Involvement of endogenous CRF in carbon tetrachloride-induced acute liver injury in rats

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Received 23 August 2001; accepted in final form 22 January 2002

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 200–240 g (Charles River Japan, Yokohama, Japan) were housed in group cages

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under conditions of controlled temperature (22–24°C) and illumination (12-h light cycle starting at 6 AM) for at least 7 days before experiments. Animals were maintained on laboratory chow and water. Before the experiment, rats were deprived of food for 24 h but given free 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 in accordance with the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.”

**Chemicals.** The following substances were used: a CRF receptor antagonist, α-helical CRF-(9–41) (Sigma, St. Louis, MO), MO (Wako Pure Chemicals, Osaka, Japan), phenol (Wako), atropine methyl nitrate (Sigma), 6-hydroxy dopamine (6-OHDA; Aldrich, Milwaukee, WI). α-helical CRF-(9–41) was dissolved in 0.9% saline (pH 7.4) before the experiment and injected intracisternally in a 10 μl volume using a 50-μl Hamilton microsyringe (Hamilton, Reno, NV).

**Experimental design.** After 24 h of fasting, rats were anesthetized with ether and mounted on ear bars of a stereotaxic apparatus (Kopf model 900, David Kopf Instruments, Tujunga, CA) and injected with α-helical CRF-(9–41) (0.125–5 μg) or saline intracisternally or intravenously through the jugular vein 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 moderate and reproducible liver injury 24 h after CCl4 in 24-h-fasted rats under our experimental conditions. Rats in the control group were injected with olive oil at a volume of 2 ml/kg. Rats were kept in individual cages, and blood samples were obtained before and 24 h after CCl4 administration from the jugular vein. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined by commercially available kits (Wako). The liver sample was removed from the hepatic median lobe 24 h after CCl4 administration and fixed in 10% formalin solution. The specimens were stained with hematoxylin and eosin. Five fields per each slide at ×75 magnification were blindly evaluated under a light microscope. Percentage of the necrotic areas surrounded by fatty degeneration (33) was measured by a computerized image analyzer. Microscopic findings were photographed with color print films (Super G 200, Fuji Film, Tokyo, Japan), converted to digital signals by an image scanner (JX-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 α-helical CRF-(9–41) on food intake, rats were pair fed with vehicle-treated rats.

To confirm an antagonistic effect of α-helical CRF-(9–41), the antagonist (2 μg) was injected intracisternally 5 min before intracisternal injection of CRF(10 μg) in CCl4-administered rats. Rats were fasted for 12 h before CCl4 (2 mg/kg) injection to obtain mild liver injury, and CRF was intracisternally injected just before and 6 h after CCl4. Liver injury was assessed by serum ALT level 24 h after CCl4.

**Effects of hepatic plexus denervation, 6-OHDA, atropine, and hepatic branch vagotomy on α-helical CRF-(9–41)-induced modulation of acute liver injury by CCl4.** Either hepatic plexus denervation or vehicle treatment was performed on animals under pentobarbital sodium anesthesia (Abbott, North Chicago, IL; 50 mg/kg ip) 7 days before the peptide injection, according to the method of Lautt (16). 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 (100 mg/kg on the 1st day, 80 mg/kg on the 4th day), and intracisternal injection of α-helical CRF-(9–41) was performed on the seventh day (32). Atropine methyl nitrate (0.15 μg/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 sodium anesthesia (50 mg/kg ip) 72 h before the peptide injection. Hepatic branch vagotomy was achieved 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 under a dissection microscope. To exclude the effect of hepatic plexus denervation, 6-OHDA, and hepatic branch vagotomy on food intake, rats were pair fed with respective vehicle-treated or sham-operated rats.

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

**RESULTS**

**Effect of intracisternal CRF receptor antagonist α-helical CRF-(9–41) on CCl4-induced liver injury.** Administration of CCl4 (2 mg/kg) induced an elevation of serum ALT level from 6 ± 1 to 330 ± 21 IU/l 24 h after CCl4 in 24-h-fasted rats (P < 0.01). Intracisternal administration of CRF receptor antagonist α-helical CRF-(9–41) (2 μg) both just before and 6 h after CCl4 injection reduced the elevation of serum ALT level induced by CCl4, although either intracisternal single injection of α-helical CRF-(9–41) just before or 6 h after CCl4 did not influence serum ALT level (Fig. 1). Intracisternal administration of α-helical CRF-(9–41) (just...
before and 6 h after CCl4 injection) dose dependently reduced the CCl4-induced elevation of serum ALT level in doses ranging from 0.25 to 2 μg (mean ± SE, IU/l): saline, 330 ± 21; 0.125 μg, 342 ± 13; 0.25 μg, 246 ± 22; 0.5 μg, 223 ± 19; 1 μg, 181 ± 20; 2 μg, 137 ± 15; 5 μg, 139 ± 3; n = 5–7; Fig. 2). Elevation of serum AST induced by CCl4 was also dose dependently reduced by intracisternal α-helical CRF-(9–41) injection (Fig. 3).

