Selecte activat of central NPY Y1 vs. Y5 recepto elicits hyperinsulm via distinct mechanisms

Jun Gao, Lorraine Ghibaudi, and Joyce J. Hwa
Department of Cardiovascular/Metabolic Diseases, Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Submitted 20 November 2003; accepted in final form 30 May 2004

Neuropeptide Y (NPY) is a 36-amino-acid neurotransmitter that is highly concentrated in the hypothalamus (8). In the hypothalamus, NPY is synthesized in the arcuate nucleus, which projects into the paraventricular nuclei and the dorsomedial nuclei to release NPY (1). In the periphery, NPY is colocalized with norepinephrine in sympathetic nerve endings (32). A variety of studies have linked NPY to physiological processes related to feeding behavior, energy homeostasis, neuroendocrine interactions, cardiovascular regulation, and emotional integration (9, 12, 15, 19, 35).

NPY is a member of the pancreatic polypeptide family, which elicits a wide variety of physiological effects via the activation of G protein-coupled receptors (Y1, Y2, Y4, Y5, and y6) (5). NPY receptor subtypes are all present in the hypothalamus (30) and have been suggested to play a role in modulating food intake and energy homeostasis (9, 12, 15, 19, 35).

The insulinemic response to hypothalamic or icv NPY is largely hyperinsulinemia in a variety of species (10, 11, 20, 26, 39, 47). Hyperinsulinemia has not been clearly elucidated. Chronic and selective activation of central NPY receptor subtypes is the most likely candidate for central NPY-induced hyperinsulinemia. Recent evidence has suggested that the Y1 and Y5 receptor subtypes may both mediate NPY-stimulated feeding. The present study attempts to further characterize the role of central NPY receptor subtypes involved in hyperinsulinemia. NPY and peptide analogs of NPY that selectively activated the NPY Y1 or Y5 receptor subtype induced feeding and hyperinsulinemia in satiated Long Evans rats, whereas NPY analogs that selectively activated the NPY Y2 or Y4 receptor subtype did not. To determine whether NPY-induced hyperinsulinemia is secondary to its hyperphagic effect, we compared the plasma insulin levels in the presence and absence of food after a 1-min central infusion of NPY and its analogs at 15, 60, and 120 min postinfusion. Our data suggest that selective activation of central NPY Y1 receptor subtype induced hyperinsulinemia independent of food ingestion, whereas the NPY Y5 receptor-induced hyperinsulinemia was dependent on food ingestion.

Central administration of the selective Y1 receptor agonist d-Arg<sup>25</sup> NPY eventually decreased plasma glucose levels 2 h postinfusion in Long Evans rats.

The aim of this study was to evaluate the effects of selective NPY Y1, Y2, Y4, and Y5 receptor agonists on plasma insulin, glucose, and glucagon levels in the presence and absence of food to determine the role of each NPY receptor subtype on NPY-induced hyperinsulinemia. The Y1 selective peptide chosen, d-Arg<sup>25</sup> NPY, binds the Y1 receptor with 12-, 82-, and 48-fold higher affinity than the Y2, Y4, and Y5 receptor subtypes, respectively (28). For Y2 receptor activation, we used C2-NPY, which is 51-, 43-, and 46-fold more selective for the Y2 receptor over the Y1, Y4, and Y5 receptor subtypes, respectively (16). To selectively activate the Y4 receptor, we used rat pancreatic polypeptide (rPP), which is 20,000-fold more selective for the Y4 receptor subtype than the Y1 and Y2 receptor subtypes and 2,000-fold more selective than the Y5 receptor subtype (16). To clarify the role of the Y5 receptor subtype on NPY-induced hyperinsulinemia, we used d-Trp<sup>34</sup> NPY, a Y5 agonist, which is 7-, 29-, and 4-fold more selective for the Y5 receptor over the Y1, Y2, and Y4 receptor subtypes, respectively (29). We also used a selective Y5 agonistic peptide reported by Cabrele et al., [cPP<sub>1–7</sub>, NPY<sub>19–23</sub>, Ala<sup>31</sup>, Aib<sup>32</sup>, Gln<sup>34</sup>hPP] (cPP NPY) (7). This peptide is 12-fold more potent than d-Trp<sup>34</sup> NPY at the Y5 receptor. In addition, cPP has over 2,000-fold greater affinity for Y5 over the Y1 or Y2 receptor, over 200-fold greater affinity for the Y4 receptor subtype, and is 3-fold more potent than the native ligand NPY.

