Intracerebroventricular Administration of Metformin Inhibits Ghrelin-Induced Hypothalamic AMP-Kinase Signalling and Food Intake

Darko Stevanovic^a Kristina Janjetovic^{b, d} Maja Misirkic^{b, d} Ljubica Vucicevic^{b, d}
Mirjana Sumarac-Dumanovic^c Dragan Micic^c Vesna Starcevic^a
Vladimir Trajkovic^b

Institutes of ^aMedical Physiology, ^bMicrobiology and Immunology, and ^cEndocrinology, Diabetes and Diseases of Metabolism, School of Medicine, and ^dInstitute for Biological Research 'Sinisa Stankovic', University of Belgrade, Belgrade, Serbia

Key Words

Metformin • Ghrelin • Food intake • Hypothalamus • AMPK

Abstract

Background/Aims: The antihyperglycaemic drug metformin reduces food consumption through mechanisms that are not fully elucidated. The present study investigated the effects of intracerebroventricular administration of metformin on food intake and hypothalamic appetite-regulating signalling pathways induced by the orexigenic peptide ghrelin. **Methods:** Rats were injected intracerebroventricularly with ghrelin (5 µg), metformin (50, 100 or 200 µg), 5-amino-imidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR, 25 µg) and L-leucine (1 µg) in different combinations. Food intake was monitored during the next 4 h. Hypothalamic activation of AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), regulatory-associated protein of mTOR (Raptor), mammalian target of rapamycin (mTOR) and p70 S6 kinase 1 (S6K) after 1 h of treatment was analysed by immunoblotting. **Results:** Metformin suppressed the increase in food consumption induced by intracerebroventricular ghrelin in a dose-dependent manner. Ghrelin increased phosphorylation of hypothalamic AMPK and its targets ACC

and Raptor, which was associated with the reduced phosphorylation of mTOR. The mTOR substrate, S6K, was activated by intracerebroventricular ghrelin despite the inhibition of mTOR. Metformin treatment blocked ghrelin-induced activation of hypothalamic AMPK/ACC/Raptor and restored mTOR activity without affecting S6K phosphorylation. Metformin also reduced food consumption induced by the AMPK activator AICAR while the ghrelin-triggered food intake was inhibited by the mTOR activator L-leucine. **Conclusion:** Metformin could reduce food intake by preventing ghrelin-induced AMPK signalling and mTOR inhibition in the hypothalamus.

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Introduction

Metformin [(1-(diaminomethylidene)-3,3-dimethylguanidine)] is an antihyperglycaemic drug widely used for the management of type 2 diabetes [1]. The glucoregulatory properties of metformin are mainly attributed to reduced hepatic glucose production and augmented glucose uptake by the peripheral tissues [1]. Metformin has also been suggested to reduce weight in diabetic and non-

diabetic patients, in contrast to sulphonylureas, thiazolidinediones and insulin, which all induce weight gain [2]. The positive effect of metformin on weight control has been associated with reduced food intake both in humans and experimental animals [3–9], but the mechanisms responsible for the metformin-mediated reduction of food consumption have not been fully clarified. Consistent with the ability of orally administered metformin to readily cross the blood-brain barrier [10], some recent data suggest that its anorexigenic effect might result from a direct action on the hypothalamic centres regulating satiety and feeding [11–13]. In diet-induced obese rats, metformin enhanced the hypothalamic phosphorylation of STAT3 induced by acute intracerebroventricular administration of the anorexigenic hormone leptin [11] and increased hypothalamic leptin receptor expression [12]. Additionally, metformin reduced glucose deprivation-triggered release of the potent orexigenic mediator neuropeptide Y (NPY) in primary hypothalamic neuronal cell cultures [13].

Peripheral metabolic effects of metformin at least partly depend on the stimulation of AMP-activated protein kinase (AMPK) [14, 15], an intracellular energy sensor that is activated by raising AMP and acts by switching on ATP-generating catabolic pathways while switching off ATP-requiring anabolic processes [3]. However, the same dose of intraperitoneally injected metformin that readily activated hepatic AMPK, failed to increase hypothalamic AMPK phosphorylation in rats [16]. Moreover, metformin completely blocked glucose deprivation-induced AMPK phosphorylation in rat primary hypothalamic neurons in vitro [13], suggesting a different regulation of central and peripheral AMPK by this antidiabetic drug.

