Central administration of metformin into the third ventricle of C57BL/6 mice decreases meal size and number and activates hypothalamic S6 kinase

Running title: Central effect of metformin on energy homeostasis

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Administration of metformin is known to reduce both body weight and food intake. While the hypothalamus is recognized as a critical regulator of energy balance and body weight, there is currently no evidence for an effect of metformin in the hypothalamus. Therefore, we sought to determine the central action of metformin on energy balance and body weight as well as its potential involvement with key hypothalamic energy sensors, including AMP-activated protein kinase (AMPK) and S6 kinase (S6K). We used meal pattern analysis and a conditioned taste aversion (CTA) test, and measured energy expenditure in C56BL/6 mice administered metformin (0, 7.5, 15, or 30 μg) into the third ventricle (I3V). Furthermore, we I3V-administered either control or metformin (30 μg) and compared the phosphorylation of AMPK and S6K in the mouse mediobasal hypothalamus. Compared to the control, I3V administration of metformin decreased body weight and food intake in a dose-dependent manner and did not result in CTA. Furthermore, the reduction in food intake induced by I3V administration of metformin was accomplished by decreases in both nocturnal meal size and number. Compared to the control, I3V administration of metformin significantly increased phosphorylation of S6K at Thr^{389} and AMPK at Ser^{485/491} in the mediobasal hypothalamus, while AMPK phosphorylation at Thr^{172} was not significantly altered. Moreover, I3V rapamycin pretreatment restored the metformin-induced anorexia and weight loss. These results suggest that the reduction in food intake induced by the central administration of metformin in the mice may be mediated by activation of S6K pathway.
Keywords

Metformin, Energy homeostasis, Hypothalamus, S6 kinase, AMP-activated protein kinase,
Meal pattern
**Introduction**

Metformin is a biguanide drug that is widely used to reduce blood glucose levels in both diabetic patients and mice (9, 26, 30). It has been proposed to reduce hepatic gluconeogenesis via activation of AMP-activated protein kinase (AMPK), or independently via other signaling molecules, such as the mammalian target of rapamycin/S6 kinase (mTOR/S6K) (9, 13, 14, 35). In addition to the beneficial action of metformin in glucose regulation, it has also been shown to reduce body weight in humans and rodents (15, 26). The weight loss induced by metformin in humans and mice has mainly been attributed to a reduction in food intake (15, 19, 27, 30). Despite the clear evidence for reduced food intake after metformin treatment, the underlying mechanism of its anorectic action remains unclear.

The hypothalamus is a key player in the brain’s regulation of food intake (1, 21, 32). Various molecules involved in the regulation of food intake are integrated via signaling molecules in hypothalamic neurons to determine food consumption. AMPK and mTOR/S6K have been proposed as the main molecular sensors to monitor energy status in neurons (1, 33). Hypothalamic AMPK plays a critical role in the alterations in energy balance induced by hormones, including leptin, ghrelin, and insulin, and by dietary nutrients, including glucose and fatty acids (18). The change in AMPK activity requires either phosphorylation of AMPK at Thr172 or Ser485/491 (7, 20). Another important player in the regulation of energy balance is hypothalamic mTOR/S6K. Hormones (such as leptin) and dietary nutrients (including branched amino acids such as leucine) have been shown to decrease food intake via stimulation of hypothalamic S6K in rats and mice (2, 6). In addition, hypothalamic mTOR/S6K has been shown to interact with AMPK through phosphorylation sites at
Ser\textsuperscript{485/491} (7). AMPK has also been hypothesized to regulate mTOR/S6K in the hypothalamus in a bidirectional, inhibitory manner (6).