Previously, our laboratory has demonstrated that central activation of either Y1 receptors by d-Arg<sup>25</sup> NPY or Y5 receptors by d-Trp<sup>34</sup> NPY induced hyperphagia in satiated Long-Evans (LE) rats (28, 29). However, selective activation of either Y2 receptors by C2-NPY or Y4 receptors by rPP did not elicit hyperphagia in satiated LE rats (16). This study further indicates that activation of central Y1 or Y5, but not Y2 or Y4, receptor subtypes induces hyperinsulinemia. However, the mechanisms involved in Y1- vs. Y5-induced hyperinsulinemia are different. Y5-induced hyperinsulinemia is dependent on food ingestion, whereas Y1-induced hyperinsulinemia is independent of the presence of food.

**METHODS**

*Animals.* Adult male LE rats (250–300 g; Charles River, MA) were housed individually and maintained in a temperature- and light-
controlled environment on a 12:12 h light-dark cycle (lights on at 4:00 AM). Animals had free access to food and water. All studies were conducted in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. The following protocols were approved by the Schering-Plough Research Institute’s Animal Care and Use Committee according to the guideline of the National Institutes of Health for the care and use of laboratory animals.

Surgery. A single 22-gauge stainless steel guide cannula (Plastics One, Roanoke, VA) was chronically implanted in the lateral ventricle of a selected kainic-amino-substituted (100:10 mg/kg ip) anesthetized by use of the following coordinates: 1.0 mm posterior to bregma, 1.5 mm lateral to midline, and 3.6 mm ventral to dura (16). The cannula was secured to the surface of the skull with jeweler’s screws and dental cement, and a 28-gauge obturator was inserted into the cannula to maintain patency. After a 3-wk recovery period, all animals were tested for cannula placement by central infusion of 0.3 nmol of NPY. Only animals demonstrating a prompt and robust feeding effect (>2.0 g intake within 1 h of infusion) were retained for the study.

Peptide and infusion protocols. About 1 wk after the NPY challenge, rats were icv infused with sterile saline during the nadir of the light cycle, and their food intake was monitored for 2 h. Cannulated rats were balanced into six study groups (NPY, Y1, Y2, Y4, Y5, and saline control) on the basis of their NPY-induced food intake and saline intake. Rats were evenly divided into the “with food” and “without food” paradigms within each study group. Human NPY, rPP, d-Trp34 NPY, d-Arg25 NPY, C2-NPY, and cPP NPY were synthesized by AnaSpec (San Jose, CA). All peptides were dissolved in 0.9% sterile saline (Sigma, St. Louis, MO) and infused icv at 1 l/min for 1 min. The infusion cannula remained inserted for an additional minute to prevent diffusion up the needle track. The chow-filled feeder (Purina Rodent Chow no. 5053, St. Louis, MO) was weighed during the infusion period and then returned to the home cage with the animal immediately after treatment. The feeder was not returned to the home cages with animals in the “without food” group. Food consumption was monitored, and blood samples were collected by heart puncture at 15, 60, or 120 min after the 1-min icv infusion. Because the protruding icv cannula may get caught in the wire cage, rats were individually housed in plastic cages with corn cob bedding (bed-o-cobs, 1/4 in.; Anderson Cob Mills, Malumee, OH). Minor powdery food spillage was not recorded, which may potentially overestimate the food intake of the treated groups at the 1-h and 2-h time points. All rats were infused at the nadir of the light cycle, which is ~6-7 h after lights on. After the study, rat brains were infused with dye, fixed in 10% Formalin saline solution, and sectioned, and injection sites were determined under light microscopy. All rats were killed between 11:00 AM and 12:00 PM (7-8 h after lights on) for blood sample collection after icv infusion (15 min, 1 h, or 2 h). Blood samples were collected into heparinized tubes, and plasma was isolated for assays after centrifugation at 3,000 rpm.