Ghrelin is a 28-amino-acid peptide that promotes food intake [17–20] mainly by acting on hypothalamic NPY and agouti-related protein systems [21]. It has been suggested that the orexigenic action of ghrelin is mediated by stimulation of AMPK [22, 23], which controls the feeding behaviour through integration of orexigenic and anorexigenic signals [23, 24]. The intracellular signals downstream of AMPK activation include phosphorylation of regulatory-associated protein of the mammalian target of rapamycin (mTOR) (Raptor) and subsequent inactivation of mTOR and its target p70 S6 kinase 1 (S6K) [25]. The activation of hypothalamic mTOR/S6K has been proposed as an important anorexigenic signal [26–28], thus making plausible that the orexigenic action of ghrelin might involve central AMPK-mediated mTOR/S6K inactivation. While metformin has recently been found to increase plasma ghrelin levels in patients with

type 2 diabetes [29], the effects of metformin on ghrelin-induced food intake and hypothalamic AMPK/mTOR signalling have not been investigated.

Based on the above findings, we hypothesized that metformin could reduce food intake by inhibiting AMPK and consequently restoring mTOR/S6K activity in the hypothalamus. To test this assumption, we examined the influence of centrally applied metformin on ghrelin-triggered acute increase in food intake and hypothalamic AMPK/mTOR signalling.

Materials and Methods

Animal Preparation

Eight-week-old male Wistar rats (body weight 200 ± 20 g) were obtained from the Institute of Biomedical Research Gale-nika (Belgrade, Serbia). They were kept in individual cages under a 12:12 h light/dark cycle, at $22 \pm 2^\circ\text{C}$, and were accustomed to daily handling for 2 weeks (body weight 252 ± 15 g). The animals were anaesthetized with intramuscular ketamine (50 mg/kg; Pfizer, New York, N.Y., USA) -xylazine (80 mg/kg; Bayer, Leverkusen, Germany) and equipped with a headset for intracerebroventricular injection, consisting of a silastic-sealed 20-gauge cannula positioned in the right lateral cerebral ventricle (1 mm posterior and 1.5 mm lateral to the bregma, and 3 mm below the cortical surface) [30]. A small stainless steel anchor screw was placed at a remote site on the skull. The cannula and screw were cemented to the skull with standard dental acrylic. Following surgery, the animals received a single subcutaneous dose of 0.28 mg/kg buprenorphin (Reckitt Benckiser Healthcare, Mannheim, Germany) and 1 week of recovery was allowed before the experiments. Only animals demonstrating progressive weight gain after surgery were used in subsequent experiments. All rats had ad libitum access to rodent chow (D.D. Veterinarski zavod Subotica, Subotica, Serbia) and water during experimental testing.

Experiment 1

Effect of Intracerebroventricular Metformin on Ghrelin-Induced Food Intake

In this experiment, the rats were treated intracerebroventricularly with 5 μg ghrelin (Bachem, Weil am Rhein, Germany), metformin hydrochloride (50, 100 or 200 μg ; 99.9% Hemofarm, Vrsac, Serbia) or ghrelin and metformin ($n = 6$ in each group). The orexigenic concentration of ghrelin (5 μg) was chosen based on a previous study [23]. The concentrations of metformin (50–200 μg) which did not cause overt neurotoxicity after a single intracerebroventricular injection were selected based on previous reports [16, 31]. Both ghrelin and metformin were applied in 2 μl of phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, Mo., USA) using a 5- μl Hamilton syringe. Metformin was administered 30 min before ghrelin. The animals in the ghrelin-only group received 2 μl PBS 30 min before treatment while those in the metformin-only group received 2 μl PBS 30 min after the treatment. Control animals received $2 \times 2 \mu\text{l}$ PBS. Food intake was measured each hour up to 4 h after the second injection.

Experiment 2

Effect of Intracerebroventricular Metformin on Ghrelin-Induced AMPK/mTOR Signalling

The rats were treated as described in experiment 1 ($n = 6$ in each group), but metformin was used at the concentration of 100 μg . One hour after the last injection, the rats were killed by decapitation under deep isoflurane anaesthesia; hypothalamic tissues were collected and immediately frozen in liquid nitrogen for immunoblot analysis.

