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Possible Involvement of Nitric Oxide in Chlordiazepoxide-Induced Feeding in the Mouse

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CZECH, D. A. *Possible involvement of nitric oxide in chlordiazepoxide-induced feeding in the mouse.* PHARMACOL BIOCHEM BEHAV **55**(3) 327–331, 1996.—Two experiments investigated a possible role of nitric oxide (NO) in chlordiazepoxide (CP)-induced feeding in nondeprived male ICR mice in independent groups designs. Experiment 1 demonstrated a dose-related decrease in CP-induced solid food intake over a 60-min test period with increasing dose (10, 25, and 50 mg/kg SC) of the NO-synthase (NOS) inhibitor, L-N^G-nitro arginine (L-NOARG), reaching statistical significance at 10 mg/kg L-NOARG when compared to vehicle control. Identical doses of L-NOARG failed to significantly affect normal feeding in vehicle treated mice. In Experiment 2, initial pretreatment with L-arginine (500 and 1000 mg/kg IP) partially or completely restored the feeding inhibitory action of a challenge dose (25 mg/kg SC) of L-NOARG; D-arginine (500 mg/kg IP) was ineffective, thus supporting a stereospecific action of arginine. Arginine isomers did not differentially affect intake in normal feeding animals. These results implicate involvement of NO in CP-induced hyperphagia; they are consistent with and extend research linking NO and ingestive behaviors. **Copyright © 1996 Elsevier Science Inc.**

Benzodiazepine Chlordiazepoxide-induced feeding D-arginine Food intake L-arginine
L-N^G-nitro arginine L-NOARG Mice Nitric oxide Nitric oxide synthase inhibitor

A prominent literature provides evidence of reliable and often pronounced effects of benzodiazepine receptor ligands on ingestive behaviors [for review, see (5)]. Benzodiazepine-induced feeding, for example, has been reported in a number of animal species under both nondeprived (4,11) and deprived (4,22) conditions, and with differing experimental protocols and diets (5,9). The benzodiazepine drug, chlordiazepoxide, was recently reported to dose dependently reverse radiation-induced attenuation of food intake in the rat (28). Further, chlordiazepoxide (CP) has been shown to enhance appetite, increasing the amount of food consumed and/or caloric intake, in humans (12,27).

It has recently been recognized that nitric oxide (NO), a naturally occurring vasodilating gas, might play an important role in mechanisms regulating feeding behavior in animals under certain conditions. There is evidence that NO functions as a neurotransmitter and intracellular messenger in both central and peripheral nervous systems (6,15). Pharmacologic manipulations purportedly interfering with production of endogenous NO have been reported to decrease food intake in food-deprived mice (18,19,21), rats (24) and chickens (3), and in genetically obese strains of mice (20) and rats (25). Similarly, morphine-stimulated food intake was thusly attenuated in mice (2). In these studies, NO production was restricted through inhibition of the catalytic enzyme, NO-synthase (NOS). Feeding could be restored to varying degree with L-arginine (L-arg), the natural substrate for NOS and NO precursor. At the same time, inhibition of NO production with NOS inhibitors has been reported not to significantly affect spontaneous feeding (2,24,25). Further, enhancement of spontaneous or treatment-induced food intake has been observed following administration of L-arg; this effect, however, is not consistently found and can require large doses of substrate (21,24).

The aim of the present research was to investigate a possible role of NO in CP-induced hyperphagia in the mouse, here also exploiting inhibition of NOS as a useful tool. Specifically, L-N^G-nitro arginine (L-NOARG), an NOS inhibitor reported

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to be highly selective for inhibiting brain NO synthesis (13), was administered. It has been demonstrated that L-NOARG can compete with L-arg for active sites on the NOS and to, thus, inhibit generation of NO from L-arg (17).

METHOD

Animals

Male ICR mice (Sasco, Omaha, NE), weighing 28–50 g, were individually housed in standard opaque polypropylene tub-type cages, and maintained on a 12 L:12 D cycle (lights on 0700–1900 h) in a temperature- and humidity-controlled colony room, with continuous access to tap water and pelleted food (Teklad rodent diet 8604). They were held in the colony room for at least 7 days before testing. Animals were tested individually, and all testing was carried out during the light period between 0900 and 1400 h.