Plasma insulin, glucagon, and glucose assays. Both insulin and glucagon were analyzed by ELISA kits from ALPCO Diagnostics (Windham, NH) and Peninsula Laboratories (San Carlos, CA), respectively. The coefficients of variation for insulin and glucagon ELISA assays were 4.9 ± 1.2 and 8.4 ± 1.9%, respectively. Glucose was analyzed by Sigma Diagnostic Glucose Trinder Agent (St. Louis, MO).

Statistical analysis. Results are given as means ± SE. For each time point, plasma insulin, glucose, and glucagon levels were compared by two-way ANOVA (peptide treatment × food access). Whenever the overall model was statistically significant, with significant food effects, data were further separated into “with food” vs. “without food” groups and analyzed against the respective saline control groups. When Bartlett’s test indicated that the group comparisons had equal variances, one-way ANOVA and Dunnett’s multiple comparison post hoc tests were used. When the group data showed unequal variances, the nonparametric Wilcoxon/Kruskal-Wallis and Dunn’s multiple comparison post hoc tests were used. Comparisons between the paired groups in the presence or absence of food were analyzed by Student’s t-tests. P < 0.05 was considered to be significant. These statistical tests were performed using JMP 4.0.4 (SAS Institute, Cary, NC) and Prism 3.0 (GraphPad Software, San Diego, CA).

RESULTS

Fifteen minutes after icv infusion of saline vs. 1 nmol of NPY or NPY analogs. Central administration of NPY and its analogs did not induce increased food intake (Fig. 1A) or any significant changes in plasma glucagon and glucose levels (Fig. 1, C and D) at the 15-min postinfusion time. However, plasma insulin levels were significantly elevated after icv infusion of the selective Y1 agonist d-Arg25 NPY (1 nmol) compared with the saline control groups [Fig. 1B, two-way ANOVA F(11,59) = 6.02, P < 0.0001]. Kruskal-Wallis and Dunn’s multiple comparison tests showed that the combined Y1 treatment effects, with and without food, were significantly different from control, P < 0.001. In contrast, plasma insulin levels were not significantly different from control with NPY or with NPY analogs that selectively stimulate Y2, Y4, or Y5 receptor subtypes (Fig. 1B).

One hour after icv infusion of saline vs. 1 nmol of NPY or NPY analogs. Central administration of 1 nmol of NPY, the Y1-selective agonist (d-Arg25 NPY), or the Y5-selective agonists (cPP NPY) produced an increase in food intake that was significantly greater than the saline control group 1 h postinfusion (Fig. 2A; Kruskal-Wallis and Dunn’s multiple comparison tests, NPY: P < 0.001, Y1: P < 0.05, Y5: P < 0.01). In the presence of food, icv infusion of NPY, Y1, or Y5 agonists resulted in elevated levels of plasma insulin that were significantly higher than the level of saline control animals [Fig. 2B; two-way ANOVA, F(11,103) = 14.0, P < 0.001; Kruskal-Wallis and Dunn’s multiple comparison tests, NPY: P < 0.01, Y1: P < 0.01, Y5: P < 0.01]. In the absence of food after icv infusion, only the selective Y1 agonist produced increased plasma insulin levels (Fig. 2B). In a different group of rats, plasma insulin levels induced by icv NPY, Y1, and Y5 agonists in animals with food were significantly higher than those in rats without food (Student’s t-test, P < 0.05; Fig. 2B).

Plasma glucagon levels in Y2 agonist-treated rats were significantly lower than the level in the saline group with or without food 1 h postinfusion [Fig. 2C; two-way ANOVA, F(11,103) = 4.34, P < 0.001; Kruskal-Wallis and Dunn’s multiple comparison tests, with food, P < 0.01; without food, P < 0.05]. However, plasma glucagon levels of all other treatments were not significantly different from control.

In a different group of rats, plasma glucagon levels were measured 1 h after icv infusion of these peptides. We did not observe any significant change of plasma glucagon levels 1 h after icv infusion (Fig. 2D).

