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Time-Course Changes of Hormones and Cytokines by Lipopolysaccharide and Its Relation with Anorexia

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Abstract: We assessed the time course effects of lipopolysaccaride (LPS) on food intake, cytokines, and hormones in rats and evaluated the relation between LPS-induced anorexia and its possible causative factors. Food intake was reduced 2 h after LPS injection (500 μg/kg, intraperitoneally) and remained decreased for 24 h. Plasma TNF-α and IL-6 levels increased by LPS administration at 0.5 and 2 h, and at 2 and 4 h, respectively. Plasma leptin and glucose levels were elevated at 8 and 16 h, and insulin levels were elevated at 2, 4, 8, and 16 h in the LPS-injected group, as compared to the counterpart controls. IL-6 levels in the CSF were elevated at 2 and 4 h. Hypothalamic cytokines tended to increase as early as 0.5 h after LPS injection and remained increased until 16 h. LPS-induced anorexia was attenuated in insulin-deficient STZ rats and was abolished by insulin treatment. The hypothalamic expression of NPY, a target of insulin's anorexic effect, was decreased 2 h after LPS administration, and central NPY injection (3 nM) prevented LPS-induced anorexia. In conclusion, cytokines, insulin, and leptin levels evidence different time courses by LPS administration. In LPS-induced anorexia, insulin may constitute a newly found causative factor, whereas leptin appears to be uninvolved in an early period in rats.

Key words: lipopolysaccharide, anorexia, cytokine, leptin, insulin.

Anorexia and body weight loss are common complications of many diseases, including infections [1, 2] and inflammation [3]. Despite the host of metabolic changes associated with infection and inflammation, reduced caloric intake appears to play a significant role in weight loss. For example, despite the hypermetabolism associated with AIDS patients [4], weight loss tends to occur only in the presence of reduced caloric intake [4, 5]. Lipopolysaccharide (LPS) has been repeatedly demonstrated to generate similar responses to infection when intraperitoneally injected [6]. Many of the physiological effects associated with LPS are mediated by cytokines, including tumor necrosis factor–alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), the levels of all being elevated as a result of LPS injection [7, 8]. LPS-induced anorexia may also be mediated by these cytokines, and this has been verified by the production of anorexia via the central injection of these cytokines [9–11]. LPS administration also induces changes in the gene expression of TNF-α and IL-1, as well as in the gene expression of food intake regulatory neurotransmitters [12–14]. LPS injection induces an elevation in energy-regulatory hormones, including plasma leptin and insulin [15–19]. Leptin, which is synthesized and released from adipose tissue and functions as a long-term signal for body adiposity, reduces food intake via hypothalamic receptors [20, 21]. However, LPS induces anorexia in genetically leptin-aberrant rodent models; thus the role of leptin in LPS-induced anorexia remains unclear [22, 23]. Insulin also functions as an adiposity signal and effects a food intake reduction when injected into the brain [24, 25]. Although it is increased as a result of LPS administration [18, 19], the role of insulin in LPS-induced anorexia has yet to be clearly elucidated. Because several cytokines and hormones have been proposed as possible candidates for LPS-induced anorexia, it is possible that not only one factor, but also several cytokines and hormones may be involved in LPS-induced anorexia in a time-dependent manner. However, few studies have attempted to evaluate the changes of these various cytokines and hormones simultaneously in association with LPS-induced anorexia. An analysis of the relationship between food intake and these food intake–regulatory factors may facilitate an elucidation of the cause of LPS-induced anorexia. Therefore in the current study we have assessed the time-course effects of LPS on food intake, cytokines, and hormones, both peripherally and centrally.

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MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were purchased from Jung-Ang Lab Animals (Seoul, Korea) and were housed in the animal unit of the College of Medicine at Yeungnam University. Two weeks prior to the commencement of the experiment, the rats were housed in a group cage within a room on a 12-h:12-h light/dark cycle, with lights off at 10:00 and lights on at 22:00, to conduct experiments during the dark phase [26]. Four days prior to the experiment, the rats were transferred to separate cages. They were fed a standard chow diet and provided with *ad libitum* access to water. Food intake was monitored via manual weighing, and spillage was taken into consideration. Food spillage was negligible (less than 1%). This study was conducted in accordance with the guidelines for the care and use of laboratory animals established by Yeungnam University, and all experimental protocols were previously approved by the ethical committee of Yeungnam University.