Drugs

Chlordiazepoxide HCl (CP), L- and D-arg, and L-NOARG were purchased from Sigma Chemical Co. (St. Louis, MO). All drugs were freshly prepared in sterile 0.9% NaCl vehicle on the morning of testing. L-NOARG (or vehicle) was injected SC, and L- or D-arg (or vehicle) IP, approximately 45 min prior to start of intake tests; CP was administered SC, 30 min before testing. All drugs were injected in a volume of 0.1 ml/10 g body weight.

Behavioral Testing Procedures

Mice were adapted to the experimental test environment, with food present, on several days prior to testing. On the day of testing, mice were weighed to the nearest 0.5 g, placed into a living cage with fresh bedding, but without food or water, and returned to the colony room. Approximately 30 min later, drug administration was started.

Experiment 1: L-NOARG Dose–Response Series. Mice (n = 15–20/group) were first pretreated with 10, 25, or 50 mg/kg L-NOARG or 0.9% NaCl vehicle SC. Fifteen minutes later, 10 mg/kg CP or vehicle was administered SC. Two series of groups were thus formed: a 10 mg/kg CP + L-NOARG (or vehicle) series and a CP vehicle + L-NOARG (or vehicle) series. Thirty minutes later, mice were placed in a bare polypropylene cage, identical to the living cage, with a preweighed food pellet (same as provided for maintenance diet) to begin the 60-min test period. The pellet was weighed at 30 min and again at test end on a balance accurate to 0.001 g (Mettler model PC-180). Water was not available during the test period. Uneaten crumbs were collected and incorporated into pellet weighing at 60 min.

Experiment 2: L- and D- Arginine Series. Mice (n = 15–19/group) were initially pretreated with L-arg (500 or 1000 mg/kg), D-arg (500 mg/kg), or vehicle control IP, along with 25 mg/kg L-NOARG or vehicle control SC. Fifteen minutes later, 10 mg/kg CP or vehicle was administered SC. Three series of groups were thus formed: 10 mg/kg CP + 25 mg/kg L-NOARG + arginine (or vehicle); CP vehicle + 25 mg/kg L-NOARG + arginine (or vehicle); and CP vehicle + L-NOARG vehicle + arginine (or vehicle). A single group administered 10 mg/kg CP, along with two injections of equal volume of vehicle, constituted a control for CP-induced feeding. Thirty minutes after last (CP or vehicle) injection, mice were transferred to test cages and food intake was measured as in Experiment 1 above. Again, water was unavailable during the test period.

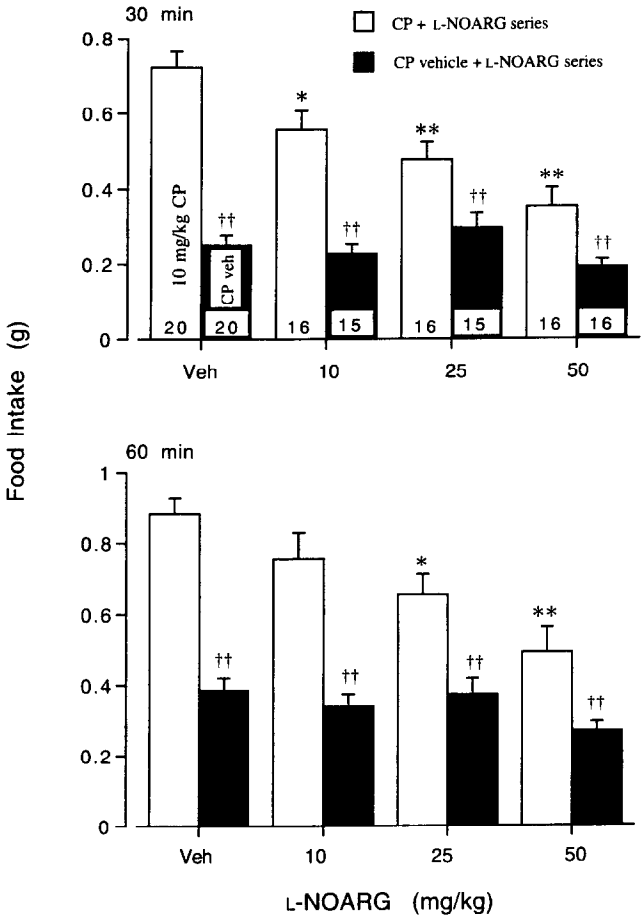

FIG. 1. Mean (± SEM) cumulative food intake in chlordiazepoxide (CP)- or vehicle-treated mice following pretreatment with L-NOARG or 0.9% NaCl vehicle at 30 min (upper panel) and 60 min (lower panel); n = 15–20/group, as indicated in base of bar. *$p < 0.05$, **$p < 0.01$ compared to 10 mg/kg CP + vehicle control group, Dunnett's t-test (one tail). ††$p < 0.01$, compared to corresponding CP + L-NOARG (or veh) group, Bonferonni protected Student's t-test.

