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INCREASED PANCREATIC ACINAR CONTENT AND SECRETION OF CATIONIC TRYPSINOGEN FOLLOWING 30-DAY CONTINUOUS ETHANOL INTOXICATION IN RATS*

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Abstract—The effects of sustained, high blood alcohol levels (216 ± 120 mg/100 ml, S.D.) for 30 days on cholecystokinin (CCK)-mediated pancreatic exocrine function were studied in a rat model that achieves both maximally controlled, optimal nutrition and high alcohol intake (~40.5% of total calories). In alcohol-fed rats, basal plasma levels of immunoreactive cationic trypsinogen (ICT) were reduced by 50% ($P < 0.05$), but intravenous doses (0–30 IDU/kg/hr; 1 IDU = ~62.5 ng CCK-8) of cholecystokinin octapeptide (CCK-8) resulted in a 3-fold greater maximal concentration of ICT and an 80% steeper slope of the dose–response curve compared to those of pair-fed control animals. Basal plasma levels of amylase were not different in the two groups at basal conditions and did not change significantly following CCK-8 administration. *In vitro* studies with isolated pancreatic acini have shown that basal secretion of ICT into the media was similar in the two groups. However, ICT secretion in response to CCK-8 (30–3000 pM) was 2-fold greater in alcohol-fed rats than in pair-fed controls, resulting in a CCK-8 EC_{50} which was about half that of controls. On the contrary, the basal and maximal amylase secretion from acini isolated from alcohol-fed rats was reduced by 67 and 43%, respectively, causing a reduction in the magnitude of the response curve with almost identical EC_{50} and slopes. Despite the marked alterations in CCK-stimulated enzyme secretion, radioiodinated CCK-33 binding to receptors on acini isolated from both control and alcohol-fed rats was similar. Cellular concentrations of ICT and amylase, however, revealed similar patterns of alterations: 2 to 3-fold increase in ICT and 70% reduction in amylase in alcoholic acini compared to controls. These results indicate that the inverse changes in amylase and ICT secretions following continuous ethanol administration are probably due to differential effects on enzyme synthesis.

Cholecystokinin (CCK) is a major gastrointestinal hormone which regulates both secretion [1, 2] and synthesis [3, 4] of pancreatic exocrine enzymes. Ethanol has been shown to exert an acute inhibitory effect on CCK-stimulated pancreatic secretion in dogs [5] and in humans [6]. The binding of CCK to acinar receptors is inhibited *in vitro* by high concentrations of ethanol in a dose-dependent manner, and this inhibition correlates with an ethanol-induced reduction in CCK-stimulated amylase secretion [7]. On the other hand, chronic alcohol intake augments the pancreatic sensitivity to CCK stimulation in dogs [8] as well as in humans [9, 10]. Of different secretory proteins examined, trypsinogen levels have been shown to increase in the pancreatic secretion of rats chronically given ethanol [11–13] and of human alcoholics [10, 14]. In addition, the injection of secretin produced a significantly greater response of serum trypsin-like immunoreactivity in human alcoholics than in controls [15]. However, a biochemical or physiological mechanism for these increased exocrine and plasma responses of proteolytic enzymes in alcoholic subjects is currently unknown. In particular, effects of chronic alcohol intoxication on CCK-mediated pancreatic exocrine function including that on the CCK binding to acinar receptors have not been characterized in detail.

In the current study, effects of chronically sustained blood alcohol levels on the following CCK-mediated functions were investigated: (1) *in vivo* response patterns of plasma amylase and immunoreactive cationic trypsinogen (ICT) levels to increasing doses of CCK-8; (2) *in vitro* acinar secretory responses of these enzymes to increasing doses of CCK-8; and (3) CCK binding to its specific receptors on acini. For this purpose, we have employed a rat model that achieves high alcohol intake and sustained blood alcohol levels and which simultaneously provides maximally controlled, optimal nutrition [16].