Experiment 3

Effect of Intracerebroventricular Metformin on 5-Aminoimidazole-4-Carboxamide 1- β -D-Ribofuranoside-Induced Food Intake

Rats were treated as described in experiment 1 ($n = 5$ in each group), except that 5-aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR; Sigma-Aldrich) (25 μg in 2 μl dimethylsulphoxide, DMSO) was used instead of ghrelin while metformin was used at 100 μg . The orexigenic dose of AICAR was chosen based on a previous study [23]. The animals in the metformin-only group received 2 μl DMSO 30 min after metformin treatment while control animals received 2 μl PBS + 2 μl DMSO. Food intake was measured each hour up to 4 h after the second injection.

Experiment 4

Effect of L-Leucine on Ghrelin-Induced Food Intake

Rats were treated as described in experiment 1 ($n = 5$ in each group), except that L-leucine (Sigma-Aldrich) (1 μg in 2 μl PBS) was used instead of ghrelin. Food intake was measured each hour up to 4 h after the second injection. The concentration of intracerebroventricular L-leucine was selected based on its ability to suppress post-fasting food intake in rats [32].

Immunoblot Analysis

Western blot followed by protein detection with specific antibodies was used to assess phosphorylation (activation) of various members of the AMPK/mTOR signalling pathway [AMPK, acetyl-CoA carboxylase (ACC), Raptor, mTOR and S6K]. The hypothalamic tissue was lysed in a RIPA buffer (Sigma-Aldrich) on ice for 30 min, centrifuged at 14,000 g for 15 min at 4°C, and the supernatants were collected. Equal amounts of total protein from each sample (10 μg for actin blot and 25 μg for all other proteins) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Marnes-la-Coquette, France). All blots were performed on separate gels. Following incubation with primary antibodies against phospho-AMPK α (Thr172), AMPK, phospho-ACC (Ser79), phospho-Raptor (Ser792), Raptor, phospho-mTOR (Ser2448), phospho-S6K (Thr389), S6K or actin (Cell Signaling Technology, Beverly, Mass., USA) and peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) as the secondary antibody, specific protein bands were visualized using enhanced-chemiluminescence reagents for Western blot analysis (Amersham Pharmacia Biotech, Piscataway, N.J., USA). The signal intensity was determined by densitometry using Image J software and the results were presented as phospho/total protein signal ratio, which was arbitrarily set to 1 in control.

Statistical Analysis

The data obtained from each sample were averaged per experimental group and the standard deviation of the mean (SD) was calculated. A one-way analysis of variance (ANOVA), followed by a Student-Newman-Keuls test for multiple comparisons, was used to assess differences between the groups. A p value of less than 0.05 was considered statistically significant.

Results

Centrally Applied Metformin Inhibits Ghrelin-Induced Food Intake

We first investigated the ability of centrally applied metformin to influence the orexigenic effect of ghrelin. In the absence of ghrelin, intracerebroventricular administration of metformin (50, 100 or 200 μg) did not significantly affect food intake (fig. 1a). Expectedly, intracerebroventricular injection of ghrelin (5 μg) caused a time-dependent increase in cumulative food intake over the 4-hour post-injection period (fig. 1b). Pretreatment with metformin reduced the orexigenic effect of ghrelin at each time point in a dose-dependent manner (fig. 1b). These data demonstrate that metformin can counteract the orexigenic effect of ghrelin at the hypothalamic level.

Metformin Modulates Hypothalamic AMPK/mTOR Signalling in Ghrelin-Treated Rats

We next investigated the influence of metformin on hypothalamic AMPK/mTOR signalling in ghrelin-treated rats. Central administration of ghrelin (5 μg) increased phosphorylation of hypothalamic AMPK and its downstream targets ACC and Raptor, which was associated with reduced phosphorylation of mTOR, but increased activation of its direct substrate S6K (fig. 2). While treatment with metformin (100 μg) did not alter hypothalamic AMPK/mTOR signalling in the absence of ghrelin, it significantly suppressed ghrelin-induced activation of AMPK/ACC/Raptor and restored the phosphorylation of ghrelin-inactivated mTOR without affecting S6K phosphorylation (fig. 2). Therefore, centrally applied metformin can interfere with hypothalamic AMPK/mTOR signalling, but not with S6K activation in rats treated with intracerebroventricular ghrelin.

