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Involvement of calcitonin gene-related peptide and capsaicin-sensitive afferents in central thyrotropin-releasing hormone-induced hepatic cytoprotection

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Abstract

The involvement of capsaicin-sensitive afferent neurons and calcitonin gene-related peptide (CGRP) in central thyrotropin-releasing hormone (TRH)-induced hepatic cytoprotection was investigated in rats. Both systemic capsaicin pretreatment and intravenous administration of CGRP receptor antagonist, human CGRP-(8–37), completely abolished the protective effect of intracisternal TRH analog (RX-77368; p-Glu-His-(3,3′-dimethyl)-Pro-NH_2, 5 ng) against carbon tetrachloride (CCl_4)-induced acute liver injury, assessed by serum alanin aminotransferase levels and histological changes. These data demonstrate the involvement of capsaicin-sensitive afferent neurons and CGRP in central TRH-induced hepatic cytoprotection.

Keywords: Neuropeptide; Vagus; Autonomic nervous system; Hepatic cytoprotection

1. Introduction

Neuropeptides acts in the central nervous system as a neurotransmitter or neuromodulator to regulate gastrointestinal functions through the autonomic nervous system (Taché et al., 1990). We have recently found that several neuropeptides act in the brain to control hepatobiliary physiological and pathophysiological regulations in animal models (Nakade et al., 2002; Yokohama et al., 1999; Yoneda et al., 1997a,b, 2001). In particular, central thyrotropin-releasing hormone (TRH) plays a variety of roles in the hepatic physiological functions through vagal-cholinergic pathways. We have recently found that intracisternal injection of TRH analog enhances hepatic blood flow and protects against experimental acute liver injury through nitric oxide synthesis as well as vagal-cholinergic pathways in rats (Sato et al., 2003; Tamori et al., 1998).

In the rat skin microvasculature, acetylcholine-induced vasodilation involves both nitric oxide and capsaicin-sensitive components (Ralevic et al., 1992). Anatomic and functional observations are consistent with the possibility that hepatic cytoprotection resulting from central vagal-cholinergic activation may also involved the recruitment of capsaicin-sensitive afferent neurons. First, capsaicin-sensitive primary sensory neurons, which contain calcitonin gene-related peptide (CGRP), innervate the liver (Goehler and Sternini, 1996). Second, the involvement of CGRP and capsaicin-sensitive afferent neurons in central TRH-induced gastric cytoprotection has been reported (Kato et al., 1994, 1996). Finally, we have recently found that CGRP and capsaicin-sensitive afferents play a role in central TRH analog-induced hepatic hyperemia in rats (Tamori et al., 1999).

These lines of evidence have prompted us to examine a possible involvement for CGRP and capsaicin-sensitive afferent neurons in central TRH-induced hepatic cytoprotection. Therefore, in this study, the effect of capsaicin pretreatment and CGRP receptor antagonist on a hepatic protection induced by intracisternal injection of TRH analog

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was investigated in carbon tetrachloride (CCl_4)-induced acute liver injury model of rats.

2. Materials and methods

The following chemicals were used: a stable TRH analog, RX-77368 (p-Glu-His-(3,3′-dimethyl)-Pro-NH_2; Reckitt and Colman, Kingston-upon-Hill, UK), human CGRP-(8–37), CGRP receptor antagonist (Peptide Institute, Osaka, Japan), capsaicin (Sigma, St. Louis, MO), CCl_4 (Sigma).

Male Wistar rats (200–240 g, Charles River Japan, Yokohama, Japan) were housed in group cages under condition of controlled temperature (22–24 °C) and illumination (12-h light cycle starting at 6 a.m.) for at least 7 days before experiments. After a 24-h fasting, rats were anesthetized with ether and mounted on ear bars of stereotaxic apparatus (Kopf model 900, David Kopf Instruments, Tujunga, CA), and RX-77368 (5 ng) or 0.9% saline vehicle was injected intracisternally. The doses of TRH analog were chosen based on our previous study (Sato et al., 2003). Rats were regained light reflex within 4 min and returned to their home cages. The accuracy of the intracisternal injection was ascertained by the aspiration of cerebrospinal fluid before and after the injection. Sixty minutes after the peptide injection, CCl_4 or vehicle was injected subcutaneously. CCl_4 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 CCl_4 based on the our previous study, because 2 ml/kg of mixed solution of CCl_4 and olive oil injected subcutaneously induced moderate and reproducible acute hepatocellular necrosis 24 h after CCl_4 in 24-h-fasted rats under our experimental conditions (Sato et al., 2003). Rats in the control group were injected with olive oil at a volume of 2 ml/kg. CGRP receptor antagonist, human CGRP-(8–37) (128 nmol/kg), was intravenously injected just before and 4 h after the intracisternal injection of the TRH analog. Rats were kept in individual cages, and blood samples were obtained from the jugular vein before and 24 h after CCl_4 administration. Serum alanine aminotransferase levels were determined by commercially available kits (Wako, Osaka, Japan). Rats were

