Original Article

Direct Action of Celastrol on Hypothalamic ARC Neurons to Increase Leptin Sensitivity

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Abstract

Leptin is an adipocyte-derived hormone that is involved in the regulation of food intake and energy expenditure. Individuals with obesity generally exhibit hyperleptinemia and leptin resistance. Leptin sensitizers, rather than leptin, are expected to act as anti-obesity drugs. Recently, celastrol was identified as a leptin sensitizer. Celastrol alleviated endoplasmic reticulum (ER) stress and restored leptin sensitivity in the hypothalamus. However, the molecular mechanisms and sites of action of celastrol remain poorly understood. In this study, after confirming the effect of celastrol on body weight, food intake, and leptin sensitivity in lean and diet-induced obese (DIO) mice, we investigated the direct effect of celastrol on arcuate nucleus (ARC) neurons isolated from the hypothalamus by monitoring the intracellular calcium concentrations ($[Ca^{2+}]i$). Celastrol treatment significantly decreased body weight gain and food intake, and significantly augmented leptin actions on food intake and hypothalamic signal transducer and activator of transcription 3 (STAT3) phosphorylation in both lean and DIO mice. In accordance with these results, celastrol treatment enhanced $[Ca^{2+}]i$ response to leptin in the ARC neurons of both lean and DIO mice. To our knowledge, this is the first report to demonstrate the direct effect of celastrol on ARC neurons. This knowledge might shed light on the molecular mechanisms underlying celastrol-induced leptin sensitization and resistance.

（Key words: arcuate nucleus neuron, celastrol, hypothalamus, leptin, obesity）

Introduction

Leptin is an adipocyte-derived hormone that regulates energy homeostasis, mainly through the hypothalamus [1–3]. In circulation, leptin enters the brain through the blood-brain barrier [4] and binds to its receptor, which is expressed in the hypothalamus, with particularly high expression within the arcuate nucleus (ARC) [5] to attenuate food intake and decrease body weight [6]. Circulating leptin levels increase in proportion to body fat content [7]. Thus, most obese individuals have high serum leptin levels; however, increased leptin levels fail to suppress the progression of obesity. Obese humans weakly respond to exogenously administered leptin in terms of body weight reduction [8]. This pathological condition is known as leptin resistance. Currently, leptin sensitizers rather than leptin itself are expected to act as anti-obesity drugs.

Although the precise mechanism underlying leptin resistance remains unclear, endoplasmic reticulum (ER) stress in the hypothalamus plays a key role in the pathogenesis of leptin resistance [9, 10]. It has also been demonstrated that chemical chaperones, such as 4-phenyl butyric acid (PBA) and tauroursodeoxycholic acid (TUDCA), which have the ability to decrease ER stress, act as leptin-sensitizing agents [10]. Celastrol, a natural compound found

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in the roots of the thunder god vine, was recently identified as a leptin sensitizer[11]. Celastrol alleviates hypothalamic ER stress and restores hypothalamic sensitivity to leptin. Thus, celastrol is a promising agent for the treatment of obesity and its associated complications. However, the molecular mechanisms by which celastrol alleviates ER stress and restores leptin sensitivity in the hypothalamus, and its site of action remain poorly understood.

Celastrol was originally identified as an inhibitor of NF-κB[12]. The effects of celastrol on NF-κB signaling pathway and inflammation has been demonstrated in various cell types of peripheral tissues including immune cells such as macrophages and T-cells[13, 14]. Celastrol has been reported to inhibit protein tyrosine phosphatase (PTP) 1 B (PTP1B) and T-cell PTP (TCPTP) in the ARC of the hypothalamus[15]. The involvement of interleukin-1 receptor 1 (IL1R1) in celastrol leptin sensitization in the hypothalamus has also been reported[16]. However, celastrol was administered systemically in these studies. On the other hand, there are no reports on the direct effects of celastrol on the hypothalamus. Furthermore, the central nervous system consists of not only neural cells but also glial cells, and both cell types are involved in the development of ER stress and leptin resistance in the hypothalamus[17]. If celastrol has a direct effect on the hypothalamus, the cell type that is the most important target is unknown.

