Central Stimulating Effects of Cerulenin on CPT-1 Activity Through Sympathetic Nervous System Activation Surpass Its Peripheral Inhibitory Effects

Thesis by

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Central Stimulating Effects of Cerulenin on CPT-1 Activity Through Sympathetic Nervous System Activation Surpass Its Peripheral Inhibitory Effects

Directed by Professor **Sung-Kil Lim**

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This certifies that the Doctoral Dissertation
of Yong-Jun Jin is approved.

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우선 이 논문시작부터 완성하기까지 지도와 격려를 아끼지 않으신 지도교수 임승길 교수님께 진심으로 감사를 드립니다. 아울러 심사위원으로 수고해 주신 백자현 교수님, 차봉수 교수님, 김동구 교수님과 김덕희 교수님께 감사의 말씀을 드립니다. 처음부터 실험을 가르쳐 주시면서 많은 조언을 아끼지 않고 관심해 주신 선배님 이송철 선생님께 깊은 감사를 전합니다. 타향살이에 자신도 바쁘면서도 항상 힘들고 지칠 때마다 큰 힘이 되여 주신 선배님이자 듬직한 큰 형님 조정산 선생님께 진심으로 감사의 말씀을 드립니다. 10년간 의리의 친구로 변함없었던 소중한 친구 심웅호와 후배 안연희에게도 감사를 전합니다.

한 실험실에서 공부하고 서로 의지하면서 슬픔과 기쁨을 함께 나눈 황난주 선생님과 이은진 선생님, 비록 시간은 짧았지만 따뜻한 웃음을 함께 나눈 정현주, 전수정 선생님, 그리고 박기청, 박민정 선생님께도 감사를 드립니다. 실험실 생활에 많은 관심을 가져주시고 항상 밝은 미소의 이유미, 김세화, 이시훈 선생님, 그리고 함께 실험을 하면서 많은 것을 함께 느끼고 생각했던 김유미 선생님께도 감사를 드립니다.

평생의 반려이자 마음속의 든든한 기둥인 사랑하는 내 아내 양미자, 힘들 때마다 짜증을 내서 미안하고, 또 말없이 받아줘서 무엇보다 감사합니다. 태여나서 함께 한 시간은 짧지만 언제나 보고만 싶었던 귀여운 아들 성흠; 항상 이 동생을 아끼고 관심해 주신 보고 싶은 형님과 형수님; 그리고 말없이 든든한 뒷심으로 걱정없이 공부하게끔 사랑으로 지켜봐 주신 부모님과 장인, 장모님께 특별한 감사를 드리면서 이 논문을 마칩니다.

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김 용 군 씀

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ABSTRACT

Central Stimulating Effects of Cerulenin on CPT-1 Activity Through Sympathetic Nervous System Activation Surpass Its Peripheral Inhibitory Effects

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(Directed by Professor **Sung-Kil Lim**)

To clarify the paradoxical effects of cerulenin, the so-called selective reduction in adipose mass despite a significant increase in the cellular levels of malonyl-CoA, we studied the *in vivo* and *in vitro* effects of cerulenin on carnitine palmitoyltransferase-1 (CPT-1) activity. A single intraperitoneal injection of cerulenin significantly reduced body weight and increased the core temperature without a significant reduction in food intake. Cerulenin's effect on CPT-1 activity was biphasic in the liver and muscle. *In vitro* treatment of cerulenin reduced CPT-1 activity, which was overcome by co-treatment with catecholamine. In contrast, an intracerebroventricular injection of cerulenin increased CPT-1 activity significantly in soleus muscle. Pretreatment with α-methyl-p-tyrosine inhibited the cerulenin-induced increase in core temperature, as well as the late phase stimulating effect of cerulenin on CPT-1 activity. This activity was significantly reduced in adrena-lectomized mice; however, it was reversed by cerulenin. An in situ hybridization study reconfirmed that a single injection of cerulenin did not affect the expression

of orexigenic neuropeptide mRNA in hypothalamus. *In vivo* treatment with cerulenin enhanced the CPT-1 activity of the muscle not in the gold thioglucose-treated (ventromedial hypothalamus lesion) mice but in the monosodium glutamate-treated (arcuate nucleus lesion) mice. All these findings suggest that cerulenin induced the late phase stimulating effects on CPT-1 activity, and energy expenditure was mediated by the activation of innervated sympathetic nervous system neurons through firing of the undefined neurons of the ventromedial hypothalamus, rather than the arcuate nucleus.

Key Words: cerulenin, obesity, hypothalamus, sympathetic nervous system, carnitine palmitoyltransferase-1, fatty acid oxidation

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Ⅰ. INTRODUCTION

Excess body weight is a major health problem in developed countries, associated with increased risk of type 2 diabetes, cardiovascular and cerebrovascular diseases, and increased mortality. The magnitude of this health problem and the limitations of successful weight-reduction therapy emphasize the need for different approaches to control body weight. Cerulenin, a new class of drug which inhibits fatty acid synthase (FAS), robustly and reversibly reduced body weight[1], and it brings hope that this family will be useful in obesity management.

Several trials on analyzing the weight reducing mechanisms of FAS inhibitors have taken place. A synthesized FAS inhibitor, C75 acts both centrally to reduce food intake and peripherally to increase fatty acid oxidation, leading to rapid and

profound weight loss, loss of adipose mass[2]. In contrast to C75, a natural FAS inhibitor, cerulenin induced the preferential loss of adipose tissue, which was not highly correlated with food intake. Treatment with cerulenin neither reduced the food intake nor attenuated the fasting-induced elevation of hypothalamic agouti-related peptide (AGRP) and neuropeptide Y (NPY) mRNA or the reduction of hypothalamic pro-opiomelanocortin (POMC) and cocaine-and amphetamine-related peptide (CART) mRNA in wild-type mice. Furthermore, cerulenin, intriguingly stimulated heat production and metabolic activity in wild-type *ad libitum* fed mice[3]. Therefore, the increased metabolic rate, rather than the reduction in food intake has been considered to contribute to a major role for weight loss, especially in the fat mass reduction induced by cerulenin.

