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Cholinergic Mediation of Alcohol-Induced Experimental Pancreatitis

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Abstract

Objectives—The mechanisms initiating pancreatitis in patients with chronic alcohol abuse are poorly understood. Although alcohol feeding has been previously suggested to alter cholinergic pathways, the effects of these cholinergic alterations in promoting pancreatitis have not been characterized. For the present study, we determined the role of the cholinergic system in ethanol-induced sensitizing effects on cerulein pancreatitis.

Methods—Rats were pair fed control and ethanol-containing Lieber-DeCarli diets for 6 weeks followed by parenteral administration of four hourly intraperitoneal injections of the cholecystokinin analogue, cerulein at 0.5 µg/Kg. This dose of cerulein was selected because it caused pancreatic injury in ethanol-fed but not in control-fed rats. Pancreatitis was preceded by treatment with the muscarinic receptor antagonist atropine or by bilateral subdiaphragmatic vagotomy. Measurement of pancreatic pathology included serum lipase activity, pancreatic trypsin and caspase-3 activities, and markers of pancreatic necrosis, apoptosis and autophagy. In addition, we measured the effects of ethanol feeding on pancreatic acetylcholinesterase activity and pancreatic levels of the muscarinic acetylcholine receptors m1 and m3. Finally, we examined the synergistic effects of ethanol and carbachol on inducing acinar cell damage.

Results—We found that atropine blocked almost completely pancreatic pathology caused by cerulein administration in ethanol fed rats, while vagotomy was less effective. Ethanol feeding did not alter expression levels of cholinergic muscarinic receptors in the pancreas but significantly decreased pancreatic acetylcholinesterase activity, suggesting that acetylcholine levels and cholinergic input within the pancreas can be higher in ethanol fed rats. We further found that ethanol treatment of pancreatic acinar cells augmented pancreatic injury responses caused by the cholinergic agonist, carbachol.

Conclusion—These results demonstrate key roles for the cholinergic system in the mechanisms of alcoholic pancreatitis.

Keywords

Pancreas; alcohol feeding; cerulein; cholinergic pathways; acetylcholinesterase

Introduction

Alcohol abuse is a key etiologic factor in the mechanisms of acute and chronic pancreatitis (Pandol et al., 2007; Pandol et al., 2008). Whereas not all heavy drinkers develop pancreatitis, the risk of developing pancreatitis is directly related to the amount and duration of alcohol drinking (Pandol et al., 2007), indicating that other environmental and/or genetic factors contribute to the risk of disease initiation. Although no specific factors that contribute to the effect of alcohol on pancreatitis have been definitively established, evidence obtained from animal models of pancreatitis indicates that alcohol consumption sensitizes the pancreas to inappropriate intrapancreatic activation of digestive enzymes, acinar cell death, inflammation and fibrosis (Deng et al., 2005a; Gukovsky et al., 2008; Hanck and Whitcomb, 2004; Pandol et al., 1999; Pandol et al., 2008; Perides et al., 2005; Satoh et al., 2006).

Although the mechanisms are not completely understood, previous work supports an important role for the cholinergic system in mediating alcohol effects in several tissues including pancreas (Deng et al., 2004; Deng et al., 2005b; Nevo and Hamon, 1995; Niebergall-Roth et al., 1998; Renner et al., 1980; Rinderknecht et al., 1985; Samuel et al., 2005). Cholinergic inputs to the pancreas provided by preganglionic fibers in both sympathetic and parasympathetic systems, and postganglionic fibers emanating from intrapancreatic ganglia regulate many aspects of pancreas physiology (Niebergall-Roth and Singer, 2001; Solomon, 1994). The intrapancreatic ganglia are the integration centers of the pancreatic exocrine secretion, with terminal axons from these ganglia surround almost every acini. Studies in humans and experimental animals have demonstrated that the cholinergic system regulates pancreatic exocrine secretion via vago-vagal reflexes. These reflexes appear to originate in afferent vagal fibers emanating from neurons within the dorsal motor nucleus, and pass via cholinergic preganglionic fibers to synapse with the intrapancreatic ganglia (Owyang, 1996; Owyang and Logsdon, 2004). Whereas preganglionic neurotransmission is mediated by acetylcholine through both nicotinic and muscarinic receptors, intrapancreatic postganglionic innervations can be mediated by numerous neurotransmitters including acetylcholine acting on muscarinic receptors on the pancreatic acinar cell (Niebergall-Roth and Singer, 2001; Singer and Niebergall-Roth, 2009).

Regulation of pancreatic enzyme secretion in human and rodents by gastrointestinal hormones arises predominantly via cholecystokinin (CCK) binding and activation of high affinity CCK₁ receptors on vagal afferents (Owyang, 1996; Owyang and Logsdon, 2004; Singer and Niebergall-Roth, 2009). In this respect, it has been shown that enzyme secretion in response to physiologic doses of CCK can be prevented in rodents and humans by specific cholinergic blockade, and by bilateral vagotomy in certain experimental settings, supporting a critical role for vago-vagal cholinergic pathways in mediating CCK effects on pancreas (Owyang and Logsdon, 2004; Singer and Niebergall-Roth, 2009). Pancreatic acinar cells contain functional CCK receptors indicating that certain redundancy is built into this signaling system (Murphy et al., 2008; Owyang, 1996). However, the *in vivo* results described above suggest that normally cholinergic pathways incorporate hormonal CCK input and thus act as a common pathway in mediating physiologic secretory responses of the pancreatic acinar cell.