In this study, after confirming the effects of celastrol on body weight, food intake, and leptin sensitivity in lean and diet-induced obese (DIO) mice, we investigated the direct effect of celastrol on ARC neurons isolated from the hypothalamus of lean and DIO mice by monitoring intracellular calcium concentrations ($[Ca^{2+}]i$). Celastrol treatment significantly decreased body weight gain and food intake, and significantly augmented leptin actions on food intake and hypothalamic signal transducer and activator of transcription 3 (STAT3) phosphorylation in both lean and DIO mice. In accordance with these results, celastrol treatment enhanced $[Ca^{2+}]i$ response to leptin in the ARC neurons of both lean and DIO mice. To our knowledge, this is the first report to demonstrate the direct effect of celastrol on ARC neurons. This knowledge might shed light on the molecular mechanisms underlying celastrol-induced leptin sensitization and leptin resistance.

Materials and Methods

Animals

Six-week-old male C57BL/6J mice were obtained from Japan SLC (Shizuoka, Japan). The mice were housed under conditions of controlled temperature (23 ℃ ± 1 ℃), humidity (55% ± 5%) and lighting (light phase 7:30–19:30) and fed an ad libitum standard diet (SD) (MF; Oriental Yeast, Tokyo, Japan). For high-fat diet loading, mice were fed an ad libitum high-fat diet (HFD) containing 20% kcal for kcal (kcal/kcal) protein, 20% kcal/kcal carbohydrate, and 60% kcal/kcal fat (D12492; Research Diets, New Brunswick, NJ, USA) beyond the age of 8 weeks. To induce diet-induced obesity (DIO) in mice, HFD loading was performed for 8 weeks. Mice of the same age fed SD were used as lean controls. All animal experiments were approved by the Institutional Animal Experiment Committee and were performed in accordance with the Institutional Regulations for Animal Experiments of Jichi Medical University.

Celastrol treatment

Celastrol (Sigma-Aldrich, St Louis, MO) was dissolved in 25% dimethyl sulfoxide (DMSO) and administered to mice at a dose of 300 μg/kg by intraperitoneal injection (100 μL) at 9:00 h once a day. Vehicle groups received 100 μl of 25% DMSO for control purposes during the course of the experiments. Body weight and food intake were measured weekly; however, because the mice were kept in 4-6 mice per cage, food intake was calculated based on the number of mice and the number of days and converted to calories.

Leptin injection experiment

Vehicle or Celastrol (150 μg/kg) was administered at 9:00 h once a day for three days to 16-week-old lean and DIO mice. The vehicle and celastrol groups were further divided into two sub-groups, and the mice were intraperitoneally injected with either saline or leptin (150 μg/kg) at 10:00 h on the third day. Food intake was monitored for 6 h after the injection of saline or leptin. The mice were fasted overnight on the second day and then re-fed after the injection of saline or leptin on the third day. For western blot analysis, whole hypothalami were obtained from the mice 1 h after saline or leptin injection.

Western blot analysis

Whole hypothalami were homogenized and lysed in a solution containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate and 1 mM sodium orthovanadate; the lysates were analyzed for protein content before SDS-PAGE analysis. Membranes were immunoblotted with a phosphotyrosine antibody against STAT3 (Tyr705) (Cell Signaling Technology, Beverley, MA, USA) and then stripped and re-blotted with antibodies against STAT3 (Cell Signaling Technology). Amersham ECL Prime (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and ImageQuant LAS 4000 mini (GE Healthcare Life Sciences) were used for detection.