The details of hypothalamic involvement in metformin-induced anorexia have not been clarified by previous studies (16, 19, 27). Some reports have shown that metformin treatment affects the hypothalamic neuropeptide Y (NPY) system in obese rats and mice, although the effect of metformin on neuropeptide systems requires further investigation (19, 27). A direct effect of metformin on hypothalamic AMPK was not observed in studies of food intake, despite the strong evidence for AMPK-mediated improvement of glucose regulation induced by metformin (16, 19, 29, 35). In addition to the association between the hypothalamus and metformin-induced anorexia, we have shown that peripheral administration of metformin may reduce food intake via neuronal activation in the hindbrain regions that mediate satiety signals from peripheral tissues (15). However, the potential involvement of the hypothalamus in the anorectic action of peripherally administered metformin cannot be excluded because it can easily cross the blood–brain barrier (17, 19). Therefore, it remains to be determined whether central administration of metformin can affect food intake via regulation of the primary molecular energy sensors in the hypothalamus.

In this study, we determined whether direct administration of a range of doses of metformin into the third ventricle affects the energy balance in mice by performing meal pattern analysis and by comparing energy expenditure. In addition, we determined whether centrally administered metformin produced either illness or food aversion by performing a conditioned taste aversion (CTA) test in mice. We also investigated whether centrally administered metformin could modulate key energy sensors in the mediobasal hypothalamus, such as AMPK and S6K, as well as signaling molecules related to leptin and insulin pathways,
such as the signal transducer and activator of transcription 3 (Stat3) and Akt.
Materials and methods

Animals

Male C57BL/6 mice were obtained from Orient (Seoul, Republic of Korea) and were maintained in a room with controlled temperature and humidity (22 °C, 60% relative humidity) before the experiments commenced. All animals were aged 8 weeks upon arrival at the laboratory and were housed individually in standard mouse cages under a 12-h light/dark cycle (lights off at 1800 hours). Water and standard laboratory chow diet (5L79 Purina rat & mouse 18% chow, Charles River Laboratories Inc., Wilmington, MA) were provided ad libitum. All experimental procedures followed the guidelines on the ethical use of animals issued by the Animal Care and Use Committee of Korea University.

Drugs

1,1-Dimethylbiguanide hydrochloride (metformin; Sigma-Aldrich, St. Louis, MO) was dissolved in artificial cerebrospinal fluid (aCSF; Tocris Bioscience, Bristol, UK). Rapamycin (Calbiochem, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). The vehicles for each drug were used as controls.

Third ventricle cannulation

The animals were anesthetized with intraperitoneal (IP) injection of pentobarbital sodium (50 mg/kg) and underwent third ventricular cannulation as previously described (4). The mice were placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A burr hole was drilled for the implantation of a 26-gauge guide cannula (Plastics One, Roanoke, VA) into the third ventricle. The tip of a guide cannula was placed just above the
third ventricle at the following coordinates (10): 0.825 mm posterior to the bregma, midline, 4.8 mm below the skull surface. A 30-gauge dummy cannula (Plastics One, Roanoke, VA) was inserted into the guide cannula to maintain patency. The animals were allowed to recover for at least 1 week following cannulation surgery before the experiments.

After the recovery period, cannula placement was verified as described previously (3). Mice were administered 1 μg of NPY (Sigma-Aldrich, St Louis, MO) at a volume of 1 μl for 30 s into the third ventricle (I3V) during the middle of the light phase. Mice that ate more than 0.5 g of food pellets in the subsequent 2 h were used for experiments.

**Single I3V administration of metformin**

For microinjection, the dummy cannula was replaced with a 33-gauge injection cannula (Plastics One, Roanoke, VA), which was connected to a Hamilton syringe (Hamilton, Reno, NV), driven by a remotely controlled infusion pump (Model 102, CMA Microdialysis AB, Stockholm, Sweden). Mice were fed prior to I3V administration of metformin (7.5, 15, or 30 μg). Control solution (aCSF) or metformin was delivered into the third ventricle at a volume of 2 μl for 1 min, and the injection cannula was withdrawn 1 min after the end of injection.