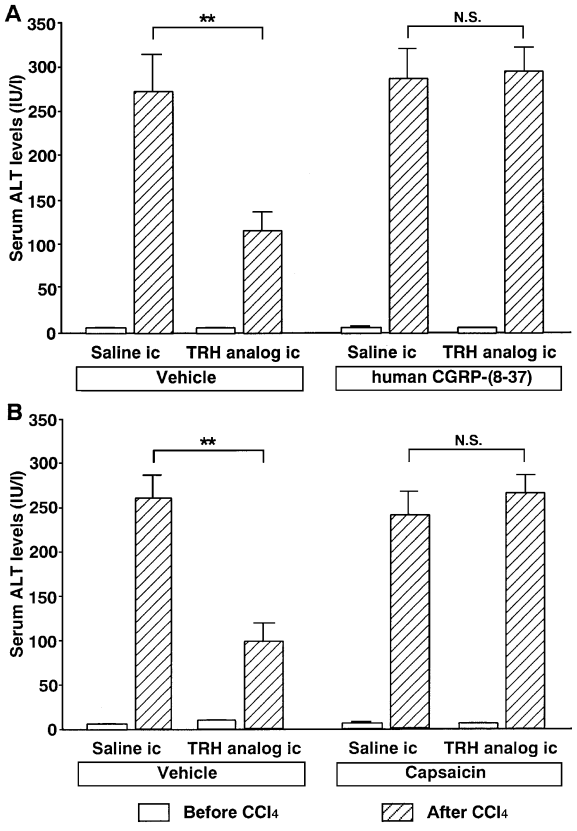

Fig. 1. Effect of calcitonin gene-related peptide (CGRP) receptor antagonist, human CGRP-(8–37) (A) and capsaicin pretreatment (B) on hepatic cytoprotection induced by intracisternal injection of TRH analog, RX-77368, assessed by serum alanine aminotransferase (ALT) levels. TRH analog (5 ng) or saline was intracisternally (i.c.) injected 60 min before subcutaneous administration of CCl_4 (2 ml/kg). Serum ALT levels were determined before and 24 h after the CCl_4 administration. Human CGRP-(8–37) (128 ng/kg) was injected just before and 4 h after the intracisternal injection of the TRH analog. Capsaicin was subcutaneously injected 10–14 days before the experiment. Each column represents mean ± S.E. $^{**}P<0.01$, N.S.: not significantly different.

