Effect of NPY in the hypothalamic paraventricular nucleus on uncoupling proteins 1, 2, and 3 in the rat

C. M. Kotz, C. F. Wang, J. E. Briggs, A. S. Levine, and C. J. Billington

The uncoupling proteins (UCP) are mediators of thermogenesis that may play a role in the modulation of energy balance (5). UCP-1 has a demonstrated role in uncoupling ATP production from mitochondrial respiration, the result of which is dissipation of energy as heat (17). Strong evidence exists to support the idea that UCP-2 and UCP-3 act in a similar manner (5). This uncoupling activity decreases efficiency of energy metabolism, and because increased metabolic rate may result in a decreased propensity to gain weight (23), alterations in UCP-3 may have a dramatic influence on energy expenditure. A very recent report indicates the existence of another UCP, UCP-4, that is present only in brain tissue (20). The role of UCP-4 has yet to be determined.

Little is known about the neural regulation of UCP-2 and UCP-3. Neuropeptide Y (NPY) is a powerful stimulator of feeding (10, 18), and several lines of evidence exist for NPY-UCP signaling. It has previously been shown that NPY injected into the hypothalamic paraventricular nucleus (PVN) decreases the gene expression of UCP-1 in BAT independent of changes in food intake and insulin levels (4). NPY decreases sympathetic activation to BAT (13), and a proposed PVN-BAT neuronal signaling pathway has been demonstrated by use of the transneuronal viral labeling technique (2). NPY administered into the ventricle decreases insulin-stimulated uptake of glucose into muscle tissue, but this effect is dependent on increased food intake and elevated insulin and does not occur during states of normoinsulinemia (28). In obese (fa/fa) rats, NPY levels are significantly increased (3), whereas muscle UCP-3 gene expression is significantly decreased (7), indicating possible modulation of muscle UCP-3 by NPY. It was recently demonstrated that ventricular NPY administration results in decreased UCP-3 mRNA in BAT, although the effect on muscle UCP-3 was not tested (27).

The current study was undertaken to determine whether NPY in the PVN alters the gene expression of UCP-2 in WAT and UCP-3 in muscle tissue. Because treatment effects on the UCPs can be dependent on tissue depot assayed (5, 16), we assayed two muscle depots: acromiotrapezius (TM) and biceps femoris muscle (BFM). These depots were chosen based on their potential for involvement in physical activity, because physical exercise influences muscle UCP-3 (6, 26). BFM is highly involved in physical activity, whereas TM is less important to exercise conditions. Because leptin has been reported to influence UCP-2 in WAT and UCP-3 in muscle (11, 12), serum leptin was also measured in this study.

Kotz, C. M., C. F. Wang, J. E. Briggs, A. S. Levine, and C. J. Billington. Effect of NPY in the hypothalamic paraventricular nucleus on uncoupling proteins 1, 2, and 3 in the rat. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R494–R498, 2000.—Neuropeptide Y (NPY) injected into the hypothalamic paraventricular nucleus (PVN) stimulates feeding and decreases uncoupling protein (UCP)-1 mRNA in brown adipose tissue (BAT). The present studies were undertaken to determine whether UCP-2 in white adipose tissue (WAT) and UCP-3 in muscle are regulated by NPY in the PVN. PVN-cannulated male Sprague-Dawley rats were injected with either saline or NPY (PVN, 117 pmol, 0.5 µl) every 6 h for 24 h. NPY in the PVN stimulated feeding and decreased UCP-1 mRNA in BAT independent of NPY-induced feeding. UCP-2 mRNA in WAT was unchanged by NPY. In acromiotrapezius muscle, NPY decreased UCP-3 mRNA, but this was reversed by restricting food intake to control levels. In biceps femoris muscle, NPY alone had no effect on UCP-3 mRNA, but UCP-3 mRNA was significantly increased in the NPY-treated rats that were restricted to control levels of intake. These results suggest that UCP-2 in WAT and UCP-3 in muscle are not subject to specific regulation by NPY in the PVN.

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measured to determine whether changes in the UCPs were correlated with leptin levels.

**METHODS**

Animals. Male Sprague-Dawley rats (Harlan, Madison, WI) weighing 280–305 g were individually housed in conventional hanging cages with a 12:12-h light-dark photoperiod (lights on at 0700) in a temperature-controlled room (21–22°C). Teklad lab chow and water were allowed ad libitum, except where noted.