The time-course changes of food intake, hormones, and cytokines in LPS-injected rats. For this experiment, rats weighing about 200 g were used. To determine the effects of LPS (055:B5, Sigma, St. Louis, MO, USA) on food intake, the rats were randomly divided into control (normal saline) and LPS-injected groups (n = 10 per group), and food intake was measured at 2, 4, 6, 8, and 24 h after LPS injection. LPS (500 μg/kg body weight, i.p.) [22, 27, 28] was administered 30 min before lights off. To determine the time-course changes of cytokines in plasma and cerebrospinal fluid (CSF) by LPS, the rats were divided into 0, 0.5, 2, 4, 8, or 16 h groups (n = 7 per group). For plasma leptin, insulin, and glucose measurements, the rats were divided into saline-injected and LPS-injected groups, and the rats in each of the groups were sacrificed at 0, 0.5, 2, 4, 8, or 16 h (n = 6 in each groups) after the intraperitoneal administration of normal saline or LPS. To circumvent the confounding effects of food intake on the plasma leptin and insulin concentrations, the food was removed upon LPS injections. CSF for IL-6 was obtained via a puncture of the 4th ventricle under stereotaxic fixation with anesthesia (mixture of 100 mg/kg body weight ketamine and 10 mg/kg body weight xylazine). Blood from the aorta and hypothalamus was obtained from each group. Insulin and leptin levels were determined via radioimmunoassay (Linco, St. Charles, MO, USA) and TNF-α and IL-6 (Endogen, Rockford, IL, USA) were measured via enzyme-linked immunoassay. Plasma glucose was determined via the enzymatic colorimetric method (Sigma, St. Louis, MO, USA).

Expression of cytokines and neuropeptide Y (NPY) in hypothalamus after LPS injection. Hypothalamic TNF-α and IL-6 mRNA expression were evaluated via RT-PCR at 0, 0.5, 2, 4, 8, and 16 h (n = 5 in each group) after LPS administrations (500 μg/kg body weight, i.p.) in rats weighing about 200 g. LPS was administered 30 min before lights off. RT-PCR kits were purchased from PerkinElmer (Norwalk, CT, USA). Hypothalamus samples were freshly acquired andinitially stored in a liquid nitrogen tank until total RNA isolation was achieved. The whole hypothalamic samples from the tank were then ground into powder on dry ice, using a hand grinder. The powdered samples were transferred to 1 ml of Trizol solution (Sigma) and 200 μl of chloroform followed by centrifugation, and the aqueous phase was combined with an equal volume of isopropanol. The precipitated pellet was washed with 70% ethanol and resuspended in diethylpyrocarbonate (DEPC)-treated water. One microgram of total RNA per sample was then reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (PerkinElmer, Norwalk, CT, USA) and oligo dT priming, in accordance with the manufacturers' instructions, for 15 min at 42°C. Amplification with specific primers was conducted using a Gene Amp PCR system 9600 (PerkinElmer) for 40 cycles with a 1 min/94°C denaturation, 1 min/55°C annealing, and 1.5 min/72°C extension profile in the case of TNF-α; for 35 cycles with a 30 s/95°C denaturation, 30 s/50°C annealing, and 1 min/72°C extension profile in the case of IL-6; and for 30 cycles with a 30 s/95°C denaturation, 30 s/60°C annealing, and 30 s/72°C extension profile in the case of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). An amplication of mRNA for the GAPDH housekeeping gene was employed as an internal quality standard. The amplified products were electrophoresed on 1.5–2% agarose gel stained with 0.5 g/ml ethidium bromide. The primer sequences were as follows: TNF-α (251 bp): sense, 5'-atgacgacagaaagcatgatcc-3'; antisense, 5'-gaagatgatctgtagtgtg-3'. IL-6 (496 bp): sense, 5'-cttccagccagttgccttct-3'; antisense, 5'-gagagcattggaagttgggg-3'. GAPDH (515 bp): sense, 5'-aatgcatcctgcaccaccaa-3'; antisense, 5'-gtagccatattcattgtcata-3'. Real-time PCR for TNF-α (0, 0.5, 2, 4, 8, and 16 h; n = 3 per group) and NPY (0 and 2 h; n = 3 per group) in the hypothalamus of rats injected with LPS (500 μg/kg body weight, i.p.) were performed using the LightCycler (Roche, Mannheim, Germany). RNA was reverse transcribed to cDNA from 1 μg of total RNA, which was then subjected to real-time PCR performed essentially according to the manufacturer's instructions. PCR was performed in triplicate in a total volume of 20 μl of LightCycler FastStart DNA SYBR Green I mix (Roche) containing primer and 2 μl of cDNA. PCR amplification was preceded by incubation of the mixture for 10 min at 95°C, and the amplification step consisted of 45 cycles of denaturation, annealing, and extension. Denaturation was performed for 10 s at 95°C, annealing for 5 s at an appropriate temperature depending on each primer, and extensionat 72°C for 10 s, with fluorescence detection at 72°C after each cycle. After the final cycle, the melting point analyses of all samples were performed within a range of