Metformin Inhibits Food Intake Induced by AMPK Activator AICAR

We next assessed whether metformin could inhibit food intake induced by intracerebroventricular injection of AICAR, a pharmacological AMPK activator with orexigenic activity in rats [23]. In comparison with con-

Fig. 1. Centrally applied metformin inhibits ghrelin-induced food intake. Rats ($n = 6$ per group) were injected intracerebroventricularly with PBS (control; **a, b**), 50–200 μg metformin (**a**), 5 μg ghrelin (**b**), or ghrelin and metformin (**b**). Cumulative food intake was

measured during 4 h at the indicated time points. The results from the same experiment (presented separately for clarity) are mean $\pm$ SD values (^a $p < 0.05$ and ^b $p < 0.05$ refer to control and ghrelin-treated rats, respectively).

trol animals, rats treated with AICAR (25 μg) consumed more food during the 4-hour follow-up period, with a significant increase in food intake observed after 3 and 4 h (fig. 3). AICAR-induced hyperphagia was significantly reduced at both time points by co-administration of metformin (100 μg).

mTOR Activator L-Leucine Inhibits Ghrelin-Induced Food Intake

Finally, we tested the ability of L-leucine, an mTOR activator with anorexigenic activity [32], to affect ghrelin-mediated food intake. Treatment with ghrelin (5 μg , i.c.v.) induced a sustained increase in food consumption for 4 h after injection (fig. 4). Intracerebroventricularly injected L-leucine (1 μg) did not interfere with food intake in control animals (fig. 4). On the other hand, it significantly reduced the ghrelin-triggered increase in food consumption at each of the time points (fig. 4).

Discussion

The present study, for the first time, demonstrates the ability of centrally applied metformin to suppress acute ghrelin-induced increase in food intake. The observed ef-

fect was associated with the inhibition of ghrelin-triggered activation of hypothalamic AMPK, as well as with the restoration of mTOR activity. These data suggest that the previously well-documented anorexigenic effect of metformin [3–9] could, at least partly, be mediated at the hypothalamic level through interference with ghrelin-induced orexigenic AMPK signalling.

Our hypothesis that the appetite-reducing effect of metformin could be due to suppression of AMPK activation is consistent with the proposed role of this intracellular energy sensor in ghrelin-mediated food intake [22, 23]. In accordance with the present study, both central and systemic ghrelin administration activate hypothalamic AMPK [22, 23], and experimental genetic activation or inactivation of hypothalamic AMPK leads to increased or decreased food intake, respectively [24]. It has been shown that the calcium/calmodulin-dependent protein kinase 2 and sirtuin 1/p53 pathway are required for hypothalamic AMPK activation by ghrelin [33–35], but the appetite-controlling signals downstream of AMPK activation have not been fully delineated. mTOR is a plausible AMPK target which has been suggested to mediate the anorexigenic effects of leptin [26–28], an important adipocyte-derived negative regulator of energy balance that counteracts the metabolic actions of

Fig. 2. Metformin modulates hypothalamic AMPK/mTOR signalling in ghrelin-treated rats. Rats ($n = 6$ per group) were injected intracerebroventricularly with PBS (control), metformin (100 μg), ghrelin (5 μg), or ghrelin and metformin. After 1 h, the levels of phosphorylated (p) and total AMPK, ACC, Raptor, mTOR and S6K were assessed by immunoblotting, with actin used as a loading control. The representative blots are presented in **a** while the data in **b** are mean $\pm$ SD values ($n = 6$) of phospho/total protein signal ratio (^a $p < 0.05$ and ^b $p < 0.05$ refer to control and ghrelin-treated rats, respectively).

ghrelin [36]. Our findings that ghrelin-induced hyperphagia coincided with downregulation of mTOR activity and was suppressed by the mTOR activator L-leucine are indeed consistent with the proposed role of hypothalamic mTOR as an anorexigenic signal [26–28]. Moreover, in our study, metformin-mediated blockade of ghrelin-triggered food intake and AMPK activation were associated with restoration of hypothalamic mTOR activity, thus indicating that the anorexigenic effect of metformin might depend on interference with AMPK-mediated mTOR downregulation in the hypothalamus. This assumption is consistent with the *in vitro* data demonstrating metformin-mediated AMPK inhibition in glucose-deprived rat primary hypothalamic neurons [13]. However, it seems that the inhibitory effect of metformin on AMPK might be restricted to the hypothalamus or might be species specific as in two recent studies metformin activated AMPK and inhibited mTOR in

mouse hippocampal slices and cultured cortical neurons [37, 38].