METHODS

Animal model. Details of the model have been described elsewhere, including the cannulation pro-

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cedure [17], the dietary regimen [18], and the blood alcohol levels achieved [16]. Central venous and gastrostomy cannulas were aseptically implanted in male Wistar rats (350–400 g). Double gastrostomy cannulas were employed for the independent continuous intragastric infusion of a liquid diet and ethanol solution. Single gastrostomy cannulas were used in pair-fed controls to infuse an isocaloric diet in which ethanol was replaced by dextrose. The use of spring coils and swivels allowed the protection of the cannulas and the free movement of animals in individual metabolism cages. The low fat liquid diet originally described by Thompson and Reitz [19] was used. In this diet, protein was provided by lactalbumin hydrolysate, carbohydrate by dextrose, and fat by corn oil. Caloric contributions of ethanol and macronutrients at an ethanol dose level of 8 g/kg/day were: 25% of the total calories as protein, 5% as fat, 38% as carbohydrate, and 32% as ethanol. The ethanol intake was progressively increased to 40.5% of total calories by raising the concentration of ethanol infused in order to maintain blood alcohol levels between 100 and 300 mg/100 ml over a period of 30 days. Optimal nutrition achieved in this model has been supported by the steady and normal weight gains of both alcoholic (15.4 ± 1.9 g/week) and pair-fed controls (15.6 ± 0.8 g/week) as reported previously [16, 18].

In vivo *experiment*. Following 30 days of alcohol intoxication, five pairs of awake rats were infused in a stepwise manner with secretin (15 CU/kg/hr); 1 CU = ~300 ng secretin) and graded doses of CCK-8 (0, 1, 5, 15, 30 IDU/kg/hr; 1 IDU = ~62.5 ng octapeptide CCK-8) through chronically implanted central venous cannulas over 30-min periods. CCK-8 (E. R. Squibb & Sons, Princeton, NJ) was reconstituted with 0.15 M NaCl containing 0.1% rat serum albumin to give a concentration of 2 μg/ml, and stored as a stock solution at −76°. Secretin (Research Plus, Inc., Bayonne, NJ) was prepared in the same manner as a 2 μg/ml stock solution and stored at −76°. Immediately before an experiment, aliquots of stock solutions were thawed and diluted to the appropriate concentrations with 0.15 M NaCl containing 0.1% rat serum albumin. During the experiment, intragastric infusion of a liquid diet and ethanol (or isocaloric dextrose solution) was interrupted. A mixed solution of secretin and CCK-8 was infused at a constant rate of 3 ml/hr using a Harvard syringe pump (Harvard Apparatus, South Natick, MA). Prior to the start of the experiment and at the end of each 30-min interval, 0.5-ml samples of heparinized blood were obtained via the venous cannulas and centrifuged for separation of plasma, which was stored at −76° until assayed for ICT and amylase.

In vitro *experiment*. Another five pairs of rats were killed following 30 days of continuous ethanol administration. Animals were infused with the diet plus ethanol or glucose up to the time they were killed. Pancreatic tissues were removed quickly, and acini were isolated by the enzymatic digestion of the pancreas as described by Williams *et al.* [20]. After 30 min of preincubation, the separate aliquots of acini were stimulated with increasing concentrations of CCK-8 (0.03, 0.06, 0.1, 0.3, 0.6, 1.0, 3.0 nM) in the media as previously described [20, 21]. Aliquots of the media were saved, following stimulation by each concentration of CCK-8, for ICT and amylase determination. The pellets obtained from 1-ml aliquots of acinar suspension were sonicated in 1 ml of distilled water and analyzed for protein, DNA, amylase, and cationic trypsinogen concentrations.

CCK binding to the acinar receptors. CCK binding to acini was determined by employing biologically active [^{125}I]CCK-33 prepared by the method of Sankaran *et al.* [21, 22]. Porcine CCK-33 (>99% pure) was obtained from Dr. Viktor Mutt, Gastrointestinal Hormone Laboratory, Karolinska Institute, Stockholm, Sweden. Since in our isolated acini, CCK-8 and CCK-33 have identical potencies in enzyme secretion and binding characteristics, we employed CCK-8 for competitive inhibition of tracer CCK-33 binding. Tracer CCK-33 (10 pM) was incubated with 1.5 ml of suspension of acini in the absence and presence of various concentrations of CCK-8 for 30 min at 37°. At the end of incubation, the hormone bound to acini was separated from the free hormone by microcentrifugation, and after two washes the radioactivity in the pellet was measured in a gamma counter.