**Meal pattern analysis**

The effect of I3V administration of metformin on spontaneous feeding behavior was evaluated by performing meal pattern analysis as described previously (15). Control or metformin (7.5, 15, or 30 μg) solutions were administered into the third ventricle of the mice 1 h before the start of experiments. The mice were individually placed into the open-field
area of a cage (200 × 200 × 370 mm) after administration of the solution. A food bin filled with food pellets and a water bottle were both freely accessible to each mouse at all times. To minimize food spillage, the wall of chamber facing the food bin was designed so that the spillage could return to the food bin. The weight of the food bins was measured every second by using an automatic data acquisition unit (National Instruments, Austin, TX). Raw data were analyzed using custom-designed software written in LabVIEW 11 (National Instruments, Austin, TX). Meal pattern analysis was started at the beginning of the dark phase (at 1800 hours) after I3V administration of control or metformin solution, and data were collected for 22 h. A meal was defined as the removal of >0.01 g from the food bin for at least 10 s and following a 5-min inter-meal interval (IMI, the time elapsed between the end of one meal and the start of the next). The meal continued until 5 min elapsed with no consumption. Meal size was calculated as the change in the weight of a food bin between the start and end of each meal. Mice that had a tendency to gnaw the pellets until they were powdered were excluded.

**Energy expenditure**

To determine whether changes in energy expenditure contributed to body weight change elicited by central administration of metformin, we assessed energy expenditure by indirect calorimetry (Oxylet; Panlab, Cornella, Spain) after I3V administration of metformin (30 μg) in a separate experiment. A pair-fed (PF) group was provided the same amount of food as the metformin-treated group every 12 h. The mice were adapted to the calorimetry chambers for 2 days prior to I3V administration. Food and water were freely available and resting energy expenditure was recorded for the following 24 h. Oxygen consumption (VO₂) and carbon dioxide production (VCO₂) were measured continuously for 3 min at 30 min intervals. VO₂ was adjusted for body weight to the power of 0.75 and respiratory quotient...
(RQ) was calculated by dividing VCO$_2$ by VO$_2$ (31).

**CTA**

To determine whether I3V administration of 30 µg of metformin caused visceral illness, we performed a CTA test in lean mice. Briefly, the animals were divided into three groups: an IP saline- and I3V control-treated (IP SAL/I3V CON) group, an IP saline- and I3V metformin-treated (IP SAL/I3V M30) group, and an IP lithium chloride (a potent aversive agent, 0.15 M)- and I3V control-treated (IP LiCl/I3V CON) group. On the conditioning day, after 22 h of water deprivation, the mice were I3V administered control or metformin solution at a volume of 2 µl for 1 min and IP administered saline or equimolar LiCl (1% body weight). Mice were given saccharin-flavored water (0.15% saccharin dissolved in water) for 2 h immediately after the administration and then the saccharin-flavored water was replaced with water. On the next day, the mice were simultaneously given access to both saccharin-flavored water and water after a 23-h water deprivation period. Fluid consumption was recorded at 1, 2, 4, and 24 h, and preference ratios were calculated by dividing saccharin-flavored water consumption by total fluid consumption.

**Double I3V administration of metformin and rapamycin**

To determine whether the anorexia induced by I3V administration of metformin was mediated by S6K activity in the hypothalamus, mice were I3V administered either rapamycin (RAPA, 50 ng) or DMSO 1 h before I3V administration of either metformin (M50, 50 µg) or control (CON, aCSF). Each compound was delivered into the third ventricle at a volume of 2 µl for 1 min. Food intake and change in mouse body weight were measured in the home cages. In this experimental design, we had to increase the dose of metformin to 50 µg to
achieve the same degree of anorexia as the single I3V administration study.