Alcohol has been long recognized to have an effect on physiological exocrine pancreatic secretion by mechanisms only partially elucidated (Niebergall-Roth et al., 1998; Renner et al., 1980; Rinderknecht et al., 1985; Sarles, 1986). Acute oral or intraduodenal administration of ethanol to rats stimulates pancreatic secretion and intestinal CCK release while acute intravenous ethanol perfusion appears to increase or reduce pancreatic secretion depending on the experimental settings (Jin and Green, 2003; Li et al., 2008; Liddle et al.,

1984; Saluja et al., 1997). Recent work from animal models suggests that high-dose alcohol feeding causes two major effects on pancreatic secretion (Deng et al., 2004; Deng et al., 2005a; Deng et al., 2005b). In one, alcohol feeding reduces the pancreatic secretion inhibitory pathways that emanate from the brain's area postrema. This effect of ethanol leads to augmented pancreatic secretory responses with administration of CCK or a meal. In the second effect, ethanol feeding potentiates the pancreatic secretory response to cholinergic agonists, suggesting an increased sensitivity of the acinar cell to cholinergic inputs. This second effect of alcohol is not accompanied by changes in cholinergic receptor affinities in pancreatic acinar cells as measured by radiolabeled ligand binding (Acosta et al., 2000). However, the mechanisms underlying these effects of alcohol feeding have yet to be determined.

In contrast to our knowledge about the effects of alcohol on the physiology of pancreatic secretion, little is known about the participation of cholinergic pathways in alcoholic pancreatitis. It is known that pancreatitis can be induced in experimental animals or in isolated pancreatic acinar cells by the application of supraphysiologic stimulation by cholinergic and cholecystokinin (CCK) agonists (Blinman et al., 2000; Chaudhuri et al., 2005; Gukovskaya et al., 1997; Gukovskaya et al., 2004; Thrower et al., 2008). We have previously shown that chronic ethanol feeding in rats made them susceptible to pancreatitis induced by a low dose of CCK that does not cause pathological damage in pancreas of control-fed rats (Pandolfi et al., 1999). Considering the interplay between CCK and cholinergic pancreatic innervations, and the potential effects of alcohol on cholinergic pathways, for the present study we tested the hypothesis that changes in the cholinergic system play a key role in mediating alcohol-induced pancreatitis.

Materials and Methods

Animals

Littermate male Wistar rats (120-150 g, Charles River, Wilmington, MA) were used in all experiments. Animals were housed in standard facilities under controlled conditions of temperature, humidity and illumination, and maintained on standard rodent chow or Lieber-DeCarli diets with free access to tap water. Animal care and all procedures were approved by the Institutional Animal Care and Use Committees of the Veterans Affairs Greater Los Angeles Healthcare System and the University of Southern California.

Ethanol feeding

For this study we used the Lieber-DeCarli model of ethanol feeding (Lieber and DeCarli, 1994). Ethanol feeding was performed in the animal core facilities of the Southern California Research Center for ALPD & Cirrhosis under the supervision of Dr Hidekazu Tsukamoto. This model was selected over other models we have used before (e.g. Tsukamoto-French intragastric infusion model) because it maintains intact the extrinsic and extrinsic innervations of the pancreas that can be affected by invasive procedures such as the ones needed for the Tsukamoto-French model. In addition, numerous studies by others and our group demonstrate that this model provides sufficient nutrient intake, and it is appropriate to study the sensitizing effects of alcohol to pancreatitis induced by various factors (Cosen-Binker et al., 2007; Deng et al., 2005a; Fortunato et al., 2009; Gukovsky et al., 2008; Perides et al., 2005).

After one week acclimatizing on standard chow rodent diet, rats were randomly distributed into two diet groups, which were pair-fed for 6 weeks with isocaloric amounts of nutritionally adequate Lieber-DeCarli liquid diet containing ethanol (36% of total calories) or maltose-dextrin as substitute of ethanol in the control diet, as previously described

(Lieber and DeCarli, 1994). The Lieber-DeCarli diets were purchased from Bio-Serv (Frenchtown, NJ) and prepared following instructions from the manufacture. Control and ethanol diets had the same nutrient composition, including fat and lipid components, and only differed with respect to the content of ethanol and maltose-dextrin. Casein was the main source of protein and provided 18% of total calories, while fat was provided by a mixture of safflower, olive and corn oils that represented 8% of total calories. The diets also contained cellulose, L-cystine, L-methionine, choline bitartrate, and a mixed of vitamins and minerals. Rats on the ethanol-containing diet were introduced gradually to increasing concentrations of ethanol, and by the 2 week they were advanced to the final diet containing 36% of total calories as ethanol, while the control group received all the time isocaloric amounts of the ethanol-free diet.

Rats were monitored daily to provide isocaloric pair-feeding, and assess general health status and alcohol intoxication. Body weight gain in rats fed ethanol diet was similar to that in rats pair-fed control diet, indicating that the nutritional state was similar in both groups of diet. For example, body weight gain per week, expressed as mean (SD) and 95% CI of mean, was Control diet = 27.9 (4.8), 1.8 CI of mean (n=30); Ethanol diet = 26.8 (7.5), 2.8 CI of mean (n=30); t-test p=0.53. Previous studies from our group using the Lieber-DeCarli model with the same diet composition used in the current study showed that rats fed control or ethanol diets for up to 8 weeks did not show signs of malnutrition or pancreas damage if not challenged with other toxic factors (Gukovsky et al., 2008).

Blood alcohol levels in rodents maintained with ethanol Lieber-DeCarli diets have been reported to be variable, with maximal concentrations during the feeding period (Perides et al., 2005; Tsukamoto et al., 1988). In our study, blood alcohol levels at sacrificed ranged from 17 to 25 mM (n=10). Animals were maintained on these diets for 6 weeks and then randomly distributed into treatment groups receiving cerulein or saline as described below, with each feeding pair receiving the same treatment.