Enzyme and chemistry assays. The radioimmunoassay methodology for measuring ICT in rat plasma and acinar media and pellet samples was similar to that described previously [23]. Purification of rat cationic trypsinogen was described previously [24]. Rabbit antisera to rat cationic trypsinogen was used at a final dilution of 1:200,000. An immobilized form of goat anti-rabbit IgG (Immunobeads, Bio-Rad Laboratories, Richmond, CA) was used to separate bound and free labeled antigen following 24 hr of incubation at 15°. Plasma amylase levels were determined as activity on an ACA Dupont Automatic Clinical Analyzer using a micro sample technique. Amylase concentrations in the media and the acinar pellets were measured by the method of Jung [25] using α-amylase (Sigma, St. Louis, MO) as an external standard to convert the catalytic activities to the amounts of the protein in a semi-quantitative manner. Total protein and DNA concentration in acini were determined by the method of Bradford [26] and that of Hinegardner [27] respectively.

Statistical analysis and curve fitting. All data are expressed as means ± S.E. The statistical significance between sample means was assessed with the paired *t*-test. The dose–response curves were logistically fitted and analyzed simultaneously for maximal and basal responses, ED_{50} (EC_{50}), and curve slope by a computer program (written in Pascal for Apple II by Dr. C. Johnson, Department of Pharmacology, University of Cincinnati) originally described by De Lean *et al.* [28].

RESULTS

In vivo *plasma responses of ICT and amylase to CCK-8 and secretin*. The basal plasma levels of amylase were not different in alcoholic and control rats (1234 ± 51 and 1138 ± 126 units/dl respectively) and were not altered significantly by the infusion of secretin and CCK-8. However, the basal ICT levels of alcoholic rats were significantly ($P < 0.05$) lower than that of controls (4.1 ± 0.7 vs 8.9 ± 1.1 ng/ml)

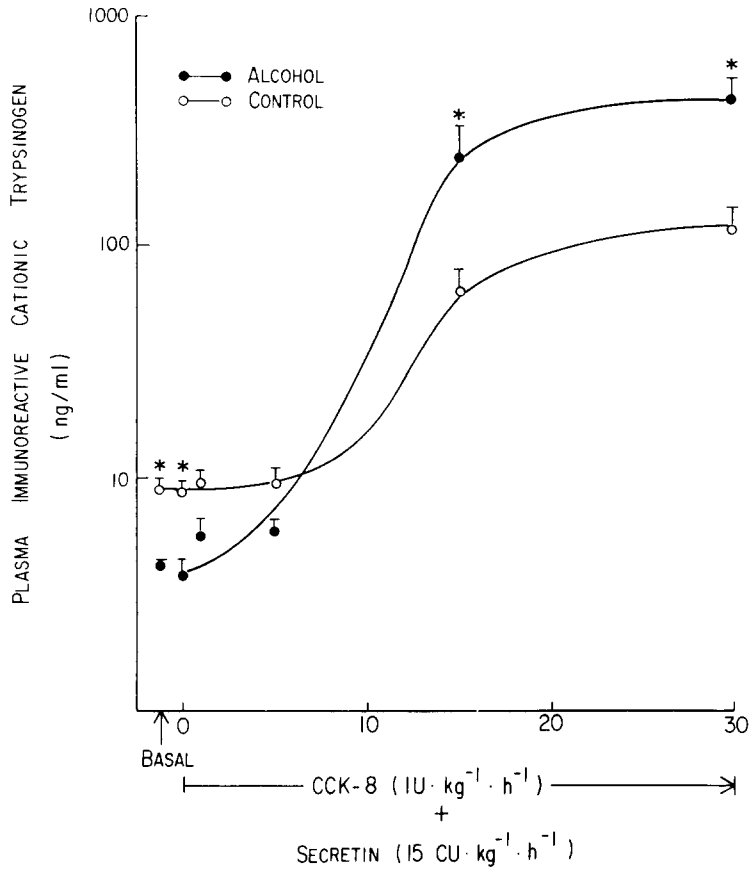

Fig. 1. Effects of continuous ethanol intoxication on the *in vivo* plasma response of immunoreactive cationic trypsinogen to secretin/CCK-8. Following 30 days of ethanol administration, five pairs of awake rats were infused intravenously with secretin (15 CU/kg/hr) and stepwise increasing doses of CCK-8 (0–30 IDU/kg/hr) at 30-min intervals. Prior to the start of the infusion and at the end of each interval, blood samples were obtained and analyzed for ICT levels. An asterisk indicates a significant difference from corresponding control values ($P < 0.05$).

