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Repeated glucoprivation delayed hyperphagic responses while activating neuropeptide Y neurons in rats

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ABSTRACT

It is well known that glucoprivation induces the release of counterregulatory hormones such as glucagon, and that the response is attenuated when the stimuli are repeated. Glucoprivation also activates orexigenic neurons and induces hyperphagic responses, although it remains unclear whether these responses are attenuated in repeated glucoprivation. In this study, we examined time course changes in feeding as well as activities of orexigenic neuropeptide Y (NPY) neurons in repeated glucoprivation in rats. Either 2-deoxy-D-glucose (2DG), which blocks glucose utilization, or isotonic saline (control) was injected subcutaneously to rats for 14 days, and food consumption for 1 and 2 h after injection was monitored throughout the experiment. While 2DG injection induced robust feeding responses during the first 1 h after injection, the response was gradually attenuated and the food consumption was significantly less on days 12–14 compared to that on day 1. On the other hand, food consumption during 2 h after 2DG injection was not changed significantly for 14 days. The transcriptional activities of NPY neurons in the arcuate nucleus and C1/A1 region of the hindbrain, measured by intronic *in situ* hybridization, were significantly enhanced after repeated 2DG injection for 14 days, while the feeding responses to intracerebroventricular injection of NPY were significantly less in the 2DG-repeated group compared to the saline-repeated group. It is thus demonstrated that repeated glucoprivation delayed hyperphagic responses while activating NPY neurons in rats. Our data also suggest that decreased feeding responses to NPY might be at least partially responsible for the delayed response.

1. Introduction

Glucoprivation induced by injection of either insulin or 2-deoxy-D-glucose (2DG), which blocks glucose utilization [8], reportedly increases food intake [39]. Although the detailed mechanisms by which glucoprivation increases food intake are not elucidated fully, there are several lines of evidence suggesting that neuropeptide Y (NPY), one of the most potent orexigenic peptides in the brain [11,27,42], is involved in the response [1,20,24,37,38,43]. NPY expressed in the arcuate nucleus [1,37,43] as well as in the hindbrain [24] is upregulated in response to glucoprivation, and hyperphagic responses to glucoprivation are abolished in NPY knock-out mice [38] or rats treated with NPY antibody [20]. On the other hand, there are also studies suggesting that NPY neurons are not prerequisite for the hyperphagic responses to glucoprivation [9,25].

Glucoprivation leads to the release of counterregulatory hormones such as glucagon [28], and the neurons in the ventromedial hypothalamic nucleus (VMN) have been implicated in the response [5,6]. In the electrophysiological experiments, some neurons in the VMN are shown to be glucose-inhibited (GI) neurons, defined as those which are excited in response to decreases in glucose levels [40]. When glucoprivation is repeated, the GI neurons in the VMN are reportedly desensitized [41] followed by the attenuated release of the counterregulatory hormones [13,17,21]. There is controversy whether or not hyperphagic responses are attenuated in repeated glucoprivation [10,30,34,35]. As some NPY neurons in the arcuate nucleus are shown to be GI neurons [15,29], it is possible that NPY neurons are desensitized, as are the VMN neurons, in repeated glucoprivation. Actually, it has been shown that expression of c-fos, an early gene which has been used as a marker of neuronal activities [26], is decreased in the arcuate nucleus in repeated glucoprivation [35].

To determine whether hyperphagic responses are attenuated when glucoprivation is repeated, we monitored feeding responses to 2DG for 2 weeks in rats. We also examined whether or not NPY

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neurons in the arcuate nucleus and hindbrain are desensitized in repeated glucoprivation.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats [270–320 g body weight (BW); Chubu Science Materials, Nagoya, Japan] were housed individually in plastic (wire bottom) cages under controlled conditions (23.0 ± 0.5 °C, lights on from 9:00 to 21:00), and had access to standard chow and water *ad libitum* until experiments. All rats were handled for one week before experiments. All procedures were performed in accordance with the institutional guidelines for animal care at Nagoya University Graduate School of Medicine and approved by the Animal Experimentation Committee.

2.2. Changes in feeding and BW in response to repeated injection of 2DG

Rats were divided into two groups: those injected (1.3 ml/kg) subcutaneously (s.c.) with 500 mg/kg 2DG (Sigma, St. Louis, MO, USA) dissolved in isotonic saline once a day at 10:00 for 14 days (2DG-repeated group), and those injected s.c. with vehicle (saline) for 14 days (saline-repeated group). Rats had access to standard chow and water *ad libitum* during all experimental days. Food consumption for 1, 2 and 24 h, respectively, after injection as well as BW was monitored throughout the experiment.

