Intracerebroventricular Administration of Leptin-Induced Apoptosis in the Rat Small Intestinal Mucosa

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The localization of leptin and leptin receptors in the stomach and small intestine has been reported. Their function is still unknown, although leptin is a hormone that regulates appetite and fat-related metabolism. The small intestine is one of the important organs for regulating metabolism. The purpose of the present study was to investigate whether leptin regulates apoptosis in the small intestinal mucosa. Intestinal apoptosis was evaluated by percent fragmented DNA, electrophoresis, TUNEL staining, and western blotting analysis of caspase-3. Mucosal apoptosis in the rat jejunum and ileum was evaluated at 0, 3, 6, 12, and 24 hrs after injection. Rats were tested after *ad libitum* feeding and 24-hr fasting to exclude the anorectic effect of leptin. Leptin was injected intraperitoneally (ip) at a dose of 200 μg/rat and infused into the rat third cerebroventricle (icv) at a dose of 8 μg/rat. Leptin at a dose of 8 μg/rat significantly induced intestinal apoptosis in the small intestine at 3 and 6 hrs after icv administration in both *ad libitum* feeding and 24-hr fasted rats. This increase in apoptosis was not attenuated by vagotomy. Intestinal apoptosis increased 12 and 24 hrs after ip injection of leptin at a dose of 200 μg/rat. The peak of the increase in apoptosis in icv rats appeared earlier than that in ip rats. Leptin induced jejunal and ileal mucosal apoptosis in the rat, indicating that leptin might control intestinal function through the regulation of intestinal apoptosis. Exp Biol Med 228:1239–1244, 2003

Key words: brain gut axis; fragmented DNA; vagal nerve; hypothalamus

Leptin plays important roles in the regulation of metabolism in living organs through several physiological functions, including feeding suppression (1, 2). With regard to the gastrointestinal tract, the localization of leptin and its receptors have been identified in the stomach (3, 4), intestine (5), liver (6), pancreas (6), and so on. The physiological roles of leptin in the gastrointestinal tract have not been well documented.

The small intestine is one of the important organs for regulating metabolism via the digestion and absorption of food. Maintenance of the absorption coefficient in the villous is important for the small intestine to maintain its function of digestion and absorption. There are several hormones secreted in peripheral organs to regulate the function and movement of the gastrointestinal tract, and several hormones act not only at the gastrointestinal tract but also at the central nervous system. A recent study revealed that leptin produces some physiological effects in several organs (7).

The aim of this study was to examine whether leptin had any effect on mucosal apoptosis in the small intestine through the central nervous system to evaluate whether leptin could regulate the energy balance through the intestinal mucosa.

Materials and Methods

Animals and Surgery. Male Sprague-Dawley rats (200–250 g) were used in this study. The animals were housed in wire-bottomed cages in a room illuminated from 8 AM to 8 PM (12-hr light-dark cycle). The rats were allowed access to water and chow *ad libitum*. Five days before testing, a cannula was implanted into the third cerebroventricle of each rat, according to a previously described method (8). Under halothane anesthesia, each rat was fixed in a stereotaxic apparatus, its skull was exposed, and two small screws were threaded into the skull to anchor the cannula. A 3-mm diameter hole was drilled in the skull on the midline 6.0 mm anterior to ear bar zero. A 29-gauge 18-mm-long stainless steel cannula was inserted into the third ventricle to a depth of 7.8 mm from the cortical surface, according to the atlas of König and Klippel (9). At testing time, the food intake

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and body weight were ascertained to have returned to the level before surgery.

Truncal vagotomy was performed in additional rats before intracerebroventricular (icv) cannulation (10–12). The esophagus was gently lifted just below the liver and diaphragm, and the mesentery and visible vagal fibers were cut within 2 cm of the esophagus. Transection of the vagal fibers on both sides was verified microscopically in each rat after the experiment.