**Western blot analysis**

To investigate the involvement of key energy sensors, such as AMPK and S6K, in the anorectic action of centrally administered metformin, the mice were killed 1 h after I3V administration of the control solution or metformin (30 μg) in a separate experiment. The mediobasal hypothalamus was quickly dissected according to the methods used in a previous study (34). Tissue was homogenized and lysed with radioimmunoprecipitation assay buffer (Triton X-100 1.25%, SDS 0.1%, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.6) supplemented with a mixture of phosphatase inhibitors, sodium fluoride (Sigma-Aldrich, St Louis, MO), and sodium pyrophosphate decahydrate (Sigma-Aldrich, St Louis, MO). A total of 20 μg of protein was separated by 10% SDS-PAGE, and then transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 8.0) with 5% skim milk or 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO) and incubated overnight at 4°C with a primary antibody against Phospho-AMPKalpha (Thr$^{172}$) (1:1000), Phospho-AMPKalpah1 (Ser$^{485}$)/AMPKalpha2 (Ser$^{491}$) (1:1000), AMPK-alpha (1:2000), Phospho-p70 S6 Kinase (Thr$^{389}$) (1:1000), p70 S6 Kinase (1:2000), Phospho-Stat3 (1:500), Stat3 (1:1000), Phospho-Akt (1:1000), Akt (1:2000), or beta-actin (1:4000). All primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). After washing in TBS containing 0.05% Tween-20 (TBST), membranes were incubated in HRP-conjugated secondary antibody (1:2000, Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. After further washing in TBST, the proteins were visualized using an ECL kit (Thermo Scientific, Rockford, IL). The intensity of the bands was analyzed using Image J (NIH, Bethesda, MD).
and normalized to the intensity of an internal control (i.e., beta-actin or alternate form of assayed protein).

**Statistical analysis**

Results are presented as means ± standard errors of means (SEMs). All data from animal studies were analyzed by two-way repeated measures ANOVA followed by Bonferroni’s multiple comparisons, one-way ANOVA followed by Tukey’s post-hoc test, or two-tailed Student’s t-test by using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). For the western blot analysis, the intensity of the bands in the groups was compared using two-tailed Student’s t-test. Statistical significance was accepted for p values of <0.05.
Results

Effect of central administration of metformin on food intake and body weight

Compared to the administration of the control, administration of 15 and 30 µg of metformin (M15 and M30, respectively) into the third ventricle significantly reduced food intake after 22 h (Figure 1A). Two-way repeated measures ANOVA performed to analyze cumulative food intake showed significant effects of time (F_{4, 172} = 173.89, p < 0.001) and doses of metformin (F_{3, 43} = 3.67, p < 0.05). Bonferroni’s multiple comparisons revealed a significant difference in food intake between the control mice and the mice that received M15 and M30, but not 7.5 µg of metformin (M7.5), over a range of time points including 4, 8, 12, and 22 h after treatment (Figure 1A).

I3V administration of metformin was found to significantly reduce food intake at various time intervals over 0–8 h when analyzed based on the time interval after metformin treatment by one-way ANOVA (F_{3, 31} = 11.59, p < 0.001 for 0–1 h time interval; F_{3, 31} = 8.88, p < 0.001 for 1–2 h time interval; F_{3, 31} = 12.19, p < 0.001 for 2–4 h time interval; and F_{3, 31} = 14.91, p < 0.001 for 4–8 h time interval). Tukey’s post-hoc test showed that compared to the control, M30 significantly reduced food intake at time intervals of 0–1 h, 1–2 h, 2–4 h, and 4–8 h, and M15 reduced food intake at intervals of 0–1 h, 1–2 h, and 2–4 h, indicating a potent effect of centrally administered metformin on food intake during the early period of treatment (Figure 1B).

Compared to the control, M30 significantly decreased body weight after 24 h (F_{3, 31} = 4.50, p < 0.01; Figure 1C). By 48 h after treatment, the decrease in body weight induced by M30 recovered to the level measured before metformin administration, indicating a transient
Effect of centrally administered metformin on meal size and meal number

For a detailed analysis of changes in feeding behavior elicited by metformin treatment, we investigated patterns of meal size and meal number based on the time intervals after I3V administration of the control solution or metformin.