65ºC to 95°C with continuous fluorescence detection. Expression levels of β-actin were used for sample normalization. The results for each gene are expressed as the relative expression level compared with β-actin. The primers were as follows: TNF-α (215 bp): sense, 5'-cccagaccctcacactcagat-3'; antisense, 5'-ttgtcccttgaagagaccctg-3'. NPY (220 bp): sesnse, 5'-tatccctgctcgtgtgtttg-3'; antisense, 5'-gttctgggggcattttctg-3'. β-actin (101 bp): sense, 5'-tactgccctggctcctagca-3'; antisense, 5'-tggacagtgaggccaggatag-3'. The levels of TNF-α and NPY mRNA were determined by comparing experimental levels to the standard curves and are expressed as a fold of relative expression.

The effect of LPS-induced anorexia on streptozotocin (STZ)-injected rats. We evaluated the LPS-induced anorexia in insulin-deficient STZ rats and saline-injected control rats (n = 10 per group). Sprague-Dawley rats weighing 300 g were intraperitoneally injected with STZ (65 mg/kg body weight) in citrate buffer (pH 4.5). The induction of diabetes was confirmed using urine glucose sticks. After confirming the induction of diabetes, food intake was measured at 2, 4, 6, and 8 h after lights off (10:00 a.m.). After LPS administration (500 μg/kg body weight, i.p.) 30 min before lights off for the STZ and control rats, food intake was measured at the same times as described above and was calculated as the percentage ratio of food intake from after to before LPS injection in each group. Food intake before LPS injection was averaged for 3 days. To confirm the involvement of insulin in LPS-induced anorexia, we measured LPS-induced anorexia in insulin-treated STZ rats (n = 6 per group). We injected STZ in Sprague-Dawley rats weighing 300 g and measured food intake the same as we explained above. For the experiment, we randomly assigned STZ rats into the saline-treated and insulin-treated groups. Insulin (5 units/rats, Humulin, Lilly, USA) was injected subcutaneously 30 min before LPS injection (500 μg/kg body weight, i.p.). The percentage ratio of food intake from after to before LPS injection in each group was calculated.

Effects of centrally injected NPY on LPS-induced anorexia. For this experiment, 300 g of rats (n = 8 in each group) were used. NPY was purchased from Phoenix, USA. The surgical procedures were conducted under pentobarbital sodium (40 mg/kg). Each anesthetized rat was mounted on a stereotaxic frame (DJ 8001, Daejong, Korea), and a stainless steel guide cannula (26 gauge) was implanted in the lateral ventricle. Coordinates were as follows, using flat skull position and bregma as the reference: A/P 1 mm, M/L 1.3 mm, D/V 3.2 mm. The stainless steel cannula was secured via stainless steel screws and acrylic resin applied to the skull. At least one week after surgery, the injections of each drug were conducted through a 31-gauge stainless steel injector that extended 1 mm beyond the end of the guide cannula. A total volume of 7 μl of NPY (3 nmol) was injected over a 5-min period. The control rats were injected with 7 μl of artificial CSF [aCSF, mM, NaCl 128, KCl 3, $CaCl_2$ 1.2, $MgCl_2$ 0.8, NaH_2PO_4 0.25, $NaHCO_3$ 20, and glucose 3.4 (pH 7.4)]. Correct cannula positioning was confirmed via cresyl violet stain. LPS (500 μg/kg body weight) was intraperitoneally administered prior to intracerebroventricular (i.c.v.) injection. Food intake was measured every two hours for 6 h after NPY injection.