2.3. Changes in gene expression of NPY and c-fos after repeated injection of 2DG

Rats were injected s.c. with 500 mg/kg 2DG or saline once a day at 10:00 for 13 days. Rats had access to standard chow and water *ad libitum* throughout these procedures. On day 14, rats in both groups were injected s.c. with either 2DG or saline and deprived of food and water after injection. The rats injected with 2DG were decapitated either 0.5 or 2 h after injection and those injected with saline were decapitated 2 h after injection.

2.4. Feeding responses to intracerebroventricular injection of NPY after repeated injection of 2DG

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg BW), and a 28-gage stainless steel cannula (Brain infusion kit II; Alzet, Palo Alto, CA, USA) was inserted stereotaxically into the right lateral ventricle while an osmotic mini-pump (Model 2004; Alzet, Palo Alto, CA, USA) connected to the cannula was placed subcutaneously as described previously [4]. Isotonic saline was constantly administered into the right lateral ventricle through intracerebroventricular (icv) cannula connected osmotic mini-pumps. Three days after the icv operation, s.c. injection of 500 mg/kg 2DG or isotonic saline once a day at 10:00 was started and continued for 13 days. On day 14, rats were anesthetized by inhalation of diethyl ether (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and NPY (5 μg/2.5 μl saline, Peptide Institute, Inc., Minoh, Osaka, Japan) or isotonic saline was administered through the icv cannula. Food intake was measured at 0.5 and 2 h after icv injection.

2.5. Indirect calorimetry

Oxygen consumption was measured on days 7 and 14 in both saline-repeated and 2DG-repeated groups with an O_2/CO_2 metabolism measuring system (model MK-5000RQ/02, Muromachikikai, Tokyo, Japan).

2.6. Probes for NPY and c-fos

The plasmids containing the cDNA for rat preproNPY were kindly provided by Dr. S. L. Sabol (Laboratory of Biochemical Genetics, National Institutes of Health, Bethesda, MD, USA). A 408 bp fragment of c-fos gene (−21∼+387) was subcloned into the pCRII-TOPO (Invitrogen, Carlsbad, CA, USA). Plasmids containing a 733-bp fragment localized entirely within intron 1 of the rat NPY gene were used to generate probes for NPY heteronuclear RNA (hnRNA). The specificity of the probe was demonstrated in a previous study [19]. High specific RNA probes were synthesized as described previously [2,22].

2.7. In situ hybridization

Brains were removed after decapitation, frozen on dry ice, and stored at −80 °C until sectioning for *in situ* hybridization. Coronal sections (14 μm) of the arcuate nucleus and the lateral hypothalamus at 2.8 mm and those of the hindbrain at 13.8 mm caudal from the Bregma, according to the brain atlas of Paxinos and Watson [32], were cut on a cryostat, mounted onto slides (catalog no. S9441, Matsunami Glass Industry, Kishiwada, Osaka, Japan), and then stored at −80 °C until *in situ* hybridization. Prehybridization, hybridization and posthybridization procedures were performed as described previously [2,22]. The optical densities (OD) of the autoradiograph were quantified using a computer image analysis system (Imaging Research, St. Catharines, Ontario, Canada). Changes in gene expression in the film images were quantified by measurements of the integrated OD (OD × area). Some hybridized sections were dipped in nuclear Kodak NTB2 emulsion (Kodak, Rochester, NY, USA), and exposed for 2 days for NPY mRNA and 6 weeks for c-fos mRNA and NPY hnRNA to be visualized. The emulsion-dipped sections were stained with cresyl violet to assist cellular localization of the hybridized signals. The c-fos expression in the arcuate nucleus was examined in adjacent sections of those hybridized with NPY mRNA probes. The area of arcuate nucleus was delineated based on the Atlas [32] and was divided into the medial and lateral parts, the former defined by the area where NPY mRNA was expressed in the adjacent section. Any neuronal cross sections with grains of more than threefold the background density were considered labeled.

2.8. Statistics

Statistical significance of the differences between groups was calculated by two-way ANOVA with repeated or non-repeated measures followed by Bonferroni's test or unpaired *t*-test. Results are expressed as means ± SE, and differences were considered significant at $P < 0.05$. The number of rats in each group was 8 unless indicated otherwise.

3. Results

3.1. Changes in feeding responses to repeated injection of 2DG

The food consumption after 2DG injection was significantly increased compared to saline injection throughout the experiment (Fig. 1A–C). However, the feeding response to 2DG injection during the first hour (0–1 h) was gradually decreased, and the absolute values were significantly lower on days 12–14 compared to those on day 1 (Fig. 1A). In contrast, the feeding response to 2DG injection during the second hour (1–2 h) was gradually increased, and the absolute values were significantly increased on day 14 compared to those on day 1 (Fig. 1B). There were no time course effects