Cannulation. Rats were anesthetized with Nembutal (40 mg/kg) and were fitted with a 26-gauge stainless steel guide cannula placed just above the PVN. Stereotaxic coordinates were determined from the rat brain atlas by Paxinos and Watson (22) and are as follows: 0.5 mm lateral and 1.9 mm posterior to bregma and 7.3 mm below the skull surface. The injector extended 1 mm beyond the end of the guide cannula. For all cannulations, the incisor bar was set at 3.3 mm below the ear bars. At least 10 days elapsed following surgery before experimental trials.

Verification of cannula placement. After the experiment, brains were dissected out and stored in a 10% formaldehyde solution for later placement verification by histological examination. Data from animals with incorrectly placed cannulas were excluded from the final analysis. A cannula is deemed incorrect if the actual injection site is farther than 0.25 mm away from the targeted site. This is based on diffusion of injectate volume delivered (21).

Drugs. NPY was purchased from Bachem (Torrence, CA) and was dissolved in artificial cerebrospinal fluid (aCSF) just prior to use.

Experimental design. Twenty-seven PVN-cannulated rats were randomly separated into three groups: 1) saline with food provided ad libitum, 2) 117 pmol NPY with food provided ad libitum, or 3) 117 pmol NPY with food restricted to that of the saline-treated animals. Rats were injected into the PVN every 6 h for 24 h, starting at 0800. Food intake was measured at 1, 2, 4, 6, 12, 18, and 24 h. Two hours after the final set of injections at 1000, the animals were killed, and blood and tissues were taken for analysis. UCP-1, -2, and -3 mRNA in BAT, UCP-2 mRNA in epididymal WAT, and UCP-3 mRNA levels in TM and BFu tissue were measured.

Food intake measurements. Food was allowed ad libitum throughout the experiment with the exception of rats in the pair-fed group. For pair-fed rats, food intake of the saline-treated rats was measured first, and then an amount, -1 g above that amount (to allow for spillage), was placed inside the NPY-treated, pair-fed rat’s cage.

Leptin RIA. Blood samples were centrifuged for 20 min at 2,000 g, and sera were stored at -4°C until use. On the day of the RIA, samples were slowly thawed and 100 µl sera were taken and added to the RIA tube for use in the rat leptin RIA kit (Linco Research, St. Louis, MO). Standard concentrations ranged between 0.5 and 50 ng/ml, which is within the limit of linearity. All samples were within this range. The assay sensitivity is 0.5 ng/ml (100-µl sample size). The cross-reactivity test, provided by Linco Research, indicated no cross-reaction with insulin, glucagon, or somatotropin release-inhibiting factor.

UCP-1, -2, and -3 and β-actin mRNA determination. Total RNA from all tissues was extracted by the rapid guanidine thiocyanate-phenol-chloroform method (9). Tissue was homogenized in a buffer containing 4 M guanidine thiocyanate with added β-mercaptoethanol and water-saturated molecular bi- 

ology grade phenol. Sarcosyl, 2 M sodium acetate, and chloroform were then added. After centrifugation, the aqueous phase was precipitated with isopropanol, resuspended in guanidine thiocyanate buffer, and reprecipitated with isopropanol. The pellet was washed with 75% ethanol. Resulting RNA was stored in 100% ethanol at -80°C.