Malonyl-CoA, in addition to its role as a substrate for FAS, is pivotal to energy regulation through its reversible inhibition of carnitine palmitoyltransferase-1(CPT-1)[4]. During situations of excess energy, the increased malonyl-CoA that is generated for fatty acid synthesis inhibits CPT-1 activity, preventing the oxidation of newly formed fatty acids bound for energy storage. However, during starvation, malonyl-CoA levels fall to permit the oxidation of fatty acids for energy. Cerulenin, known as a FAS inhibitor at the cellular level, directly increases malonyl-CoA to inhibit CPT-1, the rate-limiting enzyme of fatty acid oxidation[5]. Interestingly, in contrast to the in vitro inhibitory effects of cerulenin on fat catabolism, in vivo treatment of cerulenin causes the opposite phenomenon, a selective reduction of fat mass. This phenomenon is so called as the paradoxical effect of cerulenin.

Recently, it has been reported that FAS is also expressed in the hypothalamus: arcuate nucleus (ARC), ventromedial hypothalamus (VMH), and paraventricular nucleus (PVN)[6]. Considerable evidence has accumulated that raise the possibility of malonyl-CoA being one of the prime agents in the sensing of fuel supply in the brain and peripheral organs[7]. Meanwhile, the sympathetic nervous system (SNS) has been shown to play an important role in the regulation of both sides of the energy balance equation, but especially in the regulation of the expenditure side. Studies in which the endogenous catecholamines, norepinephrine[8,9] and epinephrine[10-12] are

infused showed significant increases in energy expenditure, lipid oxidation, and lipolysis. Furthermore, the reduction in sensitivity to certain levels of SNS activity and to lipid oxidation has also been shown in obesity[13-15].

How could the paradoxical effects of cerulenin be explained, where a significant increase in the cellular levels of malonyl-CoA caused an opposite phenomenon, lipid oxidation and a selective reduction of fat mass? In this study, we hypothesized that the modest and delayed central effects of cerulenin through SNS activation stimulated CPT-1 activity and fatty oxidation of the peripheral tissues. To prove our hypothesis, we endeavored to clarify the catabolic effects of cerulenin on adipose mass by studying ① the effects of a single intraperitoneal (I.P.) injection of cereulenin on food intake, body weight change and core temperature, ② *in vitro* and *in vivo* effects of cerulenin on CPT-1 activity, ③ the role of activation of SNS for cerulenin induced reversible fat catabolism, eg fatty oxidation, ④ the central pathway for cerulenin induced activation of SNS.

Ⅱ. Materials and Methods

1. Animal

Six-week-old C57BL/6J male mice were obtained from the Jackson Laboratory (Deahan Biolink Co., Korea). Mice were fed post-weaning through the experimental procedures. Mice were maintained in a 12-h light-dark cycle at 23C. After a 1-week acclimatization, animals were administered a single intraperitoneal (I.P.) injection at 60 mg/kg body weight. Food intake and body weight change were measured 24h after the cerulenin treatment. In the fasted mice, core temperature was measured with digital thermometer (Toshiba, Japan).

2. Drugs

Cerulenin, α-methyl-p-tyrosine (AMPT), monosodium glutamate (MSG) and gold thioglucose (GTG) were purchased from Sigma. L-[*methyl*-^{14}C]-carnitine was purchased from Amersham Pharmacia Biotech. Cerulenin was dissolved in the RPMI medium 1640 and injected I.P. at doses of 60 mg/kg body weight and intracerebroventrically (ICV) injected at doses of 10ug. AMPT was dissolved in saline and was injected I.P. at doses of 300 mg/kg body weight.

3. Adrenalectomy

Operations were carried out under ketamine (100 mg/kg; Korea United Pharm, Korea) and xylazine (10 mg/kg; Bayer Korea Ltd., Korea) anesthesia. Adrenalectomy was performed by bilateral flank incision. Control animals underwent sham operations, in which the adrenal glands were grasped but not removed. Adrenalectomized (ADX) mice were supplemented with 0.9% saline; sham mice received tap water *ad lib*. CPT-1 activity assay was carried out 7-9 days after the adrenalectomy operation.

4. Development of the ARC and VMH lesion mice

For development of the ARC lesion, pregnant C57BL/6J mice were housed singly prior to delivery. From day 1 to 7, the offsprings were injected subcutaneously with either MSG (2 mg/g body weight) or 0.9% NaCI daily. They were weaned at 21 days of age, and then housed in groups of the same sex. All animals were allowed free access to water and the standard rodent chow. Male-control and male-MSG treated mice were studied at 8 weeks of age. For development of the VMH lesion model, 4-week-old C57BL/6J male mice were single I.P. injected with GTG (0.5 mg/g body weight) or 0.9% NaCI. Animals were studied 8 weeks after injection. When the mice were killed, their brains were removed and frozen with liquid nitrogen. The brains were later sectioned through the hypothalamus on a cryostat and the brain sections were fixed with paraformaldehyde. The sections were stained with Cresyl violet and the stained sections were examined under a microscope to confirm the presence or absence of lesions in the ARC and VMH.

5. Measurement of CPT-1 activity *in vitro*

CPT-1 was measured using digitonin-treated permeabilized cells[16]. Primary hepatocytes 1×10^6 cells were plated in DMEM with 10% FBS in six-well plates in triplicate. After overnight incubation, the drugs and vehicle controls were added as indicated. After 2h, the medium was removed, and the cells were washed with PBS and then incubated with 700l of assay medium con- sisting of 50 mM imidazole, 70 mM KCl, 80 mM sucrose, 1 mM EGTA, 2 mM $MgCl_2$, 1mM DTT, 1mM KCN, 1mM ATP, 0.1% fatty acid-free BSA, 70 µM palmitoyl-CoA, 0.25 µCi of L-[*methyl*-^{14}C]-carnitine, and 40 µg of digi- tonin. Cerulenin was added as specified for each experiment. After incubation for 6 min at 37℃, the reaction was stopped by the addition of 500 µl of ice-cold 4 M perchloric acid. Cells were then harvested and centrifuged at 13,000×g for 5min. The pellet was washed with 500 µl of ice-cold 2 mM perchloric acid and centrifuged again. The resulting pellet was resuspended in 800 µl of deionized H_2O and extracted with 400l of butanol. The ^{14}C in the butanol phase was quantified and represents the acylcarnitine derivative.