(Fig. 1). Infusion of secretin alone did not alter the ICT levels in either group (the second data points from basal levels), whereas the response of plasma ICT to CCK-8 was dose dependent and significantly enhanced in alcoholic rats compared to that of controls, resulting in 3-fold greater maximal response and 80% steeper slope of the curve as shown in Table 1.

Blood alcohol levels determined in the alcoholic rats just prior to the CCK-8 and secretin infusion ranged from 50.3 to 338.0 mg/100 ml. This variation in the blood alcohol level is similar to that we previously observed and is due to the cyclical pattern of blood alcohol levels [16]. No correlation was observed between the blood alcohol levels and basal or stimulated plasma levels of enzymes.

Table 1. Comparisons of curve parameters

	In vivo plasma response of ICT to secretin/CCK-8		*In vitro** Acinar Secretory Response of ICT to CCK-8		*In vitro** Acinar Secretory Response of Amylase to CCK-8	
	Control	Alcoholic	Control	Alcoholic	Control	Alcoholic
Basal level	8.9 (ng/ml)	4.1 (ng/ml)	0.34 (μg)	0.48 (μg)	6.60 (μg)	2.16 (μg)
Maximal response	144.6 (ng/ml)	453.2 (ng/ml)	1.15 (μg)	1.96 (μg)	19.45 (μg)	11.03 (μg)
ED_{50} or EC_{50}	16.9 (IDU)	14.5 (IDU)	155.0 (pM)	70.0 (pM)	46.4 (pM)	47.8 (pM)
Curve slope	2.67	4.72	1.45	1.74	1.76	1.80

* All values of ICT and amylase secretion are expressed as μg per mg of acinar protein.

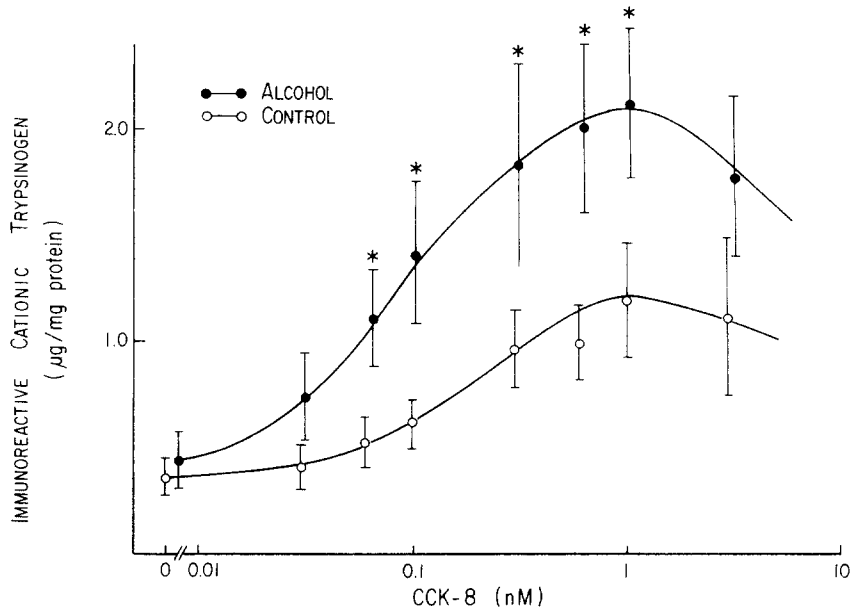

Fig. 2. Effects of continuous ethanol administration on the *in vitro* secretory response of ICT to CCK-8. After 30 days of continuous ethanol administration, another five pairs of rats were killed, and pancreatic acini were isolated. Isolated acini were incubated at 37° in the absence or presence of increasing concentrations of CCK-8 (0–3 nM). An asterisk indicates a significant difference from control values ($P < 0.05$).

In vitro *acinar secretory responses of ICT and amylase to CCK-8*. Protein concentrations per 100 μg of DNA were not significantly different in alcoholic and control acini (1.37 ± 0.43 and 1.39 ± 0.30 mg/100 μg of DNA respectively). Therefore, all data for acinar enzyme release and concentration were standardized per mg of protein concentration.