Effect of icv Administration of Leptin on Apoptosis in the Small Intestine. Leptin (R&D Systems Inc., Minneapolis, MN) was infused through the icv cannula for 10 min. Rats were administered with icv 8 μg leptin solution (8 μg in 10 μl solution) or 10 μl vehicle (0.15 M saline) at 0, 1, 3, 6, 12, and 24 hrs before euthanasia. The experiment was performed under two conditions: 24-hr fasting and *ad libitum* feeding. Six rats were tested in each group. Rats were sacrificed under halothane anesthesia between 8 and 9 AM to elucidate the influence of the circadian rhythm on mucosal apoptosis in rats (13, 14). Intestinal mucosa was collected for evaluation of % fragmented DNA, agarose gel electrophoresis, immunochemical staining (TUNEL), and western blotting analysis of caspase-3.

Effect of Intraperitoneal (ip) Injection of Leptin on Apoptosis in the Small Intestinal Mucosa. Rats were injected with 200 μl ip leptin (200 μg/200 μl) or vehicle (0.15 M saline) at 0, 3, 6, 12, and 24 hrs before euthanasia. The experiment was performed under two conditions: 24-hr fasting and *ad libitum* feeding. Six rats were tested in each group. Rats were sacrificed under halothane anesthesia between 8 and 9 AM.

Collection of Intestinal Mucosa. After the experiments, the animals were anesthetized and then sacrificed. The entire small intestine was carefully removed and placed on ice. The oral 10 cm (duodenum) was removed, and the rest of the intestine was divided into two equal segments, as the proximal (jejunum) and distal (ileum) segments. Each segment was rinsed thoroughly with physiological saline and opened longitudinally on its antimesenteric border to expose the intestinal epithelium. The mucosal layer was harvested by gentle scraping of the epithelium using a glass slide, as previously described (15).

DNA Fragmentation Assay. The mucosal scraping was processed immediately after collection to minimize nonspecific DNA fragmentation. The amount of fragmented DNA was determined as previously described (16) with modifications. Mucosal scrapings of the different intestinal segments were homogenized in 10 vol of a lysis buffer (pH 8.0) consisting of 5 mM Tris-HCl, 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100. Aliquots (1 ml) of each sample were centrifuged for 20 min at 27,000 × g to separate the intact chromatin (pellet) from the fragmented DNA (supernatant) (17). The supernatant was decanted and saved, and the pellet was resuspended in 1 ml of Tris buffer (pH 8.0) containing 10 mM Tris-HCl and 1 mM EDTA. The pellet and supernatant fractions were assayed for DNA content, using the diphenylamine reaction (18). The results were expressed as the percentage of fragmented DNA/total DNA.

Purification of Mucosal DNA and Agarose Gel Electrophoresis. DNA was extracted from the 27,000 × g fraction (17). The fragmented DNA from various fractions was extracted sequentially with a phenol-chloroform-isoamyl alcohol mixture (25:24:1, vol:vol:vol) to remove the proteins. The protein-free DNA extracts were treated with 100% ethanol in 0.1 M sodium acetate at –20°C overnight to purify the DNA. The precipitated DNA was washed with 70% ethanol and resuspended in Tris buffer (pH 8.0) containing 10 mM Tris-HCL and 10 mM EDTA. DNA samples were incubated with 100 μg/ml ribonuclease for 15 min at 37°C to remove RNA. Resolving agarose gel electrophoresis was performed using 1.5% gels containing 1.0 μg/ml ethidium bromide. Depending on the experiment, 20 μg DNA per well was loaded. DNA standards (0.5 μg per well) were included to identify the size of the DNA fragments. Electrophoresis was performed for 2 hrs at 70 V, and DNA was visualized by ultraviolet fluorescence.

Imumunohistochemical Staining. Tissue samples were removed from the jejunum and ileum, and immediately fixed in 10% neutral buffered formalin. The samples were then embedded in paraffin and sectioned. Fragmented DNA was stained by the terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick and labeling (TUNEL) method (19) with modifications using an Apop Tag Kit (Oncor, Gaithersburg, MD). Specimens were dewaxed and immersed in phosphate-buffered saline containing 0.3% hydrogen peroxide for 10 min at room temperature and then incubated with 20 μg/ml proteinase K for 15 min at room temperature. Seventy-five μl of equilibration buffer was applied directly onto the specimens for 10 min at room temperature, followed by incubation in 55 μl of TdT enzyme at 37°C for 1 hr. The reaction was terminated by transferring the slides to prewarmed stop/wash buffer for 30 min at 37°C. The specimens were covered with a few drops of rabbit serum and incubated for 20 min at room temperature, and then incubated with 55 μl of anti-digoxigenin peroxidase for 30 min at room temperature. Specimens were then soaked in Tris buffer containing 0.02% diaminobenzidine and 0.02% hydrogen peroxide for 1 min to achieve color development. Finally, the specimens were counterstained by immersion in hematoxylin.

