Yokohama, Shiro, Masashi Yoneda, Kimihide Nakamura, and Isao Makino. Effect of central corticotropin-releasing factor on carbon tetrachloride-induced acute liver injury in rats. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G622–G628, 1999.—Central neuropeptides play important roles in many instances of physiological and pathophysiological regulation mediated through the autonomic nervous system. In regard to the hepatobiliary system, several neuropeptides act in the brain to regulate bile secretion, hepatic blood flow, and hepatic proliferation. Stressors and sympathetic nerve activation are reported to exacerbate experimental liver injury. Some stressors are known to stimulate corticotropin-releasing factor (CRF) synthesis in the central nervous system and induce activation of sympathetic nerves in animal models. The effect of intracisternal CRF on carbon tetrachloride (CCl4)-induced acute liver injury was examined in rats. Intracisternal injection of CRF dose-dependently enhanced elevation of the serum alanine aminotransferase (ALT) level induced by CCl4. Elevations of serum aspartate aminotransferase, alkaline phosphatase, and total bilirubin levels by CCl4 were also enhanced by intracisternal CRF injection. Intracisternal injection of CRF also aggravated CCl4-induced hepatic histological changes. Intracisternal CRF injection alone did not modify the serum ALT level. Intravenous administration of CRF did not influence CCl4-induced acute liver injury. The aggravating effect of central CRF on CCl4-induced acute liver injury was abolished by denervation of hepatic plexus with phenol and by denervation of noradrenergic fibers with 6-hydroxydopamine treatment but not by hepatic branch vagotomy or atropine treatment. These results suggest that CRF acts in the brain to exacerbate acute liver injury through the sympathetic-noradrenergic pathways.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 280–320 g (Charles River Japan, Yokohama, Japan) were housed in group cages under conditions of controlled temperature (22–24°C) and illumination (12-h light cycle starting at 6:00 AM) for at least 7 days before experiments. Animals were maintained on laboratory chow and water. Experiments were performed in rats deprived of food for 12 h (starting at 6:00 PM) but given access to water up to the beginning of the study. Protocols describing the use of rats were approved by the Animal Care Committee of Asahikawa Medical College and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chemicals. The following substances were used: rat CRF (Peptide Institute, Osaka, Japan), CCl4 (Wako Pure Chemical Industries, Osaka, Japan), phenol (Wako), atropine methyl nitrate (Sigmar, St. Louis, MO), and 6-hydroxydopamine (6-OHDA; Aldrich, Milwaukee, WI). CRF was dissolved in 0.9% saline (pH 7.4) before the experiment and injected intracisternally in 10 µl using a 50-µl Hamilton microsyringe (Hamilton, Reno, NV).

Experimental design. After 12 h of fasting, rats were anesthetized with ether and mounted on ear bars of a stereotaxic apparatus (model 900, David Kopf Instruments, Tujunga, CA) and injected with CRF (0.5–20 µg) or saline intracisternally or intravenously just before and 6 h after CCl4 administration. CCl4 was mixed with an equal volume of olive oil and injected subcutaneously in a volume of 2 ml/kg.
We chose the dose and administration method for CCl₄ by pilot experiments, because 2 ml/kg of mixed solution of CCl₄ and olive oil injected subcutaneously induced mild and reproducible liver damage 24 h after CCl₄ in 12-h-fasted rats under our experimental conditions. Rats in the control group were injected with olive oil at a dose of 2 ml/kg. Rats were kept in individual cages, and blood samples were obtained from the jugular vein before and 24 h after CCl₄ administration. **P < 0.01 compared with respective control group.

Microscopic findings were photographed with color print films (Super G 200, Fuji Film, Tokyo, Japan), converted to digital signals by an image scanner (J X-330, Sharp Electric, Tokyo, Japan), and analyzed by a computer (Power Macintosh 8100, Apple Computer, Cupertino, CA) equipped with National Institutes of Health Image analyzer software. To exclude the effect of intracisternal injection of CRF on food intake, rats were pair fed with vehicle-treated rats.