Blood alcohol levels determined prior to the isolation of pancreatic acini had a range similar to that seen at the time of the *in vivo* CCK-8/secretin experiment. These blood alcohol levels had no apparent effects on the *in vitro* acinar parameters examined in this study. The basal secretion of ICT by isolated acini was similar in the two groups (Fig. 2 and Table 1). The CCK-8-stimulated ICT secretion

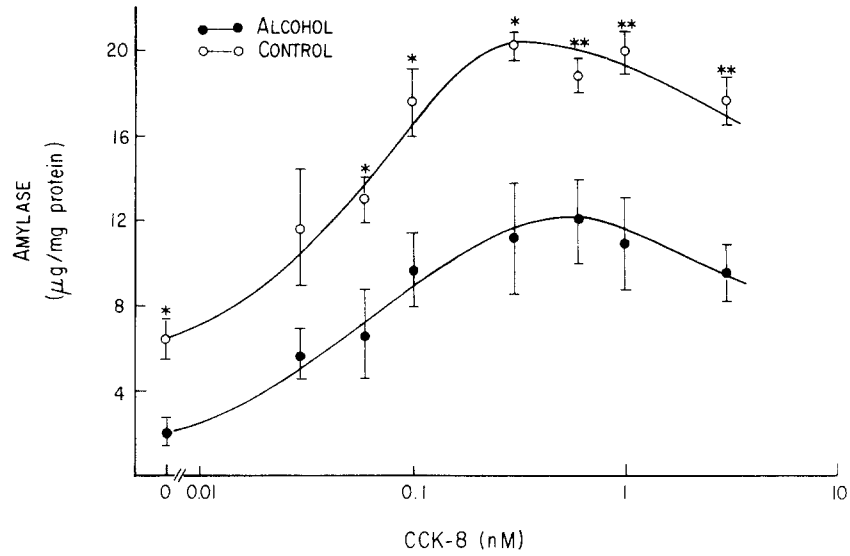

Fig. 3. Effects of continuous ethanol administration on the *in vitro* secretory response of amylase to CCK-8. The response curve of acini isolated from alcohol-fed rats was shifted downward, resulting in about a 50% reduction in maximal response but maintaining a similar CCK-8 EC_{50} and curve slope. Asterisks indicate a significant difference when compared to corresponding control values: (*) $P < 0.05$ and (**) $P < 0.01$.

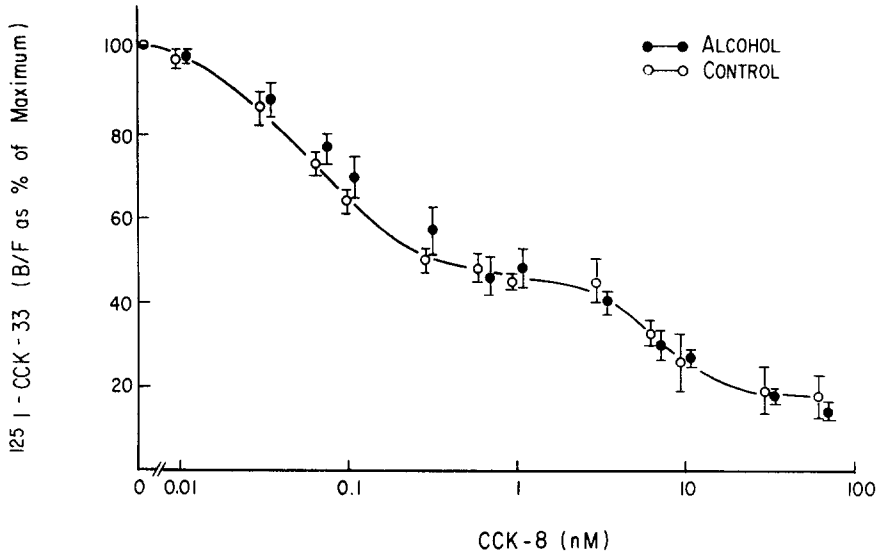

Fig. 4. Effects of continuous ethanol administration on the competitive inhibition of [^{125}I]CCK-33 binding to isolated rat acini by CCK-8. Tracer CCK-33 (10 pM) was incubated with acini suspension in the absence or presence of various concentrations of CCK-8 at 37° for 30 min. At the end of incubation, the hormone bound to acini was separated from the free hormone by microcentrifugation, and after two washes the radioactivity in the pellet was measured in a gamma counter.