One-way ANOVA followed by Tukey’s post hoc test revealed that compared to the control, M15 and M30 significantly decreased both meal size (F_{3, 31} = 10.83, p < 0.001) and meal number (F_{3, 31} = 13.73, p < 0.001) during the first 1 h after treatment. Afterward, compared to the control, M30 significantly reduced meal size at the time intervals of 1–2 h (F_{3, 31} = 5.99, p < 0.01) and 2–4 h (F_{3, 31} = 3.91, p < 0.05), and reduced meal number at intervals of 1–2 h (F_{3, 31} = 8.52, p < 0.001) and 4–8 h (F_{3, 31} = 3.90, p < 0.05) (Figure 1D and E). These results indicated that administration of metformin into the third ventricle decreased food intake by reducing both meal size and meal number during the early period of treatment, and that the reduced food intake was maintained until 22 h after treatment.

Effect of centrally administered metformin on CTA

CTA was produced in the IP LiCl/I3V CON group, but not in the IP SAL/I3V CON group or the IP SAL/I3V M30 group. The preference ratio in the IP LiCl/I3V CON group was significantly lowered compared to that in the other two groups (F_{2, 14} = 7.352, p < 0.01). However, no significant difference was observed in the preference ratios between the IP SAL/I3V CON group and the IP SAL/I3V M30 group (Figure 1F). These results indicated that central administration of metformin was not associated with aversion or illness.
Effect of centrally administered metformin on energy expenditure

Nocturnal RQ ratios were significantly lower in the M30-treated and PF groups than in the control mice ($F_{2, 21} = 9.39$, $p < 0.01$; Figure 2A). However, no differences in diurnal RQ ratios were observed between the groups (Figure 2B). Nocturnal and diurnal energy expenditure expressed as oxygen consumption ($V_O^2$) adjusted for calculated body mass was also not significantly different between the groups (Figure 2C and D). However, compared to the control-treated mice and the PF group, the M30-treated group showed significantly reduced energy expenditure and RQ ratios during the first 3 h after I3V administration ($F_{2, 21} = 8.90$ and $F_{2, 21} = 10.59$, respectively, all $p < 0.01$; Figure 2E and F).

Effect of centrally administered metformin on hypothalamic energy sensors

To investigate the direct effect of metformin on key hypothalamic energy sensors, we compared changes in phosphorylation of AMPK and S6K 1 h after I3V administration of metformin at the dose and time point that potently reduced food intake in the meal pattern study. M30 slightly decreased phosphorylation of AMPK at Thr$^{172}$ in the mediobasal hypothalamus, which was associated with an increase in AMPK activity ($p = 0.092$; Figure 3A). However, metformin significantly increased the phosphorylated form of AMPK at Ser$^{485/491}$ in the mediobasal hypothalamus, which inhibited AMPK activity ($p < 0.05$; Figure 3B).

We next determined whether hypothalamic S6K, another key energy sensor, was associated with metformin-induced anorexia. M30 significantly increased S6K phosphorylation at Thr$^{389}$ in the mediobasal hypothalamus ($p < 0.001$; Figure 4). Furthermore, M30 decreased both Akt and Stat3 phosphorylation in the mediobasal hypothalamus ($p <$
0.05; Figure 5A and B), indicating that the hypothalamic leptin and insulin signaling pathways may not be directly involved in metformin-induced anorexia.

**Effect of rapamycin pretreatment on metformin-induced anorexia**

We chose a subthreshold dose of rapamycin (50 ng) that did not influence body weight or food intake in this experiment. Compared to both the DMSO- and CON-treated (DMSO/CON) group and the RAPA- and CON-treated group, the DMSO- and M50-treated (DMSO/M50) group showed a significant decrease in food intake and body weight 24 h after I3V administration of metformin. Pretreatment with rapamycin, a selective mTOR inhibitor, mitigated the weight loss and anorexia observed in the DMSO/M50 group ($F_{3,37} = 14.59$ and $F_{3,37} = 9.59$, respectively, all $p < 0.001$; Figure 4B and C), suggesting an important role for hypothalamic mTOR/S6K signaling in metformin-induced anorexia.
4. Discussion