Western Blotting Analysis of Caspase-3. The mucosal scrapings were immediately homogenized in 10 vols of lysis buffer (pH 7.6) consisting of 50 mM Tris-HCl, 300 mM NaCl, 0.5% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1.8 mg/ml iodoacetamide, and lysed at 4°C. Insoluble material was removed by centrifugation at 4°C for 15 min at 14,000 × g, and the protein concentration of the soluble fraction was determined. Equal quantities (20 μg) of lysates were resolved by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a nitrocel-

lulose membrane (Trans-Blot, Bio-Rad, Hercules, CA). After blocking with phosphate-buffered saline containing 0.1% polyoxyethylene sorbitan monolaurate and 5% dry milk at 4°C overnight, the membrane was incubated with a polyclonal rabbit anti-caspase-3 antibody (1:1000; Santa Cruz Biotech, CA), which recognizes both procaspase-3 (inactive form) and cleaved caspase-3 (active form) (20), for 2 hrs. Antigen–antibody complexes were detected with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000, Santa Cruz Biotech). Chemiluminescence was detected with ECL western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK), according to the supplier's recommendations.

Statistics. Results are expressed as the means ± SE. Data were evaluated by analysis of variance in which multiple comparisons were carried out by the least significant difference method. Differences were considered significant if the probability of the difference occurring by chance was less than 5 in 100 ($P < 0.05$).

Results

Figure 1 shows the % fragmented DNA in the jejunal and ileal mucosa after icv infusion of 8 μg leptin in *ad libitum* feeding rats. The % fragmented DNA increased 3 and 6 hrs after icv administration of leptin in the jejunum and ileum (3 hrs and 6 hrs: $P < 0.05$ in each), although increases in fragmented DNA were not observed 1 hr after infusion. The increase in apoptosis returned to the control level 12 hrs after the infusion in the small intestinal mucosa. To exclude an effect of leptin on food consumption, leptin was infused in 24-hr fasted rats. As shown in Figure 2, intestinal apoptosis in the jejunum and ileum increased significantly 3 and 6 hrs after icv administration of leptin in 24-hr fasted rats (3 hrs and 6 hrs: $P < 0.05$ in each), the same as in the *ad libitum* feeding rats.

We demonstrated that 8 μg leptin induced apoptosis in the intestinal mucosa after icv infusion, but 2 μg leptin had no effect on the intestinal apoptosis and this dose could not suppress feeding behavior (data not shown). In addition, ip injection of 8 μg had no effect on intestinal apoptosis. Furthermore, we examined the effect of truncal vagotomy on the leptin-induced intestinal apoptosis. Truncal vagotomy had no effect on the intestinal apoptosis induced by icv infusion of leptin (% fragmented DNA in the jejunal mucosa of vagotomized fasted rats after icv infusion of 8 μg leptin; 3 hrs: 40.4 ± 3.2 and 6 hrs: 32.0 ± 3.7).

Resolving agarose gel electrophoresis was performed to evaluate the nature of the fragmented DNA in the jejunal mucosa after icv administration of leptin. As shown in Figure 3, agarose gel electrophoresis of the fragmented DNA obtained from the jejunal mucosa revealed DNA ladders. These ladders were characteristic of apoptosis (16, 17), al-