Statistical analysis. The results were expressed as the means ± SEM. Differences between two groups were assessed via Student's t-tests. Time- course differences in saline-injected or LPS-injected groups and differences among groups in the NPY-injected study were assessed via one-way analysis of variance (ANOVA) followed by LSD t-tests. All statistical analyses were conducted using the SPSS system.

RESULTS

Food intake, plasma cytokines, and hormones in LPS injected rats

Food intake was decreased 2 h after LPS administration, and remained to be decreased for 24 h as compared to the saline-injected control rats (Fig. 1). Plasma TNF-α levels were elevated at 0.5 and 2 h after LPS administration and reached a maximum level at 2 h. Plasma IL-6 levels were elevated at 2 and 4 h and reached a maximum level at 2 h (Fig. 2). Plasma leptin, insulin, and glucose levels were reduced at 8 and 16 h as compared to 0 h in the saline- injected group as a result of the fasting. However, LPS administration increased leptin levels at 8 h and insulin at 2, 4, and 8 h compared to the corresponding 0 h. In both cases, the increased levels returned to the level of 0 h at 16 h. Glucose was temporarily reduced at 2 h as com-

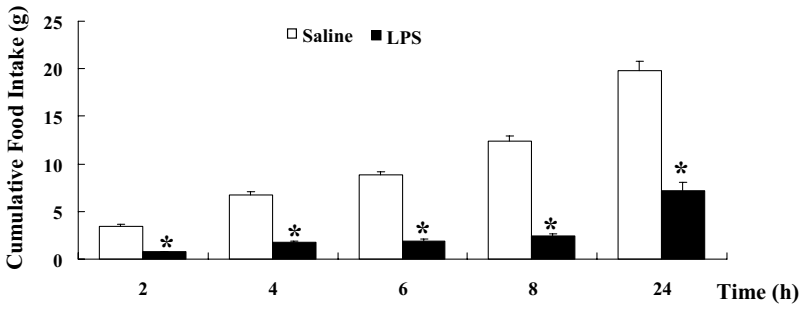

Fig. 1. Cumulative food intake after lipopolysaccharide (LPS) injection (500 μg/kg, i.p.). Control rats were injected with saline. Food intake was significantly decreased in LPS-injected rats. The difference between the two groups was analyzed with Student's t-test. *$p < 0.05$ vs. saline.

pared to 0 h as a result of higher insulin levels, but it returned to the level of 0 h at 4, 8, and 16 h, although plasma insulin levels remained elevated. Plasma leptin and glucose levels were elevated at 8 and 16 h, and insulin levels were elevated at 2, 4, 8, and 16 h in the LPS-injected group as compared to the counterpart controls (Fig. 3).

IL-6 in CSF and IL-6 and TNF-α gene expressions in hypothalamus

We also measured IL-6 levels in the CSF after LPS injection. They were elevated at 2 and 4 h and returned to the level of 0 h at 8 h. IL-6 reached maximum levels at 4 h in the CSF. Hypothalamic TNF-α and IL-6 mRNA expression levels by RT PCR were markedly elevated at 2 h. We also evaluated TNF-α mRNA in hypothalamus by real-time PCR and found that TNF-α mRNA tended to increase as early as 0.5 h after LPS injection ($p = 0.08$), and the increase persisted until 16 h after LPS administration (Fig. 4).