Histological studies showed marked centrilobular necrosis and fatty degeneration (steatotic hepatocytes) (Fig. 4). Intracisternal α-helical CRF-(9–41) (2 μg) injection decreased necrotic areas surrounded by fatty degeneration (Fig. 4 and Table 1). Intracisternal α-helical CRF-(9–41) (2 μg) injection alone did not influence serum ALT level when α-helical CRF-(9–41) was injected with olive oil vehicle (2 ml/kg sc) instead of CCl4 (Table 2). Intravenous administration of α-helical CRF-(9–41) (2 μg) did not influence the CCl4-induced elevation of serum ALT level (Table 3).

Intracisternal injection of CRF (10 μg) aggravated CCl4-induced liver injury, and preinjection of α-helical CRF-(9–41) (2 μg) completely abolished these effects of CRF (Table 4).

Effect of hepatic plexus denervation, 6-OHDA, atropine, and hepatic branch vagotomy on serum ALT level 24 h after CCl4 administration in response to intracisternal α-helical CRF-(9–41). Denervation of hepatic plexus by 85% phenol (7 days before) or denervation of noradrenergic fibers by 6-OHDA intraperitoneal injection (100 mg/kg, 7 days before and 80 mg/kg ip, 4 days before) by itself partially reduced the elevation of serum ALT level 24 h after CCl4 administration, but the serum ALT level was still abnormally high in rats with these pretreatments (Fig. 5, A and B). Intracisternal injection of α-helical CRF-(9–41) did not induce any improvement on the elevated serum ALT level in rats with hepatic plexus denervation or 6-OHDA pretreatment (Fig. 5, A and B). 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 effect of intracisternal injection of α-helical CRF-(9–41) (2 μg).
Table 1. Degenerative and necrotic areas in the liver 24 h after CCl4 administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Degeneration and Necrosis Area, % n</th>
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</thead>
<tbody>
<tr>
<td>Saline</td>
<td>69 ± 1 5</td>
</tr>
<tr>
<td>α-Helical CRF-(9-41)</td>
<td>41 ± 3* 5</td>
</tr>
</tbody>
</table>

Values are means ± SE of n fields. Corticotropin-releasing factor (CRF) antagonist, α-helical CRF-(9-41) (2 μg), or saline was injected intracisternally just before and 6 h after carbon tetrachloride (CCl4) administration. The 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 the degeneration and necrosis areas surrounded by fatty degeneration were measured by a computerized image analyzer. *P < 0.01 vs. saline.

(9–41) on the CCl4-induced elevation of serum ALT level (Fig. 5, C and D).

DISCUSSION

In the present study, we demonstrate that the CRF receptor antagonist α-helical CRF-(9–41) injected intracisternally lessens CCl4-induced acute liver injury in conscious rats assessed by serum ALT and AST levels and by liver histology. The reduction of CCl4-induced serum ALT and AST level elevation by intracisternal α-helical CRF-(9–41) was dose related in doses ranging from 0.25 to 2 μg. Administration of 5 μg of α-helical CRF-(9–41) did not further inhibit the CCl4-induced increase of serum ALT and AST levels, indicating that the maximal effective dose of α-helical CRF-(9–41) injected intracisternally on CCl4-induced liver injury is 2 μg and the maximal effect was a 58 and 71% reduction of serum ALT and AST, respectively. In contrast, when injected intravenously at the dose that was maximally effective when given intracisternally, α-helical CRF-(9–41) did not influence CCl4-induced liver injury. These results indicate that α-helical CRF-(9–41) injected into the cisterna magna acts in the central nervous system to lessen CCl4-induced acute liver injury, although not through leakage into the peripheral circulation. Intracisternal administration of α-helical CRF-(9–41) (2 μg) alone just before and 6 h after olive oil vehicle administration instead of CCl4 did not influence serum ALT level, suggesting that α-helical CRF-(9–41) does not have any ability to influence serum ALT level by itself. Although intracisternal injection of α-helical CRF-(9–41) (2 μg) both just before and 6 h after CCl4 administration lessened CCl4-induced acute liver injury, α-helical CRF-(9–41) injection only just before or 6 h after CCl4 administration did not influence it. These results indicate that continuous or intermittent blocking of central CRF action by α-helical CRF-(9–41) is essential to lessen CCl4-induced acute liver injury.

