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Nitric Oxide Synthase Inhibition and Food Intake: Effects on Motivation to Eat and in Female Mice

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MORLEY, J. E., S. A. FARR, M. D. SUAREZ AND J. F. FLOOD. *Nitric oxide synthase inhibition and food intake: Effects on motivation to eat and in female mice.* PHARMACOL BIOCHEM BEHAV **50**(3) 369-373, 1995.—Recent studies have demonstrated that nitric oxide may play an important role in the regulation of food intake. The studies reported here extend these findings by demonstrating that N^G-nitro-arginine-methylester, *N*-Arg(ME), a nitric oxide synthase inhibitor, decreased intake of a highly palatable substance (i.e., milk), though at a higher dose than necessary for decreasing consumption of food pellets. *N*-Arg(ME) failed to inhibit lever press for milk reward in nonprefed mice, but decreased lever pressing in prefed mice. *N*-Arg(ME) decreased food intake in female mice, being most potent in proestrus. These studies suggest that nitric oxide synthase inhibition decreases food intake without inducing aversion or illness.

Anorexia Estrogen Estrous cycle Female Food intake Hunger Lever press Mice Motivation Nitric oxide Progesterone

IN 1987 nitric oxide was demonstrated to be synthesized and released from vascular endothelial cells and to produce relaxation of vascular smooth muscle (16). Since then, nitric oxide has been found to be a mediator, messenger, or regulator of cell function in a variety of physiological systems, including the central nervous system (6). We have reported that inhibition of nitric oxide synthesis decreased food intake and produced weight loss in mice (11,12). This has been confirmed by others (4). Further, nitric oxide synthase inhibition results in weight loss in genetically obese (ob/ob) mice (13) and in Zucker fatty rats (17).

When a drug decreases food intake, it is possible that the decrease is due to a nonspecific effect such as decreased locomotion or is due to "illness" (2,7). Flood et al. (8) have utilized an experimental paradigm where mice have to work to get food (i.e., press a lever) coupled with and without prefeeding to allow the motivational aspects of drug suppression of feeding to be more fully studied.

Sex hormones have been demonstrated to modulate food intake (18). Estrogen decreases food intake and progesterone blocks the anorexic effect of estradiol administration in ovariectomized rats. Most studies on the effects of substances that modulate food intake are done in male animals to obviate potential effects of the estrous cycle on food intake.

The purposes of the studies reported here were to further our understanding of the effects of nitric oxide synthase inhibition on food intake by studying its effects on (i) the intake of a highly palatable substance, (ii) motivational effects on food intake utilizing the lever press paradigm, and (iii) food intake in females during the different phases of the estrous cycle.

METHOD

Subjects

Male TAC (SW) mice 2-3 months of age, obtained from Taconic Farms Inc. (Germantown, NY), or female CD-1 mice 2-3 months of age, obtained from Charles Rivers Breeding Laboratories (Wilmington, MA), served as subjects. They were individually housed in plastic cages and maintained on a 12L : 12D schedule (lights off at 1800 h) under controlled temperature (21-23°C). Water and food (Purina Rodent Lab-

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oratory Chow No. 5001) were available ad lib except as indicated.

Habituation to Milk Solution

Rodents are reluctant to consume novel foods. Therefore, prior to beginning either feeding studies with milk solution or lever press conditioning for milk reinforcement, mice were habituated to a milk solution consisting of one part evaporated milk and two parts water. Habituation to the milk solution was accomplished by providing milk in place of food and water overnight (from 1400 to 0700 h) and replacing food and water in the morning. So that the mice would not lose more than 10% body weight, milk solution was provided for 2 consecutive nights followed by 1 night of standard rodent laboratory chow. After 1 week mice readily consumed most of the 40 ml provided per night. The milk solution contained 0.5 cal/ml.