Effect of hepatic plexus denervation, 6-OHDA, atropine, and hepatic branch vagotomy on CRF-induced modulation of acute liver injury by CCl₄. Either hepatic plexus denervation or vehicle treatment was performed under pentobarbital anesthesia (Abbott, North Chicago, IL; 50 mg/kg ip) 7 days before and 6 h after CCl₄ administration.
before the peptide injection, according to the method of Lautt (27). Denervation of hepatic plexus (anterior plexus and posterior plexus) was achieved rapidly (<20 min) by phenol (85%) applied to the region where the hepatic artery and the portal vein run in close apposition. 6-OHDA dissolved in saline was intraperitoneally injected twice (100 mg/kg on the first day, 80 mg/kg on the fourth day), and intracisternal injection of CRF was performed on the seventh day (50). Atropine methyl nitrate (0.15 mg/kg) dissolved in saline was injected intraperitoneally 30 min before the peptide injection in a 1.0 ml/kg volume. Either hepatic branch vagotomy or sham operation was performed under pentobarbital anesthesia (50 mg/kg ip) 72 h before the peptide injection. Hepatic branch vagotomy was achieved under a dissection microscope by selective section of the hepatic branch of the vagus nerve branching off from the anterior vagal trunk a few millimeters proximal to the cardia (49). To exclude the effect of hepatic plexus denervation, 6-OHDA, atropine methyl nitrate, and hepatic branch vagotomy on food intake, rats were pair fed with respective vehicle-treated or sham-operated rats.

Statistical analysis. All results are expressed as means ± SE. Comparison between two independent groups was made by Mann-Whitney U-test. Comparison of the values before and after CCl4 was made by paired Student’s t-test. Multiple group comparisons were performed by ANOVA followed by Fisher’s least significant difference test. P < 0.05 was considered statistically significant.

RESULTS

Effect of intracisternal CRF administration on CCl4-induced acute liver injury. Twenty-four hours after administration of CCl4 (2 ml/kg), the serum ALT level was 10.2 ± 0.3 U/L, which is significantly higher than the baseline level of 3.0 ± 0.2 U/L (P < 0.01). The result is consistent with previous studies (27, 28).

Table 1. Aggravating effect of intracisternal CRF injection on degeneration and necrosis in liver 24 h after CCl4 administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Degeneration and Necrosis Area, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>8.1 ± 1.9</td>
<td>5</td>
</tr>
<tr>
<td>CRF</td>
<td>33.5 ± 4.8*</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SE of n fields. Corticotropin-releasing factor (CRF; 10 µg) or saline was injected intracisternally just before and 6 h after CCl4 administration. Liver tissue was obtained 24 h after CCl4 and stained with hematoxylin and eosin. Five fields (×75 magnification) per slide were blindly evaluated under a light microscope, and degeneration and necrosis areas surrounded by fatty degeneration were measured by a computerized image analyzer. *P < 0.01 compared with saline-injected group.

Fig. 4. Effect of intracisternal CRF on CCl4-induced hepatic histological changes. Saline or CRF (10 µg) was injected intracisternally just before and 6 h after CCl4 (2 ml/kg) administration, liver tissues were obtained 24 h after CCl4 administration, and specimens were stained with hematoxylin and eosin. A: intracisternal saline injection (×75). B: intracisternal saline injection (×150). C: intracisternal CRF injection (×75). D: intracisternal CRF injection (×150).
was significantly elevated, from 6 ± 1 to 29 ± 8 IU/l (P < 0.01). Intracisternal administration of CRF (10 µg) just before and 6 h after CCl₄ injection enhanced the elevation of serum ALT levels induced by CCl₄, although intracisternal administration of CRF either just before or 6 h after CCl₄ did not influence serum ALT levels (Fig. 1). Intracisternal injection of CRF (just before and 6 h after CCl₄ injection) dose dependently enhanced the CCl₄-induced elevation of serum ALT levels (means ± SE, in IU/l: saline, 29 ± 8; 0.5 µg CRF, 38 ± 8; 1 µg CRF, 55 ± 11; 3 µg CRF, 85 ± 38; 5 µg CRF, 119 ± 36; 10 µg CRF, 140 ± 28; 20 µg CRF, 135 ± 39; n = 7–10; Fig. 2). Elevation of serum AST, ALP, and T-Bil levels induced by CCl₄ was also enhanced by intracisternal CRF injection (Fig. 3). Histological studies showed marked fatty degeneration (steatotic hepatocytes) with minimal necrosis (Fig. 4, A and B). Intracisternal CRF (10 µg) injection increased necrotic areas surrounded by fatty degeneration (Fig. 4, C and D, and Table 1). Intracisternal CRF (10 µg) injection alone did not influence serum ALT level when CRF was injected with olive oil vehicle (2 ml/kg sc) instead of CCl₄ (Table 2).

### Table 2. Effect of intracisternal CRF injection on serum ALT levels with olive oil treatment instead of CCl₄

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT, IU/l</th>
<th>Before</th>
<th>24 h After</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6 ± 1</td>
<td>29 ± 8</td>
<td>7 ± 1</td>
<td>4</td>
</tr>
<tr>
<td>CRF</td>
<td>7 ± 1</td>
<td>38 ± 8</td>
<td>119 ± 36</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SE of n fields. ALT, alanine aminotransferase. Instead of CCl₄, olive oil (2 ml/kg) was injected subcutaneously. CRF (10 µg) or saline was injected intracisternally just before and 6 h after olive oil administration. Blood samples were collected just before and 24 h after CCl₄ administration.