The pathways through which central administration of α-helical CRF-(9–41) lessened CCl4-induced acute liver injury were investigated in this study. Previous reports showed that central CRF affects peripheral organs in part through the autonomic nervous system (29). In regard to the digestive system, central CRF inhibits gastric secretion and motility and excocrine secretion of the pancreas through the sympathetic-noradrenergic nervous system and the central CRF receptor antagonist partially reverses these effects (1, 17, 28). Meanwhile, we recently demonstrated that intracisternal injection of CRF aggravates CCl4-induced acute liver injury through the sympathetic-noradrenergic nervous system (33). In the present study, the effect of intracisternal α-helical CRF-(9–41) was abolished by denervation of the hepatic plexus by phenol and 6-OHDA pretreatment, whereas hepatic branch vagotomy or atropine methyl nitrate treatment had no effect. The treatment of hepatic plexus with phenol is known to dominantly denervate the hepatic sympathetic nerve and 6-OHDA treatment chemically depletes noradrenergic nerve fibers via biosynthetic adrenergic intermediates (16, 32). Chemical sympathectomy by 85% phenol or noradrenergic nerve de
ternal injection of α-helical CRF-(9–41) (2 μg) was injected intracisternally just before and 6 h after CCl4 administration. Blood samples were collected just before and 24 h after CCl4 administration.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT, IU/I n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before 24 h After</td>
</tr>
<tr>
<td>Saline</td>
<td>6 ± 1 5</td>
</tr>
<tr>
<td>α-Helical CRF-(9-41)</td>
<td>5 ± 1 5</td>
</tr>
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</table>

Values are means ± SE of n fields. ALT, alanine aminotransferase. Instead of CCl4, olive oil (2 ml/kg) was injected subcutaneously. α-Helical CRF-(9-41) (2 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 olive oil administration.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT, IU/I n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before 24 h After</td>
</tr>
<tr>
<td>Saline</td>
<td>6 ± 1 5</td>
</tr>
<tr>
<td>α-Helical CRF-(9-41)</td>
<td>6 ± 1 5</td>
</tr>
</tbody>
</table>

Values are means ± SE of n fields. CRF antagonist, α-helical CRF-(9–41) (2 μg), or saline was injected intracisternally just before and 6 h after CCl4 administration. Blood samples were collected just before and 24 h after CCl4 administration. *P < 0.01 vs. vehicle.

Table 4. Effect of intracisternal α-helical CRF on intracisternal CRF-induced aggravation of liver injury by CCl4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT (IU/ml) n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before 24 h After</td>
</tr>
<tr>
<td>Vehicle sc + Saline ic + Saline ic</td>
<td>6 ± 2 4</td>
</tr>
<tr>
<td>CCl4 sc + Saline ic + Saline ic</td>
<td>48 ± 8* 4</td>
</tr>
<tr>
<td>CCl4 sc + CRF ic + Saline ic</td>
<td>151 ± 30* 4</td>
</tr>
<tr>
<td>CCl4 sc + CRF ic + α-helical CRF ic</td>
<td>52 ± 12* 4</td>
</tr>
</tbody>
</table>

Values are means ± SE of n fields. CRF (10 μg) or saline was injected intracisternally (ic) just before and 6 h after CCl4 administration. CRF antagonist, α-helical CRF (2 μg), or saline was injected intracisternally 5 min before the each CRF injection. Blood samples were collected just before and 24 h after CCl4 administration. *P < 0.01 vs. vehicle.
vation by 6-OHDA by itself incompletely reduced CCl4-induced elevation of serum ALT level in the present study, indicating that sympathetic and noradrenergic nerve tone may play a role in aggravating CCl4-induced acute liver injury. These findings are very consistent with a previous report that indicated that chemical sympathectomy improved CCl4-induced liver injury in spontaneously hypertensive rats in which the sympathetic nerve tone is thought to be activated (12). Although chemical sympathectomy and noradrenergic nerve denervation lessened CCl4-induced liver injury by ~50% assessed by serum ALT level, serum levels 24 h after CCl4 in rats with these pretreatments were still abnormally high compared with vehicle treatment. However, intracisternal injection of α-helical CRF-(9–41) did not induce any improvement on the elevated serum ALT level in these rats, indicating that the partially reducing effect of central α-helical CRF-(9–41) on serum ALT was at least in part mediated through the sympathetic-noradrenergic nervous system. From these findings, it is suggested that during CCl4-induced liver injury the sympathetic tone is activated, resulting in aggravation of the liver injury and endogenous CRF in the brain may play a role in the activation of the sympathetic tone.

