Peptides that Regulate Food Intake
Repeated administration of the anorectic factor prolactin-releasing peptide leads to tolerance to its effects on energy homeostasis

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10.1152/ajpregu.00237.2003.—Central administration of a single dose of prolactin-releasing peptide (PrRP) causes a reduction in both fast-induced and nocturnal food intake and body weight gain. The aim of this study was to examine the effect of repeated administration of PrRP on energy homeostasis, including a measure of the expression of the mitochondrial uncoupling protein-1 (UCP-1) in brown adipose tissue. Conscious, free-feeding animals received central injections of PrRP (4 nmol icv) or vehicle. A single injection at 1000 caused a sustained hyperthermia over the 4-h test period and an increase in the expression of UCP-1 mRNA. Repeated, twice daily injection caused a reduction in body weight gain greater than that seen in pair-fed animals for the first 48–72 h. After 72 h, the animals became refractory to the actions of PrRP. The pair-fed group showed a reduction in UCP-1 mRNA expression at 48 h, which was reversed by PrRP treatment. This study indicates that PrRP exerts its effects on energy homeostasis in the short-medium term by reducing food intake and increasing energy expenditure.

ProLactin-ReleaseIng Peptide (PrRP) was discovered in 1998 by reverse pharmacology from extracts of bovine hypothalamus (12). It has a limited distribution in the brain and the periphery. In the brain, PrRP is expressed in the dorsomedial hypothalamus and in the nucleus of the solitary tract and the ventrolateral medulla of the brain stem (3, 7, 14, 18, 20), but the lack of PrRP-immunoreactive fibers in the external region of the median eminence argues against it being a hypothypothalamic hormone (18). Rather, the localization of PrRP in regions associated with the regulation of energy balance suggests an alternative role. This was confirmed when it was demonstrated by our group and others that a single, central injection of PrRP inhibits fast-induced and nocturnal food intake and body weight (16, 17, 29). The contribution of endogenous PrRP in the regulation of energy homeostasis was supported when it was shown that PrRP mRNA is downregulated during states of negative energy balance, such as fasting and lactation (6, 16), and in chronic genetic obesity (6).

Of the peptide factors known to regulate appetite and body weight, some have actions in the short term to mediate the initiation and termination of feeding incidences, whereas others are involved in longer-term regulation, modulating energy expenditure, and the expression of other regulatory neuropeptides. An example of a peptide that has short-term effects to induce satiety is CCK. CCK causes a reduction in food intake in rats (9) and a number of other species, including humans (5, 8, 13, 25), through its action on vagal sensory afferents from the gut to the brain stem (33). However, chronic administration of CCK in rats results in the rapid development of tolerance (4). Other factors shown to have predominantly short-term effects on energy homeostasis include orexin-A (11, 28, 35), galanin (32), and melanin concentrating hormone (MCH; 27). In contrast, leptin is effective over the longer term by acting on both the brain (36), to reduce food intake and increase energy expenditure, and peripheral tissues. Leptin causes weight loss due to specific depletion of adipose tissue (10), activation of lipid oxidation (30), and apoptosis of adipocytes in rats (26).

There are similarities in the responses to single intracerebroventricular injections of PrRP and leptin, including a reduction in food intake and body weight, accompanied by an increase in core body temperature, a surrogate marker of heightened energy expenditure (6, 16). However, there are also similarities with the behavioral satiating effects of CCK. Indeed, brain stem PrRP neurons are activated by systemic injection of CCK, but not by the nonphysiological anorexigen, lithium chloride (17). The aim of the present study was to examine whether PrRP is able to exert its anorexigenic effects only in the short term as a satiety factor or whether it is effective in the longer term in a similar manner to leptin. Our previous experiments have suggested that PrRP can increase thermogenesis. Thus we predicted an increase in the expression of brown adipose tissue (BAT) uncoupling protein-1 (UCP-1), a
marker for activation of the sympathetic nervous system (1, 22).

METHODS

Animals and materials. All experiments were performed using adult male Sprague-Dawley rats (250–300 g, Charles River Laboratories, Sandwich, UK). Animals were kept in a 12:12-h light/dark cycle (lights on 0800–2000) at 21 ± 1°C with 45 ± 10% humidity and free access to food (Beekay International, Hull, UK) and water. All experiments were performed in accordance with the United Kingdom’s Animals (Scientific Procedures) Act (1986). Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich, Poole, UK).

