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The effect of metformin on neuronal activity in the appetite-regulating brain regions of mice fed a high-fat diet during an anorectic period

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HIGHLIGHTS

- We identified brain regions involved in metformin-induced anorexia.
- Metformin caused weight loss and anorexia in obese mice fed a high fat diet.
- Acute metformin activated neurons in the PVN, AP, and CeA.
- Repeated metformin activated neurons independent of weight loss and anorexia.
- Repeated metformin administration activated neurons in the VMH, NTS, GCL, and Acb.

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ABSTRACT

Metformin reduces body weight by decreasing food intake in humans and animals. However, the brain regions involved in metformin-induced anorexia remain unclear. Therefore, we investigated c-Fos expression (FOS), a marker of neuronal activity, in the appetite-regulating brain regions after oral administration of metformin (PO, 300 mg/kg daily for 1 or 3 days) or vehicle.

The body weight and food intake decreased in mice treated with metformin for 3 days (RM group) and mice that had the same amount of food as the RM group (Pair-fed group; PF) compared to the control group. FOS expression levels increased in the paraventricular nucleus, area postrema, and central amygdala of mice administered an acute single dose of metformin (SM group) compared to the control mice. In the nucleus tractus solitarius, the FOS expression levels increased in both the SM and RM groups compared to the control group. The FOS expression levels also increased in the nucleus accumbens of the RM group compared to other groups. The FOS expression levels decreased in the ventromedial hypothalamic nucleus in the PF group, but not the RM group, compared to the control group, suggesting a potential hypothalamic area involvement for metformin-induced anorexia.

These results suggest that both the hypothalamic and extra-hypothalamic regions are associated with metformin-induced anorexia, which is dependent on metformin treatment duration.

1. Introduction

The prevalence of obesity has increased rapidly in both developing and industrialized countries, predisposing obese individuals to cardiovascular disease, diabetes, and cancer [1]. Metformin has been commonly used as a diabetes treatment in obese patients with diabetes, not only for glycemic control but also for body weight control [2,3]. Substantial evidence in various rodent models [4–8] and in normal and diabetic humans [9,10] has shown that metformin reduces body weight by decreasing food intake, with no effect on energy expenditure. Furthermore, this weight loss can be maintained for over 10 years in humans [11], suggesting that metformin treatment may continue to affect appetite over long periods of time.

Multifactorial mechanisms are involved in the anorectic effect of metformin [12]. Several specific brain regions have been hypothesized to play a role in metformin-induced anorexia. Many brain regions, including the hypothalamus, brainstem, limbic system, and mesolimbic pathway, are involved in appetite regulation [13,14]. It is reasonable to assume metformin is activating the brain regions that are involved in appetite regulation. In previous studies, peripherally administered

Abbreviations: VMH, ventromedial hypothalamic nucleus; PVN, paraventricular nucleus; DMH, dorsomedial hypothalamic nucleus; ARC, hypothalamic arcuate nucleus; LH, lateral hypothalamic area; CeA, Central amygdala; BLA, basolateral amygdala; MeA, medial amygdala; GCL, granule cell layer of the hippocampal dentate gyrus; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; VTA, ventral tegmental area; NTS, nucleus tractus solitarius; AP, area postrema.

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metformin activated appetite-regulating brain regions in rats, including NPY/AgRP neurons in the hypothalamus [4,6] and neurons in the nucleus tractus solitaries [8]. However, the precise regions involved in the anorectic effect of metformin still remain unclear. To identify these regions, we analyzed c-Fos expression, a marker of neuronal activity, in the appetite-regulating brain regions after metformin treatment. Furthermore, to determine the duration of metformin-induced neuronal activation, we compared c-Fos expression at the beginning of treatment to the third day of treatment, which is a time point when metformin has significant effects on body weight and food intake [8].

2. Experimental procedures

2.1. Animals

Adult male C57BL/6 mice were purchased from Orient (Seoul, Republic of Korea) and maintained in a temperature- and humidity-controlled room (22 ± 2 °C, 60% relative humidity). All animals were 7 weeks old upon arrival and were housed individually in standard mouse cages under a 12 h light/dark cycle (lights on at 0600 h). After a habituation period, mice were fed a high-fat diet consisting of 45% fat, 20% protein, and 35% carbohydrates (4.73 kcal/g, D12451; Research Diets, New Brunswick, NJ) for 16 weeks before metformin treatment. Mice were fed a high-fat diet (HFD) since metformin's effects are more potent in obese rodents than in lean ones [5,6]. Water and food were provided ad libitum. Body weight was assessed once a week. All experimental procedures followed the guidelines on the ethical use of animals issued by the Animal Care and Use Committee of Korea University and were approved by Institutional Animal Care & Use Committee of Korea University. All efforts were made to minimize the number of animals used and animal suffering.

2.2. Drugs and experimental procedures

1,1-Dimethylbiguanide hydrochloride (Metformin; Sigma, St. Louis, MO) was dissolved in 0.9% saline (vehicle). All animals received a daily injection of vehicle or metformin (300 mg/kg body weight) for 3 days, by gastric gavage at a volume of 0.5% body weight. Administration occurred immediately prior to the dark cycle (1800 h).