Samples were analyzed by the slot-blot method using nylon membranes (Zeta-Probe, Biorad, Hercules, CA). Aliquots of total RNA were dissolved in 7.4% formaldehyde 6× standard citrate (SSC; 1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) and denatured for 10 min at 68°C. Duplicates of 2-µg total RNA of each sample were slot-blotted onto 6× SSC-soaked nylon membrane (Zeta-Probe, Biorad). The membranes were then placed under ultraviolet (UV) light, and even loading of samples was verified by shadowing of the nucleic acids (25). The RNA was fixed onto the nylon after air drying by UV cross-linking. The slot-blot membranes were prehybridized for 24 h at 42°C in 50% formamide, 5× SSC, 10× Denhardt’s solution, 0.1% SDS, and denatured salmon sperm DNA in 50 mM Na phosphate, pH 6.5. For the UCP-1 hybridization, we used UCP-365 probe, generously supplied by Dr. Daniel Ricquier (Meudon, France). For the UCP-2 hybridization, we used a cDNA probe specific for rat UCP-2 (generously donated by Dr. Craig Warden (Univ. of CA, Davis, CA)). For the UCP-3 hybridization, we used a cDNA probe specific for rat UCP-3 (generously donated by Dr. Bradford Lowell, Beth-Deaconess Hospital, Boston, MA). For the β-actin hybridization, we used β-actin probe obtained from Oncor (Gaithersburg, MD). The hybridization medium (16 ml/tube) was 50% formamide, 5× SSC, 2× Denhardt’s solution, 0.2% SDS, denatured salmon sperm DNA and yeast t-RNA in 50 mM Na phosphate, pH 6.5 with the addition of 106 counts·min-1·µl-1 of [32P]2-deoxycytidine 5'-triphosphate (specific activity = 3,000 Ci/mm) random primer labeled probe. After hybridization for 24 h at 42°C, the nylon membranes were subjected to high stringency washes (50% vol/vol, 1 h, 70°C). The membranes were then scanned using a Storm imager (Molecular Dynamics), and samples were quantified using ImageQuaNT software (Molecular Dynamics). Levels of mRNA are expressed in optical density (OD) units. The membranes were stripped of probe and radioactive label with 10 mM Na2HPO4 and deionized formamide solution (50% vol/vol, 1 h, 70°C). After determining that the membranes were negative for [32P] signal, the membranes were subsequently labeled with β-actin (24 h at 42°C), and levels were quantified as described above for UCP. To normalize the data for overall changes in gene expression and minor individual variability in RNA loading onto the slot blots, UCP mRNA levels were divided by β-actin mRNA levels such that data are expressed as UCP mRNA/β-actin mRNA.

Statistical analysis. Data were analyzed by one-factor ANOVA followed by Fisher’s protected least significant t-test to compare treatment means. Data are represented as means ± SE.

**RESULTS**

As shown in Fig. 1, NPY significantly increased 24-h food intake (P < 0.01). Although the pair-feeding protocol resulted in these animals consuming somewhat less than controls, this difference was not significant (P = 0.1589, Fig. 1). NPY treatment resulted in a significant decrease in UCP-1 mRNA levels in BAT in both the NPY-treated ad libitum-fed rats and the NPY-treated pair-fed rats (P = 0.0136 and P = 0.0262, respectively; Fig. 2). There was no effect of NPY treatment on BAT mRNA levels of UCP-2 (saline: 1.8 ± 0.1; NPY: 1.7 ± 0.2; NPY pair fed: 1.7 ± 0.2, OD units) or UCP-3 (saline: 5.3 ± 0.3; NPY: 5.0 ± 0.6; NPY pair fed:
5.5 ± 0.4, OD units). There was no effect of NPY treatment on UCP-2 mRNA in WAT in either the ad libitum-fed or pair-fed groups (Fig. 3). NPY in the PVN decreased UCP-3 mRNA levels in TM (P = 0.0414, Fig. 4). However, this decrease was reversed by restricting food intake to control levels (P = 0.2326), indicating that the effect observed in the NPY-treated animals is due to the increased feeding in this group (Fig. 4). In the BFM, NPY treatment alone had no effect on gene expression of UCP-3 (P = 0.6074, Fig. 5). However, there was a significant induction of UCP-3 in the NPY-treated pair-fed animals (P = 0.0189, Fig. 5). Serum leptin was increased by NPY treatment (P = 0.0119, Fig. 6). This effect appears to be due to the increased food intake in these animals, because serum leptin levels in the NPY-treated rats with food intake paired to control levels were the same as the levels in the controls (P = 0.9953, Fig. 6).

DISCUSSION

NPY in the PVN significantly stimulated feeding and decreased UCP-1 gene expression in BAT (Figs. 1 and 2). This decrease was independent of NPY-stimulated feeding, because there was no difference in UCP-1 mRNA between the NPY-treated ad libitum-fed and pair-fed groups (Fig. 2), a result that confirms previous studies (4). There was no difference in UCP-2 and -3 mRNA levels in BAT in response to NPY treatment, suggesting specific regulation of BAT UCP-1 by NPY in the PVN. A recent report indicates that ventricular NPY results in decreased UCP-3 in BAT (27). These somewhat discrepant results may be due to the difference in site of NPY injection, because a ventricular injection distributes injectate further and to several sites close to the ventricular space, and the neural origin of stimulus is unknown. Together, these data suggest the possibility of other NPY-responsive sites outside of the PVN that regulate UCP-3 in BAT.