Determining Food Pellet and Milk Solution Consumption

To determine how much food or milk mice consumed, a food pellet (Purina 5001) or a 40-ml centrifuge tube of milk solution with cork and dripless spout was weighed prior to beginning the experiment. After the mice were injected, a food pellet was placed in the cage or a tube with milk was inserted through the wire cage lid. Following the test period, consumption was determined by weighing the pellet or tube and determining the weight difference. It might be argued that milk is fluid. However, to a hungry mouse this is apparently food because we have previously reported that neuropeptide Y increases the consumption of food and milk but not water (14). In addition, a mouse consumes an average of about 5 ml of water overnight and will drink 40 ml of milk solution over the same time period. The food pellets contained 3.04 cal/g of metabolizable energy.

Lever Press Training and Testing

TAC(SW) mice were trained in fully automated lever press boxes consisting of a small test cage (18.0 × 16.5 cm and 207 cm deep, Coulbourn Instruments Inc. Model E10-11) with one wall containing a lever (E22-01) situated 1.7 cm above the stainless steel grid floor. On the wall opposite the lever, a dipper module (E14-05) delivered 100 ml of milk solution (two parts water to one part evaporated milk) when the mouse pressed the lever. Mice were initially trained to lever press for milk reinforcement after 18-h food and water deprivation. They were trained at 2- to 3-day intervals to press for milk reinforcement on a continuous reinforcement schedule. Each training session lasted 40 min. Mice were trained for 4 days; those reaching a criterion of at least 30 presses in each of the 10-min periods of a training session were used in the experiment.

To test for the effect of the drug on lever press performance, mice were injected and then placed into the lever press apparatus. The number of lever presses made was recorded for a 30-min period with data automatically collected every 10 min. In this paradigm, prefeeding was used to reduce hunger to determine if an anorectic compound would exert a greater effect in mice that were not completely food deprived. Prefeeding consisted of giving the mice free access to the milk solution for 30 min. Testing in the lever press apparatus followed a 30-min period during which no food, water, or milk was provided.

Determining Phase of the Estrous Cycle

In one of the studies below, we determined if the phase of the estrous cycle influenced consumption of a food pellet and whether this interacted with the anorectic effect of *N*-Arg(ME). To determine which phase of the estrous cycle CD-1 female mice were in, cotton swabs prepared from tooth picks and sterile cotton were used to take vaginal smears. The mouse's tail was raised and the cotton swab was inserted into the vagina and rotated. When the swab was removed, it was rolled onto a microscope slide. After microscopic examination, mice were classed as being in proestrus, estrus, metestrus, or diestrus based on the number of cells, the aggregation, and their condition (1). This was repeated daily for three cycles so we could predict when each female would be in a particular part of the cycle. Immediately after the feeding study, vaginal smears were used to confirm the part of the cycle the mouse was in during the feeding study.

Statistics

All results are expressed as mean ± SEM. Statistical significance was determined by analysis of variance (ANOVA). Duncan's multiple range test or Tukey's *t*-test was used to test for significant differences among group means (10,22).

Drugs

N^G-Nitro-arginine-methylester (*N*-Arg(ME), a nitric oxide synthase inhibitor, was obtained from Sigma Chemicals (St. Louis, MO). It was prepared fresh daily and was administered subcutaneously at a volume of 0.01 ml/g body weight.

RESULTS

Experiment 1: The Effect of N-Arg(ME) on Food Pellet and Milk Consumption

TAC(SW) male mice were habituated to milk solution as described above. They were then food deprived overnight (14 h) and at 0800 h were injected with saline or 25 or 50 mg/kg of *N*-Arg(ME). Food or milk solution was provided as indicated above for 60 min following drug administration. Food and milk consumption was determined at the end of the test pe-