The pathophysiological effect of stressors and the autonomic nervous system on the liver has been reported. Some stressors or enhancement of the sympathetic nervous activity exacerbate experimental liver injury (6, 11–13, 33). It has been shown that some physiological stressors increase CRF mRNA expression and CRF immunoreactivity in the hypothalamus and amygdala (8, 15) and endogenous CRF regulates stress-induced alteration of the gastrointestinal functions through the autonomic nervous system (1, 18, 20). In this study, we investigated a role of endogenous CRF in hepatic pathophysiological regulations using CRF receptor antagonist α-helical CRF-(9–41) and demonstrated that α-helical CRF-(9–41) acts in the central nervous system and lessens CCl4-induced acute liver injury at least partially through the sympathetic-noradrenergic nervous system in rats. These findings establish a pathophysiological role of endogenous CRF in the brain in experimental liver injury. Because the sick condition induced by CCl4 liver injury can be a stress for animals and may stimulate brain CRF synthesis resulting in sympathetic-noradrenergic activation, it is of interest to investigate CRF mRNA expression in the brain after CCl4 administration.
CRF nerve fibers and receptors are widely distributed in the central nervous system (4), and the site of action of CRF on experimental liver injury remains to be investigated because microinjection of CRF into the specific brain nuclei was not performed. In the present study, the dose of α-helical CRF-(9–41) to induce a maximal effect is relatively low compared with previous studies (1, 18, 20), and we injected the antagonist into the cisterna magna, which is close to the medulla. Therefore, it can be suggested that the site of action for CRF antagonist is near the medulla, because CRF nerve terminals and receptors are located in the nuclei in this area (4).

CRF mediates its actions through activation of specific seven-transmembrane domain receptors, which are coupled to a guanine nucleotide stimulatory factor signaling protein, resulting in increased intracellular cAMP levels (3). To date, two CRF receptor subtypes, designated CRF1 and CRF2 receptors, have been identified through molecular cloning from distinct genes in the rat and human (3, 19). CRF2 receptor is located on brain neurons, whereas the CRF2 receptor is found in nonneuronal brain tissue and in the periphery (19, 24). We found that intracisternal injection of urocortin, endogenous CRF2 receptor agonist, aggravates CCl4-induced liver injury, suggesting an involvement of CRF2 receptor in the brain (34).

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·−) probably proceeds via the microsomal cytochrome P-450 reductase and NADPH-dependent reductive pathways. Formation of free radicals may cause lipid peroxidation and subsequent membrane injury (25). A decrease in hepatic blood flow is suggested as one of the important factors in aggravation of experimental liver injury induced by stimulation of hepatic sympathetic nerve (13). Because central injection of CRF decreases hepatic blood flow through the sympathetic nerve (22), it may be suggested that activation of sympathetic nerves by central endogenous CRF decreases hepatic blood flow and reduces oxygen supply to hepatocytes, resulting in aggravation of CCl4-induced injury, and intracisternal injection of α-helical CRF-(9–41) abolishes these events.

The liver injury induced by CCl4 in this study was severe compared with that in our previous study (33). The difference of the study protocol between the present study and our previous study was the duration of fasting state. Because in the pilot study we found that severity of liver injury induced by CCl4 was partially dependent on fasting time, we chose a longer fasting duration (24 h) than that of the previous study (12 h) to induce relatively severe liver injury in the present study.

Because some hepatotoxic agents have been reported to stimulate the medullary nuclei (10) and several cytokines in the liver are thought to play important roles in experimental liver injury (14, 23), it is of interest to study an effect of central neuropeptides on the expression of these cytokines in the liver.

The liver is known to be richly innervated, and there is abundant evidence that indicates important roles of the central and autonomic nervous system in hepatic function (7, 9, 16, 26). Although a little is revealed about central neuropeptides as a neurotransmitter inducing modulation of hepatic function (5, 31, 33–37), nothing is known about the role of endogenous neuropeptides in hepatic physiological and pathophysiological regulations. In the present study, we found that central administration of CRF receptor antagonist induces a partial hepatic cytoprotection against experimental liver injury through sympathetic-noradrenergic pathways and speculated that endogenous CRF acts in the brain as neurotransmitter to induce central modulation of experimental acute liver injury.

In summary, the present study indicates that a CRF receptor antagonist injected intracisternally acts in the brain to induce a partial hepatic cytoprotection at least partially through sympathetic-noradrenergic pathways. These findings provide the first evidence for a role of endogenous neuropeptides in the central nervous system in hepatic pathophysiological regulations.

This work was supported in part by the grant-in-aid for Scientific Research from Japan Society for the Promotion of Science (No. 12670512).

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