Animals underwent lateral cerebroventricular cannula (0.8 mm posterior and 1.5 mm lateral to bregma and 3.0 mm down from dura; coordinates according to Ref. 24) under halothane anesthesia. After 1 wk of recovery, animals were housed individually and left to acclimatize. The free-moving, conscious rats were given intracerebroventricular injections of 4 nmol PrRP1–31 (Peptide Institute, Osaka, Japan) or vehicle (isotonic sterile saline) in a volume of 2 μl at the times stated. At the end of the experiment, animals were killed using a rising concentration of CO2 followed by cervical dislocation. Interscapular BAT and epididymal white adipose tissue (WAT) were collected and weighed.

Quantification of UCP-1 mRNA levels in BAT. RNA was extracted from BAT using Trizol (Invitrogen/NEN Life Science, Hounslow, UK): 0.5 μg DNase I-treated RNA was incubated with 1× Taqman reverse transcriptase buffer, 5.5 mM MgCl₂, 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, 1.25 U/μl Multiscribe reverse transcriptase in a total volume of 50 μl. The reverse transcription reaction was performed in a thermocycler under the following conditions: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. PCR was performed in a total volume of 25 μl in a 96-well plate; 5 μl cDNA and 12.5 μl Taqman universal PCR master mix containing 300 nM forward and reverse primers and 200 nM probe made up to 25 μl with nuclease-free water. The sequence of the primers and probe for UCP-1 and the housekeeping gene, human hypoxanthine phosphoribosyl transferase (HPRT), were as follows: UCP-1, forward: 5'-GAAACGCCTGCCTCTTTGG-3', reverse: 5'-GTGAAGGTCAGAATGCAAGCAC-3', probe: 5'-CGAGCCGACCGGTTCTG-3'; and HPRT, forward: 5'-CGAGCCGACCGGTTCTG-3', reverse: 5'-CATAACCTGGTTCATCACTAACATC3', probe: 5'-CATGTCGACCCTCAGTCCCGACG3'.

RESULTS

Experiment 1: acute effect of PrRP. A single, intracerebroventricular injection of 4 nmol PrRP caused a rapid and transient hypothermia (nadir 10 min after injection), followed by a small, but extended, hyperthermia as reported after nighttime PrRP injection (6, 16). This hyperthermia was apparent from 30 min after the injection to the end of the 4-h recording period and was significantly different from vehicle-treated animals (AUC: vehicle 0.5 ± 0.05°C/h, PrRP 1.0 ± 0.1°C/h; P < 0.05, Fig. 1A). The increase in BAT UCP-1 expression was significant using a one-tailed Student's t-test (relative expression: vehicle 1.0 ± 0.1, PrRP 1.6 ± 0.3; P < 0.05; Fig. 1B).

Experiment 2: effect of repeated administration of PrRP (48 h). Administration of PrRP twice daily produced a reduction in cumulative food intake in the 24 h after the first injection, but this reduction was not statistically significant compared with vehicle-treated animals (vehicle 27.1 ± 1.4 g, PrRP 23.1 ± 1.9 g; P > 0.05; Fig. 2A). However, there was a significant reduction in cumulative body weight gain over the 24 h after the first injection (vehicle 10.1 ± 1.7 g, PrRP 6.2 ± 2.7 g; P < 0.01; Fig. 2B). Pair-fed animals that received the same amount of food as was consumed by the PrRP-treated group, gained significantly less body weight in the first 24 h after the initial injection than vehicle-treated animals, but this reduction was not as great as that for the PrRP-treated group (pair-fed 4.7 ±
Water intake was unaffected by any of the treatments (results not shown).

Food intake in PrRP-treated animals remained lower than that of the control group during the 48-h period, although again this did not reach statistical significance (vehicle 55.0 ± 1.6 g, PrRP 48.4 ± 3.4 g; P > 0.05; Fig. 2B). However, there was again a significant difference in body weight gain (vehicle 21.3 ± 1.1 g, PrRP 11.0 ± 3.0 g; P < 0.05; Fig. 2B). Pair-fed animals showed a significantly reduced body weight gain compared with vehicle, but not PrRP-treated animals (pair-fed 13.4 ± 2.1 g; P < 0.01 vs. vehicle, P > 0.05 vs. PrRP; Fig. 2B).

There was no significant difference in the interscapular BAT (vehicle 0.06 ± 0.005%, PrRP 0.06 ± 0.005%, and pair-fed 0.07 ± 0.005%; P > 0.05) or epididymal WAT (vehicle 0.41 ± 0.04%, PrRP 0.37 ± 0.03%, and pair-fed 0.36 ± 0.02%; P > 0.05) mass, expressed as percentage of total body weight, between groups after 48 h of twice daily administration of PrRP. UCP-1 mRNA expression was significantly reduced in the pair-fed animals (vehicle 1.0 ± 0.17, pair-fed 0.38 ± 0.07; P < 0.05 compared with vehicle-treated group; Fig. 2C). PrRP-treated animals did not show this decrease (PrRP 0.95 ± 0.12; P > 0.05 and P < 0.01 compared with vehicle-treated and pair-fed groups, respectively; Fig. 2C).