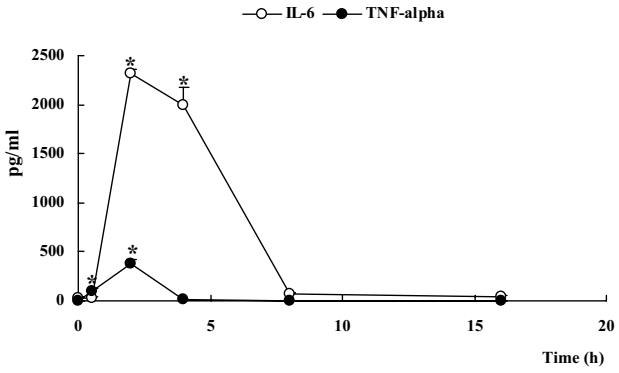

Fig. 2. Time-dependent change (0, 0.5, 2, 4, 8, and 16 h) in the plasma concentration of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) after lipopolysaccharide (LPS) injection (500 μg/kg, i.p.). The differences among six groups in IL-6 or TNF-alpha were assessed via one-way analysis of variance (ANOVA) followed by LSD *t*-tests. *$p < 0.05$ vs. corresponding 0 h.

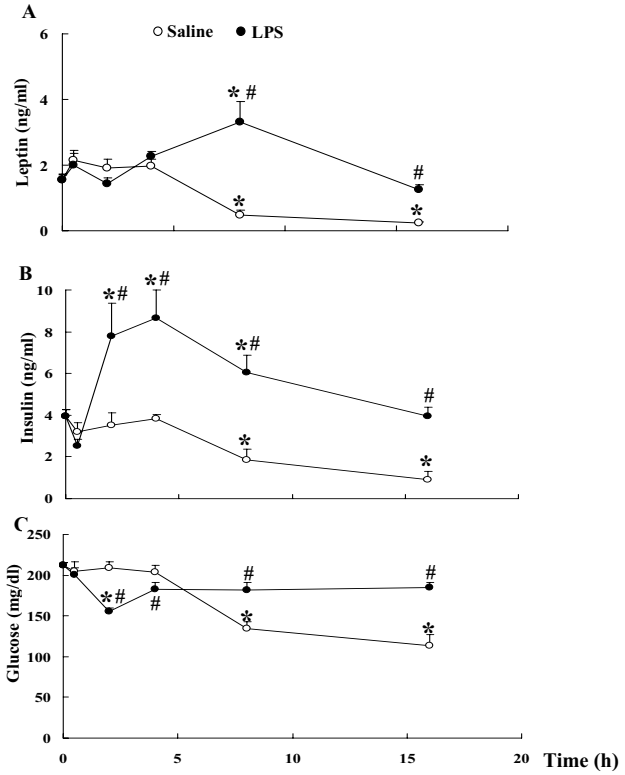

Fig. 3. Time-dependent changes (0, 0.5, 2, 4, 8, and 16 h) in plasma concentration of leptin (**A**), insulin (**B**), and glucose (**C**) in control (saline)- and lipopolysaccharide (LPS; 500 μg/kg, i.p.)-injected rats. The differences among six time points were assessed via one-way analysis of variance (ANOVA) followed by LSD *t*-tests. The differences between saline and LPS groups in each time point was analyzed with Student's *t*-test. *$p < 0.05$ vs. corresponding 0 h. #$p < 0.05$ vs. counterpart saline.