Instead of CCl₄, olive oil (2 ml/kg) was injected subcutaneously. CRF (10 µg) or saline was injected intracisternally just before and 6 h after olive oil administration. Blood samples were collected just before and 24 h after CCl₄ administration.

Fig. 5. Effect of hepatic plexus denervation (A), 6-hydroxydopamine (6-OHDA; B), hepatic branch vagotomy (C), and atropine methyl nitrate (D) on intracisternal CRF-induced enhancement of elevation of serum ALT levels (means ± SE) by CCl₄. Hepatic plexus denervation by 85% phenol was performed 7 days before, 6-OHDA was injected 7 days before (100 mg/kg) and 4 days before (80 mg/kg), hepatic branch vagotomy was performed 3 days before, and atropine methyl nitrate (0.15 mg/kg ip) was injected 30 min before CCl₄. Saline or CRF (10 µg) was injected intracisternally just before and 6 h after CCl₄ (2 ml/kg) administration. *P < 0.05, **P < 0.01 compared with respective control group.

### Table 3. Effect of intravenous injection of CRF on CCl₄-induced elevation of serum ALT level

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT, IU/l</th>
<th>Before</th>
<th>24 h After</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6 ± 1</td>
<td>37 ± 13</td>
<td>24 ± 5</td>
<td>6</td>
</tr>
<tr>
<td>CRF</td>
<td>6 ± 1</td>
<td>24 ± 5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SE of n fields. CRF (10 µg) or saline was injected intracisternally just before and 6 h after CCl₄ administration.
2). Intravenous administration of CRF (10 µg) did not influence the CCl₄-induced elevation of serum ALT level (Table 3).

Effect of hepatic plexus denervation, hepatic branch vagotomy, 6-OHDA, and atropine on intracisternal CRF-induced enhancement of acute liver injury by CCl₄. Denervation of hepatic plexus by 85% phenol (7 days before) or denervation of noradrenergic fibers by 6-OHDA intraperitoneal injection (100 mg/kg injected 7 days before and 80 mg/kg injected 4 days before) completely abolished the aggravating effect of intracisternal administration of CRF (10 µg) on the CCl₄-induced elevation of serum ALT level (Fig. 5, A and B) and the histological changes (Fig. 6). On the other hand, hepatic branch vagotomy (3 days before) or atropine methyl nitrate (0.15 mg/kg ip; 30 min before) did not influence the aggravating effect of intracisternal injection of CRF (10 µg) on the CCl₄-induced elevation of serum ALT level (Fig. 5, C and D).

DISCUSSION

In the present study, we demonstrated that intracisternal injection of CRF exacerbated CCl₄-induced acute liver injury in rats. We measured serum ALT, AST, T-Bil, and ALP levels and also examined hepatic histological changes. Intracisternal CRF dose dependently enhanced the CCl₄-induced elevation of serum ALT levels. Similarly, intracisternal CRF enhanced the CCl₄-induced elevation of serum AST, T-Bil, and ALP levels. The increase of serum ALT levels by intracisternal injection of CRF was dose related in doses ranging from 0.5 to 10 µg. Administration of 20 µg of CRF did not further enhance the CCl₄-induced increase of serum ALT level, indicating that the maximal effective dose of CRF injected intracisternally on CCl₄-induced liver injury is 10 µg. In contrast, when injected intravenously at the dose that was maximally effective when given intracisternally, CRF did not influence CCl₄-induced liver injury. These results indicate that CRF injected into the cisterna magna acts in the central nervous system to aggravate CCl₄-induced acute liver injury and not through leakage into the peripheral circulation (35). Intracisternal administration of CRF (10 µg) alone just before and 6 h after olive oil vehicle administration instead of CCl₄ did not influence serum ALT level, suggesting that CRF does not have any ability to cause liver injury by itself. Although intracisternal injection of CRF (10 µg) just before and 6 h after CCl₄ administration aggravated CCl₄-induced acute liver injury, CRF injection either just before or 6 h after CCl₄ administration did not influence the liver injury. These results indicate that continuous stimulation by CRF is essential to enhance CCl₄-induced acute liver injury. This effect of central CRF on liver injury is supported by previous reports that showed CCl₄-induced liver injury was aggravated by continuous stress for 6 h (22).