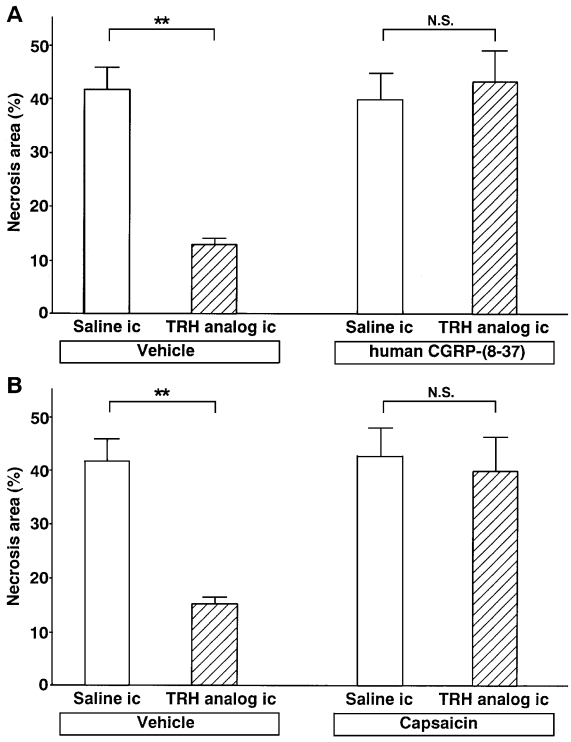

Fig. 2. Effect of calcitonin gene-related peptide (CGRP) receptor antagonist, human CGRP-(8–37) (A) and capsaicin pretreatment (B) on hepatic cytoprotection induced by intracisternal injection of TRH analog, RX-77368, assessed by histological changes. The liver sample was obtained 24 h after CCl_4 administration and stained with hematoxylin and eosin. Percentage of the necrotic area was measured by a computerized image analyzer. For details, see Fig. 1. Each column represents mean ± S.E. $^{**}P<0.01$, N.S: not significantly different.

sacrificed by CO_2 inhalation, and the liver sample was obtained from the hepatic median lobe 24 h after CCl_4 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 (Nakade et al., 2002; Sato et al., 2003; Yokohama et al., 1999) 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.

In another experiment, the protective effect of intracisternal injection of the TRH analog was studied in systemic capsaicin-pretreated rats. Capsaicin treatment was performed 10 to 14 days before the experiment. Capsaicin was dissolved in absolute ethanol, Tween 80, and 0.9% saline (10:10:80 vol/vol/vol), and injected subcutaneously under ether anesthesia at three times at 12-h intervals (25, 50, and 50 mg/kg, respectively). The control group received the same regimen of injection except that the vehicle instead of capsaicin was injected. Effect of capsaicin on afferent nerve was confirmed by the disappearance of the corneal chemosensory reflex to a drop of a 0.1% NH_4OH.

Protocols describing the use of rats were approved by the Animal Care Committee of Dokkyo University School of Medicine and Asahikawa Medical College, and in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*.

All results are expressed as means ± S.E. Comparison of the values between before and after CCl_4 was calculated by Wilcoxon signed-rank test. Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. A P value <0.05 was considered statistically significant.

3. Results

Administration of CCl_4 (2 mg/kg) induced an elevation of serum alanin aminotransferase levels from 6 ± 0 to 272 ± 42 IU/l 24 h after the injection. Intracisternal administration of TRH analog, RX-77368, at a dose of 5 ng lessened the elevation of serum alanin aminotransferase levels induced by CCl_4 (Fig. 1). This protective effect of the TRH analog against CCl_4-induced acute liver injury was abolished by intravenous administration of human CGRP-