Preparation of single ARC neurons and $[Ca^{2+}]i$ measurement

The brains were removed from 16-week-old lean control mice fed a SD and DIO mice fed a HFD for 8 weeks. Brain slices containing the ARC were prepared and the entire ARC on the left and right sides was punched out. The dissected

tissues were incubated in HKRB (HEPES-buffered Krebs–Ringer bicarbonate buffer solution) supplemented with 20 units/ml papain, 0.015 mg/ml deoxyribonuclease, and 0.75 mg/ml BSA for 16 min at 36 ℃ in a shaking water bath followed by gentle mechanical trituration. The cell suspension was then centrifuged at 100 × g for 5 min. The pellet was resuspended in HKRB and distributed onto coverslips. The cells were kept at 30 ℃ in dishes with or without 3 nM celastrol till $[Ca^{2+}]i$ measurements for up to 3–6 h. Following incubation with 2 μM fura-2 AM (DOJINDO, Kumamoto, Japan) for 30 min at 30 ℃, the cells were mounted in a chamber and superfused at 1 ml/min with HKRB containing 5 mM glucose at 30 ℃. Data were obtained from single cells that were identified as neurons: relatively large diameter (≧10 μm), clear and round cell bodies on phase-contrast microscopy, and $[Ca^{2+}]i$ responses to KCl (55 mM). Leptin (10–100 nM) was administered under superfusion conditions. Fluorescence ratio images were obtained using Aquacosmos ver. 2.5 (Hamamatsu Photonics). When $[Ca^{2+}]i$ changed within 5 min after the addition of the agents and their amplitudes were at least twice as large as the baseline fluctuations, they were considered responses. In all experiments, neurons from at least three separate preparations were analyzed.

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics software (IBM, Chicago, IL, USA). Comparisons between or among groups were assessed using the Student's *t* test or two-way repeated-measures measures ANOVA. $P < 0.05$ was considered statistically significant. Data are expressed as mean ± SEM.

Results

The effect of celastrol treatment on body weight and food intake in mice under SD or HFD feeding

We investigated the effects of celastrol on body weight and food intake in mice fed a SD or HFD. Celastrol was administered to the mice once daily from 8 to 16 weeks of age. Celastrol treatment significantly inhibited body weight gain even under SD conditions, and effectively suppressed body weight gain under HFD conditions (Figure 1A and 1C). The body weight of mice fed HFD with celastrol treatment was almost the same as that of mice fed SD with vehicle treatment throughout the experiment. Consistent with these results, celastrol treatment decreased food intake in both diet groups of mice fed a SD and HFD (Figure 1B and 1D). Especially in mice fed a HFD, food intake was decreased by celastrol treatment to the degree of mice fed a SD with vehicle treatment.