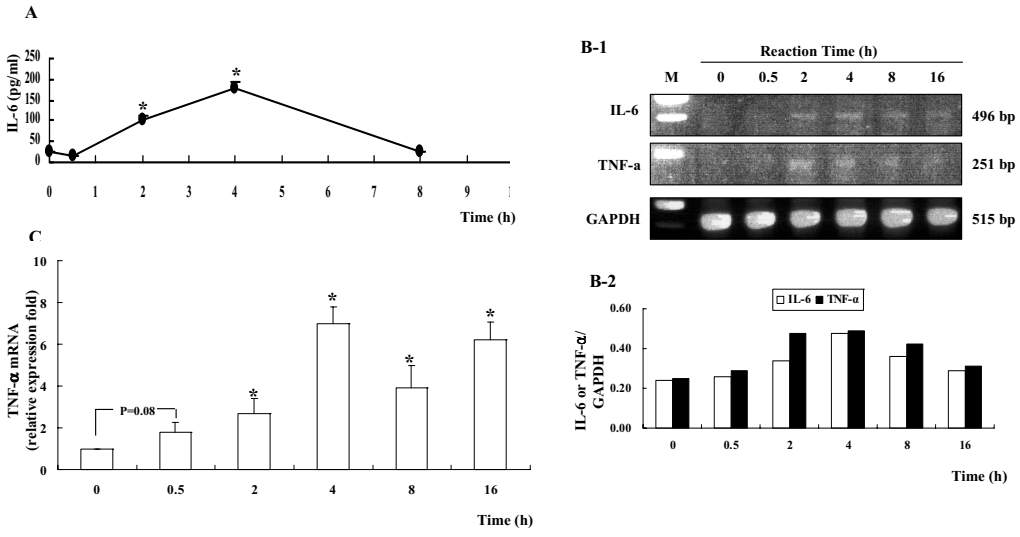

Fig. 4. A concentration of interleukin-6 (IL-6) in cerebrospinal fluid (**A**) and a mRNA expression of cytokines in the hypothalamus via RT-PCR (**B-1**) and real-time PCR (**C**) after lipopolysaccharide (LPS) injection (500 μg/kg, i.p.). The results of RT-PCR were measured with densitometry and presented in a histogram (**B-2**). The results of TNF-α mRNA were calculated as the relative expression level compared with β-actin and presented as fold of relative expression (**C**). The differences among groups were assessed via a one-way analysis of variance (ANOVA) followed by LSD *t*-tests. M: 100 bp DNA ladder. *$p < 0.05$ vs. 0 h.

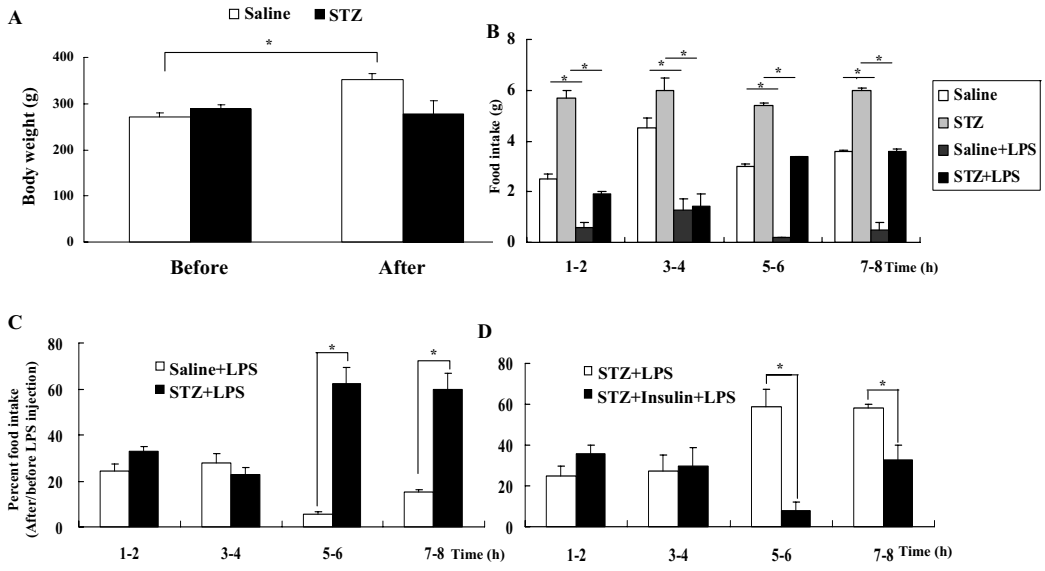

Fig. 5. Body weight (**A**), food intake (**B**), and percentage ratio of food intake from after- to before-lipopolysaccharide (LPS) injection (**C**, **D**) in control (saline)- and streptozotocin (STZ, 65 mg/kg, i.p.)-injected rats. Body weight was measured before saline or STZ injection (before in A) and 2 weeks after saline or STZ injection (after in A). LPS was injected (500 μg/kg, i.p.) 2 weeks after the saline or STZ injection. Food intake was measured at 2, 4, 6, and 8 h after lights off (10:00 a.m.) before and after LPS injection (B). Food intake was calculated as a percentage ratio of food intake from after- to before-LPS injection (C, D). Insulin (5 units) was injected subcutaneously before LPS injection (D). The difference between the two groups was analyzed with Student's *t*-test, and the differences among more than two groups were assessed via one-way analysis of variance (ANOVA) followed by LSD *t*-tests. **p* < 0.05.