Two hours after icv infusion of saline vs. 1 nmol of NPY or NPY analogs. Rats given NPY, the Y1 agonist (d-Arg25 NPY), or the Y5 agonist (d-Trp34 NPY) consumed significantly more food than the saline control group 2 h after icv infusion [one-way ANOVA, F(5,50) = 11.7, P < 0.0001, with Dunn’s multiple comparisons posttests, NPY: P < 0.01, Y1: P < 0.01, Y5: P < 0.05; Fig. 3A]. However, only NPY and the Y1-selective agonist (d-Arg25 NPY) induced a significant in-
crease in plasma insulin levels 2 h postinfusion compared with the saline control group with food [Fig. 3B; two-way ANOVA, \( F(11,59) = 5.18, P < 0.0001 \); Kruskal-Wallis and Dunn’s multiple comparison tests with food, NPY: \( P < 0.01 \); Y1: \( P < 0.05 \)]. In the absence of food, the plasma levels of insulin remained the same across all groups (Fig. 3B). In the presence of food, the plasma insulin levels were significantly higher than without food 2 h after NPY or Y1 agonist infusions (Kruskal-Wallis and Dunn’s multiple comparison tests with food, NPY: \( P < 0.01 \); Y1: \( P < 0.01 \), Fig. 3B).

Plasma glucagon levels in NPY-treated groups were significantly increased in the presence of food [two-way ANOVA, \( F(11,59) = 3.82, P < 0.001 \); Kruskal-Wallis and Dunn’s multiple comparison tests with food, NPY: \( P < 0.05 \), Fig. 3C]. In addition, there was a significant difference in NPY-induced plasma glucagon levels in the presence and absence of food (ANOVA with Student’s \( t \)-test, \( P < 0.05 \), Fig. 3C).

Two hours after icv infusion of the Y1 or Y2 agonist, plasma glucose levels were significantly decreased compared with control group in the presence of food [two-way ANOVA,
NPY RECEPTOR SUBTYPE IN ICV NPY-INDUCED HYPERINSULINEMIA

The present study is the first to delineate the central NPY receptor subtype(s) involved in the regulation of plasma insulin, glucagon, and glucose levels by the use of selective NPY Y1, Y2, Y4, or Y5 receptor agonists. Our data demonstrate that a selective Y1 agonist, D-Arg25 NPY, induced a significant elevation of plasma insulin levels in satiated rats at all time points measured. D-Arg25 NPY also significantly raised plasma insulin levels in the absence of food 1 h after icv infusion, suggesting that Y1-induced hyperinsulinemia does not completely depend on the ingestion of food. The Y1-induced hyperinsulinemia may contribute to the significantly reduced plasma glucose levels at 2 h after icv infusion with or without food. In contrast, a selective Y5 agonist only increased plasma insulin levels in the presence of food 1 h after icv infusion. The Y5-induced hyperinsulinemia was significantly reduced in the absence of food, indicating that Y5-induced hyperinsulinemia was secondary to the ingestion of food. In addition, our data confirm our previous finding that the selective Y2 agonist C2-NPY and the selective Y4 agonist rPP do not elicit feeding behavior during the nadir of the light cycle. Furthermore, central administration of the selective Y2 agonist significantly reduced plasma glucagon levels with or without food at 1 h postinfusion and decreased plasma glucose levels 2 h postinfusion without food, whereas the Y4 agonist does not have a significant effect on plasma insulin, glucagon, or glucose levels.

Pancreatic insulin and glucagon secretion is a tightly regulated process that maintains energy fuel homeostasis. In addition to the close control of blood glucose levels, the secretion of insulin is in part regulated by the vagus nerve. Activation of the parasympathetic nervous system can stimulate the secretion of insulin (22, 44) and enhance the insulin secretory response to glucose (37). It has been suggested that central administration of NPY may act directly on the nucleus of solitary tract, which may mediate the direct link to the pancreas via a vagal pathway to enhance insulin secretion (10). It has been demonstrated that the muscarinic receptor antagonist atropine or vagotomy can block icv NPY-induced insulin release or gastropancreatic secretion, which may be considered as a cephalic phase of secretion (11, 43). High densities of Y1, Y2, and Y4 NPY receptor subtypes have been reported within the dorsal vagal complex, including nucleus of the solitary tract and dorsal motor nucleus of vagus (3, 18, 21). Recent studies have also indicated that Y1 receptors in the dorsal vagal complex may be involved in NPY-induced bile secretion as well as peptide YY-induced gastric acid secretion (45, 46). Our data suggest that selective activation of the Y1 receptor, but not Y2, Y4, or Y5 receptors, significantly elevated plasma insulin levels independent of food ingestion. Because we administered these peptides by lateral ventricle icv infusion, these peptides may have multiple sites of action in the brain. Therefore, additional studies including fourth ventricle or site-specific injection of NPY or a Y1 selective peptide will be necessary to provide further insight into the role of NPY and the Y1 receptor on insulin response.