Figure 1

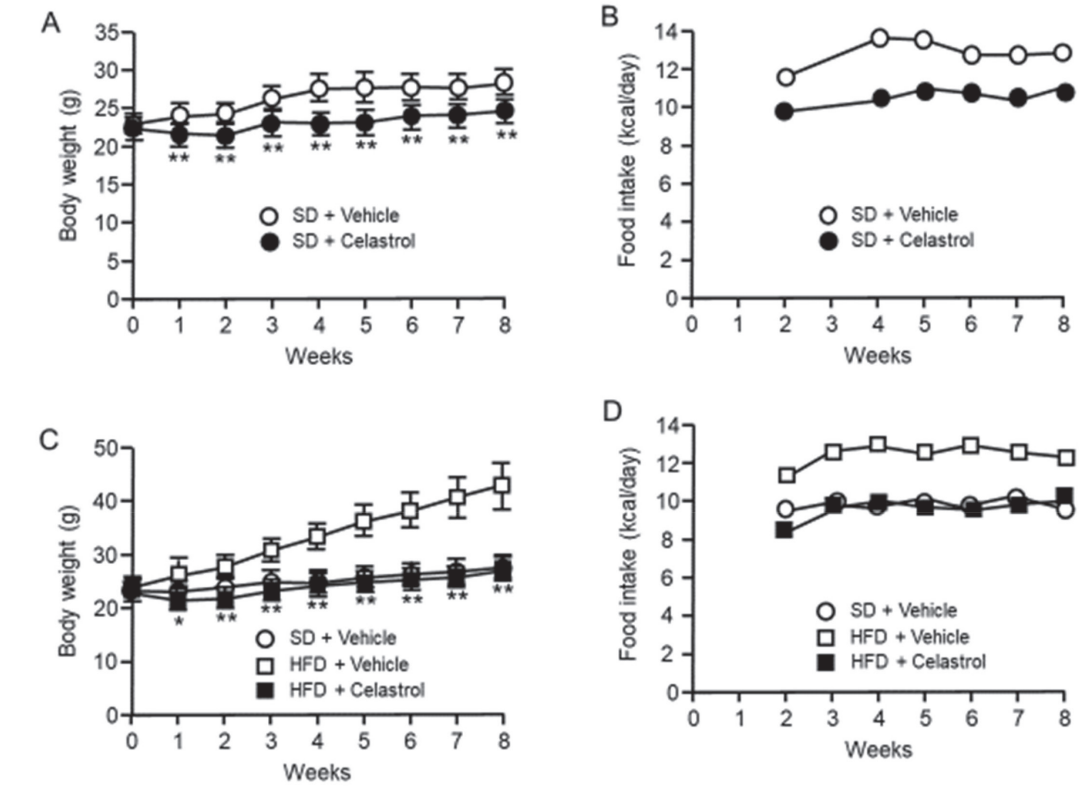

Figure 1. Effect of celastrol on body weight and food intake in mice under SD (A, B) and HFD (C, D). (A, C) Body weight change in mice treated with vehicle or celastrol under SD or HFD. $*P < 0.05$, $**P < 0.01$ *vs* vehicle treatment (ANOVA). Values are means ± SEM (n = 5–9 per group). (B, D) Food intake in mice treated with vehicle or celastrol under SD or HFD.

The effect of celastrol treatment on leptin actions in lean and DIO mice

To examine the acute effect of celastrol treatment on leptin activity in lean and DIO mice, the mice were administered vehicle or celastrol once daily for three days, followed by an intraperitoneal injection of leptin. Celastrol treatment for three-day did not significantly change the body weight or blood glucose concentrations in either lean or DIO mice (data not shown). In lean mice, leptin injection significantly decreased food intake in both the vehicle and celastrol groups, and the reduction rate in the celastrol group was greater than that in the vehicle group (40% vs. 30%) (Figure 2A). Furthermore, the leptin injection effectively increased the phosphorylation of hypothalamic STAT3, and the rate of increase in the celastrol group was greater than that in the vehicle group (254% vs. 22%) (Figure 2B and 2C). In the DIO mice, leptin injection did not significantly decrease food intake in vehicle group, indicating leptin resistance in DIO mice. In contrast, the leptin injection significantly decreased food intake by 44% in the celastrol group (Figure 2D). Furthermore, leptin injection significantly increased the phosphorylation of hypothalamic STAT3 in the celastrol group by 166% but not in the vehicle group (Figure 2E and 2F). These results demonstrate that celastrol augmented leptin activity in both lean and DIO mice.

The effect of celastrol on [Ca^{2+}]i response to leptin in single ARC neurons from lean and DIO mice

To examine the direct effect of celastrol on hypothalamic ARC neurons, [Ca^{2+}]i in single ARC neurons from lean and DIO mice was measured using fura-2 microfluorometry. Figure 3A and 3D show representative records of the [Ca^{2+}] i response to the sequential addition of leptin at 10 nM and 100 nM in ARC neurons from lean and DIO mice, respectively. In each figure, the top panel shows the result with vehicle treatment, and the bottom panel shows the result with 3 nM celastrol treatment that was administered 3h prior to [Ca^{2+}]i measurements and was present in the superfusion through the end of the experiments. In experiments with ARC neurons from both lean and DIO mice, the incidence of ARC neurons with a $[Ca^{2+}]_i$ response to leptin increased in a concentration-dependent manner. Celastrol treatment increased the incidence of ARC neurons with a $[Ca^{2+}]_i$ response to both 10 and 100 nM leptin. Among 24 single ARC neurons exhibiting [Ca2+] increase evoked by KCl, 5 neurons (21% of cells) responded to 10 nM leptin and 9 neurons (38% of cells) responded to 100 nM leptin in the vehicle-treated lean mice. Among 27 single ARC neurons exhibiting [Ca2+] increase evoked by KCl, 8 neurons (30% of cells) responded to 10 nM leptin and 11 neurons (41% of cells) responded to 100 nM leptin in the celastrol-treated