Cerulein-induced Pancreatitis in ethanol and control fed rats

To study mechanisms of ethanol-sensitizing effects on pancreatitis, at the end of the 6-week feeding period rats received four hourly i.p. injections of 0.5 µg/kg cerulein (American Peptide Company, Sunnyvale, CA). Control rats received comparable injections of 0.9 % sodium chloride (saline). The doses and regime of cerulein administration used throughout this study was chosen in order to induce pancreatitis in ethanol-fed but not in control-fed rats. Preliminary dose-dependent studies to establish such doses are described in the Results section and in Figure 1.

To determine the participation of cholinergic pathways in ethanol-induced pancreatitis in response to cerulein administration, pancreatitis was preceded by treatment with the non selective muscarinic receptor antagonist atropine sulfate or by acute bilateral subdiaphragmatic vagotomy. Atropine sulfate was administered in conscious rats twice as an intraperitoneal bolus of 40 µg/kg, 30 min before the first and third injections of cerulein. In some experiments, atropine was given i.p. at the same indicated doses, but 15 min after the first and third injection of cerulein. Bilateral subdiaphragmatic vagotomy was performed in rats anesthetized with urethane (1.25 g/kg, Sigma-Aldrich, St Louis, MO) as previously described (Li et al., 2001). Briefly, an abdominal midline incision was used to expose the stomach and the distal esophagus. The subdiaphragmatic vagal trunks were exposed halfway between the diaphragm and the gastric cardia, and both the left and right vagus trunks were sectioned. Subsequently, the skin was closed with sutures and one hour later pancreatitis was induced under urethane anesthesia as indicated above (4 i.p. hourly injections of 0.5 µg/kg cerulein). In sham operated rats, both branches of the vagus were exposed and isolated from surrounding tissue but not transected.

Rats from all treatment groups were euthanized one hour after the last cerulein or saline injection and, immediately, blood samples were collected from the inferior vena cava and processed for measurements of lipase activity. Pancreata were removed, weighed and samples immediately frozen in liquid nitrogen and store at -80°C for subsequent biochemical analysis, or fixed in 10 % buffered formalin phosphate or 2 % glutaraldehyde for histological analysis.

Incubation of Rat Acinar Cells with Ethanol and/or Carbachol

Dispersed pancreatic acini were isolated from male rats by collagenase digestion as described before (Lugea et al., 2003). Briefly, the pancreas was digested with CLPS grade collagenase (Worthington Biochemicals, Freehold, NJ); and the resulting preparation of acini was suspended in 199 medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 10 $\mu\text{g/ml}$ soybean trypsin inhibitor. Acini were preincubated at 37°C in a 5 % CO_2 -air humidified atmosphere with 100 mM ethanol for 3 h, and then incubated for 0.5-2 h more with carbamoylcholine chloride (1-100 μM carbachol, Sigma-Aldrich, St Louis, MO). At the end of the incubation period, cells were harvested and prepared for histological analysis and intracellular trypsin activation.

Histological Analysis

Formalin-fixed pancreatic sections from at least 4 rats per group were embedded in paraffin. Five μm sections were stained with H&E and examined by light microscopy for assessment of parenchymal structure, inflammation and acinar cell death. Two experienced pathologists who were blinded to treatments examined in multiple random, non-overlapping sections acinar cell necrosis, cell vacuolization, edema and inflammatory cell infiltration. In addition, ultrastructure of acinar cells was examined by standard transmission electron microscopy. Briefly, small sections (2 mm^3) of pancreatic tissue were fixed with 2.5% glutaraldehyde in a 0.1 mol/L sodium cacodylate buffer, pH 7.3. Cells were postfixated with 1% osmium tetroxide for 30 minutes and embedded in Epon-Spurr resin after dehydration. After uranyl acetate and lead citrate staining, ultrathin sections (80 nm) were examined using a Hitachi 600 electron microscope (Hitachi Limited, Tokyo, Japan) transmission electron microscope.

Acinar cell vacuolization was evaluated in H&E stained pancreatic tissue sections by morphometric analysis of digitized pictures obtained from multiple random, non-overlapping sections under a high-power field ($\times 400$ -magnification, 6-10 random fields per section). Images were captured with all exposures manually set at equal times with a Nikon Eclipse E600 microscope equipped with a digital camera using the SPOT imaging software (Diagnostic Instruments, Sterling Heights, MI). The area occupied by vacuoles (stained in white or pale pink) in each acinus was quantified using a computer-assisted image analysis system (MetaMorph imaging system; Universal Imaging Corporation, Downingtown, PA), and expressed as percentage of area occupied by all the vacuoles per total examined area. In this way, we quantified vacuolization taking in account the number and the size of the vacuoles.

Acinar cell necrosis was determined by counting the number of necrotic cells, and expressed in the figures as percentage of necrotic cells per total pancreatic area. Cells with swollen cytoplasm, loss of plasma membrane integrity, and leakage of organelles into the interstitium were considered necrotic. Apoptotic cells were identified as cells exhibiting DNA fragmentation (TUNEL positive cells) in conjunction with characteristic morphological changes related with apoptosis, such as shrinkage and membrane blebbing ($\times 400$ -magnification). DNA fragmentation was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) procedure (Apoptosis Detections kit TA4625; R&D Systems, Minneapolis, MN).

LC3B staining in pancreatic tissue sections

LC3B processing, as indicative of autophagosome accumulation and the extent of autophagy, was evaluated in formalin-fixed pancreatic tissue sections by conventional immunofluorescence techniques. Pancreatic tissue sections were stained with primary antibody against LC3B (# 2775, Cell Signaling Technology, Danvers, MA) and Texas Red-conjugated anti-rabbit secondary antibody (T-2767, Invitrogen Corporation, Carlsbad, CA). Nuclei were counterstained with DAPI. Fluorescence images were obtained with a fluorescence microscope (Nikon Eclipse E600) equipped with a monochrome camera (CoolSnap, 20 mHz, Roper Scientific, Germany) and the MetaMorph imaging system (Universal Imaging Corporation, Downingtown, PA). Accumulation of autophagosomes was estimated by morphometric analysis of total area of LC3 stained bright dots relative to the total nuclei area using ImageJ 1.42I software (from NIH), and expressed as average LC3 dot area per nuclei.