6. Measurement of CPT-1 activity in the liver and muscle

Animals were killed by decapitation and the liver and soleus muscle were homogenized in 0.25 M-sucrose medium containing 10 mM Tris-HCI (pH 7.4) and 1 mM EDTA, and 0.15 M-KCI medium containing 10 mM Tris-HCI (pH 7.4) and 1 mM EDTA, respectively. Mitochondria were isolated according to Saggerson and Carpenter[17,18] and finally resuspended in 0.3 M sucrose buffer. Mitochondrial protein was determined following the method of Lowry *et al.*[19]. Enzyme activity was determined within 15 minutes of the mitochondria isolation by measuring the incorporation of L-[*methyl*-^{14}C]-carnitine into the *n*-butanol soluble product[20]. Mitochondria (150 to 250 micrograms of protein, depending on the tissue) were preincubated at 25℃ for 4 min in 1.0 ml containing 150 mM sucrose, 60 mM KCl, 25mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 40 μM palmitoyl-CoA and 1.3 mg/ml albumin (fatty acid free). Reactions were started by the addition of 25 μl containing 1 μCi (0.4 μmol) of L-[*metyhl*-^{14}C]-carnitine, then carried out for up to 4 min and stopped with 1.0 ml ice-cold 1N HCl. Blank values were determined by replacing the mitochondria with an equal volume of resuspension buffer.

7. In situ hybridization

The mouse brain cryostat sections (10 μm) were prepared and hybridized with the specific antisense riboprobes. The antisense and sense RNA probes were labeled with [^{35}S]CTP (>800 Ci/mmol; Amersham) by the standard conditions using T3, T7 or SP6 polymerased (Promega). *In situ* hybridization was performed as previously described[21]. The dried sections were first exposed to Kodak XAR-OMAT film for autoradiography and subsequently dipped in a Kodak NTB2 nuclear emulsion. The slides were exposed at 4℃ for 24 h or 1 week, developed in Kodak D19, fixed with Kodak Unifix and counterstained with Toluidine Blue. The specificity of the *in situ* hybridization results was confirmed by sense strand riboprobes, which showed no detectable signals. The dark-field images were processed using an Imaging Technology image digitalizer to quantify the in situ hybidizations. The digitalized images were then analyzed on a MCID image-analysis system (Imaging Research

Inc., Canada). The autoradiograms were analyzed in parallel by a digital scanning densitometer (Luminescent image analyzer, LAS-1000 CH, Fuji, Japan), operating using the image acquisition and analysis program, TINA on BAS 2500 (Fuji, Japan).

8. Statistical analysis

All values are reported as mean ± SEM. Statistical significance was determined by Student's *t* test and one-way analysis of variance (ANOVA) followed by Tukey post hoc test, using GraphPad Software.

Ⅲ. RESULTS

1. Effects of cerulenin on food intake, body weight change and core temperature

To investigate the physiological consequences of the *in vivo* inhibition of fatty acid synthesis on the global fat metabolism, we administered cerulenin [60 mg/kg body weight] to mice by a single I.P. injection. 24h after injec- tion, the food intake and body weight were measured. As previously described, cerulenin produced a significant decrease in body weight; the effect on food intake was reduced but did not quite reach a significant level (Fig. 1A and B). Fasted mice treated with cerulenin lost 15% more weight than did the fasted control mice (Fig. 1C). This result suggested that cerulenin treatment might allow the maintenance of normal energy utilization or the enhancement of energy expenditure even in a fasting state.

To investigate cerulenin's effects on energy expenditure, we measured the core temperature in the fasted mice following cerulenin or RPMI treatment. The core temperature of the control mice given RPMI was found to drop dramatically during the 24-h period after food deprivation (Fig. 1D). In contrast, a single administration of cerulenin significantly prevented this reduction in the core temperature at 3h and 5h after treatment. Tyrosine hydroxylase (TH) was the rate-limiting enzyme of catecholamine synthesis. To test whether the effects of cerulenin on the core temperature of the fasted mice were mediated through the activation of SNS, mice were pretreated with AMPT, an inhibitor of TH. As presented in figure 1D, the cerulenin-induced elevation of the core temperature was blocked in the AMPT pretreated mice. These data suggested that the cerulenin-induced elevation of core temperature could be mediated through the activation of the SNS, which contributed to weight loss.

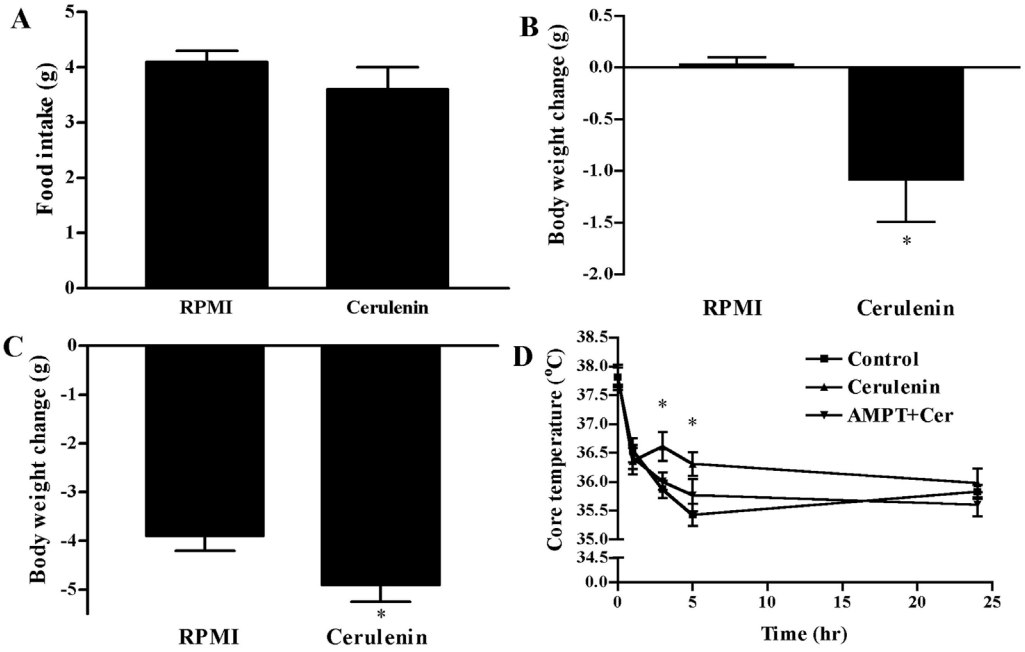

Fig. 1. Effects of vehicle(RPMI) and cerulenin on food intake, body weight change and core temperature. Cerulenin was single I.P. injected at doses 60 mg/kg body weight. After 24h, the food intake (**A**) and body weight change were measured in fed (**B**) and fasted (**C**) mice. The core temperature was measured during the 24h after cerulenin treatment in fasted mice (**D**). *, $P<0.05$ vs. control.