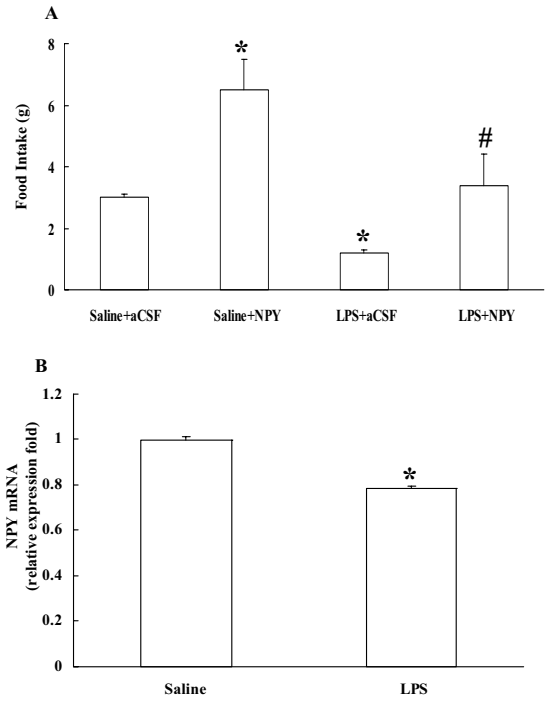

Fig. 6. Food intake for 2 h after the intracerebroventricular (i.c.v.) injection of neuropeptide Y (NPY) (3 nM) (**A**) and the mRNA expression of NPY in hypothalamus in lipopolysaccharide (LPS; 500 μg/kg, i.p.)-injected rats. LPS was injected 30 min prior to the NPY injection. The results of NPY mRNA were calculated as the relative expression level compared with β-actin and presented as fold of relative expression (**B**). The differences among the groups were assessed via one-way analysis of variance (ANOVA), followed by LSD *t*-tests. The differences of NPY mRNA between saline and LPS was assessed with a Student's *t*-test. **p* < 0.05 vs. saline + aCSF or saline. #*p* < 0.05 vs. LPS + aCSF.

The effect of LPS injection on food intake in insulin-deficient STZ rats

To evaluate the relation between LPS-induced anorexia and insulin, we injected LPS in insulin-deficient diabetic STZ rats (insulin levels: 2.6 ± 0.8 vs. 0.5 ± 0.07 ng/ml in saline and STZ rats, respectively) and measured food intake. The initial body weight before STZ injection was not different between saline and STZ-injected rats, but it significantly lowered in STZ-injected rats at 2 weeks after STZ injection (Fig. 5A). Food intake ultimately reached almost 2 times that of the saline-injected rats at the end of 2 weeks after STZ injection. In both the saline and STZ rats, food intake was reduced significantly by LPS. We observed no differences between the two groups with regard to percentage ratio of food intake from after- to before-LPS injection until 4 h; however, LPS-induced anorexia was clearly attenuated in the STZ rats from 5 and 8 hours (Fig. 5, B and C). Insulin treatment increased insulin level (0.6 ± 0.02 vs. 16 ± 0.1 ng/ml in STZ with saline and STZ with insulin, respectively) and abolished this attenuation of LPS-induced anorexia in STZ rats (Fig. 5D).

Effects of centrally injected NPY on LPS-induced anorexia

The expression of NPY mRNA by real time PCR at 2 h after LPS injection was reduced compared with saline-treated rats. Food intake and body weight gain in rats were reduced as the result of the cannulation procedure, but returned to preoperation values 3 days after surgery (data not shown). At least 1 week after surgery, NPY was injected into the lateral ventricle. Food intake was measured for 2 h. NPY injection induced an increase in food intake as compared to the aCSF-injected rats. LPS administration

effected a reduction in food intake in the aCSF-injected rats by 60%, but NPY injection returned food intake to normal (Fig. 6).

DISCUSSION

In this study, we have shown an increase of plasma insulin by LPS in an early period and an attenuation of LPS-induced anorexia in insulin-deficient STZ rats, which was reversed by insulin treatment. These results suggest the possible role of insulin in LPS-induce anorexia.