Immunoblot Analysis

Briefly, tissue samples were homogenized in 20 mmol/L potassium phosphate buffer (pH 7.4) containing 1 mmol/L EDTA, and thereafter resuspended in X-100 triton, 0.1 % SDS and a freshly added mixture of protease inhibitors (50 µg/mL each of aprotinin, pepstatin, leupeptin, antipain, chymostatin and 1 mmol/L phenylmethylsulfonyl flouride). Protein extracts (40-60 µg total proteins) were resolved by SDS-PAGE electrophoresis, and then blotted to nitrocellulose membranes following standard methods. Primary antibodies used included anti-muscarinic receptor subtypes 1, mAChR M1 (M 9808, Sigma Chemical, St. Louis, MO), and 3, mAChR M3 (sc-9108 Santa Cruz Biotechnology, Santa Cruz, CA); and anti-LC3B (L7543 for rat LC3B, Sigma Chemical, St. Louis, MO) as a marker of autophagosome accumulation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, MA) or ½ ERK (Cell Signaling Technology, Danvers, MA) primary antibodies were used as a loading control for total protein cell content. Membranes were then incubated with horseradish peroxidase-conjugated specific secondary antibodies (Cell Signaling Technology, Danvers, MA). Immuno-reactive bands were visualized using chemiluminescence detection reagents (Pierce, Rockford, IL) in the Alpha Innotech FlourChem HD2 imaging system (San Leandro, CA), and quantified by densitometry using FlourChem software (Alpha Innotech, San Leandro, CA).

Trypsin Activity Assay

Active trypsin in pancreatic tissue homogenates was measured as described previously (Lugea et al., 2006). Briefly, the tissue was homogenized on ice in a buffer containing 5 mmol/L MES, 1 mmol/L MgSO₄ and 250 mmoles/L sucrose (pH 6.5). A 25-µl aliquot of the homogenate was incubated at 37°C for 300 sec in assay buffer containing 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1 mmol/L CaCl₂ and 0.1 mg/ml BSA and Boc-Gln-Ala-Arg-AMC as a specific substrate for trypsin. Cleavage of this substrate by trypsin releases 7-amino-4-methylcoumarin (AMC), which emits fluorescence at 440 nm (λ_{em}) with excitation at 380 nm (λ_{ex}). Trypsin activity in each sample was determined using a standard curve for purified trypsin (Sigma Chemical, St. Louis, MO).

Caspase 3 Activity Assay

Caspase 3 activity was measured using a fluorogenic assay as we described previously ((Wang et al., 2006). Briefly, pancreatic tissue was homogenized in a lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.5% Igepal CA-630, and 0.5 mmol/L EDTA, and soluble fractions were collected after centrifugation for 15 min at 16,000 × g. Caspase activity was measured in the soluble fractions at 37°C in a buffer containing 25 mmol/L HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 10 mmol/L dithiothreitol, and Ac-

DEVD-AMC, a caspase-3 specific substrate. Cleavage of Ac-DEVD-AMC by caspase 3 releases 7-amino-4-methylcoumarin (AMC), which emits fluorescent signal with excitation at 380 nm and emission at 440 nm. Caspase activity in the samples was determined relative to a standard curve for AMC and expressed as pmol of AMC. min⁻¹.mg⁻¹ of total protein.

Acetylcholinesterase activity Assay

Acetylcholinesterase (AChE) activity was measured in soluble fractions of pancreatic tissue homogenates by the colorimetric Ellman method (Ellman et al., 1961) with some modifications. Samples were diluted in 100 mmol/L phosphate buffer, pH 8, containing 125 µmol/L 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and 0.5 µmol/L acetylthiocholine iodide as a synthetic substrate for AChE. Color development was measured at 412 nm every 30 min during 3 min. AChE activity was expressed as pmol AChE.min⁻¹.mg⁻¹ of total protein.

Other Assays

Plasma amylase and lipase levels were measured in a Hitachi 707 analyzer (Antech Diagnostics, Irvine, CA). Total protein concentration in tissue extracts was measured by the Bradford assay (Bio-Rad Laboratories, Richmond, CA). Total DNA in tissue samples was quantified by spectrofluorometric assay using Hoeschst-33258 stain and calf thymus DNA as a standard (λ_{ex} 356 nm; λ_{em} 458 nm) (Lugea et al., 2006).

Statistical analysis

Data are presented as means ± SEM of values obtained from at least five rats per group. Histology images and immunoblots are representative of at least four rats per group. The standard error of the mean (SEM) was selected because takes in account the variability of the sample (standard deviation) and the sample size, and is a good estimator of how close the data mean can predict the sample mean. Statistical analysis of the data was performed using the SigmaStat software. Parametric ANOVA tests were the primary option for data following a Gaussian distribution and presenting equal variance between groups, and were taken in account only when the power of the selected ANOVA test was above 0.8, with alpha set at 0.05. If data was not normally distributed, the Kruskal Wallis non parametric test (or ANOVA on ranks) was used instead. When significance between treatment groups was demonstrated by the ANOVA tests, Bonferroni tests were selected for multiple comparisons versus the control group (as indicated in Figure 1), or post-hoc Tukey tests for all pairwise comparisons of the mean groups (as indicated in Figures 2-7). A value of P<0.05 was considered statistically significant.