It has been shown that lipoprotein lipase activity in WAT is influenced by PVN NPY (4), and a direct signaling pathway has been demonstrated through the use of retrograde labeling techniques (1). Thus there is basis for suspecting that genes present in WAT, with relevance to energy metabolism, may be influenced by neuromodulation within the PVN, a major site of
sympathetic nervous system (SNS) outflow. However, in the current study, there was no effect of NPY in the PVN on UCP-2 mRNA in epididymal WAT (Fig. 3). On the basis of accumulating evidence that UCP responses may be tissue and depot dependent (5), it is possible that results may be different in other WAT depots. However, future studies will be needed to verify this possibility.

The response of muscle UCP-3 mRNA is dependent on muscle depot assayed. In TM, NPY significantly decreased UCP-3 gene expression (Fig. 4). However, unlike NPY regulation of UCP-1 in BAT, this effect appears to be due to the enhanced food intake in these rats, because restricting the food intake of NPY-treated rats to the level of that in the control rats completely abolished the effect of NPY on UCP-3 (Fig. 4). Thus it appears that NPY does not specifically regulate the UCP-3 gene in TM. In the BFM, a different pattern emerges. NPY treatment and alterations in food intake alone had no effect on the UCP-3 gene, but there was a significant interaction between NPY treatment and food restriction on the UCP-3 gene, as UCP-3 mRNA was significantly increased in this group (Fig. 5). Although this result is difficult to interpret, one possibility may be related to the level of activity in these animals. In the current study, the NPY pair-fed rats were provided with the same amount of food as the control animals and thus are not considered to be food restricted in the conventional sense. However, this controlled level of intake is not what the rats would consume if allowed ad libitum access to food, as evidenced by the induction of feeding observed in the rats given NPY with ad libitum access to food (Fig. 1). Thus it is likely that, in these animals, there was a perception of hunger, causing them to search the cage for more food. Because physical activity induces muscle UCP-3 gene expression (26), this may explain the induction of the UCP-3 gene in the BFM of the NPY-treated pair-fed rats. However, additional studies assessing activity levels in these animals will be necessary to verify this hypothesis.

Although it has previously been demonstrated that response of the UCPs to various treatments is dependent on tissue type (5), differences in UCP-3 response within different muscle depots has not been previously reported. The mechanism behind the different patterns observed in the two muscle depots may be related to several factors, including level of innervation by the SNS, type of muscle (glycolytic/oxidative), and relative role of the muscle in physical activity. One might expect that UCP-3 in leg muscle, such as the BFM, may be more influenced by factors related to physical activity than is UCP-3 in TM, which, due to its interscapular location, may be less involved in physical exertion. The decrease in UCP-3 mRNA in TM due to overconsumption indicates that during periods of overeating, this muscle tissue may become more efficient at using energy, allowing less dissipation as heat and may contribute to the obesity observed in rats chronically stimulated with NPY (24). These alterations in the UCP-3 gene may have profound consequences for energy balance due to the relatively large proportion of muscle mass in humans. However, as demonstrated in the current studies, this pattern is not consistent across muscle depots.

The serum leptin results indicate that leptin is not specifically involved in any NPY effects observed in the current study (Fig. 6). Rather, serum leptin appears to reflect levels of food intake regardless of treatment group. Low level of intake results in decreased serum leptin and high level of intake results in elevated serum leptin. Although measurement of serum leptin does not directly reflect leptin activity within adipose tissue or leptin action in the brain, this measurement does provide evidence for overall leptin activity.

In conclusion, NPY administration in the PVN decreases UCP-1 gene expression in BAT, whereas UCP-2 and -3 in BAT are unaffected by NPY in the PVN. We had hypothesized that NPY would have similar effects on all three UCPs. However, this was not the case, and our findings are discussed in the manuscript. UCP-2 in WAT and UCP-3 in muscle do not appear to be specifically influenced by NPY in the PVN, although changes in food intake influence the muscle UCP-3 gene depending on muscle depot assessed. Thus the effect of NPY on UCP-2 and -3, regardless of tissue depot, is different than its effect on UCP-1 in BAT. Further studies examining the role of caloric excess and physical activ-
ity on the UCP-3 gene in several muscle depots are needed to clarify the role of the UCPs in energy balance.

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Address for reprint requests and other correspondence: C. Kotz, Veterans Affairs Medical Center, Research Service (151), One Veterans Drive, Minneapolis, MN 55417. (E-mail: kotzz004@tc.umn.edu)

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