2. Effects of cerulenin on the expression of hypothalamic orexigenic neuropeptides mRNA in mice

To investigate whether cerulenin alters the level of neuropeptides known to regulate food intake, the mRNA expression of the AgRP, NPY and melanin-concentrating hormone (MCH) were assessed by *in situ* hybridyzation. *Ad libitum* fed mice were single I.P. injected with either RPMI or cerulenin and then killed 3 h after injection. A previous study reported that cerulenin treatment did not reduce hypothalamic AGRP and NPY mRNA and did not elevate POMC mRNA when compared to the control group[3]. In the present study, cerulenin treatment did not reduce hypothalamic orexigenic neuropeptide, AgRP, NPY and MCH mRNA (Fig.2).

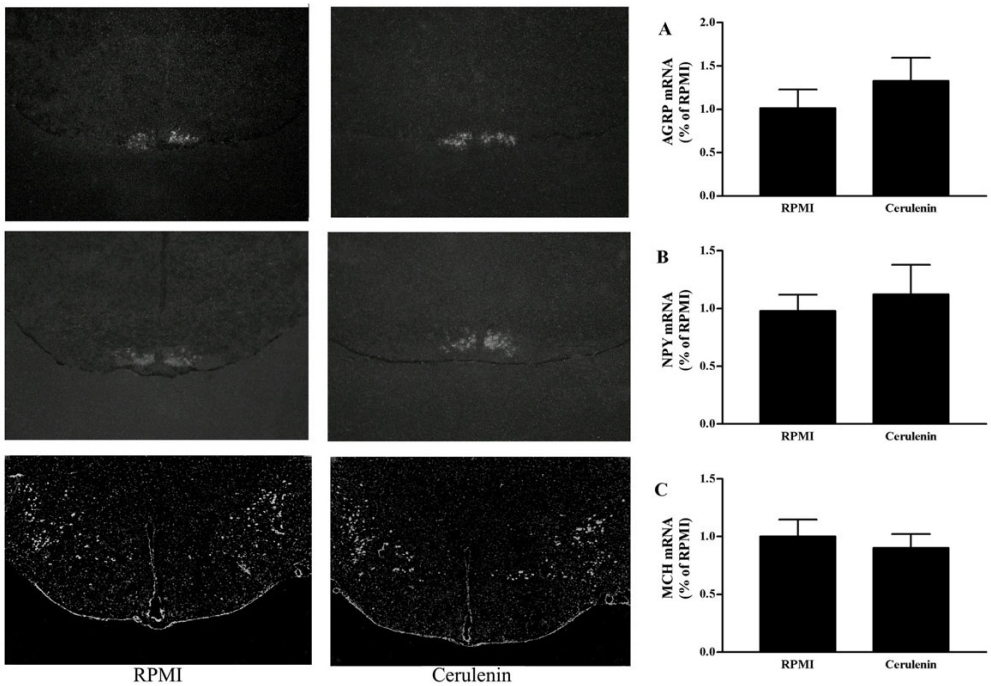

Fig. 2. Effects of cerulenin on mRNA levels of neuropeptides, AgRP (**A**), NPY (**B**), and MCH (**C**). Mice were treated with either vehicle(RPMI) or cerulenin and killed 3h after the injection. Data are expressed as a percentage of mean RPMI-injected *ad libitum* fed levels ± SEM.

These results support that cerulenin's acute effects were not effective in reducing food intake.

3. Effects of cerulenin on the CPT-1 activity *in vivo* and *in vitro*

Prior study with cerulenin reported that cerulenin preferentially reduced fat mass[3]. To investigate cerulenin's effects on fat catabolism, e.g., fatty acid oxidation, we measured the activity of CPT-1, the rate-limiting enzyme of fatty acid β-oxidation, in the liver and soleus muscle. Mice were I.P. injected with cerulenin 60 mg/kg body weight, and the CPT-1 activities were measured at 0h, 0.5h, 1h, 3h and 5h after cerulenin treatment. In the liver, the CPT-1 activity was reduced significantly at 0.5h after treatment, but recovered 1h after treatment (Fig. 3A). More interestingly,

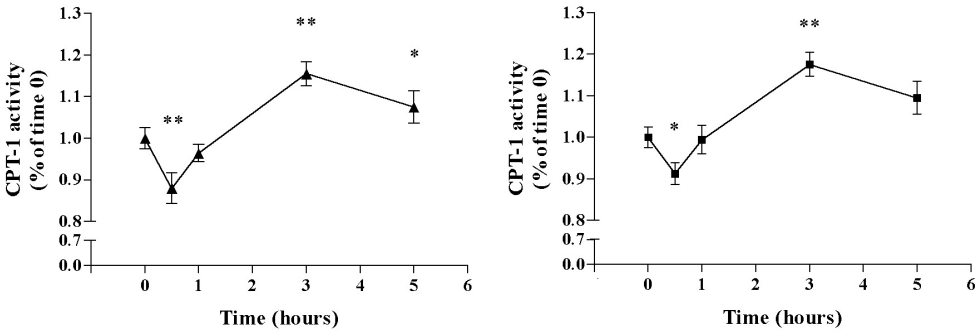

Fig. 3. Effects of cerulenin on CPT-1 activity in the liver and soleus muscle *in vivo*. Mice were treated with cerulenin at doses 60 mg/kg bw. 0h, 0.5h, 1h, 3h and 5h after the injection, CPT-1 activities were measured in the liver (**A**) and soleus muscle (**B**). *, $P<0.05$ and **, $P<0.01$ vs. 0h.

the CPT-1 activities were significantly elevated 3h and 5h after treatment (16% and 9% greater from 0h, respectively). The effect of cerulenin on muscular CPT-1 activity was also similar to the effects in the liver (Fig. 3B). Based on these results, we assessed CPT-1 activity 3h after cerulenin treatment in all the other experiments.