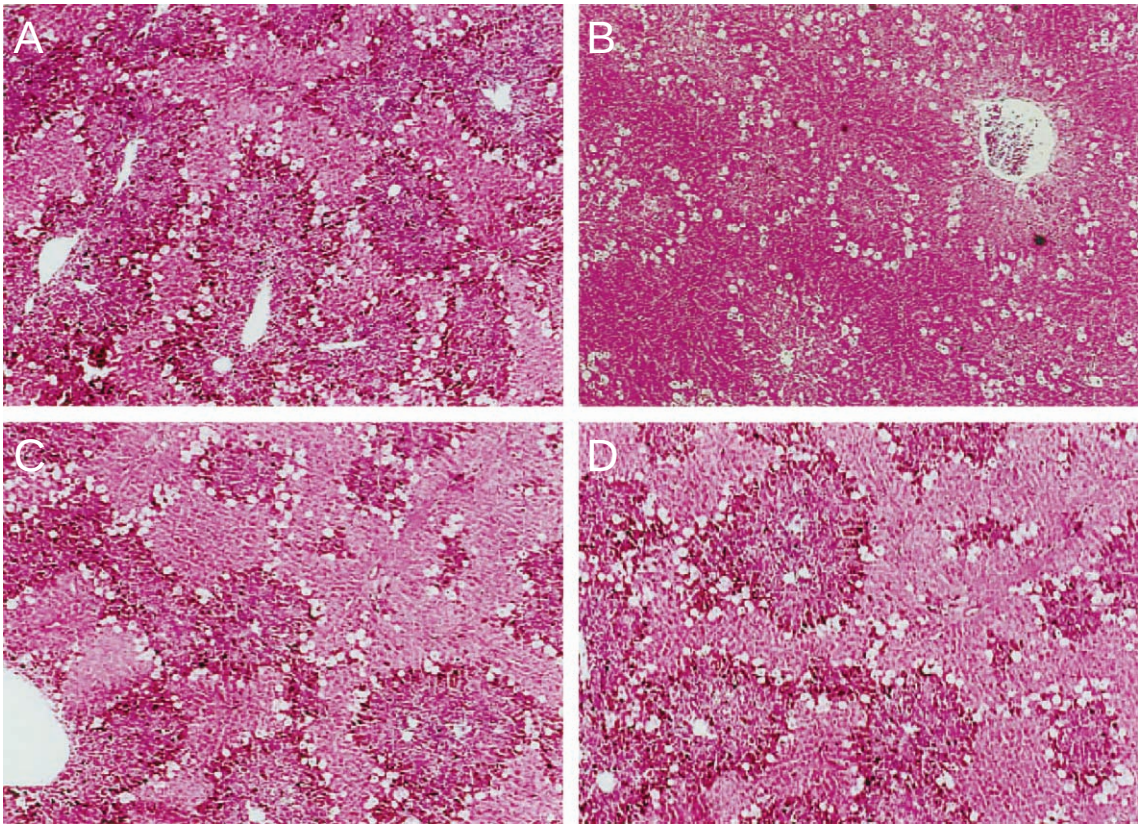

Fig. 3. Hepatic histological changes 24 h after CCl_4 administration. The liver sample was obtained 24 h after CCl_4 administration and stained with hematoxylin and eosin (×75). Intracisternal saline + intravenous vehicle injected group (A), intracisternal RX 77368 (5 ng) + intravenous vehicle injected group (B), intracisternal saline + intravenous human CGRP-(8–37) injected group (C), intracisternal RX 77368 (5 ng) + intravenous human CGRP-(8–37) injected group (D).

(8–37) (Fig. 1). Intravenous administration of human CGRP-(8–37) per se did not modify CCl_4-induced liver injury (observation in intracisternal saline injection animals; Fig. 1). The antagonistic effect of human CGRP-(8–37) against CCl_4 liver injury was also ascertained by hepatic histological change, i.e. necrosis areas surrounded by fatty degeneration (Figs. 2 and 3).

In rats pretreated with vehicle for capsaicin, subcutaneous administration of CCl_4 induced an elevation of serum alanin aminotransferase levels from 7 ± 0 to 256 ± 20 IU/l. Although intracisternal administration of the TRH analog (5 ng) inhibited the elevation of serum alanin aminotransferase levels induced by CCl_4 in vehicle-pretreated rats, this protective effect of the central TRH analog against CCl_4-induced acute liver injury was not observed in capsaicine-pretreated rats. The antagonistic effect of capsaicin pretreatment was also confirmed by the hepatic histological change (Fig. 2). Capsaicin pretreatment on its own did not influenced CCl_4-induced liver injury (observation in intracisternal saline injection animals; Fig. 1).

4. Discussion

Intracisternal injection of the stable TRH analog RX-77368 at 5 ng induced a cytoprotective effect against CCl_4-induced acute liver injury as assessed by serum alanin aminotransferase levels and histology in rats. Likewise, our previous report indicates that RX-77368 injected into the cisterna magna at 5 ng induced a maximal protective effect against experimental acute liver injury (Sato et al., 2003). Several evidence demonstrated that the hepatic cytoprotective effect in response to central injection of TRH is mediated by vagal efferent cholinergic pathways. First, injection of TRH or TRH analog into the cerebrospinal fluid stimulates efferent activity of the vagal nerve (O-Lee et al., 1997; Somiya and Tonoue, 1984). Second, hepatic branch vagotomy completely abolished the hepatic protection induced by TRH analog injected centrally (Sato et al., 2003). Finally, peripheral administration of muscarinic antagonist, atropine, completely suppressed the hepatic protection induced by central injection of TRH analog (Sato et al., 2003).