from alcoholic acini was significantly greater than that of controls with a 2-fold higher maximal response, resulting in an EC_{50} which was about half that of controls (70 vs 155 pM). The stimulated amylase secretion, however, was quite the opposite (Fig. 3 and Table 1). The basal secretion of amylase from acini of alcohol-fed animals was reduced to one-third that of controls. The maximal response was also reduced to half that of controls. As a result, the amylase dose–response curve of acini isolated from alcohol-fed rats was reduced significantly in magnitude with almost identical EC_{50} and slopes.

Enzyme concentrations in pancreatic acini and CCK binding. Concentrations of amylase and ICT in pancreatic acini are shown in Table 2. They show similar patterns of alterations as those of acinar secretory responses: the amylase content was reduced significantly to a level which represented about 30% that of controls; and the ICT content revealed a significant 2 to 3-fold increase in acini isolated from alcohol-fed rats compared to that in controls. The competitive inhibition by CCK-8 of [^{125}I]CCK-33 binding to isolated rat acini is shown in Fig. 4. There was no statistical difference between the two competitive inhibition curves, suggesting no alterations in the binding characteristics of CCK to its specific receptors.

Table 2. Acinar concentrations of ICT and amylase

	ICT (μg/mg protein)	Amylase (μg/mg protein)
Control	8.0 ± 3.6	131.7 ± 54.0
Alcoholic	18.3 ± 10.4*	37.0 ± 26.2*

* Significantly different from control values ($P < 0.05$).

DISCUSSION

At present, *ad lib.* consumption of an ethanol liquid diet is widely used to investigate the effects of alcohol in the rat [29]. However, with this type of pair-feeding, control rats consume the more palatable control liquid diet rapidly, resulting in relative starvation compared to ethanol-fed rats [12]. As shown in previous studies [30, 31], the amount and temporal pattern of nutrient intake are major factors influencing pancreatic enzyme secretion. In fact, we have shown recently [32] that pancreatic acini isolated from rats pair-fed the control diet exhibited an abnormal pattern of CCK-stimulated enzyme synthesis similar to that observed previously in fasted animals [4]. In addition, weight gains of rats fed the ethanol or control liquid diet have been shown to be 30% less than those of rats fed chow *ad lib.* [12]. There is also inevitable variability in the amount of alcohol and diet voluntarily consumed by rats. In the present study, we have employed the continuous intragastric infusion of ethanol and a liquid diet to induce a high degree of uninterrupted alcohol intoxication as well as to provide maximally controlled, optimal nutritional intake. Using this model, continuously high blood alcohol levels of 216 ± 120 mg/100 ml (S.D.) and simultaneous optimal growth similar to that of chow-fed rats have been achieved [16, 17].

The present study has demonstrated a significant potentiation of CCK-8-induced plasma response of ICT in the alcoholic rats. The maximal plasma levels achieved by intravenous infusion of CCK-8 plus secretin in these animals were 3-fold higher than those observed in pair-fed controls. Studies on *in vitro* acinar secretory function were performed to determine whether functional alterations at the cellu-

lar level correlated with the plasma response observed *in vivo*. Use of isolated pancreatic acini has permitted detailed characterization of both acinar enzyme secretion and CCK binding. CCK-8-stimulated secretion of ICT from acini obtained from alcoholic rats showed a 2-fold alteration coordinate with that observed for *in vivo* plasma response. This finding strongly suggests that the higher levels of circulating ICT in alcoholic rats during CCK-8 administration were due to an increased plasma entry of this enzyme from the pancreas. Interestingly, this magnitude of increase in ICT secretion was very similar to that found for pancreatic trypsinogen in rats fed an ethanol liquid diet [12] and that observed in pancreatic secretion of human chronic alcoholics [14].