To determine how cerulenin initially reduced and then elevated the CPT-1 activity, we examined the direct effects of cerulenin on CPT-1 activity in the primarily incubated hepatocytes. Cerulenin treatment reduced the CPT-1 activity of primary hepatocytes dose-dependently (Fig. 4A). At 40ug/ml of dose, cerulenin reduced the CPT-1 activity of the hepatocytes by up to 50% of the control. Therefore, we speculated that the initial reduction in CPT-1 activity might have been mediated through the direct effects of the elevated malonyl-CoA induced by FAS inhibition, and that the delayed elevation of CPT-1 activity might have been mediated through another mechanism. The biphasic response of CPT-1 activity to cerulenin speculated us that this delayed effect might be the indirect central effects of cerulenin. To conform that mice were ICV administered with 10 μg of cerulenin, and CPT-1 activity of soleus muscles were measured at 0h, 0.5h, 1h, 3h and 5h after cerulenin treatment. The CPT-1 activity was significantly elevated at 1h and maintained at 3h

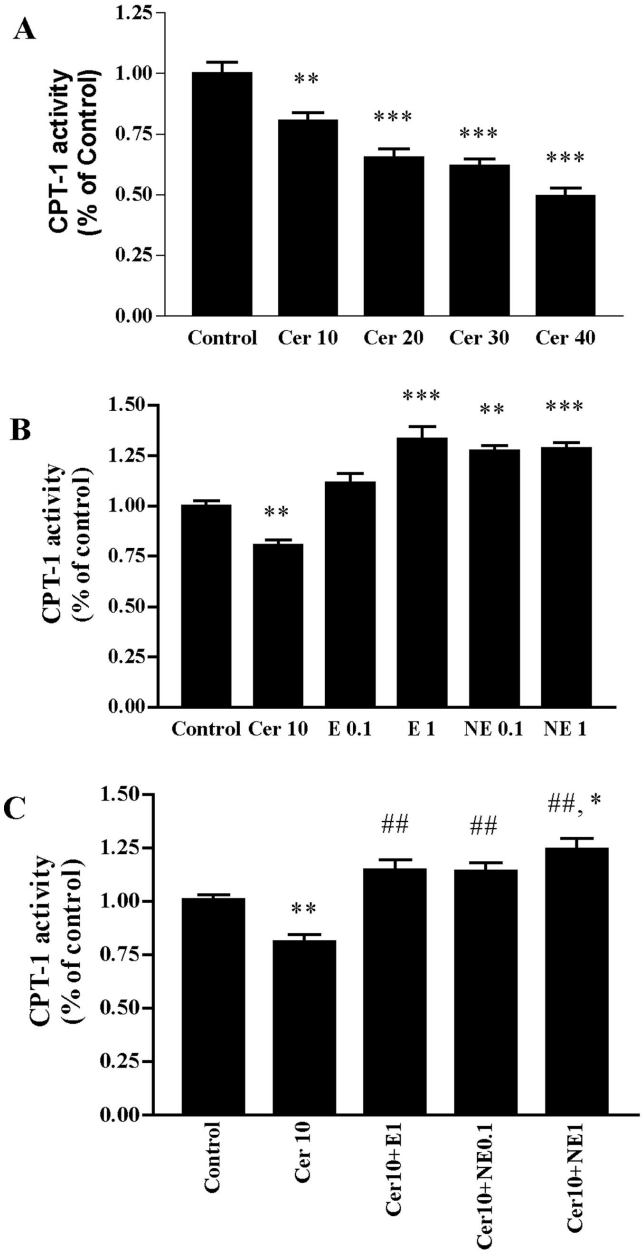

Fig. 4. Effects of cerulenin (Cer) and catecholamines (E and NE) on CPT-1 activity in primary cultured hepatocytes *in vitro*.

Primary cultured hepatocytes were treated with cerulenin at 10, 20, 30 and 40 μg/ml and the CPT-1 activities were measured (**A**). Hepatocytes were treated with epinephrine and norepinephrine at 0.1 and 1 μg/ml in the presence (**C**) and absence (**B**) of inhibitory concentration of cerulenin (10 μg/ml). *, $P<0.05$, **, $P<0.01$ and ***, $P<0.001$ vs. control. ##, $P<0.01$ vs. Cer 10.

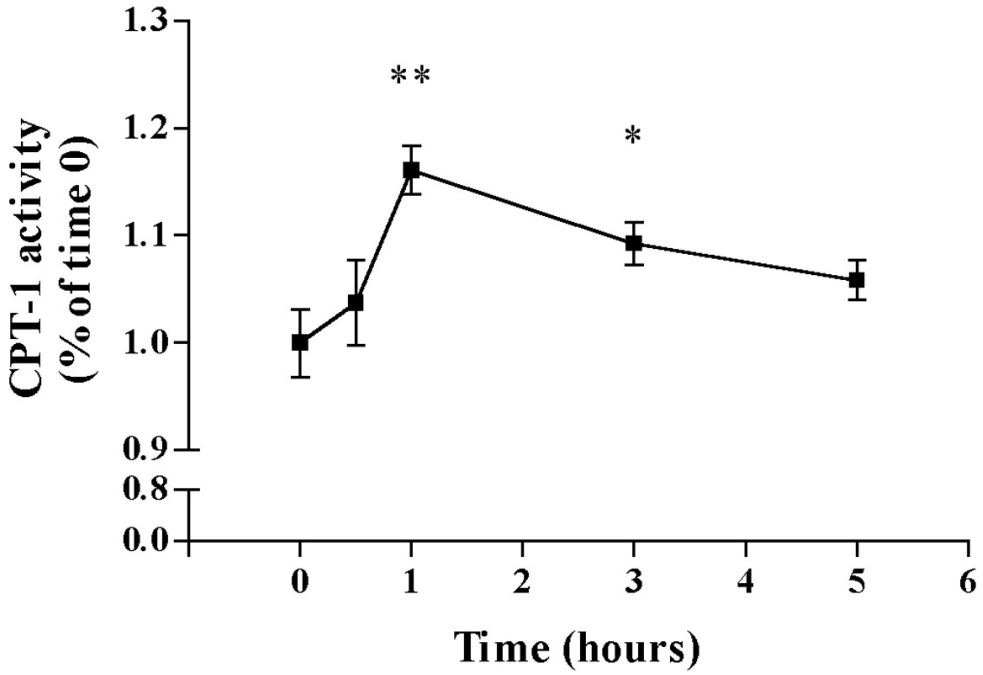

Fig. 5. Effects of ICV administered cerulenin on muscular CPT-1 activity. Mice were ICV administered with cerulenin at doses 10 μg. 0h, 0.5h, 1h, 3h and 5h after the injection, CPT-1 activities were measured in the soleus muscle. *, $P<0.05$ vs. 0h.

after cerulenin treatment (Fig. 5). These results support that the late stimulating effect of cerulenin on CPT-1 activity was through central nervous system.