The aim of this study was to determine the direct hypothalamic effect of metformin on food intake and key energy sensors, such as AMPK and S6K, by its direct administration into the third cerebral ventricle in the mice. In the meal pattern analysis, we showed that I3V administration of metformin significantly reduced food intake in a dose-dependent manner, which manifested as reduction in both meal size and meal number during the early period of treatment. Of particular importance was the observation that I3V administration of metformin at a dose eliciting a profound reduction in food intake did not produce aversion or sickness in the mice. Furthermore, we provided evidence that this reduction in food intake was accompanied by increased phosphorylation of AMPK (at Ser\(^{485/491}\)) and S6K (at Thr\(^{389}\)) in the mediobasal hypothalamus, and that metformin-induced anorexia was attenuated by inhibition of mTOR/S6K signaling within the hypothalamus. These results suggest the involvement of key energy sensors such as S6K in the anorectic action of centrally administered metformin.

The findings of our study clearly show evidence of metformin’s action in the hypothalamus. Despite the endeavors of previous researchers, indications of the involvement of the hypothalamus in metformin-induced weight loss, as well as the details of the underlying mechanism have been conflicting (16, 19, 27). Moreover, acute administration of metformin into the lateral cerebral ventricle had no effect on food intake in rats (29). However, since it is possible that metformin can affect the hypothalamus by crossing the blood–brain barrier (17, 19, 21), it was more reasonable to investigate metformin’s effects on hypothalamic regulation of food intake by administering it into the third ventricle (1, 12). In this study, we found that I3V administration of metformin adjacent to the hypothalamus...
decreased food intake in mice. In addition, I3V administration of metformin decreased both meal size and meal number, mainly during the nocturnal period. Energy intake is a function of meal size and number, which are associated with meal termination and initiation, respectively. These factors have been shown to have reciprocal compensatory action via independent mechanisms (28). Therefore, the decreases in both meal size and meal number support a potent inhibitory action of central metformin on food intake. Moreover, it should be noted that the reduced food intake was mediated via physiological changes rather than by toxic effects of metformin treatment, as shown by the results of the CTA test (8).

Overall, nocturnal and diurnal energy expenditures were unchanged, but rather transiently decreased during the early period after metformin treatment. Consistent with the reduced food intake, the nocturnal RQ ratio was lower in the metformin-treated group, implying that fat was used as an energy substrate. These results suggest that the change in the energy expenditure did not contribute to the body weight loss observed after metformin treatment.

Activation of AMPK induced by metformin improves glucose regulation via an increase in fatty acid oxidation, glucose uptake, and a decrease in lipogenesis in liver and skeletal muscle (11, 35). Hypothalamic AMPK activity contributes to the regulation of food intake, i.e., activation of AMPK in the hypothalamus increases food intake in vivo and increases NPY in vitro (5, 20). In line with a rational hypothesis, the involvement of hypothalamic AMPK in the anorectic action of metformin has been intensively studied (5, 19, 29). In cell cultures using primary rat hypothalamic neurons, metformin was shown to suppress an increase in AMPK induced by low glucose, supporting the role of hypothalamic AMPK in metformin-induced anorexia (5). However, peripheral administration of metformin
does not affect hypothalamic AMPK activity in obese rats despite reducing food intake (19).

In addition, administration of metformin into the lateral cerebral ventricle does not change hypothalamic AMPK phosphorylation in rats (29). Therefore, it is unclear whether administration of metformin reduces food intake via hypothalamic AMPK (5, 19). Consistent with these previous findings, we also observed no difference in hypothalamic AMPK phosphorylation at Thr^{172} 1 h after I3V administration of metformin. Thus, despite the strong evidence for an association between metformin and AMPK activity in hypothalamic neuronal cell cultures, phosphorylation of hypothalamic AMPK at Thr^{172} that leads to increased AMPK activity may not be directly involved in the anorectic action of metformin in vivo.