All research protocols were reviewed and approved by Marquette University's Institutional Animal Care and Use Committee (IACUC) and are in compliance with the USDA Animal Welfare Act.

Statistical Analyses

Cumulative food intake data were evaluated separately at 30 and 60 min with independent one-way and two-way ANOVAs. Pair-wise comparisons were made with Dunnett's t-tests or Bonferonni protected Student's t-tests, or with Duncan's multiple range procedures. Minimally acceptable alpha level was set at $p < 0.05$.

RESULTS

Experiment 1: L-NOARG Dose–Response Series

Cumulative food intake data from Experiment 1 are summarized in Fig. 1. As seen in the left-most pair of means, a challenge dose of 10 mg/kg of CP induced, as expected, a

prominent feeding response [Veh/Veh vs. Veh/10 mg/kg CP; $t(38) = 9.08$ and 8.65, both $p < 0.01$, at 30 and 60 min, Student's t-test]. The two-factor ANOVAs yielded statistically significant main effects at both time periods for CP, $F(1, 126) = 121.06$ and 105.72, respectively, at 30 and 60 min; both $p < 0.001$, and for L-NOARG dose, $F(3, 126) = 11.01$ and 9.45, respectively, at 30 and 60 min, both $p < 0.001$. The CP × L-NOARG interactions were also significant, $F(3, 126) = 7.68$, $p < 0.001$, and 3.31, $p < 0.022$, respectively, at 30 and 60 min. Simple effects analyses confirmed that the interaction was due to L-NOARG significantly attenuating the feeding stimulatory effect of 10 mg/kg of CP in a dose-related manner, $F(3, 64) = 11.56$ and 7.45 at 30 and 60 min, both $p < 0.001$, while L-NOARG did not significantly affect normal/spontaneous feeding, $F(3, 62) = 2.60$ and 2.54 at 30 and 60 min, both $p > 0.06$. When compared to L-NOARG vehicle condition, cumulative food intake in mice administered 10 mg/kg CP was significantly lower at all doses of L-NOARG used at 30 min and at the two higher doses at the end of 60 min ($p < 0.05$ or $p < 0.01$, Dunnett's test). L-NOARG's blocking effect was, however, partial over the entire dose range tested; except for the largest L-NOARG dose (50 mg/kg) at 60 min, intakes remained significantly higher than baseline control (Veh/Veh) group level at all doses and measurement periods; $p < 0.05$ or $p < 0.01$, Student's t-test (these differences are not identified symbolically in Fig. 1). Further, intake in all CP-treated groups was significantly higher than in their corresponding normal feeding groups (all $p < 0.01$).

Experiment 2: L- and D-Arginine Series

Cumulative food intake data from Experiment 2 are summarized in Fig. 2. The feeding-stimulatory effect of 10 mg/kg of CP was again partially, but significantly, attenuated by 25 mg/kg of L-NOARG at 30 min ($p < 0.01$). Reduced intake at 60 min failed to reach statistical significance criterion, achieving only $p < 0.08$. CP-stimulated food intake under 25 mg/kg L-NOARG (left-most open bar) dropped to 56.5 and 75.1% of intake under L-NOARG vehicle condition (crosshatched bar) at 30 and 60 min. Pretreatment with 500 mg/kg of L-arg partially reversed this L-NOARG effect at 30 min ($p < 0.05$), while 1000 mg/kg L-arg resulted in nearly complete reversal ($p < 0.01$)—thereby reflecting a dose–response pattern. Complete reversal was achieved at both doses of L-arg by 60 min. In contrast, 500 mg/kg of the inactive isomer, D-arg, was without effect in reversing the L-NOARG antagonism of CP-induced feeding. Analyses also revealed that arginine did not significantly affect spontaneous feeding under either vehicle or 25 mg/kg L-NOARG conditions. The one-way ANOVAs for the arginine + double vehicle series (solid bars) yielded $F(3, 57) = 0.81$ ($p > 0.4$) and 1.635 ($p > 0.2$), at 30 and 60 min; the ANOVAs for the CP veh + L-NOARG + arginine series (stippled bars) yielded $F(3, 62) = 0.97$ ($p > 0.4$) and 0.28 ($p > 0.8$), at 30 and 60 min. Finally, all groups not given CP (solid and stippled bars) consumed significantly less food than did their corresponding CP-treated (open bars) groups ($p < 0.05$ or $p < 0.01$).