Figure 2

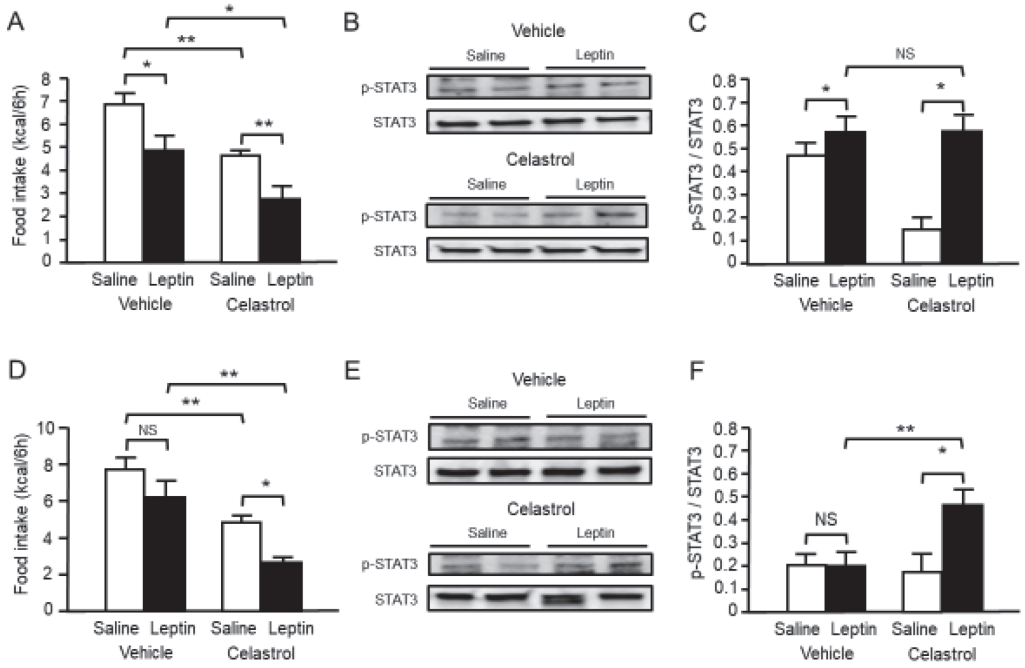

Figure 2. Effect of celastrol on leptin action in lean (A, B, C) and DIO mice (D, E, F). (A, D) 6-h food intake after saline or leptin injection in mice treated with vehicle or celastrol. (B, E) Western blot analyses for phospho-STAT3 and total STAT3 protein levels 1 hour after saline or leptin injection in the hypothalamus from mice treated with vehicle or celastrol. (C, F) Ratio of signal intensities of phospho-STAT3 to total STAT3 in B and E. NS, not significant, $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). Values are means ± SEM (n = 3–4 per group).

lean mice. Among 28 single ARC neurons exhibiting [Ca2+] increase evoked by KCl, 3 neurons (11% of cells) responded to 10 nM leptin and 8 neurons (29% of cells) responded to 100 nM leptin in the vehicle-treated DIO mice. Among 28 single ARC neurons exhibiting [Ca2+] increase evoked by KCl, 6 neurons (21% of cells) responded to 10 nM leptin and 11 neurons (39% of cells) responded to 100 nM leptin in the celastrol-treated DIO mice (Figure 3B and 3E). Celastrol treatment also significantly increased the amplitude of the $[Ca^{2+}]i$ responses to 10 nM leptin in ARC neurons from both lean and DIO mice, although the increase in amplitude with 100 nM leptin did not reach statistical significance (Figure 3C and 3F). These results clearly demonstrate that celastrol acts directly on ARC neurons to increase leptin sensitivity.