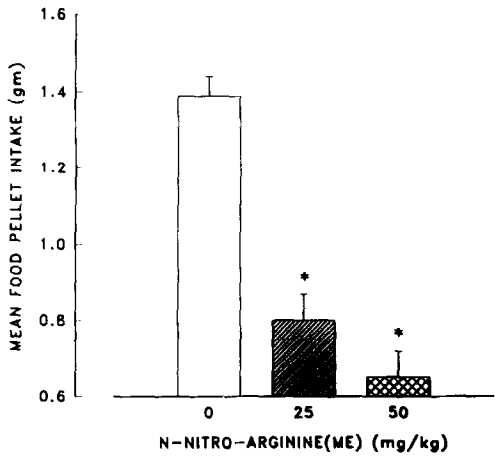

FIG. 1. *N*-Arg(ME) inhibited consumption of food pellets. The asterisk indicates a significant mean difference from the control (0 mg/kg) at $p < 0.01$ by Tukey's *t*-test. The SEM is indicated on each bar.

riod. Mice were randomly assigned to groups of 16. Separate groups of mice were used for the food pellet and milk consumption studies.

The data were analyzed in separate one-way ANOVAs. In the food pellet study, *N*-Arg(ME) had a significant effect on food intake, $F(2, 45) = 28.96$, $p < 0.001$ (Fig. 1). Dunnett's *t*-test indicated that the means of the group administered 25 and 50 mg/kg of *N*-Arg(ME) were significantly lower than the means of the group given saline ($t = 5.59$, $t = 7.27$, $p < 0.01$). In the milk study, *N*-Arg(ME) also had a significant effect on consumption, $F(3, 60) = 14.10$, $p < 0.001$ (Fig. 2). Groups receiving 50 or 100 mg/kg of drug, but not 25 mg/kg, had significantly lower means than did the saline control ($t = 4.73$, $t = 5.01$, $p < 0.01$).

Experiment 2: Effect of N-Arg(ME) on Lever Press Performance as a Function of Prefeeding or no Prefeeding With Milk Solution

TAC(SW) male mice were habituated to the milk solution and trained to lever press for milk reinforcement as described above. Mice were food and water deprived overnight. The following morning half the mice were prefed for 30 min with milk solution while the other half continued to fast. Half the mice in each of these groups received saline or 100 mg/kg of *N*-Arg(ME) and then were placed immediately into the lever press apparatus. The test session was 30 min long. There were 14–17 mice per group.

A three-way ANOVA indicated significant main effects of prefeeding status, $F(1, 162) = 49.11$, $p < 0.001$, and drug state, $F(1, 162) = 22.14$, $p < 0.001$, but not for the time over which the data was collected (Figs. 3 and 4). The interaction of prefeeding status and drug state was significant, $F(1, 162) = 5.15$, $p < 0.05$. The interaction was significant because *N*-Arg(ME) inhibited lever pressing in prefed mice but not among those that were not prefed. The interaction of time by prefeeding status and time by drug state were not significant, nor was the three-way interaction. The significant effect of prefeeding mice was to reduce the number of lever presses by 30% among saline-treated mice relative to the lever presses made by the mice that were not prefed with milk solution.

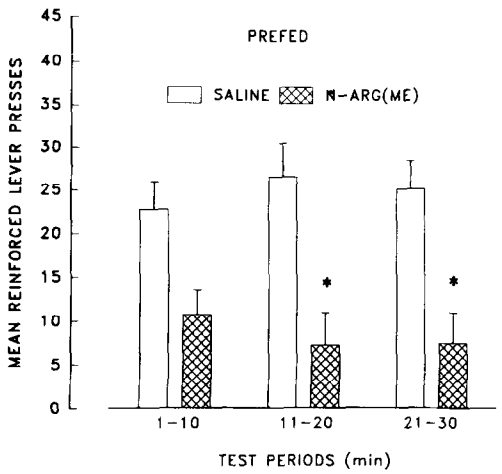

FIG. 3. *N*-Arg(ME) reduced lever pressing in mice prefed with milk solution. The asterisk indicates a significant mean difference from the control (0 mg/kg) at $p < 0.01$ by Tukey's test. The SEM is indicated on each bar.