LPS (or cytokines) is known to induce an increase in insulin levels. It has been shown that the central injection of insulin-induced anorexia [29–32] and deficiencies in insulin receptors could induce hyperphagia [33]. Peripherally injected insulin causes hyperphagia via the induction of severe hypoglycemia [34], whereas in our study, plasma glucose levels were maintained or even higher compared with control rats, even though plasma insulin levels increased four fold. Since several cytokines are well known to cause insulin resistance [35–37], the increase in TNF-α and IL-6 by LPS may inhibit insulin's action, which maintains or increases plasma glucose level in spite of a higher insulin level. The mechanism by which insulin induces hypophagia involves the suppression of hypothalamic NPY mRNA [38, 39], an orexigenic neuropeptide [40]. We showed that LPS administration reduced hypothalamic NPY mRNA and that NPY injection into the brain attenuated LPS-induced anorexia. Thus we suggest that the anorexic effect of LPS may be mediated by the reduction of hypothalamic NPY associated with an increase in insulin concentration. This notion is supported byLPS-induced anorexia being attenuated in insulin-deficient STZ-injected rats, which was reversed by insulin treatment. Previous study also suggests that insulin, but not leptin, may partly contribute to the anorexia of cytokines [41], and a recent study has shown that LPS increases the transport of insulin through the blood brain barrier by about three fold [42]. Therefore insulin is a possible candidate for LPS-induced anorexia in rats. However, no significant attenuation of LPS-induced anorexia for the first 4 h in STZ rats suggests the multiple involvements of cytokines and hormones in LPS-induced anorexia. Leptin is known to be increased by LPS [15, 16] and has been well established to reduce food intake when intraperitoneally [43] or centrally [44] injected. Several previous studies have reported increased plasma leptin concentration and/or expression of the leptin gene in LPS-treated rats [28, 45]. However, evidence against the essential role of leptin in LPS-induced anorexia also reported that plasma leptin levels remained unchanged or reduced in LPS-treated or cytokine-treated rats, even though food intake had been clearly reduced in male rats [41, 46]. Furthermore, the induction of a food intake reduction by LPS in genetically leptin or leptin receptor-aberrant rat and mice models is not supportive of leptin's role in LPS-induced anorexia [22, 23]. The current study showing that plasma leptin levels were elevated at 8 h after LPS injection, but that food intake was reduced at 2 h, indicated that leptin plays no essential role in the early phases of LPS-induced anorexia. Time discrepancy between the increase in leptin levels and the reduction in food intake has also been observed in previous study [28]. In our study, leptin may play a role in anorexia at 8 h after LPS injection, and further studies are needed to clarify the role of leptin in LPS-induced anorexia.

The increase in plasma cytokines was fast and earlier than energy-regulating hormones, which is consistent with previous studies. Intraperitoneal LPS injection in mice resulted in an increase in plasma IL-6 and TNF-α levels at 1.5 h [47]. The intramuscular injection of LPS increases plasma IL-6 and TNF-α levels within 45 min in guinea pigs [48]. The intraperitoneal administration of LPS induces an increase of TNF-α mRNA earlier than an increase of leptin in mice [49]. It has been suggested that one of the possible mechanisms to induce anorexia by LPS is the indirect modulation of food intake by cytokines via the elevation of energy-regulating plasma hormones. This notion could be supported by the increase of cytokines prior to leptin and insulin in our study and the increase of these hormones by cytokines [50, 51]. However, the direct modulation of food intake by cytokines could also be possible. The increases of hypothalamic cytokines expression by peripheral LPS administration [48] and the induction of anorexia and changes in the levels of feeding-regulatory neurotransmitters in the hypothalamus via the central injection of various cytokines [9, 10] support this hypothesis. In our study, a fairly early increase in hypothalamic TNF-α mRNA expression suggests the possibility of a direct involvement of cytokines in LPS-induced anorexia. Further studies are needed to confirm the direct involvement of cytokines in anorexia by antibodies against each cytokine. In conclusion, cytokines, insulin, and leptin levels evidence different time-course changes as the result of LPS administration. In LPS-induced anorexia, insulin may constitute a newly discovered causative factor, whereas leptin appears to be uninvolved in an early period in rats. LPS induces anorexia by multiple factors, rather than by a singular factor.

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