Discussion

The present study clearly demonstrates the direct effect of celastrol on hypothalamic neurons using single ARC neurons and the Fura 2 calcium imaging technique. Although celastrol has been demonstrated to improve leptin sensitivity in the hypothalamus following systemic administration, the site of action remains unclear. Leptin regulates food intake and energy expenditure, mainly through the hypothalamus [1–3]. Although the hypothalamus is the center controlling energy homeostasis, it is an aggregate of diverse neuronal nuclei. Even for those related to the leptin system, there are many nuclei, including the ARC, ventromedial hypothalamus (VMH), lateral hypothalamus (LH), and paraventricular nucleus (PVN)[18]. In addition to the uncertainty of the target nucleus, the target cell type is also unclear because the central nervous system consists of both neural and glial cells, and both cell types are involved in the development of ER stress and leptin resistance in the hypothalamus [17]. Celastrol may also affect leptin sensitivity in the hypothalamus through peripheral tissues, such as adipose tissues, which are known to excrete various signaling molecules [19]. Celastrol activates heat shock factor1 (HSF1) - peroxisome proliferators-activated receptor-γ co-activator-1α (PGC1α) transcriptional axis and increases energy expenditure in white and brown adipose tissues and skeletal muscles [20]. However, the present study indicates that ARC neurons are at least one of the direct sites of action of celastrol.

In the first report of celastrol as a leptin sensitizer, celastrol administration did not significantly reduce body weight in lean mice, but potently reduced body weight in DIO mice [11]. In this study, we observed a mild but significant effect of celastrol administration on the body

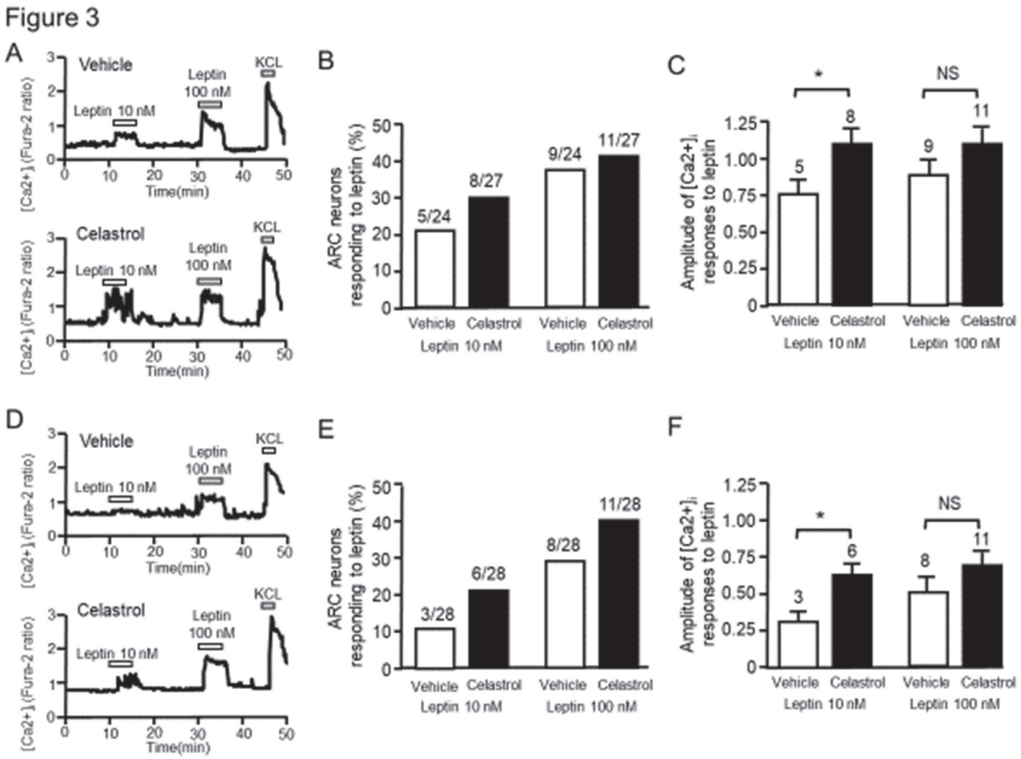

Figure 3. Effect of celastrol on $[Ca^{2+}]_i$ response to leptin in single ARC neurons from lean (A, B, C) and DIO mice (D, E, F). (A, D) Sequential addition of leptin at 10 nM and 100 nM increased $[Ca^{2+}]_i$ in an ARC neuron treated with vehicle (top) or 3 nM celastrol (bottom) for 3 hours. (B, E) Incidence of ARC neurons with $[Ca^{2+}]_i$ response to 10 nM and 100 nM leptin with or without celastrol treatment. (C, F) Amplitude of leptin-induced $[Ca^{2+}]_i$ increases in ARC neurons with or without celastrol treatment. The numbers on each bar indicate the numbers of neurons that responded to leptin. NS, not significant, $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). Values are means ± SEM (n = 3-11).