To determine whether the delayed stimulating effects of cerulenin on CPT-1 activity might be mediated through the activation of the SNS, we applied adrenergic agonists, epinephrine and norepinephrine to the primary incubated hepatocytes. Epinephrine significantly enhanced the CPT-1 activity at a 1 μg/ ml dose and norepinephrine also significantly enhanced the CPT-1 activity at 0.1 and 1 μg/ml doses (Fig. 4B), whereas cerulenin inhibited the CPT-1 activity (80% of control at 10 μg/ml). When catecholamines were applied along with cerulenin to the incubation buffer, cerulenin's inhibitory effects on the CPT-1 activity were not observed, even at high concentrations, 1 μg/ml of norepinephrine, the CPT-1 activity was still higher than the control. (Fig. 4C). To examine whether the increased CPT-1 activity by

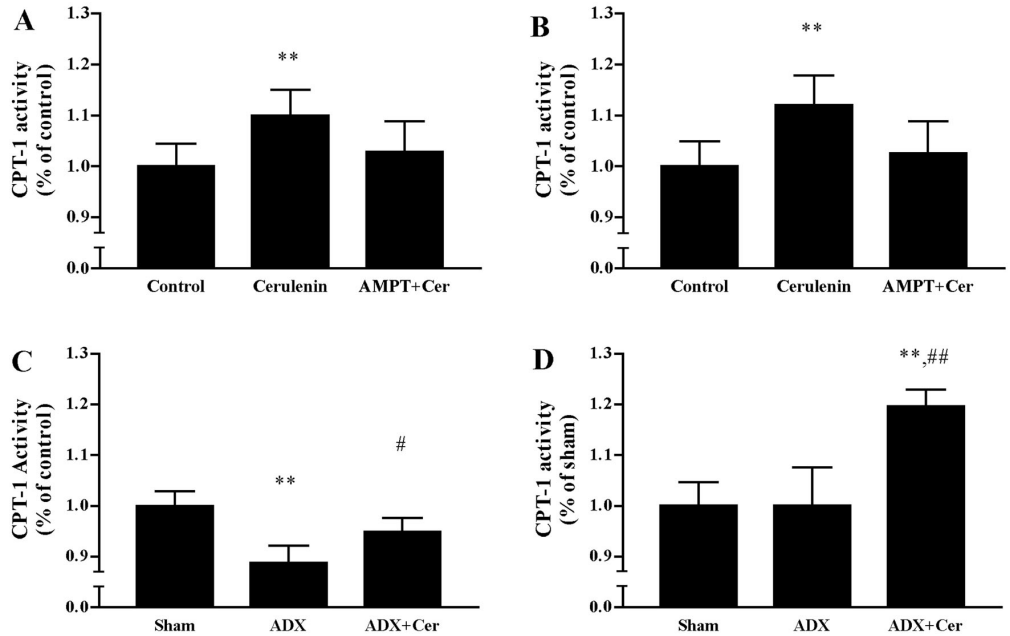

Fig. 6. Effects of cerulenin (Cer) on CPT-1 activity in AMPT pretreated and ADX mice. Cerulenin was administered in doses as described above in either AMPT pretreated or ADX mice. 3h after cerulenin treatment, the CPT-1 activity was measured in the liver (**A** and **C**) and soleus muscle (**B** and **D**). *, $P<0.05$ and **, $P<0.01$ vs. control, #, $P<0.05$ and ##, $P<0.01$ vs. ADX.

cerulenin was mediated through the activation of the SNS, we pretreated mice with AMPT prior to cerulenin treatment. Pretreatment with AMPT attenuated cerulenin's stimulating effects on CPT-1 activity in the liver and muscle (Fig. 6A and B). These results indicate that cerulenin, through the activation of the SNS, increases catecholamine levels and thus, enhances CPT-1 activity.

Catecholamines are known to be secreted from two main sources: the sympathetic nerves and the adrenal glands. To determine whether the adrenal production of catecholamines was involved in the regulation of the CPT-1 activity in cerulenin-treated mice, we analyzed mice in which the adrenal medulla, the main source of circulating epinephrine, was surgically removed. The CPT-1 activity of the liver was significantly reduced in ADX mice, whereas the CPT-1 activity of the

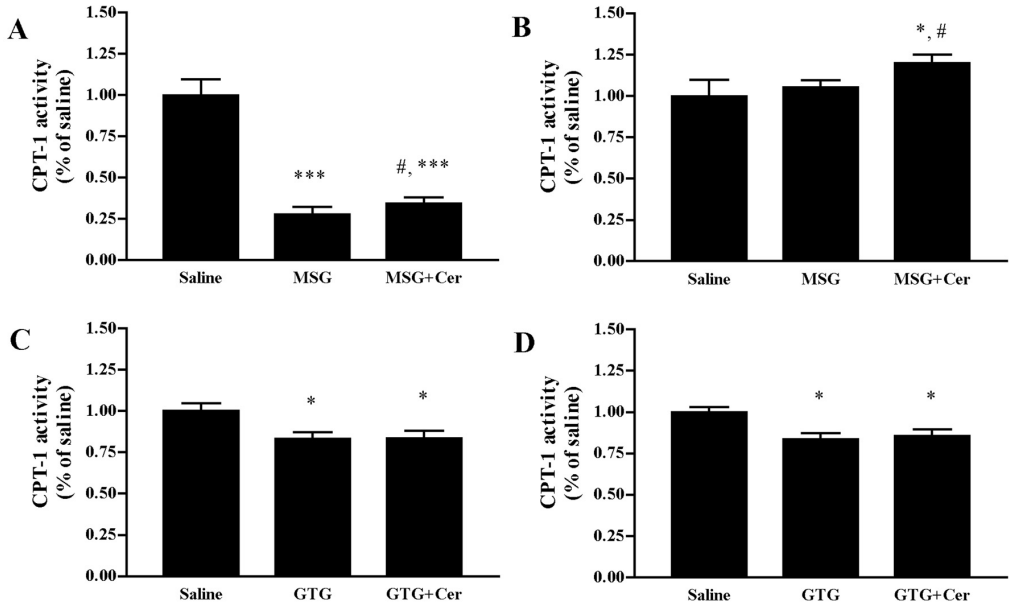

Fig. 7. Effects of cerulenin (Cer) on CPT-1 activities in MSG-treated and GTG-treated obese mice models. MSG-induced (**A**) and GTG-induced (**B**) obese mice were treated with cerulenin, and the liver (**A** and **C**) and soleus muscle's (**B** and **D**) CPT-1 activity was measured. *, $P<0.05$ and ***, $P< 0.001$ vs. saline, #, $P<0.05$ vs. MSG.

muscle was not significantly different between the ADX and sham operated mice (Fig. 6C and D). Treatment with cerulenin significantly stimulated the CPT-1 activity in the ADX mice, and the muscular CPT-1 activity reached similar levels to the non-ADX mice that were treated with cerulenin. These data suggest that the CPT-1 activity of the liver and muscle was more affected by the direct activation of innervated sympathetic nerves, rather than by adrenal catecholamines.