Results

In the present study we used the CCK analogue cerulein to induce pancreas damage in alcohol fed rats and as a tool to investigate the role of the cholinergic system in alcohol-induced pancreatitis. As indicated in the introduction, CCK is known to interact with and utilize the cholinergic system to mediate physiologic exocrine secretion in humans and rodents. It is also known that CCK and its analogues, including cerulein, induce experimental pancreatitis at supraphysiologic doses by mechanisms that are thought to be due to direct interaction with CCK receptors on acinar cells. Previous studies from our group demonstrated that more physiologic doses of CCK-8 can cause pancreatitis in rats fed ethanol-containing diets for several weeks but not in rats fed control diets, suggesting that ethanol sensitizes pancreas to CCK-induced pancreatitis (Pandol et al., 1999). Whether the cholinergic system participates in this form of alcohol-induced pancreatitis is currently unknown.

In order to choose a dose and regime of cerulein administration that does not cause pancreatic damage in control-fed animals, we first performed the dose-response studies illustrated in Fig. 1. Rats were given 4 hourly intraperitoneal (i.p.) doses of cerulein and sacrificed one hour after the last cerulein administration. Results showed that doses of up to 0.5 $\mu\text{g/kg}$ cerulein did not have detectable evidence of pancreatitis, as measured by the concentration of lipase in blood lipase and pancreatic water content, while greater doses of cerulein significantly increased these features of pancreatitis. In addition, cerulein at 0.5 $\mu\text{g/kg}$ elicited a robust pancreatic secretion into the pancreatic duct (20 fold over basal, not shown), as opposed to higher supraphysiological concentrations that are known to cause pancreatic secretion blockade. Thus, in all experiments in the present study we used cerulein at 0.5 $\mu\text{g/kg}$.

Effects of cholinergic receptor blockade with atropine on pathological responses to cerulein in pancreas from ethanol-fed rats

To investigate the participation of the cholinergic pathway in alcohol-induced pancreatitis, rats were pair-fed with control or ethanol-containing Lieber-DeCarli diets for 6 weeks. Then, rats received saline (as control) or cerulein as described above (0.5 $\mu\text{g/kg}$, 4 i.p. injections), and were sacrificed one hour after the last cerulein injection. As indicated in the Material and Methods section, rats pair-fed control or ethanol Lieber DeCarli diets alone had similar body weight gain during the whole feeding period. Saline-treated rats fed control or ethanol diets did not display any evidence of pancreatitis, as measured by blood lipase levels, intrapancreatic trypsin activation, pancreatic edema, inflammatory cell infiltration and pathological changes in acinar cells (see Figs. 2-5). As expected, in animals receiving the control diet, cerulein administration induced only minimal (not statistically significant) increases in plasma lipase levels and intrapancreatic trypsin activity (Fig. 2), and no histological damage to acinar cells (Fig. 3).

In contrast to the results found in control fed rats, low cerulein doses induced several pathological responses in rats receiving the ethanol diet. As illustrated in Fig. 2, cerulein administration resulted in robust and statistically significant increases in both blood lipase levels (70-fold vs. basal) and intrapancreatic trypsin activation (10-fold vs. basal). This result corroborates a similar experiment we reported previously (Pandolfi et al., 1999). Importantly, administration of atropine dramatically (more than 70%) attenuated cerulein-induced pancreatitis in ethanol fed animals (Fig. 2), indicating that cerulein exerts its pathological effects on the exocrine pancreas at least in part through cholinergic pathways. Of note, atropine was effective in blocking cerulein effects whether administered 30 min before cerulein administration (see Figs. 2-4) or 15 min after the first and third cerulein injections (not shown). These results support a critical role for cholinergic inputs in mediating CCK responses on acinar cells during chronic alcohol consumption.

In ethanol-fed rats, atropine also blocked cerulein-induced histological damage in pancreatic acinar cells (Fig. 3). As mentioned above, cerulein either alone or in combination with atropine had no pathological effects in pancreas of control fed rats (Fig. 3A). In contrast, the combination of ethanol feeding and administration of low doses of cerulein promoted the accumulation of large cytoplasmic vacuoles, dilation of rough endoplasmic reticulum, and occasional cell necrosis (Figs. 3A and 3B). Other histological features of acute severe pancreatitis such as inflammatory cell infiltrate and interstitial edema were almost absent in these experimental settings. In fact, acinar cell vacuolization was the most striking histological feature of cerulein-induced pancreatitis in ethanol-fed rats, with vacuoles occupying up to 20% of the cellular volume (Fig. 3B). As illustrated in Figs. 3A and 3B, cholinergic blockade elicited by atropine was effective in reducing both the number and size of cytoplasmic vacuoles in ethanol fed rats.

Although we have not extensively characterized these vacuoles in our model of alcoholic pancreatitis, electron microscopy analysis revealed that some vacuoles have characteristics of dilated endoplasmic reticulum (not shown). In addition, other vacuoles exhibited features of autophagosomes with double membranes and partially digested organelles, while others had single membranes with sparse digested or undefined cellular material (Fig. 3C). These results are consistent with observations of abundant cytoplasmic vacuolization in both human alcoholic pancreatitis and in experimental animal models of pancreatitis (Adler et al., 1985; Helin et al., 1980; Ohmuraya et al., 2005). More recently, these vacuoles have been proposed to represent dysfunctional macroautophagy (Fortunato et al., 2009; Mareninova et al., 2009).