Obese mice (>48 g) were assigned into 4 groups according to their initial body weight prior to the start of metformin treatment: obese mice treated with vehicle for 3 days (control, n = 4), obese mice treated with a single dose of 300 mg/kg of metformin following 2 days of vehicle treatment (SM, n = 5), obese mice treated daily with 300 mg/kg of metformin for 3 days (RM, n = 5), and mice pair-fed to the RM group and treated with vehicle for 3 days (PF, n = 5). The PF group was included to discriminate the effect of metformin on neuronal activation from the effect caused by the change in body weight and food intake after metformin treatment. Control, SM, and RM groups were fed ad libitum. The PF group was provided the same amount of food as was consumed by the RM group twice a day (0700 h and 1800 h). Body weight and food intake were measured twice a day during the experimental period.

Animals were sacrificed 2 h after the last metformin administration on day 3 when metformin-induced anorexia was most prominent, as previously described [8]. Food was removed after the last metformin administration (2 h before the sacrifice).

2.3. c-Fos immunohistochemistry

To evaluate neuronal c-Fos expression, c-Fos immunohistochemistry was performed as previously described [8]. Coronal sections of the hypothalamic regions were analyzed, including the following: the paraventricular nucleus (PVN; 0.82–0.94 mm posterior to bregma; All the following coordinates are posterior to bregma), arcuate nucleus (ARC; 1.46–1.82 mm), dorsomedial hypothalamic nucleus (DMH; 1.46–1.94 mm), ventromedial hypothalamic nucleus (VMH; 1.46–1.94 mm), and lateral hypothalamic area (LH; 1.70–1.94 mm), several hindbrain regions including the nucleus tractus solitarius and area postrema (NTS and AP; 7.48–7.76 mm), the limbic system including the central amygdala (CeA; 1.06–1.46 mm posterior to bregma), basolateral amygdala (BLA; 1.06–1.34 mm), medial amygdala (MeA; 1.34–1.58 mm), and the granule cell layer (GCL) and hilus of the hippocampal dentate gyrus (1.82–2.06 mm), and the mesolimbic pathway including the nucleus accumbens core and shell (AcbC and AcbSh; 1.18–1.10 mm), and the ventral tegmental area (VTA; 2.98–3.10 mm). Images were captured using a ProGress C14 camera (Jenoptik, Jena, Germany) mounted on an Olympus BX-50 microscope (Olympus, Tokyo, Japan) and analyzed using the NIH image-based ImageJ 1.44p program (National Institutes of Health, Bethesda, Maryland). The c-Fos-positive cells were counted following 2 criteria, the threshold gray values and a limited cellular diameter [15] (Fig. 2).

2.4. Statistical analysis

Normally distributed results are presented as mean ± standard error of the mean (SEM). Results that were not normally distributed (the SM group in VTA) are presented as the median ± interquartile range. All analyses were carried out using SPSS 22.0 (IBM Inc., New York, US) and visualized using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). The food intake and body weight changes over 3 days were analyzed by a repeated measures ANOVA followed by a Fisher's least significant difference test. Other normally distributed data were analyzed by a one-way ANOVA followed by a Fisher's least significant difference test. Statistical significance was set at a p-value of less than 0.05. Data that was not normally distributed were analyzed by a Kruskal–Wallis H test. Since there was no statistical difference in the VTA, a post-hoc test was not performed.

3. Results

3.1. The effect of an acute or repeated metformin administration on body weight and food intake

A repeated measures ANOVA showed a significant main effect of treatment on body weight ($F_{3,14} = 22.64$, $p < 0.001$). A Fisher's least significant difference test revealed a significant reduction in body weight in the RM and PF groups compared to the control and SM groups on day 3 of treatment (Fig. 1B). The changes in body weight in the PF group were similar to the RM group across the 3 days of treatment, while the body weight of the SM group was similar to the control group.

Consistent with the changes in body weight, the cumulative food intake of the RM group decreased significantly compared to the control and SM groups (Fig. 1C). A repeated measures ANOVA revealed a significant main effect of treatment on the cumulative food intake ($F_{3,14} = 25.65$, $p < 0.001$). The cumulative food intake in the RM and PF groups was significantly lower than in the control and SM groups during the experiment. The amount of consumed food in the SM group was similar to the control group.

3.2. The effect of an acute and repeated metformin administration on c-Fos expression in the hypothalamic subregions

In the VMH, the number of c-Fos-positive cells was similar in the SM, RM, and control groups. The number of c-Fos-positive cells was significantly lower in the PF group than in the control group ($F_{3,15} = 5.16$, $p < 0.05$; Fig. 3A).

In the PVN, the number of c-Fos-positive cells was higher in the SM group compared to the other groups ($F_{3,14} = 5.41$, $p < 0.05$; Fig. 3D). No differences were observed in the neuronal activation between the control, RM, and PF groups. There were no metformin-induced differences in the neuronal activation within the DMH, ARC, and LH (Fig. 3B, 3C, 3E).