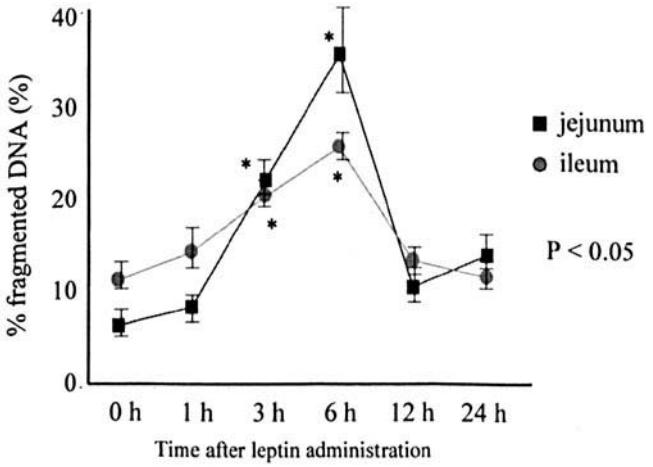

Figure 1. Time course of percent fragmented DNA in the rat small intestine after icv infusion of 8 μg leptin icv in *ad libitum* feeding rats. Jejunum: (■). Ileum: (●). Values are the mean ± SE. Six rats were studied in each group. *$P < 0.05$, compared with the corresponding value of the control rats.

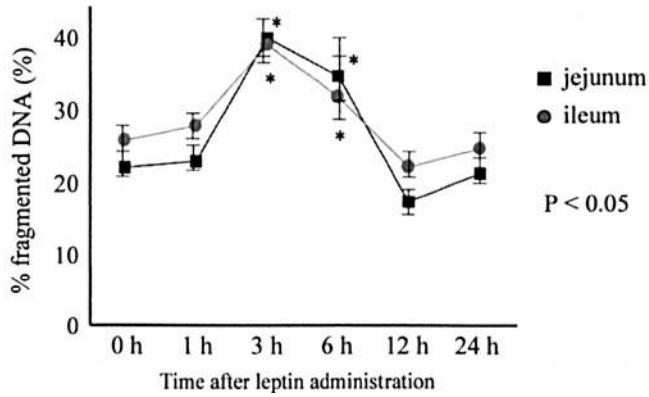

Figure 2. Time course of percent fragmented DNA in the rat small intestine after icv infusion of 8 μg leptin in 24-hr fasted rats. Jejunum: (■). Ileum: (●). Values are the mean ± SE. Six rats were studied in each group. *$P < 0.05$, compared with the corresponding value of the control rats.

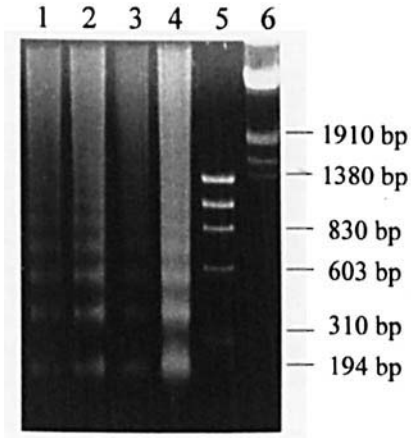

Figure 3. Agarose gel electrophoresis of fragmented DNA from the jejunal mucosa. A total of 20 μg fragmented DNA was loaded. (Lane 1) An *ad libitum* feeding rat before icv leptin. (Lane 2) 6 hrs after icv infusion of 8 μg leptin in an *ad libitum* feeding rat. (Lane 3) A 24-hr fasted rat before icv leptin. (Lane 4) 3-hr after icv infusion of 8 μg leptin in a 24-hr fasted rat. The DNA ladder characteristic of apoptosis is clearly found in each lane. Lanes 5 and 6 contain marker DNAs from a × 174 Hae Lamda EcoRIII digest (Wako, Japan), respectively.

though they were not useful for quantitative analysis because the same doses of fragmented DNA were loaded in each lane.

The immunohistochemical staining (TUNEL) of the jejunal mucosa in the tested groups is shown in Figure 4. A few apoptotic cells were observed at the villous tips in the *ad libitum* control group, whereas an increase in the number of apoptotic cells in the villous tips was seen 3 hrs after leptin icv administration. The same results were obtained from 24-hr fasted rats (data not shown).

The results of the western blotting analysis of caspase 3 in the jejunal mucosa are shown in Figure 5. In the 24-hr fasted rats, icv administration of 8 μg leptin induced 17kDa caspase-3 (active form of caspase-3) 3 hrs after the infusion. There was no significant difference in procaspase-3 after the leptin infusion. The increase in the caspase-3 protein was observed in *ad libitum* feeding rats 3 hrs after icv leptin infusion (data not shown).