CCl_4 is a well-known hepatotoxic chemical. The main cause of acute hepatocellular necrosis by CCl_4 is free radicals of its metabolites. Cleavage of the CCl_3–Cl bond by superoxide (O_2^-) 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 (Popper, 1988). Oxygen strongly inhibits the hepatic cytochrome *P*-450-mediated formation of free radicals from CCl_4, and CCl_4-induced injury is protected by hyperbaric oxygen in vitro and in vivo studies (Burk et al., 1984, 1986). It is conceivable that central TRH lessens CCl_4-induced acute hepatocellular necrosis through an increase in hepatic blood flow because intracisternal injection of TRH analog increases hepatic blood flow through nitric oxide-dependent pathways (Tamori et al., 1998) and this stimulated hepatic blood flow may increase oxygen supply to hepatocyte. This hypothesis is supported by previous study that showed that nitric oxide improved microcirculation and protected against ethanol-induced acute hepatocellular necrosis in perfused rat livers. (Oshita et al., 1994).

Several peptides exert a vasodilatory effect; however, CGRP is established to be one of the most potent vasodilators of vascular beds in many organs including the hepatobiliary system (DiPette et al., 1987; Fletcher et al., 1990). Calcitonin gene-related peptide is composed of 37 amino acids, the structure of which has been predicted on the basis of alternative processing of the primary transcript of rat calcitonin gene (Rosenfeld et al., 1983). Immunohistochemical analysis revealed a wide distribution of CGRP-like immunoreactivities in the central and peripheral nervous systems, including hepatobiliary system (Goehler and Sternini, 1996). In the present study, intravenous infusion of CGRP receptor antagonist, human CGRP-(8–37), completely inhibited the protective effect of intracisternal injection of TRH analog against CCl_4-induced acute liver injury, suggesting that peripheral CGRP plays a role in this protective effect induced by central TRH. These findings are very consistent with that in gastric cytoprotection in response to intracisternal injection of TRH analog (Kato et al., 1994, 1996). We administered CGRP receptor antagonist in a same dose as previously reported by Kato et al. (1994), and we confirmed that intravenous administration of CGRP receptor antagonist by itself does not affect hepatic injury.

In the present study, the involvement of capsaicin-sensitive afferent neurons in central TRH-induced hepatic cytoprotection was also investigated by systemic ablation of capsaicin-sensitive afferent neurons. Then, we found that systemic capsaicin pretreatment completely abolished the protective effect of intracisternal injection of TRH analog against CCl_4-induced liver injury, such as that observed in acute effect of CGRP receptor antagonist on central TRH-induced hepatic cytoprotection. Capsaicin acutely evokes and stimulates release of CGRP from afferent nerve terminals, and chronically diminishes CGRP immunoreactive nerve fibers after overstimulation of CGRP release inducing exhaustion of CGRP content (Goehler and Sternini, 1996; Holzer et al., 1990; Suzuki et al., 1997). Although the mechanisms responsible for releasing CGRP from capsaicin-sensitive afferents after intracisternal injection are not known, recent study by Adelson et al. (1999) shows intracisternal injection of TRH analog induces an excitation of gastric splanchnic afferent neurons. Afferent neurons of hepatic vagal or sympathetic nerve can be stimulated by central injection of TRH analog and it is of interest to confirm this phenomenon by electrophysiologic techniques. In our previous study, nitric oxide synthase inhibitor prevents central TRH-induced hepatic cytoprotection (Sato et

al., 2003), suggesting a possible interaction between nitric oxide and CGRP in the action of central TRH to induce hepatic cytoprotection. From our present results together with the previous report that presented the involvement of CGRP and capsaicin-sensitive afferent neurons in central TRH-induced hepatic hyperemia and gastric protection (Kato et al., 1994, 1996; Tamori et al., 1999), the hepatic protective effect in response to central vagal activation induced by intracisternal TRH analog may be mediated by local effector function of capsaicin-sensitive afferent neurons releasing CGRP (Holzer, 1988).

As far as we know, direct effect of capsaicin and CGRP on experimental liver injury has not been evaluated, and it is of interest to investigate whether acute administration of capsaicin and CGRP protect against liver injury. However, systemic acute administration of capsaicin and CGRP may affect variety of physiological regulations. Therefore, it might be better to inject them intraportally. To resolve this problem, it is important to establish different animal model, in which intraportal administration of capsaicin and CGRP is feasible.

In conclusion, our present studies suggest that CGRP-containing and capsaicin-sensitive afferent nerves are involved in the hepatic cytoprotection induced by central TRH, and that central vagal stimulation can activate the ''efferent function'' of capsaicin-sensitive afferent neurons in the liver.

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