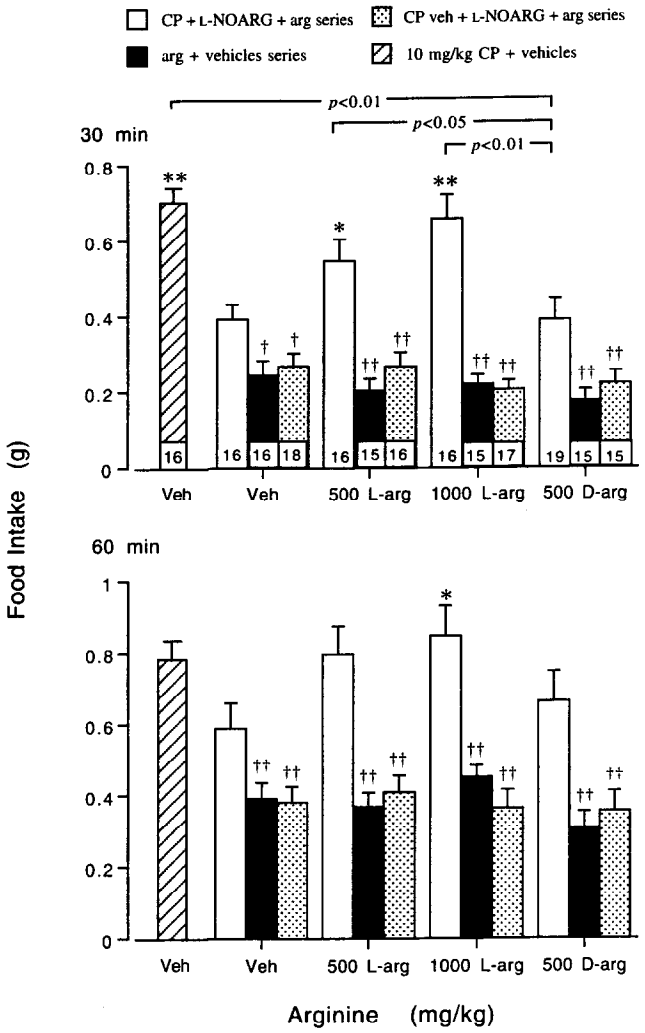

FIG. 2. Mean (± SEM) cumulative food intake in mice administered CP only (crosshatched bars), L- or D-arginine (or veh) only (solid bars), or combined treatment with CP + L-NOARG + L- or D-arginine (or veh) (open bars) or with CP veh + L-NOARG + L- or D-arginine (or veh) (stippled bars), at 30 min (upper panel) and 60 min (lower panel); n = 15–19/group, as indicated in base of bar. Doses: 10 mg/kg CP, 25 mg/kg L-NOARG, 500 or 1000 mg/kg L-arg, 500 mg/kg D-arg, or 0.9% NaCl vehicle. $*p < 0.05$, $**p < 0.01$ compared to CP + L-NOARG + arginine vehicle group, Duncan's multiple range test. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared to corresponding CP + L-NOARG + arginine series group; Bonferonni protected Student's t-test. Additional pertinent significant differences are indicated by brackets (Duncan's test).

DISCUSSION

The aim of the present study was to systematically investigate a possible role of nitric oxide in the well-documented feeding stimulatory effect of benzodiazepine receptor agonists, here using chlordiazepoxide. Two major findings, to the author's knowledge not previously reported, emerged. First, the CNS nitric oxide synthase inhibitor L-NOARG attenuated CP-induced feeding in a clearly dose-related manner. In contrast, identical doses of L-NOARG did not significantly affect normal/spontaneous feeding, although a dose-related trend in the direction of reduced feeding was, indeed, evident. The significant drug interaction, reflected by different dose–response curves for the CP- and vehicle-treated series, indicates that reduced food intake was not simply a consequence of L-NOARG producing a nonspecific anorexia. Failure to observe a significant effect of L-NOARG on normal feeding is consistent with the literature, where NOS inhibitors adminis-