Recent work from the Kahn lab has shown that constitutive activation of S6K or recombinant S6K increases phosphorylation of AMPK at Ser^{485/491} in neuronal cell cultures. However, activation of S6K does not affect phosphorylation at Thr^{172}, suggesting a new role for S6K as an AMPK kinase, distinct from liver kinase B1 and calcium/calmodulin-dependent protein kinase kinase 2 (7). Therefore, we hypothesized that metformin-induced anorexia might be associated with an increase in hypothalamic AMPK phosphorylation at Ser^{485/491}, since no difference in AMPK phosphorylation at Thr^{172} was observed. Consistent with this hypothesis, AMPK phosphorylation at Ser^{485/491} significantly increased in the hypothalamus of I3V metformin-treated mice relative to controls.

Next, we investigated the involvement of another key energy sensor, S6K, in decreased food intake after I3V metformin administration. The hypothalamic mTOR/S6K pathway has been shown to contribute to the anorectic action of hormones, including leptin and insulin, as well as the branched amino acid leucine, and constitutive activation of S6K in the mediobasal hypothalamus has been shown to decrease both food intake and body weight.
in rats (2, 6). In the present study, I3V administration of metformin increased phosphorylation of hypothalamic S6K at Thr\textsuperscript{389}. Phosphorylated forms of mTOR and S6K have been shown to be co-localized with the majority of the NPY/Agouti-related peptide neurons in the arcuate nucleus of the hypothalamus (~90%) (6). Furthermore, the activation of hypothalamic mTOR/S6K signaling by leucine, or by adenoviruses that lead to a constitutively active S6K mutant, selectively decreases NPY gene expression in the hypothalamus of rats (2, 6). This might be related to the previous finding that oral administration of metformin decreases hypothalamic NPY gene expression with no change in POMC gene expression in rats (19). It is important to note that in the present study, we showed that pretreatment with rapamycin reversed a decrease in food intake induced by metformin. In conjunction with previous studies, this finding suggests that the interaction of S6K-AMPK at Ser\textsuperscript{485/491} might be associated with the anorectic action of metformin, given that inhibition of AMPK activity following activation of S6K has been shown to suppress food intake (7, 20).

Interestingly, we observed that the phosphorylation of critical signaling molecules related to leptin and insulin action, including Stat3 and Akt, decreased in the mediobasal hypothalamus after I3V administration of metformin compared to that of the control. Leptin and insulin are considered adiposity signals and are used to monitor energy levels in the body (32). Central administration of leptin or insulin decreases food intake via activation of hypothalamic Stat3 or Akt, which mediates their anorectic action (22-24). Our study suggests that the key molecules related to leptin and insulin signaling might not directly participate in metformin-induced anorexia. Furthermore, the decrease in hypothalamic Akt phosphorylation induced by metformin may be associated with the negative feedback control of enhanced mTOR/S6K signaling (25), although the detailed interaction and role of these signaling
molecules needs further investigation.
Perspectives and Significance

Our study showed that central administration of metformin into the murine third ventricle near the hypothalamus, a key region for the regulation of food intake, decreased food intake mainly via reduction in both the size and number of meals. Notably, while metformin treatment caused profound anorexia, it did not produce taste aversion in mice. In addition, our study suggests that metformin-induced anorexia may be mediated by an increase in S6K phosphorylation at Thr\textsuperscript{389}, and presumably by subsequent AMPK phosphorylation at Ser\textsuperscript{485/491}, although the interaction of these specific pathways should be investigated in future studies. Furthermore, we postulate that key molecules related to leptin and insulin action, Stat3 and Akt, are not directly associated with metformin-induced anorexia. Therefore, we have provided novel data to help clarify the mechanism underlying the anorectic action of centrally administered metformin. Further detailed studies of the mechanism of metformin-induced anorexia based on these results may enhance their clinical application.
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References


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Figure legends

**Figure 1.** Administration of metformin into the third ventricle (I3V) decreases body weight and energy intake.

I3V administration of metformin decreases cumulative food intake over the course of 22 h (A), food intake during designated time intervals (B), and body weight change after 24 h (C). I3V administration of metformin decreases both nocturnal meal size and number (D and E). Data are presented as mean ± SEM. * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. CON. + p < 0.05, ++ p < 0.01, and +++ p < 0.001 vs. M7.5. # p < 0.05 vs. M15. CON, control (aCSF)-treated group (n = 8); M7.5, 7.5-µg-metformin–treated group (n = 9); M15, 15-µg-metformin–treated group (n = 9); M30, 30-µg-metformin–treated group (n = 9).