The effect of ip leptin infusion on intestinal apoptosis was evaluated in 24-hr fasted rats. As indicated previously, 8 μg of leptin had no effect on intestinal apoptosis after ip infusion. As shown in Figure 6, 200 μg of leptin increased intestinal mucosal apoptosis after ip injection, but less than 200 μg leptin had no effect on the apoptosis after ip injection. The % fragmented DNA in the intestinal mucosa increased 6 hrs after ip infusion, which was delayed 3 hrs in comparison to the icv infusion. This delayed effect on apoptosis after ip leptin was also observed in *ad libitum* feeding rats (data not shown).

Discussion

Leptin was identified by Zhang *et al.* (1) as a peptide suppressing appetite and preventing obesity. Many reports have shown physiological and/or pharmacological functions

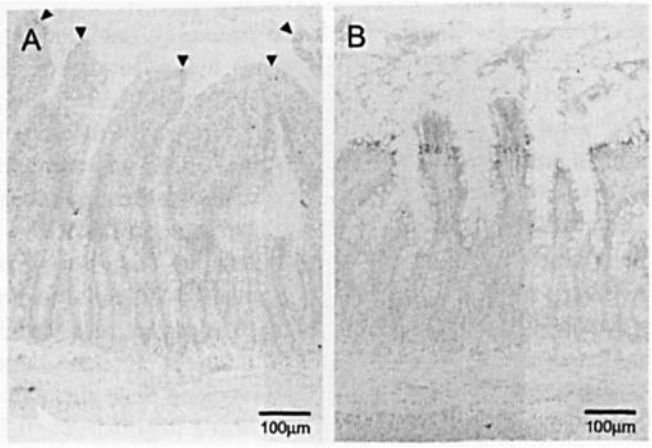

Figure 4. Light micrographs of jejunum stained by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. (A) *Ad libitum* feeding control rat. (B) *Ad libitum* feeding rat 3-hrs after icv infusion of 8 μg leptin. Magnification, × 50. In each intestinal segment, apoptotic nuclei are stained dark brown and non-apoptotic nuclei are stained light blue. Some apoptotic cells are observed in the villous tips in the control rat (arrows). In panel B, apoptotic enterocytes are prominently visible at the upper part of the villi.

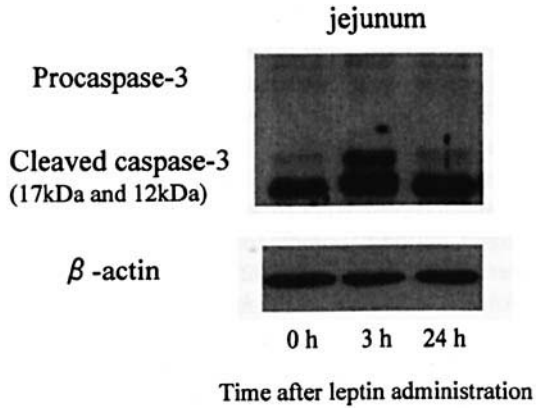

Figure 5. Processing of caspase-3 in the jejunal mucosa after icv infusion of leptin in 24-hr fasted rats. Expression of the 17kDa subunit of caspase-3 (active form of caspase-3) was amplified 3 hrs after icv infusion of leptin compared with the value before infusion. This activation returned to the control level at 24 hrs after the infusion.