While, as discussed above, metformin might target hypothalamic AMPK/mTOR signalling to block the orexigenic action of ghrelin, it should be noted that the changes in the activation of the mTOR substrate S6K in our experiments did not correlate with the activation status of mTOR. Namely, ghrelin-mediated mTOR downregulation was paradoxically associated with S6K activation, which was not further altered by restoring mTOR phosphorylation with metformin. These data actually concur with the recent findings by Villanueva et al. [39], who reported activation of S6K in the arcuate nucleus of mice treated intracerebroventricularly with ghrelin or exposed to fasting, a state associated with an increase in the levels of circulating ghrelin [40, 41]. Moreover, both S6K1- and S6K2-knockout mice responded to ghrelin by increasing food consumption comparably to their wild-

Fig. 3. Metformin inhibits AICAR-induced food intake. Rats ($n = 5$ per group) were injected intracerebroventricularly with PBS (control), metformin (100 μ g), AICAR (25 μ g), or AICAR and metformin. Cumulative food intake was measured during 4 h at the indicated time points. The results are presented as mean $\pm$ SD values (^a $p < 0.05$ and ^b $p < 0.05$ refer to control and AICAR-treated rats, respectively).

Fig. 4. L-Leucine inhibits ghrelin-induced food intake. Rats ($n = 5$ per group) were injected intracerebroventricularly with PBS (control), L-leucine (1 μ g), ghrelin (5 μ g), or ghrelin and L-leucine. Cumulative food intake was measured during 4 h at the indicated time points. The results are presented as mean $\pm$ SD values (^a $p < 0.05$ and ^b $p < 0.05$ refer to control and ghrelin-treated rats, respectively).

type counterparts, indicating that S6K modulation is dispensable for ghrelin-induced hyperphagia [Stevanovic et al., submitted for publication]. While the role of hypothalamic S6K in the orexigenic effect of ghrelin remains to be fully elucidated, the dissociated mTOR and S6K activation status in our experiments clearly indicates that ghrelin could activate S6K independently of mTOR. Accordingly, mTOR-independent regulation of S6K has previously been reported [42]. In addition, our data imply that the putative involvement of mTOR inhibition as an orexigenic signal downstream of AMPK is mediated independently of S6K, or that alternatively, mTOR-independent mechanisms are responsible for the orexigenic action of AMPK. The latter assumption is supported by previous demonstration that ghrelin-mediated activation of hypothalamic AMPK increases food intake through a hypothalamic fatty acid oxidation pathway involving ACC/malonyl-CoA, carnitine palmitoyltransferase 1 and uncoupling protein 2 [43–45]. Indeed, we have observed an increase in ACC activation upon intracerebroventricular injection of ghrelin, and this effect was efficiently counteracted by co-administration of metformin. It is therefore possible that hypothalamic ACC and/or other

AMPK downstream targets different from mTOR/S6K could be responsible for the anorexigenic action of metformin. In the present study, we did not investigate the interference of metformin with ghrelin orexigenic signals that are further downstream of hypothalamic AMPK activation, such as NPY release. In contrast to the *in vitro* inhibitory effect of metformin on NPY release by glucose-deprived rat hypothalamic neurons [13], the anorexigenic effect of metformin in genetically obese Zucker rats is independent of the changes in hypothalamic NPY content [46]. We are currently investigating the role of NPY modulation in metformin-mediated suppression of ghrelin-induced hyperphagia.

Finally, it is interesting to note that metformin treatment increased plasma ghrelin concentrations in patients with type-2 diabetes [29]. This is somewhat unexpected considering the orexigenic action of ghrelin [17–20] and the well-documented inhibitory effect of metformin on food intake [3–9]. Our data, however, resolve this discrepancy by demonstrating that metformin could actually block the action of ghrelin in the hypothalamus, thus presumably counteracting the stimulatory effect on circulating ghrelin levels.

In conclusion, our data indicate a novel mechanism of the anorexigenic action of metformin involving down-regulation of ghrelin-induced activation of the AMPK signalling pathway. A different regulation of AMPK activation in the hypothalamus and peripheral tissues might contribute to the beneficial metabolic effects of metformin as peripheral AMPK activation will increase energy expenditure while hypothalamic AMPK inhibition will reduce food intake. It should be noted, however, that the metformin concentrations used in our study and in previous studies [16, 31] seem rather high compared with those applied therapeutically [47]. Nevertheless, lower concentrations of chronically administered metformin might still affect hypothalamic AMPK signalling. There-

fore, further studies are required to explore the mechanisms underlying central AMPK downregulation by metformin and its potential therapeutic significance in metabolic disorders.

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