The pathways through which central administration of CRF enhanced CCl₄-induced acute liver injury were investigated. Previous reports have shown that central CRF affects peripheral organs in part through the autonomic nervous system (46). In regard to the digestive system, central CRF inhibits gastric secretion and motility and exocrine secretion of the pancreas through the sympathetic-noradrenergic nervous systems (2, 14, 30, 44). Meanwhile, central CRF stimulates colonic motility through the parasympathetic nervous system (32, 34). In the present study, the enhancement of CCl₄-induced acute liver injury by intracisternal injection of CRF was completely abolished by denervation of hepatic plexus by phenol and by 6-OHDA pretreatment, whereas hepatic branch vagotomy or atropine methyl nitrate treatment had no effect. Because the treatment of hepatic plexus with phenol is known to dominantly denervate the hepatic sympathetic nerve and because 6-OHDA treatment chemically depletes noradrenergic nerve fibers via biosynthetic adrenergic intermediates (27, 50), these results indicate that CRF acts in the brain to enhance CCl₄-induced acute liver injury in rats through the sympathetic-noradrenergic nervous systems.

The pathophysiological effect of stressors and the autonomic nervous system on the liver has been reported. Some stressors and enhancement of the sympathetic nervous activity exacerbate experimental liver injury (11, 20–23). Recently, it has been shown that
Some physiological stressors increase CRF mRNA expression and CRF immunoreactivity in the hypothalamus and amygdala, which are important sites for the sympathetic nervous system (15, 16, 26) and that endogenous CRF regulates stress-induced alternation of the gastrointestinal functions through the autonomic nervous system (2, 31, 33, 43, 45, 51). In this study, we have investigated the relationship between central CRF and hepatic pathophysiological changes and demonstrated that CRF acts in the central nervous system and exacerbates CCl4-induced acute liver injury through the sympathetic-noradrenergic nervous systems in rats. This is further supported by previous reports that indicate that stimulation of the hypothalamus and activation of sympathetic nerves aggravate experimental liver injury (20, 21, 23). It is of interest to investigate the role of endogenous CRF in stress-induced aggravation of liver injury using a potent CRF antibody or antagonist in an experimental stress model.

CCl4 is a well-known hepatotoxic chemical. The main cause of liver injury by CCl4 is free radicals of its metabolites. Cleavage of the CCl3-Cl bond by superoxide (O2•−) is thought to proceed via the microsomal cytochrome P-450 reductase and NADPH-dependent reductive pathways. Formation of free radicals may cause lipid peroxidation and subsequent membrane injury (36). Decrease in hepatic blood flow is suggested to be one of the important factors in aggravation of experimental liver injury induced by stimulation of the hepatic sympathetic nerve (23, 24). Oxygen strongly inhibits the hepatic cytochrome P-450-mediated formation of free radicals from CCl4, and CCl4-induced liver injury is protected against hyperbaric oxygen in vitro and in vivo studies (6, 7). It may be suggested that activation of sympathetic nerves by central CRF decreases hepatic blood flow and reduces oxygen supply to hepatocytes, inducing aggravation of CCl4-induced liver injury. Because stimulation of sympathetic nerves decreases activity of superoxide dismutase (SOD), which is the scavenger of free radicals (19), activation of sympathetic nerves by central CRF also may induce activation of free radicals in the liver, eliciting aggravation of liver injury.

The liver is known to be richly innervated (8, 9, 18, 37, 40), and abundant evidence indicates important roles of the central and autonomic nervous system in hepatic function (1, 3, 12, 13, 17, 25, 27, 39). Very little is known about the central neuropeptides involved in the modulation of hepatic function (10, 52, 54, 55). In the present study, we have found that central administration of CRF induces the enhancement of CCl4-induced acute liver injury through the sympathetic-noradrenergic pathways and have speculated that CRF acts in the brain as a neurotransmitter to induce central modulation of experimental acute liver injury. In a previous study, we have also shown that central thyrotropin-releasing hormone enhances hepatic blood flow (48) and hepatic proliferation (53) and protects against CCl4-induced acute liver injury through the vagal-cholinergic pathways in rats (38). It is very interesting that several neuropeptides act in the central nervous system and control physiological and pathological regulation of the liver through the different autonomic nervous pathways.

In conclusion, the present study indicates that CRF injected intracisternally acts in the brain to induce a potent enhancement of CCl4-induced acute liver injury in rats. The peptide action is mediated through the sympathetic-noradrenergic pathways. Central injection of CRF provides a useful tool to further investigate brain sites that influence sympathetic regulation of liver injury. It is also of interest to study the role of endogenous neuropeptide in stress-induced aggravation of liver injury by using CRF antagonists or antibodies.

This research was supported in part by Ministry of Education, Science, and Culture of Japan Grants-in-Aid 0767554 and 09670503. Address for reprint requests and other correspondence: M. Yoneda, Div. of Gastroenterology, Medical Center, International University of Health and Welfare, Kitakamamoru 2600-6, Otawara 324-0011, Japan (E-mail: yoneda@iwhw.ac.jp). Received 31 March 1998; accepted in final form 5 November 1998.

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