To further study the central pathways for the activation of the SNS mediated by cerulenin, we treated newborn pups with MSG, which predominantly damages the ARC nucleus structures[22,23]. Baseline CPT-1 activity of the muscle in 8-week-old MSG-treated mice was not significantly different from that of the saline-treated mice. I.P. injection of cerulenin in MSG-treated mice significantly elevated the muscular CPT-1 activity as in the control mice. In MSG-treated mice, the liver CPT-1 activity was even reduced to 25% of the control, and cerulenin treatment

also enhanced the activity (Fig. 7A and B). This indicates that MSG-sensitive neurons were not the ones firing for the activation of the SNS. Immunohistochemical studies showed that MSG treatment did not damage the VMH neurons and the other outside neurons of the hypothalamus (data not shown). To study the roles of VMH neurons in mediating the cerulenin-induced activation of the SNS, we treated 4-week-old mice with GTG, a compound that destroys glucose-sensitive neurons in the VMH[24]. Mice were treated with cerulenin and the liver and muscular CPT-1 activity was analyzed at 8 weeks after GTG treatment. GTG treatment significantly reduced CPT-1 activity, however, cerulenin failed to stimulate the CPT-1 activity (Fig. 7C and D). These data strongly suggested that VMH neurons were essential for the activation of the SNS and further stimulation of the CPT-1 activity after cerulenin treatment in the muscle.

Ⅳ. DISCUSSION

Recently, there has been considerable interest in the role of fatty acid oxidation in the development of obesity and insulin resistance[25-28]. Children with decreased fatty acid oxidation due to nutritional stunting developed obesity with a higher prevalence[27]. One aspect of insulin resistance has been explained by the abnormal accumulation of triglycerides in non-classic fat-storing tissues such as muscle and beta cells. Since fatty acid flux into the muscle was similar between the obese and lean subjects, it has been speculated that triglyceride accumulation in the skeletal muscle in the obese subjects might have resulted from reduced fatty acid oxidation[25]. Accordingly, many studies have focused on the potential links between changes in fatty acid oxidation and the development of obesity.

Malonyl-CoA is a prime agent in sensing the fuel supply in the brain and peripheral organs[7]. Malonyl-CoA also affects fatty acid oxidation by the inhibition of CPT-1, which controls fatty acid entry into the mitochondrion for oxidation. Cerulenin (2,3-epoxy-4-oxo-6-dodecadienoylamide), an antifungal antibiotic found in culture of Cephalosporium caerulens, has been known as a strong inhibitor of fatty acid synthesis, blocking the condensation of acetyl and malonyl-CoA[29]. A synthetic FAS inhibitor, C75, acted centrally to reduce food intake by alterations in the level of hypothalamic NPY levels and other hypothalamic peptides, leading to decreased appetite[30]. In contrast to C75, a single injection of cerulenin did not reduce food intake significantly in this study. *In situ* hybridization study reconfirmed that a single injection of cerulenin did not affect the expression of hypothalamic AGRP and NPY mRNA. MCH is also one of the strong orexigenic neuropeptides. However, any alteration of the expression of MCH was not observed after treatment of cerulenin in this study. We could not explain this discrepancy between C75 and cerulenin on food intake and it is only a single injection study. However, in the previous studies[1,3], cerulenin also caused a preferential loss of adipose tissue without any high correlation with food intake. Without significant reduction of food intake, meanwhile, a single injection of cerulenin significantly reduced body weight in *ad libitum*-fed mice. Furthermore, even in a fasted state, cerulenin reduced body weight

by 15% more than that of the control group. The core temperature of the control mice given RPMI was found to drop dramatically during the 24h period after food deprivation (Fig. 1D), which is consistent with the normal response of reducing energy utilization with fasting[31]. However, a single administration of cerulenin significantly prevented this reduction of core temperature at 3h and 5h after injection. Taken together, these results indicated again that cerulenin induced weight loss by increasing energy expenditure rather than reducing food intake.

The FAS inhibitor induced preferential reduction of adipose mass[32] and fatty liver[1] in the setting of the cellular increased malonyl-CoA levels followed by the direct reduction of fatty oxidation was well clarified by the recent beautiful work of Jagan *et al.*[2]. C75 stimulated paradoxically activated fatty acid oxidation, ATP levels, and CPT-1 activity. However, there is no evidence that cerulenin bind to CPT-1 directly. Previous *in vitro* studies using primary culture and cell line models revealed that it decreased fatty acid oxidation through elevation of malonyl CoA indirectly. The difference was explained by dicarbonyl groups and amphipathic nature of both C75 and malonyl coA which are required for direct contact for CPT-1, while cerulenin contains only a single dicarbonyl group in cyclized and not amphipathic form [33,34]. The previous study revealed cerulenin had no effect on CPT-1 activity[5]; however, in this study, CPT-1 activity was inhibited dose dependently. Even though cerulenin does not bind to CPT-1 directly, it is a natural FAS inhibitor increasing cellular malonyl CoA and therefore, it should decrease the CPT-1 activity indirectly. The discrepancy might be explained by the dosage of cerulenin, and only one dose of 10 μg/ml cerulenin was tested in previous study. Interestingly, a single I.P. injection of cerulenin revealed a biphasic response, where the early phase suppression was followed by the late phase stimulation of CPT-1 activity. The biphasic response of CPT-1 activity to cerulenin speculated us that this delayed effect might be the indirect central effects of cerulenin. ICV administered results conformed that cerulenin act centrally to enhances CPT-1 activity. (Fig. 5) Taken together, these findings suggested that the central stimulating effects of cerulenin overcame the early peripheral inhibitory effects on CPT-1 activity, and that the modest and delayed central effects of cerulenin might be the major contributor of the energy expenditure and the preferential reduction of adipose mass.