Alternatively, NPY-induced hyperinsulinemia may be partly caused by decreasing insulin clearance. Obesity and high levels of free fatty acids can reduce insulin clearance by lowering hepatic insulin extraction and inhibiting insulin-degrading enzyme (13, 36, 41). Chronic central infusion of NPY can induce an obesity syndrome, which includes hyperphagia, hyperinsulinemia, hypertriglyceridemia, hypercorticosteronemia, and weight gain in rodents (33). It is known that the proportions of insulin, proinsulin, and conversion intermediates in plasma and pancreas are not affected by chronic central infusion of NPY (33). It is not clear whether acute icv infusion of NPY or its analogs may have any effect on insulin clearance. Hyperinsulinemia induced by central administration of NPY in

DISCUSSION

NPY is an important regulator of energy balance in mammals through its orexigenic, antithermogenic, and hyperinsulinemic actions. The present study is the first to delineate the central NPY receptor subtype(s) involved in the regulation of plasma insulin, glucagon, and glucose levels by the use of selective NPY Y1, Y2, Y4, or Y5 receptor agonists. Our data demonstrate that a selective Y1 agonist, D-Arg25 NPY, induced a significant elevation of plasma insulin levels in satiated rats at all time points measured. D-Arg25 NPY also significantly raised plasma insulin levels in the absence of food 1 h after icv infusion, suggesting that Y1-induced hyperinsulinemia does not completely depend on the ingestion of food. The Y1-induced hyperinsulinemia may contribute to the significantly reduced plasma glucose levels at 2 h after icv infusion with or without food. In contrast, a selective Y5 agonist only increased plasma insulin levels in the presence of food 1 h after icv infusion. The Y5-induced hyperinsulinemia was significantly reduced in the absence of food, indicating that Y5-induced hyperinsulinemia was secondary to the ingestion of food. In addition, our data confirm our previous finding that the selective Y2 agonist C2-NPY and the selective Y4 agonist rPP do not elicit feeding behavior during the nadir of the light cycle. Furthermore, central administration of the selective Y2 agonist significantly reduced plasma glucagon levels with or without food at 1 h postinfusion and decreased plasma glucose levels 2 h postinfusion without food, whereas the Y4 agonist does not have a significant effect on plasma insulin, glucagon, or glucose levels.

Pancreatic insulin and glucagon secretion is a tightly regulated process that maintains energy fuel homeostasis. In addition to the close control of blood glucose levels, the secretion of insulin is in part regulated by the vagus nerve. Activation of the parasympathetic nervous system can stimulate the secretion of insulin (22, 44) and enhance the insulin secretory response to glucose (37). It has been suggested that central administration of NPY may act directly on the nucleus of solitary tract, which may mediate the direct link to the pancreas via a vagal pathway to enhance insulin secretion (10). It has been demonstrated that the muscarinic receptor antagonist atropine or vagotomy can block icv NPY-induced insulin release or gastropancreatic secretion, which may be considered as a cephalic phase of secretion (11, 43). High densities of Y1, Y2, and Y4 NPY receptor subtypes have been reported within the dorsal vagal complex, including nucleus of the solitary tract and dorsal motor nucleus of vagus (3, 18, 21). Recent studies have also indicated that Y1 receptors in the dorsal vagal complex may be involved in NPY-induced bile secretion as well as peptide YY-induced gastric acid secretion (45, 46). Our data suggest that selective activation of the Y1 receptor, but not Y2, Y4, or Y5 receptors, significantly elevated plasma insulin levels independent of food ingestion. Because we administered these peptides by lateral ventricle icv infusion, these peptides may have multiple sites of action in the brain. Therefore, additional studies including fourth ventricle or site-specific injection of NPY or a Y1 selective peptide will be necessary to provide further insight into the role of NPY and the Y1 receptor on insulin response.