weight of lean mice. Since the body weight of the lean mice used in this study was relatively higher than that in a previous study [11], the degree of celastrol's effect may be dependent on endogenous leptin levels. Consistent with this expectation, we observed that the effect of celastrol in lean mice was comparable to that in DIO mice, not only on the inhibitory action on food intake, but also on hypothalamic STAT3 phosphorylation by exogenous leptin injection. Furthermore, we observed a substantial effect of celastrol on single ARC neurons in both lean and DIO mice. These results indicated that celastrol exerts its leptin-sensitizing effect on ARC neurons, irrespective of HFD loading.

Although celastrol acts directly on ARC neurons for leptin sensitization, the molecule-based mechanism underlying the leptin-sensitizing effect of celastrol remains unclear. Recently, it was reported that IL1R1 is required for the leptin sensitization and anti-obesity effects [16]. However, the precise role of ILIR1 in celastrol-induced leptin sensitization remains unknown. In an experiment using the Fura 2 calcium imaging technique, whether the leptin-sensitizing effect of celastrol disappears with the use of single ARC neurons from IL1R1 deficient mice is an intriguing future issue. Celastrol administration reduced food intake and body weight in melanocortin 4 receptor (MC4R)-null mice compared with DIO mice, indicating that the effect of celastrol is independent of MC4R [21]. However, the downstream leptin receptors involved in the leptin-sensitizing effect of celastrol remain unknown. Analysis of the difference between ARC neurons with and without a leptin response enhanced by celastrol may lead to an understanding of the target molecule of celastrol.

Although the expression of the leptin receptor is the most abundant in the ARC, other hypothalamic nuclei such as the LH and PVN also express leptin receptors and play roles in leptin action [22]. However, the direct effects of celastrol on these nuclei remain unclear. In the present study, we used only leptin as a stimulator of ARC neurons. The effect of celastrol on neural responses to other anorexigenic or orexigenic peptides will also be investigated in the future. The involvement of ER stress in the direct effects of celastrol observed in this study is another issue that needs to be addressed.

In conclusion, the present study clearly demonstrates that celastrol acts directly on ARC neurons in the mouse hypothalamus and enhances sensitivity to leptin. Celastrol exerted this effect irrespective of HFD loading. These findings may shed light on the molecular mechanism underlying the leptin-sensitizing effect of celastrol and, therefore, leptin resistance.

Declaration of interest: The authors have no conflicts of interest to declare.

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セラストロールは視床下部弓状核ニューロンに直接作用してレプチン感受性を高める

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要　約

レプチンは体重調節に重要な役割を有している。しかし肥満者ではレプチン抵抗性の状態にあり，肥満治療においてはレプチン抵抗性の改善が重要である。レプチン抵抗性改善薬として天然化合物のセラストロールが見出されたが，その作用経路及び作用メカニズムは不明である。今回，セラストロールの個体レベルでのレプチン感受性改善作用と共に，視床下部弓状核ニューロンへの直接作用を検討した。セラストロールは非肥満及び肥満マウスのいずれにおいても，体重増加と摂食量を有意に抑制し，レプチンによる視床下部STAT3のリン酸化作用の増強を認め，単離視床下部弓状核ニューロンのレプチンに反応するニューロンの数の増加及びニューロンの細胞内カルシウム濃度の有意な増加を認めた。これらの結果から，セラストロールは視床下部弓状核ニューロンへ直接作用し，ニューロンが由来するマウスの肥満度に関わらずレプチンに対する感受性を高める事が明らかとなった。
（キーワード：弓状核ニューロン，セラストロール，視床下部，レプチン，肥満）

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