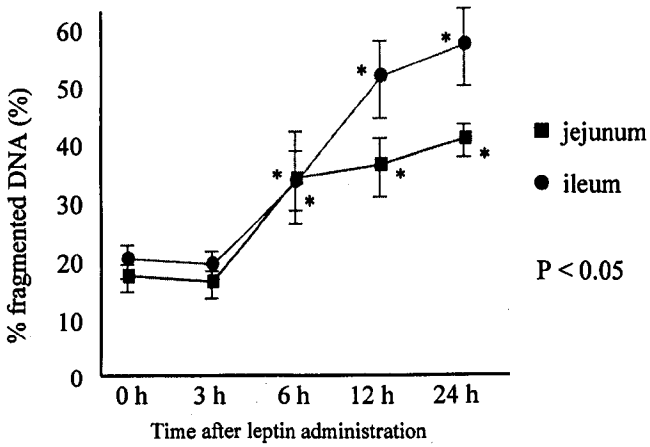

Figure 6. Time course of percent fragmented DNA in the small intestine after ip injection of 200 μg leptin in 24-hr fasted rats. Jejunum: (■). Ileum: (●). Values are the mean ± SE. Six rats were studied in each group. *$P < 0.05$, compared with the corresponding value of the control rats.

of the hormone (21), and the localization and distribution of its receptor (6). In this study, we demonstrated that leptin induced apoptosis in the rat intestinal mucosa mainly through the central nervous system. Several studies indicated that leptin suppressed feeding at a dose of 1 to 20 μg/rat after icv infusion (21–24) and approximately 100 μg/rat after ip injection (21, 22). In this study, icv infusion of leptin at a dose of 2 μg/rat had no effect on feeding behavior or intestinal apoptosis, and we infused 8 μg/rat of icv leptin in the experiments on intestinal apoptosis.

Leptin mRNA and/or protein has been identified in adipose tissue (25), placenta (26), fetal tissues (27), gastric mucosa (3, 4), and hepatic stellate cells (28). Leptin receptors are expressed in many tissues including the hypothalamus (29), small intestine (5), gastric mucosa (3, 4), liver (30), lung (31), kidney (31), muscle (31), adipose tissue (32), and so on. This peptide can participate in several physiological functions such as fetal growth (33), satiation (34), immune and/or proinflammatory responses (35), re-

production (36), nutrient intestinal absorption (5), angiogenesis (37), and lipolysis (38). The results in this study indicated that leptin induced intestinal apoptosis through the central nervous system, but there was a possibility that leptin had a direct effect on intestinal apoptosis. In this study, we did not show which nucleus leptin acted on, although other reports suggested that the arcuate nucleus, paraventricular nucleus, ventromedial nucleus, and lateral hypothalamus were important (39–42).

This study indicated that leptin might stimulate/suppress the central nervous system around the hypothalamus and the signal might pass via the efferent sympathetic nerve, but not via the parasympathetic nerve, to induce apoptosis in the intestinal mucosa. Eight μmol leptin induced intestinal apoptosis 3 to 6 hrs after icv injection, but had no effect after ip injection. Leptin induced apoptosis at 12 to 24 hrs following 200 μmol ip injection. These results suggested that leptin might induce apoptosis via the central nervous system. This effect was observed in both *ad libitum* feeding rats and 24-hr fasted rats, indicating that the apoptotic effect of leptin was independent of its effect on feeding behavior. A previous study reported that exogenous leptin exerted a potent gastro-protective action depending on vagal activity (43). Our previous study showed that the increase in ornithine decarboxylase activated by the central nerve system was suppressed by truncal vagotomy (10, 11, 44). Aizawa-Abe *et al.* (12) indicated that transgenic skinny mice (overproduction of leptin) controlled blood pressure and metabolism mainly via the sympathetic nerve. In this study, truncal vagotomy had no effect on leptin-induced apoptosis in the small intestine, suggesting that the sympathetic nerve might be important as an efferent nerve for intestinal apoptosis.

It is well known that leptin increases in the blood and cerebrospinal fluid after feeding (45), which might induce intestinal apoptosis during a postprandial period. This study showed that leptin induced intestinal apoptosis several hours after the injection. The concentration of leptin in the blood and cerebrospinal fluid decreases in a hunger period, which might suppress the intestinal apoptosis. We did not evaluate cell proliferation in this study, but Chaudhary *et al.* (46) indicated that leptin ip injection had no effect on the proliferation of intestinal mucosa in the mouse. However, they indicated that a decrease in villous area in the small intestine was observed after injection of leptin (46).

In conclusion, this study indicated that leptin induced apoptosis in the small intestine mainly through the central nervous system. Leptin is involved in the regulation of energy homeostasis partly via the regulation of intestinal apoptosis.

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