Alternatively, NPY-induced hyperinsulinemia may be partly caused by decreasing insulin clearance. Obesity and high levels of free fatty acids can reduce insulin clearance by lowering hepatic insulin extraction and inhibiting insulin-degrading enzyme (13, 36, 41). Chronic central infusion of NPY can induce an obesity syndrome, which includes hyperphagia, hyperinsulinemia, hypertriglyceridemia, hypercorticosteronemia, and weight gain in rodents (33). It is known that the proportions of insulin, proinsulin, and conversion intermediates in plasma and pancreas are not affected by chronic central infusion of NPY (33). It is not clear whether acute icv infusion of NPY or its analogs may have any effect on insulin clearance. Hyperinsulinemia induced by central administration of NPY in

\[ F(11,59) = 2.74, P < 0.01; \text{with Dunnett’s multiple comparisons posttests,} P < 0.05, \text{Fig. 3D}. \]
rats can be prevented by adrenalectomy, a surgery that eliminates glucocorticoid production in the adrenal gland. This effect was associated with a concomitant reduction in Y1 and Y5 receptor mRNA expression in the ventral medial hypothalamus (43). Glucocorticoids may have a direct effect in controlling insulin release via NPY-regulated pathways, since the Y1 receptor gene is known to have a putative glucocorticoid response element (2).

In addition to the centrally mediated hyperinsulinemic effect of NPY, it has been known that NPY regulates insulin secretion by a peripheral mechanism. NPY can inhibit insulin secretion when given intravenously or when applied directly to isolated pancreatic islets (31, 40). Expression of the Y1 receptor gene is known to have a putative glucocorticoid response element (2). It has been suggested that selective activation of the central Y1 receptors can induce hyperinsulinemia independently of food, whereas the Y5-mediated hyperinsulinemia is dependent on food ingestion.

REFERENCES


6. Burrell R, Brunner H, Seydoux J, Thorenza B, and Pedrazzini T. Increased insulin concentrations and glucose storage in neuropeptide Y Y1 receptor-deficient mice also confirm the tonic role of the peripheral Y1 receptor in plasma insulin regulation (6).

7. Central administration of NPY has a number of effects on glucose metabolism. In addition to NPY-induced hyperinsulinemia, NPY also increases hepatic glucose output (42), stimulates glucose turnover (23), and changes peripheral tissue responsiveness to insulin (48). The opposing effects of increasing hepatic glucose output and stimulating glucose turnover may partly explain why we did not observe significant changes in plasma glucose levels at 15 or 60 min postinfusion. These data were consistent with literature reports that plasma glucose levels did not change within 90 min after acute NPY infusion in nondiabetic animals (42). However, we have noticed a reduction in plasma glucose levels and an increase in plasma glucagon levels after NPY infusion at the 2-h time point. Because one of the physiological functions of glucagon is to counter hypoglycemia, it is likely that the increase in glucagon levels was not directly induced by receptor activation, but was triggered by the decrease in plasma glucose levels. Alternatively, central infusion of NPY has been shown to increase glucagon secretion in the hyperinsulinemic euglycemic clamp, suggesting that a direct effect of icv NPY on glucagon secretion is possible (42).

Although hypothalamic Y1 and Y5 receptors are both involved in icv NPY-induced hyperphagia and hyperinsulinemia, our data suggest that the mechanisms by which these receptors regulate insulin secretion may not be the same. Y1 agonist-induced hyperinsulinemia was not solely dependent on food intake, whereas Y5 agonist-induced hyperinsulinemia was dependent on food ingestion. In addition, plasma glucose levels were significantly reduced by central infusion of the Y1 agonist, but not the Y5 agonist. In summary, central administration of NPY and its analogs could stimulate insulin release through activation of Y1 or Y5, but not Y2 or Y4, receptors. Our data suggest that selective activation of the central Y1 receptors can induce hyperinsulinemia independently of food, whereas the Y5-mediated hyperinsulinemia is dependent on food ingestion.