Experiment 3: effect of repeated administration of PrRP (5 days). Animals again received twice daily administration of PrRP, and the experiment was continued until the animals became tolerant to the actions of PrRP. As with the previous experiment, there was no significant difference in cumulative food intake between groups at any time point (Fig. 3A). Tolerance to

Fig. 1. Effect of acute administration of prolactin-releasing peptide (PrRP) in satiated animals on change in core-body temperature (A) and uncoupling protein (UCP-1) mRNA levels in brown adipose tissue (BAT; B). Animals received a single intracerebroventricular injection of PrRP (4 nmol) or vehicle at 0800 and were killed 4 h later. Core body temperature was measured continuously by remote radiotelemetry and UCP-1 mRNA expression by quantitative real-time PCR. Data are expressed as means ± SE; n = 8 per group. $P < 0.05 compared with vehicle group, 1-tailed Student’s t-test.
the effect of PrRP appeared to occur at around 72 h after the first injection. Forty-eight hours after the initial injection, animals treated with PrRP showed a 32.5 ± 8.4% reduction in overnight food intake compared with vehicle-treated animals. The reduction in overnight food intake in the PrRP-treated group did not quite reach statistical significance at 72 h (P = 0.053) and, by 96 h, the overnight food intake of the PrRP-treated animals was indistinguishable from vehicle-treated animals and remained so for the duration of the experiment.

In the 24 h after the first injection, PrRP-treated animals gained 6.1 ± 1.8 g compared with vehicle-treated animals, which gained 10.7 ± 1.0 g (P < 0.05; Fig. 3B). By 48 h after the start of the experiment, animals treated with PrRP showed an overall loss in cumulative body weight, −1.5 ± 4.4 g, whereas animals that were treated with vehicle continued to show an increase in cumulative body weight, 17.4 ± 1.9 g. The pair-fed animals that received the same amount of food as the PrRP-treated animals did not show the same degree of weight loss as the PrRP-treated animals and overall gained 11.9 ± 1.2 g in weight at 48 h, which was significantly different from the PrRP-treated group (P < 0.05; Fig. 3B). After 72 h, there was still a significant difference in cumulative body weight gain between PrRP- and vehicle-treated groups (vehicle 25.8 ± 1.5 g, PrRP 5.5 ± 5.6 g; P < 0.05; Fig. 3B), but at this time point there was no significant difference when compared with pair-fed animals (pair-fed 11.6 ± 1.6 g; P > 0.05). After 96 h and for the duration of the experiment there was no significant difference in cumulative body weight gain between groups (Fig. 3B).

There was no significant difference in the BAT (vehicle 0.10 ± 0.07%, PrRP 0.08 ± 0.009%, and pair-fed 0.09 ± 0.008%; P > 0.05) and WAT (vehicle 0.38 ± 0.02%, PrRP 0.36 ± 0.03%, and pair-fed 0.38 ± 0.01%; P < 0.05) mass, expressed as percentage of total body weight, between groups. After 5 days of twice daily PrRP administration, there was no significant difference in the expression in BAT UCP-1 mRNA, compared with vehicle-treated or pair-fed animals (P < 0.05; Fig. 3C).

**DISCUSSION**

We previously showed that PrRP has a transient effect on food intake, measurable in the first hours after injection (6, 16). Although no effect on cumulative food intake is measured at 24 h, a single injection does appear to have a longer-lasting effect on body weight gain. As demonstrated here and previously with the pair-feeding groups, PrRP had additional effects on body weight that could not be attributed purely to its anorectic action. We have confirmed that PrRP has an apparent thermogenic effect, which is independent of whether the animal is eating. As during normal free feeding and fast-induced refeeding (6, 16), daytime injection of PrRP caused a long-lasting hyperthermia in satiated rats (experiment 1). Raised core body temperature is an indirect indication that energy expenditure has increased. More direct evidence for this is the fact that PrRP increases oxygen consumption (C. B. Lawrence, unpublished observations) and, as demonstrated herein, acutely increases the expression of UCP-1 mRNA in BAT. This suggests that PrRP may mediate changes in body weight gain via the modulation of UCP-1 expression, which in turn alters energy expenditure. Although changes in UCP-1 mRNA levels occurred after administration of PrRP, this does not
necessarily reflect a change in UCP-1 protein expression or mitochondrial activity. There have been reports in the literature to suggest that under certain circumstances changes in UCP mRNA levels are not reflected by changes in protein expression (31). It will be important to confirm that there are consistent changes in mitochondrial activity.