tered systemically at doses comparable to those used in the current study are reported not to have reduced intakes in normal feeding mice (2) and rats (24,25). At the same time it should be noted that NOS inhibitor differed, and that there was variability in effective dosing, across these studies. Squadrito et al. found no effect at (24) or below (25) 50 mg/kg of L-NOARG, while Calignano et al. (2) report an anorexic effect at 30 mg/kg of L-NAME. The latter authors (2), however, also report that 10 and 20 mg/kg of L-NAME completely inhibited morphine (2.5 mg/kg)-induced food intake; blocking of morphine-induced feeding at a lower dose (10 mg/kg) of NOS inhibitor is consistent with the present finding for CP-induced feeding. The second major finding was that L-arg, the substrate for synthesis of NO, acted to restore CP-induced feeding blocked by L-NOARG, and did so in a dose-related manner. Further, this action was stereospecific, the inactive D-isomer being ineffective—thereby providing clear suggestion of NO involvement. At the same time, L-arg did not significantly alter normal feeding. Calignano et al. (2) also found no change in normal feeding in mice administered L-arg, although the dose used was relatively low (50 mg/kg) in comparison with most of the literature. Overall, the present findings are in general agreement with the recent literature for deprivation- and morphine-induced feeding, as well as for normal feeding, in animals pretreated with NOS inhibitors.

A question arises concerning the possibility of an indirect effect of inhibiting NO production on feeding being responsible for current findings, as for example, a shift in general arousal (or exploratory activity—which could also reflect motor impairment). This, however, appears unlikely. Although decreases in horizontal locomotion in rats (23) and mice (16,26) in unfamiliar or novel environments and altered exploratory patterns (16,23) have been linked to systemic injection of L-NAME, these were observed only at relatively high doses (e.g., $\geq$ 100 mg/kg). Unpublished data (not shown) from our laboratory are in general accord; we found that horizontal and vertical (rears) locomotion in an open field were significantly attenuated at 50 and 100 mg/kg of L-NOARG ($p < 0.01$) in ICR mice, while lower doses (10 and 25 mg/kg) were without effect. In the present study, however, a significant drop in food intake was observed at 10 mg/kg of L-NOARG in acclimated animals in a familiar environment using familiar protocols. A related consideration is whether a general malaise or other debilitating circumstance might have led to reduced food intake; this is generally a concern whenever an activated state/condition is attenuated. The related work of Morley's group would seem to argue against such interpretation. These authors report that while the classical illness-inducing agent, lithium chloride, reduced lever pressing for milk reinforcement equally in partially sated and in 18-h fasted mice (10), L-NAME affected lever pressing differentially under these conditions (18). Further, the presently observed differential effect of L-NOARG on CP-induced and normal feeding would argue against a discomfort factor. Mice in the present study exhibited no obvious signs of distress or other unusual behavior.

The literature points to altered NO activity both peripherally and centrally that could contribute to shifts in feeding behavior. NOS inhibitors have been shown to abolish reflexive relaxation of the stomach to accommodate intake of liquid or solid food in guinea pig (8), and to antagonize the lower esophageal sphincter muscle relaxation response to swallowing and to vagal stimulation in opussum (29). Both were reversed only by the active isomer, L-arg. Reduced ingestion is arguably consistent with inhibition of either of these gastrointestinal responses. Squadrito et al. (24,25) suggest brain NO involvement in a central serotonergic system regulating food intake, reporting that depressed levels of serotonin (5-HT) and 5-hydroxyindoleacetic acid in diencephalon following 24-h food deprivation were reversed/increased by systemic injection of L-NOARG, and that selective antagonism of 5-HT receptor subtypes abolished L-NOARG-induced hypophagia in deprived animals. Involvement of serotonin in feeding behavior is well documented [for reviews, see (1,14)]. De Luca et al. (7) most recently reported opposing shifts in oxygen consumption, and in activity of sympathetic nerves innervating and temperature of brown adipose tissue in 24-h fasted rats following central or peripheral administration of L-NAME, while both resulted in depressed food intake—suggesting multiple mechanisms of NOS-linked inhibition of feeding.

In summary, these experiments provide further evidence for involvement of NO in the regulation of feeding behaviors, extending the work of others with deprivation- and morphine-induced feeding in laboratory animals. It should be instructive to systematically probe possible contributions of change(s) in factors such as gastric emptying and taste, and in meal parameters, in future investigations.

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