To further characterize atropine effects on acinar cell vacuolization induced by cerulein, we assessed pancreatic levels of the autophagosomal marker microtubule-associated protein 1 light chain (LC3). LC3 levels were determined in pancreatic tissue sections by immunofluorescence (detected as bright punctuate staining), and in pancreatic tissue homogenates by Western blotting using an antibody that recognizes both cytoplasmic LC3 (LC3-I) and autophagosome-associated lipidated LC3 (LC3-II). As illustrated by the data in fig. 4, LC3 staining and LC3 lipidation was low in control fed or ethanol fed rats treated with saline and/or atropine. Cerulein treatment increased LC3 staining (Figs. 4A and 4B) and LC3 lipidation (Figs. 4C and 4D) in both groups of diet, but this effect was more prominent in ethanol fed rats, supporting our histological data that pointed to a greater accumulation of autophagosomes in ethanol- compared to controls-fed rats (Figs 3A and 3C). Interestingly, in contrast with the robust effect of atropine in reducing the volume of acinar cell vacuolization, as measured by light microscopy image analysis (Figure 3B), atropine decreased only 20% the extent of LC3 staining or LC3 lipidation induced by cerulein in ethanol fed rats (Fig 4). These data suggest that blockade of cholinergic signaling by atropine disrupts steps in addition to or downstream of LC3 processing during the autophagic flux, and thereby prevents the formation of mature autophagic vacuoles. In addition, our data indicate that besides autophagosomes, the combination of ethanol feeding and cerulein treatment promoted the accumulation of non-autophagosomal vacuoles whose formation was blocked by atropine.

We next examined whether atropine affects cerulein-induced acinar cell death in ethanol treated rats. Necrosis was evaluated in H&E stained pancreatic sections as indicated in *Material and Methods*. Apoptosis was assessed by TUNEL staining and by analysis of caspase 3 activity in pancreatic tissue homogenates. As illustrated in Fig. 5A, low doses of cerulein induced in ethanol fed rats a moderate but significant increase in acinar cell necrosis (3% necrotic acinar cells) compared to the very low levels seen in control fed rats treated with cerulein (0.4% necrotic acinar cells), an effect that was completely prevented by atropine. Necrosis was the predominant mode of cell death in ethanol fed rats treated with cerulein, although sporadic TUNEL positive nuclei could be observed in pancreatic sections obtained from these animals (not shown). In contrast, no apoptotic cells were found in TUNEL stained pancreatic sections of control fed rats. Next, we measured caspase 3 activity in pancreas homogenates. Fig. 5B shows that, as we have described before (Wang et al., 2006), caspase 3-activity was 40% lower in basal conditions in ethanol fed rats compared to control fed rats. As expected, cerulein treatment did not change pancreatic caspase 3 activity in control fed animals, but augmented 3-fold this activity in the ethanol fed animals, an effect that was reduced by atropine. In conclusion, the combined results in Fig. 5 indicate that in our experimental settings ethanol feeding augments cerulein effects on necrosis and apoptosis death pathways in the acinar cell by a cholinergic mediated mechanism.

Effects of subdiaphragmatic bilateral vagotomy on pathological responses to cerulein in pancreas from ethanol-fed rats

In order to determine whether the cholinergic pathways necessary for the pathologic findings described above are vagally mediated, we examined the consequences of subdiaphragmatic bilateral vagotomy on measures of pancreatic injury induced by low doses of cerulein. As described in the Material and Methods section, subdiaphragmatic bilateral vagotomy or sham operation was performed in anesthetized rats one hour before cerulein administration (0.5 µg/kg; 4 i.p. hourly injections). Animals were sacrificed one hour after the last cerulein injection.

As seen in Fig. 6, neither sham nor vagotomized rats treated with saline presented any features of pancreatitis. Compared to controls, ethanol feeding enhanced in sham-operated rats the pathologic effects described above for cerulein on pancreas, i.e. increased blood lipase levels, intrapancreatic activation of trypsin and marked acinar cell vacuolization (Fig. 6). In ethanol fed rats, vagotomy partially prevented the effects of cerulein treatment on blood lipase levels and intrapancreatic trypsin activation (Figs 6A and 6B), and reduced only slightly the increased in vacuolization observed in sham ethanol-fed animals treated with cerulein (see micrographs in figure 6C).

Taken together, the combination of findings showing that atropine almost completely blocked the pathologic effects of cerulein in ethanol-fed animals, whereas vagotomy had only a partial preventative effect suggests that the ethanol feeding augments both extrinsic, vagally-mediated cholinergic input to the pancreas, and intrinsic cholinergic innervations to the acinar cell. In addition, ethanol-induced sensitizing effects could be related with a greater response of the pancreatic acinar cell itself to the pathologic effects of CCK-mediated cholinergic stimulation.

Effects of chronic ethanol feeding on pancreatic acetylcholinesterase activity and expression levels of muscarinic acetylcholine receptors

In order to determine whether chronic ethanol treatment altered pancreatic cholinergic input, we measured levels of acetylcholinesterase (AChE) activity in pancreatic homogenates. We found that compared to controls, pancreatic AChE activity was significantly reduced by 20% in chronic ethanol-fed rats, an effect that was not reversed by cerulein and/or atropine treatment (Fig. 7A). Although the physiologic relevance of these changes can not be evaluated with the experimental approaches of our study, the data suggest that chronic ethanol exposure could increase the level and duration of action of acetylcholine in the vicinity of the acinar cell. We next examined whether ethanol feeding alters the expression of muscarinic acetylcholine receptors (mAChRs) 1 and 3, two receptor subtypes that are known to regulate enzyme secretion in rat pancreatic acinar cells (Schmid et al., 1998). We found that, independently of potential effects on receptor function, chronic ethanol feeding did not alter pancreatic expression levels of these muscarinic receptors (Fig. 7B). This data are consistent with previous work showing that ethanol feeding does not alter binding of radiolabelled acetylcholine to pancreatic acinar cells (Schmidt, 1986).