The main aim of the study was to examine the effects of chronic administration of PrRP on energy homeostasis. Vergoni and colleagues (34) reported an experiment whereby rats were injected twice daily with PrRP. Surprisingly, they saw no effect on acute feeding on the first day, but did see a reduction on days 2 and 3. Unfortunately, they did not extend their experiment beyond this point. Experiment 2 in this study shows a significant effect of PrRP on body weight gain at 24 and 48 h of repeated injection. Thus we decided to carry out a further study whereby the animals were to be treated until they became unresponsive to PrRP’s effects or until such a time that the degree of weight loss was detrimental to their welfare (experiment 3). In fact, the animals became refractory to the anorectic and body weight actions of PrRP after 72 h of repeated injections. This finding indicates that PrRP has effects on energy homeostasis in the short to medium term. Tolerance to the effects of chronic administration has been reported in response to a number of other anorectic and orectic peptides, including CCK (4), orexin-A (11, 28, 35), galanin (32), and MCH (27). This result supports our hypothesis that PrRP mediates some of the effects of CCK on satiety (17), although other possibilities need consideration. First, it is possible that the apparent tolerance is secondary to other endocrine changes following weight loss, such as reduced plasma leptin and the activation of counter-regulatory mechanisms. However, if this were the case, then we might expect to see a rebound hyperphagia. Second, repeated injections may lead to a gradual increase of stress to the animals that would mask any small effects of PrRP on feeding at later time points. This would seem unlikely, because, if stress were a major factor, then we would expect to see increasing weight loss in the control group.

An alternative strategy to using repeated injections would have been to use osmotic minipumps that continuously infuse into the ventricle. These pumps remove the need for repeated handling of the animals and produce a constant elevation of peptide, which may or may not create a more physiologically relevant effect depending on the mode of action of the peptide under study. In the case of PrRP, the peptide may not be stable for long periods of time at 37°C, making the pumps impractical. However, the main disadvantage of this alternative system is that the pumps are active immediately after implantation and, thus, do not allow the animals to recover body weight lost due to the surgery before the start of the experiment. We opted for repeated injection because it probably reflects better the mode of action of a satiety peptide and because it also allows the data to be compared directly with acute studies.

The mechanism by which the tolerance to PrRP is mediated is not known, for example, whether it has a physiological or a pharmacological basis, or both. Crawley and colleagues (4) proposed a number of possible mechanisms for the behavioral tolerance that develops in response to chronic administration of CCK. At a physiological level, they proposed that tolerance might develop to the conditioned satiety component of gastric distension. At a pharmacological level, it was postulated that tolerance might occur due to the development of receptor subsensitivity, a change in receptor number, tolerance to second messengers, or receptor desensitization. These pharmacological mechanisms could also account for the tolerance seen after repeated administration of PrRP. Whatever the mechanism of the tolerance, this result would argue against PrRP having long-term actions on body weight like, for example, leptin, although these two peptides do have very similar acute effects (6).

Animals that undergo fasting, or food restriction in a relevant pair-fed group, show a reduction in BAT UCP-1 mRNA that contributes to the fall in energy expenditure (31). PrRP treatment for 48 h prevented the reduction in BAT UCP-1 mRNA caused by food restriction seen in the pair-fed group. This reversal of effects of pair-feeding on UCP-1 is well documented in the literature and is seen after administration of other anorectic factors, including leptin (31), urocortin (15), and the melanocortin-receptor agonist, MTH (2). After 5 days of treatment the animals were no longer responding to PrRP, and both the PrRP-treated and pair-fed animals had food intake and body weight equivalent to vehicle-treated controls. Thus, as expected, UCP-1 mRNA expression was not different among the three groups at this time point.

In summary, the data presented here suggest that PrRP exerts its effects on energy homeostasis in the short-medium term by inhibiting food intake and reducing body weight gain by increasing thermogenesis. The latter is indicated by an increase in the expression of UCP-1 mRNA and presumably mitochondrial activity in BAT. In rats, the main neural projections to BAT originate from the brain stem and hypothalamus, particularly the paraventricular nucleus (PVN), lateral hypothalamic area, perifornical area, and retrochiasmatic nucleus. Neurochemical characterization of paraventricular hypothalamic neurons projecting to preganglionic efferent cells indicates that some are immunoreactive for CRH or oxytocin (23). PrRP neurons of the brain stem project to the PVN (21), where they form synaptic contact with CRH- and oxytocin-containing cells (18, 19). This suggests that PrRP may be exerting an effect on BAT via connections with oxytocin- and CRH-expressing neurons in the PVN.

DISCLOSURES

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