Since the *in vitro* basal secretion of ICT was similar to that of controls, and the difference in ICT secretion was found only after CCK-8 stimulation, it appears that CCK-8-regulated mechanisms are responsible for the observed difference. CCK binding studies revealed that there was no significant difference in the binding of tracer CCK-33 to receptors on acini obtained from both groups. Furthermore, the competitive inhibition curves obtained for both groups were identical, suggesting no apparent changes in either the affinity or the capacity of CCK-8 receptors to bind CCK. Since CCK-regulated biological functions, especially enzyme secretion, have been shown to be mediated by CCK interaction with its receptors on acini [21], it is likely that CCK-manipulated membrane phenomena or responses are not the factors responsible for the differences observed for CCK-stimulated ICT and amylase secretion from acini. This interpretation is further supported by the fact that CCK-stimulated amylase secretion from alcoholic acini did not show an increase parallel to that of ICT but rather had a marked decrease.

The finding that the changes in secretory responses are coordinated with the cellular concentrations of ICT and amylase in alcoholic acini strongly suggests that the observed secretory changes resulted from non-parallel alterations in the rate of synthesis or packaging of these enzymes in alcoholic acini. A significantly reduced pancreatic concentration of amylase has been reported previously in rats fed an ethanol liquid diet [12, 33] and in rats exposed to an ethanol-enriched atmosphere [34]. However, this effect cannot be conclusively regarded as an ethanol effect since rats fed an ethanol liquid diet always had 36% less dextrin-maltose compared to pair-fed controls and rats intoxicated with ethanol-rich air had a 30% decrease in total dietary intake. In the current study, alcoholic rats were also infused with 32–40% less dextrose compared to pair-fed controls. Indeed, as first suggested by Grossman *et al.* [30] and demonstrated by others (see Ref. 35 for a review), reduced carbohydrate intake results in a decreased amylase synthesis rate.

Despite the similar basal secretion of ICT in the two groups, significantly reduced basal plasma levels of ICT were found in alcoholic rats. This difference is difficult to explain. Lower plasma basal levels of ICT can result either from a decreased entry rate of ICT into the bloodstream or from an increased plasma clearance rate of this protein. It has been shown that the kidney is a major site for clearance and catabolism of circulating pancreatic cationic trypsinogen in humans [36] and rats [24]. In order for a change to occur in the renal clearance rate of ICT, either the glomerular filtration rate or the polyanionic glycoprotein structure of the glomerular capillary wall has to be altered. However, neither the renal cortical blood flow [37] nor the glomerular filtration rate [38] has been observed to change after ethanol administration. Furthermore, since the negatively charged glomerular capillary wall facilitates the faster clearance of positively charged proteins such as ICT [24, 39], it appears unlikely that any change in the charge of the glomerular structure causes a faster clearance rate which would account for the reduction of plasma ICT levels to half. Thus, alcohol-induced inhibition of the entry of ICT into plasma appears to be a possible reason for the lower basal levels of ICT in alcoholic rats. Alternatively, induction of an extrarenal clearance mechanism by alcohol intoxication could have the same consequence. Further studies on ICT clearance in intoxicated animals are needed to clarify this point.

In summary, we have demonstrated a significant increase in acinar concentration and CCK-8-stimulated secretion of pancreatic cationic trypsinogen in rats intoxicated with ethanol for 30 days. These alterations were reflected in a 2 to 3-fold increase in response patterns of plasma ICT and *in vitro* secretion of ICT to CCK-8. We have also shown significantly reduced acinar concentration and CCK-8-induced amylase secretion from alcoholic acini. The characteristics of CCK binding to specific receptors on acini in both groups were not altered. Thus, these non-parallel functional changes appear to have been due to alterations in synthesis or packaging of pancreatic ICT and amylase. Further studies are in progress to confirm this hypothesis and to clarify whether changes in amylase concentrations reflect ethanol effects or dietary effects. Nevertheless, our finding on pancreatic trypsinogen may represent a possible biochemical mechanism for the elevated levels of this enzyme previously demonstrated [14] and recently confirmed [10] in the pancreatic juice of human alcoholics. As previously suggested by others [10, 12], this increase in secretion and content of trypsinogen may be an important pathophysiological event associated with induction of alcoholic pancreatitis. This is of particular interest in our model since the incidence of interstitial edema was demonstrated by histopathology in about two-thirds of the alcoholic rats used in this study.*

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* H. Tsukamoto *et al.* manuscript in preparation.

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