Effects of ethanol on cholinergic stimulation in rat pancreatic acini

To investigate whether ethanol sensitizes the acinar cell to pathologic cholinergic stimulation, we performed *in vitro* experiments using freshly dispersed pancreatic acini isolated from untreated rats. Acini were preincubated for 3 h with and without 100 mM ethanol and then cells were stimulated for 0.5-2 h with the cholinergic agonist carbachol at physiologic (0.1 and 1 µM) and supraphysiologic (10 and 50 µM) concentrations. The ethanol concentration selected for these *in vitro* studies was higher than the blood ethanol levels found at sacrifice (12-2 pm) in rats fed ethanol diet, but within the maximal values

found in rodents fed ethanol-containing Lieber-DeCarli diets (Perides et al., 2005; Tsukamoto et al., 1988). We found that carbachol alone, at concentrations higher than 10 μ M induced a >2 fold increase in intracellular trypsin activation (Fig. 8A). Ethanol treatment did not change the levels of trypsin activation elicited by supraphysiological doses of carbachol, but significantly augmented acinar cell responses to low physiological doses of carbachol (1 and 5 μ M) that by themselves were not pathological (Fig. 8A) and elicit maximal secretion of amylase in isolated rodent pancreatic acinar cells (Thrower et al., 2009). Similarly, ethanol treatment in combination with carbachol resulted in marked cellular vacuolization (Fig. 8B) and accumulation of autophagosomes (Figs. 8C and 8D) compared with treatments with carbachol alone. In summary, our *in vitro* data indicate that ethanol exposure can alter cholinergic signaling within the pancreatic acinar cell leading to pathological consequences such as intracellular trypsin activation and vacuolization.

Discussion

The results of the present study support those of previous ones showing that chronic alcohol feeding sensitizes rats to pancreatitis caused by doses of CCK analogues that do not cause pancreatitis in control fed animals (Pandol et al., 1999). Importantly, we found that the effect of ethanol feeding on the pancreas is dependent on animal's endogenous cholinergic pathways. This conclusion is based on findings that the pathologic effects caused by low doses of the CCK analogue, cerulein, in ethanol-fed rats were prevented in large part (more than 70%) by cholinergic blockade with the muscarinic receptor antagonist atropine (Figs. 2-5) and to a lesser extent by vagotomy (Fig. 6). The cholinergic blockade prevented many pathologic features of pancreatitis including increases in serum lipase, pancreatic trypsin activity, acinar cell vacuolization, and activation of acinar cell death pathways. Moreover, in isolated pancreatic acinar cells the combination of acute ethanol treatment and stimulation with the cholinergic agonist carbachol reproduced some of the pathologic findings found in pancreas from ethanol-fed rats treated with low doses of cerulein (Fig. 8). Previously we have showed activation of inflammatory signaling systems in the pancreas of alcohol-fed rats given low doses of CCK (Pandol et al., 1999). Further, it is known that the inflammatory response can mediate digestive enzyme activation and necrosis of pancreatitis (Gukovskaya et al., 1996; Noel-Jorand and Bras, 1994). Thus, the present results combined with those previously reported lead to the conclusion that in alcohol-fed animals, CCK-mediated cholinergic stimulation can lead to the pathobiologic signals and responses of pancreatitis.

An important conclusion that can be made from these results is that there is sufficient capacity of the endogenous cholinergic system to cause pancreatitis in alcohol-fed animals. That is, the endogenous cholinergic system and its activation by submaximal doses of CCK or other undefined activating factors are necessary and sufficient to cause the responses of pancreatitis observed in alcohol-fed animals. This conclusion is important in the field of experimental pancreatitis because of significant concern that widespread application of exogenously administered supraphysiological doses of CCK analogues such as cerulein to cause pancreatitis may not represent a scenario consistent with initiation of pancreatitis in humans (Pandol et al., 2007).

Endogenous activation of the cholinergic system sufficient to cause pancreatitis may occur with certain dietary intakes. Thus, some studies investigating associations between dietary patterns and pancreatitis in alcoholic subjects reported a greater intake of fat and proteins in those developing pancreatitis compared to those not developing pancreatitis (Noel-Jorand and Bras, 1994). Although such associations are not consistently found across all populations similarly studied (Wilson et al., 1985), the increase in fat and protein intake would be expected to cause a greater increase in intestinal CCK production and CCK-

mediated cholinergic stimulation (Owyang, 1996). In addition, short-term oral and intravenous ethanol administration has been reported to increase intestinal CCK-8 release in rats (Liddle et al., 1984; Saluja et al., 1997). Thus, we speculate that the additive effects of an increased nutrient load with an augmented CCK release and cholinergic response due to chronic alcohol ingestion, combined with acinar cells sensitized to cholinergic stimulation, converts a physiologic secretory response to a pathologic one.

An important finding in the present study is that atropine was more effective than vagotomy in preventing the pathologic responses observed in the pancreas of rats fed alcohol and treated with maximal doses of cerulein. It is now generally accepted that CCK binding sites are distributed along all the vagal branches (Moran et al., 1987), and that vago-vagal enteropancreatic reflexes play an important role in mediating a significant portion of CCK-stimulated pancreatic secretion in rodents and humans (Owyang and Logsdon, 2004; Singer and Niebergall-Roth, 2009). However, the role of vagal innervations in the mechanisms mediating experimental CCK-induced alcoholic pancreatitis in rodents is unknown. Here, we found that in ethanol-fed rats, acute bilateral vagotomy (performed one hour prior to parenteral cerulein administration) reduces cerulein-induced intrapancreatic trypsin activation and blood lipase levels by 30% compared to sham-operated rats treated with cerulein. The efficiency of vagotomy in blocking cerulein effects contrasted with the results achieved by atropine treatment in vagally intact rats, which reduced by more than 70% the values obtained for several markers of pancreatitis. Although we recognize that the time post-vagotomy prior to initiating cerulein administration may have an impact on the efficiency of vagotomy (Li and Owyang, 2003), we conclude that pancreatitis induced by maximal doses of cerulein in ethanol fed rats is mediated by different cholinergic pathways. One is a vagal-mediated cholinergic pathway likely emanating from the CNS, which is blocked by vagotomy. Others have showed that chronic alcohol feeding alters CNS mediated cholinergic input to the pancreas (Deng et al., 2004; Deng et al., 2005a; Deng et al., 2005b). Our studies extend these observations and suggest that ethanol feeding acts to cause pancreatic damage at least in part by aberrant vagally-mediated cholinergic input to the exocrine pancreas.