Among those mice receiving *N*-Arg(ME), prefed mice pressed, on the average, 72% less than those not prefed.

Experiment 3: Effect of N-Arg(ME) on Food Pellet Consumption as a Function of the Estrous Cycle

Groups of 9–10 mice were formed based on the phase of the estrous cycle they should be in on the following morning, as described above. They were food deprived overnight. In the morning, they received either saline or 25 or 50 mg/kg of *N*-Arg(ME) and a food pellet was placed in the cage. Food intake was measured after 60 min. Following the feeding experiment, vaginal smears were taken to confirm the estrous cycle status of each mouse. A total of 40 mice were used at 3-day intervals.

The results of a two-way ANOVA run on grams of food

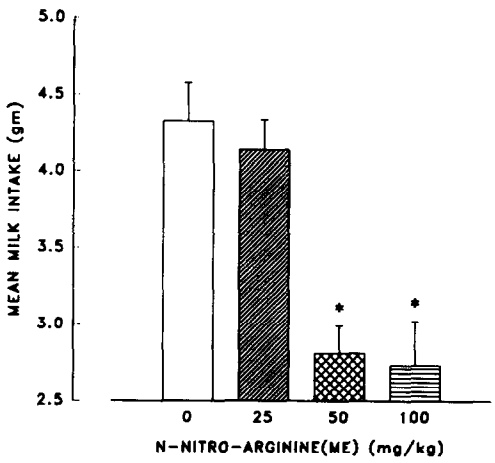

FIG. 2. *N*-Arg(ME) inhibited consumption of milk solution but required higher doses than for food pellets. The asterisk indicates a significant mean difference from the control (0 mg/kg) at $p < 0.01$ by Tukey's *t*-test. The SEM is indicated on each bar.

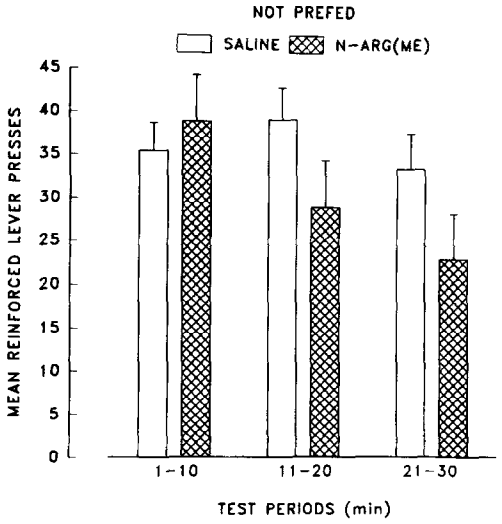

FIG. 4. *N*-Arg(ME) failed to affect lever pressing for milk reinforcement in mice that were not prefed. The SEM is indicated on each bar.

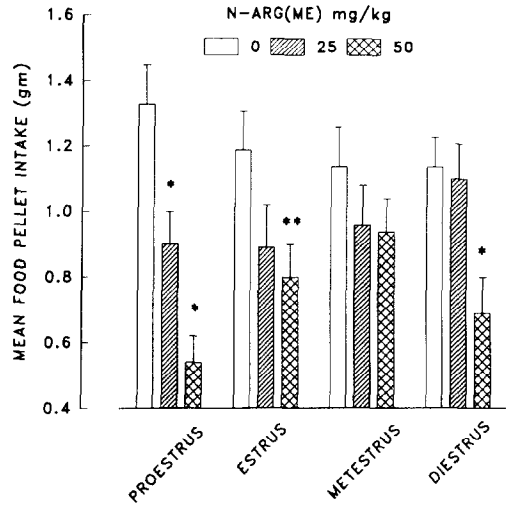

FIG. 5. The phase of the estrous cycle failed to alter food pellet consumption in these intact mice. *N*-Arg(ME) at 100 mg/kg suppressed feeding to some degree in each part of the cycle except during metestrus. The asterisk(s) indicates a significant mean difference from the control (0 mg/kg) at $*p < 0.01$ and $**p < 0.05$ by Duncan's multiple range test.. The SEM is indicated on each bar.