The autonomic nervous system is involved in processes where the brain controls energy expenditure. To get some clues of the central pathway responsible for the biphasic response of cerulenin to fatty oxidation or increased energy expenditure, we tested the following: ① The *in vitro* effects of catecholamine, epinephrine and norepinephrine on CPT-1 activity in the presence and absence of cerulenin. ② Mice were also pretreated with AMPT, an inhibitor of TH that was the rate-limiting enzyme of catecholamine synthesis. ③ The adrenal glands were removed before treatment with cerulenin. As we had expected, cerulenin-induced inhibition of CPT-1 activity was overcome by co-treatment with epinephrine or norepinephrine. Pretreatment with AMPT inhibited the cerulenin-induced increase in core temperature, and the late phase stimulating activity of cerulenin on CPT-1 activity in both the liver and muscle. In ADX mice, cerulenin treatment enhanced the CPT-1 activity in both the liver and muscle prepared from these mice. These findings suggest that a major portion of the cerulenin-induced late phase stimulating effects on CPT-1 activity occurred through the activation of innervated SNS neurons. Cerulenin induced delayed activation of CPT-1 might be also explained by the recent observation of leptin induced delayed activation of 2 AMPK through SNS, because the activation of α2 AMPK reduces acetyl-CoA carboxylase (ACC) activity and results in the stimulation of CPT-1 activity[35].

FAS is also expressed in the hypothalamus: ARC, VMH and PVN[6]. Although fasting and treatment with a FAS inhibitor significantly down-regulated the FAS activity in the liver, mRNA expression of FAS remained high in the hypothalamus, indicating that FAS in the brain is regulated differently[6]. VMH neurons mediated leptin-induced increase in the secretion of catecholamines[36], and chemical lesion reduced the SNS activities[37]. To further elucidate the central pathways involved in the cerulenin-mediated activation of the SNS, we used the well-established MSG-treated (Arc lesion) and GTG-treated (VMH lesion) mice models. Cerulenin treatment enhanced CPT-1 activity in the liver and muscle of MSG-treated mice; however, it did not enhance the activity in the GTG-treated mice. Interestingly, the initial hepatic CPT-1 activity decreased in the MSG-treated mice. Previous studies[38-41] already showed that the MSG-treated mice became significantly obese and revealed

higher levels of serum blood glucose, total cholesterol, and cholinesterase. Furthermore, when compared to the control mice, the MSG-treated mice revealed high levels of triglycerides in the liver or even showed a fatty liver. In this way, MSG treatment contributed to this unexpected phenomenon. Therefore, although FAS is expressed in the ARC nucleus with VMH and PVN, our results suggested that the enhanced CPT-1 activity by cerulenin was mediated through the VMH activation, rather than through the ARC nucleus activation to activate the SNS. However, both GTG and MSG influence several populations of neurons and both change basal CPT-1 activity. Therefore interpreting the ability of cerulenin to alter CPT-1 activity from these animal models needs caution and has some limitation. Further study is necessary to elucidate mechanism of cerulenin on fat metabolism.

Ⅴ. CONCLUSION

Taken together, our findings provided insight into the paradoxical effect of a FAS inhibitor, cerulenin, which resulted in a biphasic response on CPT-1 activity by a direct inhibitory effect in the peripheral and an indirect stimulating effect in the central hypothalamic-SNS, and found that the central stimulating effects surpass the peripheral inhibitory effects.

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국문요약

교감신경의 활성화를 통한 Cerulenin의 CPT-1에 미치는 영향

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Cerulenin은 FAS(fatty acid synthase) 억제제로 세포 내 malonyl CoA를 증가시키고 CPT-1 활성을 떨어뜨려 지방산의 베타 산화를 억제한다. 그러나 cerulenin을 in vivo로 주사하면 FAS 억제제 임에도 불구하고 지방을 우선적으로 감소시킨다. cerulenin이 상기 역설적 효과를 나타내는 기전을 연구하기 위하여, 마우스를 cerulenin으로 처치하고 식이 및 체중에 미치는 영향 및 CPT-1 활성도에 미치는 영향을 조사하였다. cerulenin을 복강 내로 주사하였을 때 일일 식이 섭취는 의미 있게 감소하지 못하였지만 체중이 의미 있게 감소되었고 체온은 의미 있게 증가되었다. 또한 cerulenin을 복강 내로 투여할 경우 마우스의 간장과 근육의 CPT-1 활성도 변화는 이상성 반응(biphasic response)을 나타내었다. 즉 투여 직후에는 CPT-1 활성도가 억제되었지만 투여 3시간과 5시간 사이에는 대조 군에 비해 의미 있는 증가를 보였다. 간장 조직을 분리한 이후 간세포에 cerulenin을 처리하면 농도 의존적으로 CPT-1 활성이 억제되지만 catecholamine과 동시에 cerulenin을 처리하였을 때는 대조 군에 비해 의미 있게 CPT-1 활성도가 증가하는 것을 관찰하였다. 반면에 ICV로 cerulenin을 투여할 경우 soleus muscle에 측정한 CPT-1 활성이 1시간 이후부터 증가하며 3시간까지 유지되는 것을 관찰하

였다. Catecholamine의 합성에 속도조절효소인 tyrosine hydroxylase를 AMPT로 억제한 후 cerulenin을 처치할 경우 cerulenin 처치시 관찰되던 체온의 증가 및 CPT-1 활성의 증가 효과를 관찰 할 수 없었지만, 부신을 제거하고 코티졸을 주사한 마우스에서 cerulenin은 여전히 CPT-1 의 활성을 증가시켰다. 복강 내 cerulenin의 투여 후 *in situ* hybridization으로 조사한 orexigenic neuropeptide인 AgRP, NPY와 MCH의 mRNA 표현은 대조 군과 비교하여 의미 있는 차이를 보이지 못하였다. MSG로 전 처치하여 arcuate nucleus가 파괴된 마우스에서 cerulenin은 여전히 간장과 근육에서 CPT-1의 활성을 증가 시켰지만, GTG로 전 처치하여 ventromedial hypothalamus가 파괴된 마우스에서는 CPT-1의 활성을 증가시키지 못하였다. 이상의 결과로 볼 때 cerulenin이 FAS억제제 임에도 불구하고 CPT-1활성과 energy expenditure를 증가 시키는 기전은, cerulenin이 간장 및 근육 조직에 직접적으로 영향을 미친 초기 억제 효과와 central hypothalamic-SNS를 활성화에 따른 지연자극 효과의 가중에 의한 결과일 것으로 사료된다.

핵심되는 말: cerulenin, 비만, hypothalamus, 교감신경, CPT-1, 지방산산화