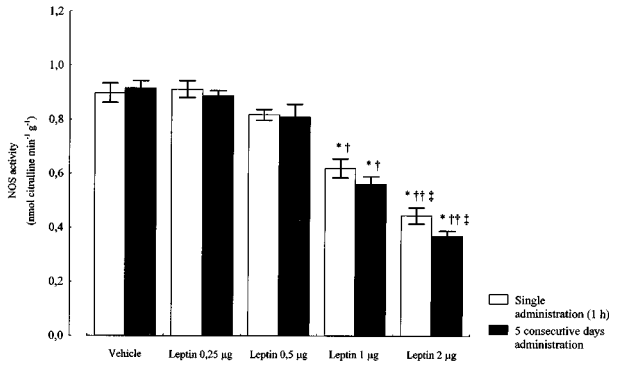

Figure 4 The effect of a single dose or consecutive doses of leptin administration over 5 days on diencephalic nitric oxide synthase activity. Each column represents the mean ± s.e.mean for six animals. $*P<0.001$ vs vehicle and leptin (0.25 μg). †$P<0.01$, ††$P<0.001$ vs leptin (0.5 μg). ‡$P<0.05$ vs leptin (1 μg).

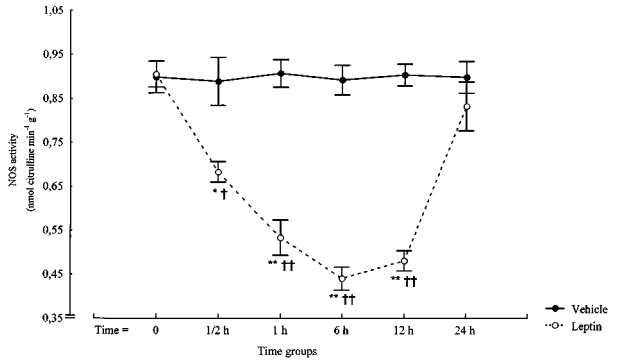

Figure 5 Time course of diencephalic nitric oxide synthase activity after i.c.v. vehicle or leptin (2 μg) injection. Each time point represents the mean ± s.e.mean for six animals. $*P<0.01$, $**P<0.001$ vs Time = 0. †$P<0.01$, ††$P<0.001$ vs vehicle.

occurring after glutamate receptor activation (Lowenstein *et al*., 1994). Much evidence indicates that NO is implicated in several central nervous system functions including neuroendocrine functions (Costa *et al*., 1993), long-term potentiation (Haley *et al*., 1992) and ingestive behaviour (Calapai & Caputi 1996; Stricker-Krongrad *et al*., 1996). NOS oxidizes the guanidino group of L-arginine in a process that utilizes five electrons and co-produces NO and citrulline (Zhang & Snyder 1995), and can be classified into three isoforms (two constitutive and one inducible)(Forstermann & Kleinert 1995). Neuronal NOS (nNOS) is constitutive and calcium-dependent (Fukuto & Chauduri 1995) and is relatively highly expressed in the diencephalic region, in particular in the hypothalamic paraventricular and supraoptic nuclei (Bredt *et al*., 1990; Dawson *et al*., 1991).

Our experiments performed by injecting leptin in the lateral cerebral ventricle of mice confirm that the ob gene protein reduces food intake and body weight gain and show that these effects can be antagonized by i.c.v. pretreatment with the basic amino acid, L-arginine, which is the substrate for NOS (Knowles *et al*., 1989). Thus, an interaction between leptin and nitric oxide in the mechanisms regulating food consumption may be hypothesized.

This hypothesis is sustained by data obtained from the evaluation of neuronal NOS activity following central administration of leptin. NOS can be divided in two distinct classes: constitutive (cNOS) and inducible (iNOS) (Fukuto & Chauduri 1995). The latter was not detectable in our experiments. Diencephalic regions of mice sacrificed 1 h after leptin administration showed a reduction in cNOS activity. Moreover, the inhibitory effect of leptin appears rapidly and is maintained during the first 12 h after i.c.v. injection. Since previously reported findings demonstrate that inhibition of neuronal NOS activity reduces food intake (Morley & Flood, 1991), a similar mechanism could be suggested to explain the anorectic effects of leptin.

Another effect shared by leptin and antagonists of NOS is the ability to reduce body weight gain (Squadrito *et al*., 1993). Since our results demonstrate that the fall in body weight gain following leptin administration was antagonized by L-arginine administration, but not by D-arginine (not a substrate for NOS), one could suggest that nitric oxide may also have a role in modulating energy expenditure.

Nitric oxide may regulate neurotransmitter release through activation of cGMP-dependent protein phosphorylation cascades. Furthermore, inhibitors of NOS block NMDA receptor-mediated neurotransmitter release (Montague *et al*., 1994). Moreover, it has been observed that NPY mRNA and NOS are colocalized in neurons of rat hypothalamus (Whale *et al*., 1993). Thus, a role for the L-arginine/NO pathway in leptin-mediated effects on NPY secretion is proposed.

Recently the relationship between leptin and NO in the central nervous system has been studied. This report shows that leptin acts at the hypothalamic and pituitary level to stimulate (not to inhibit) NO release in the rat (Yu *et al*., 1997). Although of interest, it should be borne in mind that these results were obtained in an entirely different system and using a different species of laboratory animal.

As previously reported, central leptin administration suppresses feeding behaviour and promotes weight loss. Furthermore, the co-administration of leptin with L-arginine restores normal feeding behaviour and body weight gain. Finally, central leptin administration reduces diencephalic neuronal NOS activity. These data taken together suggest that the brain L-arginine/NO pathway may be involved in central leptin effects.

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