I3V administration of metformin did not cause illness (F). Preference ratio was calculated by dividing the volume of saccharin intake by the total volume of fluid intake after I3V and IP administrations. IP SAL/I3V CON, IP saline- and I3V control-treated group (n = 6); IP LiCl/I3V CON, IP lithium chloride- and I3V control-treated group (n = 6); IP SAL/I3V M30, IP saline- and I3V metformin-treated group (n = 5); IP, intraperitoneal administration. Data are presented as mean ± SEM. * p < 0.05; ** p < 0.01 vs. IP LiCl/I3V CON.

**Figure 2.** Change in overall energy expenditure does not contribute to metformin-induced anorexia.

I3V administration decreases nocturnal RQ (A), but not diurnal RQ (B). I3V administration of metformin does not change overall nocturnal or diurnal VO₂ (C and D). However, RQ and VO₂ are decreased during the first 3 h after I3V administration of metformin (E and F).
Data are presented as mean ± SEM. *p < 0.05 vs. CON, control-treated group (n = 8); M30, 30-µg-metformin–treated group (n = 8); PF, group pair-fed to M30 (n = 8); RQ, respiratory quotient; VO₂, oxygen consumption adjusted for estimated body mass.

**Figure 3.** I3V administration of metformin increases phosphorylation of AMPK at Ser\(^{485/491}\), but not at Thr\(^{172}\).

(A) Ratio of the phosphorylated form of AMPK at Thr\(^{172}\) to total AMPK
(B) Ratio of the phosphorylated form of AMPK at Ser\(^{485/491}\) to total AMPK

Data are presented as mean ± SEM. *p < 0.05 vs. CON. CON, control-treated group (n = 8); M30, 30-µg-metformin–treated group (n = 8).

**Figure 4.** S6K signaling contributes to metformin-induced anorexia.

(A) Ratio of the phosphorylated form of S6K at Thr\(^{389}\) to total S6K.

Data are presented as mean ± SEM. ***p < 0.001 vs. CON. CON, control-treated group (n = 8); M30, 30-µg-metformin–treated group (n = 8).

Rapamycin pretreatment blunts metformin-induced anorexia (B) and weight loss (C) 24 h after double I3V administration of rapamycin (50 ng in 2 µl) followed by metformin.

Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. DMSO/M50. DMSO/CON, DMSO- and CON-treated group (n = 7), DMSO/M50, DMSO- and 50-µg-metformin–treated group (n = 12), RAPA/CON, 50-ng-rapamycin- and CON-treated group (n = 11); RAPA/M50, rapamycin- and metformin-treated group (n = 11)

**Figure 5.** I3V administration of metformin decreases activation of Akt and Stat3.
(A) Ratio of the phosphorylated form of Akt at Ser$^{473}$ to total Akt

(B) Ratio of the phosphorylated form of Stat3 at Tyr$^{705}$ to total Stat3

Data are presented as mean ± SEM. * p < 0.05 vs. CON. CON, control (aCSF, n = 8); M30, 30-μg metformin (n = 8).
Figure 1
Figure 2
Figure 3

(A) p-AMPK/AMPK (Thr$^{172}$)

(B) p-AMPK/AMPK (Ser$^{485/491}$)
Figure 4

Panel A: p-S6K/S6K (Thr^{389})

Panel B: % Change in Food Intake Compared to DMSO/CON

Panel C: % Difference of Initial Body Weight
Figure 5

(A) p-Akt/Akt (Ser^{473})

(B) p-Stat3/Stat3 (Tyr^{705})