consumed indicated that the main effect of drug state was significant, $F(2, 107) = 19.49$, $p < 0.001$), but the main effect of the estrous cycle was not significant, $F < 1$. Among saline (0 mg/kg)-treated mice, those in proestrus tended to eat more than those in other phases of the estrous cycle but the difference was not significant. Among the mice in the proestrous phase, *N*-Arg(ME) significantly reduced feeding in a dose-dependent manner (Fig. 5) with significant suppression of eating at 25 and 50 mg/kg. For mice in the estrous and diestrous phases, only 50 mg/kg suppressed feeding, and no dose significantly reduced feeding for those in metestrus.

DISCUSSION

These studies demonstrate that the nitric oxide synthase inhibitor, N^G-Arg(ME): (i) has to be utilized at a higher dose to inhibit a highly palatable food (milk) compared to regular food pellets (Exp. 1); (ii) has a greater effect in partially satiated mice (prefed) than in fasted mice (not prefed) when lever pressing for milk reinforcement (Exp. 2); and (iii) inhibits food intake in female mice, with the effect being most prominent during proestrus. These studies strongly support the concept that the decreased feeding following nitric oxide synthase inhibition is specific to the degree of "hunger" experienced by the animal. This supports the concept that nitric oxide plays a physiological role in the regulation of food intake.

Previous studies have examined a variety of methods to determine whether food inhibition by drugs alters appetite or produces gustatory aversion (i.e., induces illness). The classical method to determine this has been the utilization of the conditioned taste aversion paradigm (5,7). We have previously argued that this phenomenon is dependent more on the novel pairing of a drug and food than due to illness per se (8); drugs that do not otherwise affect feeding will suppress feeding in this paradigm. Billington et al. (2) developed the differential satiety paradigm in which it was found that true satiety substances decreased food intake to a greater extent in animals that were less hungry (shorter periods of food deprivation) than in those that were more hungry. The lever press paradigm with and without prefeeding represents the reverse of this paradigm. Flood et al. (8) demonstrated that the classical aversive agent, lithium chloride, decreased lever pressing equally in prefed and nonprefed mice. Although no single experiment can unequivocally rule out illness as the reason for reduction of food intake, the results of Experiments 1 and 2 suggest that inhibition of nitric oxide synthase reduces appetite. Thus, higher doses of *N*-Arg(ME) were needed to suppress food intake of the more palatable milk solution than the standard rodent chow. Mice with a reduced level of hunger due to prefeeding showed suppression of lever pressing when given *N*-Arg(ME), whereas those not prefed show no significant reduction of lever pressing. If *N*-Arg(ME) caused significant "illness," then it would be expected to reduce lever pressing in both groups, as previously reported for lithium chloride.

Female hormones have been found to alter food intake in ovariectomized rats; those given estrogens show a decrease in food intake, whereas progesterone blocked anorexia induced by estradiol (18). Previous studies have shown that female hormones may modulate the effects of naloxone (15,20) and peptide hormones (19,21) on food intake. Our study, using intact female mice, did not detect an effect of estrous cycle during the 60-min test. The discrepancy with previously reported results may be due to species differences, the short period of time over which food intake was measured, or to the fact that intact female rodents show relatively small differences in the levels of estrogen and progesterone as the cycle changes (3). The differing effects of nitric oxide synthase inhibitors on food intake at different phases of the estrous cycle suggest that nitric oxide effects may be modulated by female hormones. The effects of nitric oxide on vasodilation are more marked in females than males (9).

In conclusion, the studies reported here support a physiological role for nitric oxide in the modulation of food intake. Sex steroid hormones may have a modulatory effect on nitric oxide effects on food intake.

ACKNOWLEDGEMENT

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