Besides a vagally mediated cholinergic pathway, our data are consistent with other cholinergic pathways mediating cerulein effects in ethanol fed rats. These pathways are blocked by atropine and could be located within the pancreas at the level of the pancreatic ganglia and/or the acinar cell. The pancreatic ganglia are a ganglionated plexus spread throughout the pancreas with postganglionic cholinergic fibers innervating the exocrine pancreas (Ceyhan et al., 2009; Kiba, 2004; Owyang and Logsdon, 2004). Although it is accepted that pancreatic ganglia integrate signals from vagal preganglionic, sympathetic postganglionic, and enteric fibers, and enable a certain degree of independence of the pancreas from the central nervous system and the gut (Singer and Niebergall-Roth, 2009), it is still unclear how they generate or integrate neural input to and from the acinar cell during physiological or pathological responses. Our studies do not address the participation of pancreatic ganglia in mediating cerulein effects during rat pancreatitis. However, previous studies have showed that CCK-8 induces depolarization in isolated pancreatic ganglia of the cat (Ma and Szurszewski, 1996a; Ma and Szurszewski, 1996b), suggesting that pancreatic ganglia can mediate CCK effects on the exocrine pancreas. Future studies should address the electrophysiological characteristics of the pancreatic ganglia and their role in alcoholic pancreatitis.

Here we report initial approaches to understand how alcohol feeding sensitizes the animals to pancreatitis caused by CCK-mediated cholinergic stimulation. We envisioned that the mechanisms of action of ethanol on pancreatic cholinergic input could include changes in synaptic transmission along cholinergic pathways, changes in the density and quality of

cholinergic input to the pancreas, altered functionality of CCK₁ receptors within cholinergic fibers projecting to the pancreas, or effects on acetylcholine signaling within the acinar cell.

Our present data show that chronic ethanol feeding significantly reduced pancreatic acetylcholinesterase activity (Fig. 7). Others have showed similar effects in brain and liver of chronic ethanol red rats (Ruano et al., 2000). Although our experimental approach does not allow us to evaluate the physiologic relevance of the observed reduction in pancreatic AChE activity, it is tempting to speculate that ethanol abuse alters cholinergic synaptic transmission within the pancreas by inhibiting acetylcholinesterase activity that in turn increases the level and duration of action of acetylcholine in the vicinity of the acinar cell, leading to cholinergic hyperstimulation and cell injury. This notion is supported by previous experimental work showing that pancreatitis can be induced by conditions of cholinergic hyperstimulation of the pancreas (Chaudhuri et al., 2005; Cosen-Binker et al., 2007; Cosen-Binker et al., 2008; Samuel et al., 2005; Yuan et al., 2008). Furthermore, acetylcholinesterase inhibition caused by organophosphate poisoning can result in pancreatitis in humans (Ikizceli et al., 2005; Zamir and Novis, 1994), presumably through high levels of cholinergic stimulation of the exocrine pancreas. Interestingly, recent histopathological analysis of pancreatic tissue samples from patients with chronic pancreatitis, including alcoholic pancreatitis, have revealed an slight increase in the density of cholinergic fibers within the pancreas of chronic pancreatitis compared to normal pancreatic tissue samples (Ceyhan et al., 2009). Although the role of this increased density in intrapancreatic cholinergic fibers on the development and progression of chronic pancreatitis needs further investigation, these data emphasize the importance of neuronal innervations and, in particular, the cholinergic system as key players in pancreatic pathobiology.

Another effect of ethanol feeding reported here is an augmented response of the acinar cell to cholinergic stimulation *in vitro*. We found that ethanol treatment increased susceptibility of acinar cells to inappropriate intracellular trypsin activation and intracellular vacuolization induced by physiologic and supraphysiologic concentrations of carbachol (Fig. 8). We have previously reported that ethanol treatment sensitizes cultured acinar cells to disordered basolateral exocytosis when stimulated with physiologic doses of cholinergic agonists (Lam et al., 2007), which results in inhibition of normal secretion mimicking the effects of pathologic supraphysiologic doses of cholinergic agonists (Schmidt and Pandol, 1990). Of note, we did not find that alcohol feeding altered the expression of cholinergic receptor subtypes in the pancreas consistent with previous radiolabelled ligand studies (Acosta et al., 2000). These sensitizing effects of ethanol can be mediated by PKC signaling downstream to cholinergic receptors. In this respect, we have demonstrated that in isolated acinar cells acute ethanol treatment augments PKC activation (Satoh et al., 2004; Satoh et al., 2006), and that cerulein- and carbachol-induced stimulation of PKC delta and PKC epsilon activities modulate intracellular trypsin activation and NF- κ B activation (Thrower et al., 2008; Yuan et al., 2008). Taken together, our *in vitro* and *in vivo* data suggest that alcohol abuse alters the pancreatic acinar cell so that it is more sensitive to the pathologic responses induced by cholinergic overstimulation caused by ethanol.

In conclusion, our findings reveal key roles for the cholinergic system in the mechanism of alcoholic pancreatitis. Our results, when considered in the context of previous investigations of ethanol effects on the cholinergic system, suggest that ethanol feeding increases central cholinergic as well as intrapancreatic cholinergic input to the exocrine pancreas. In addition, our data indicate that ethanol might decrease the threshold of the acinar cell to cholinergic stimulation, rendering cells susceptible to factors triggering cholinergic hyperstimulation. In this setting it is conceivable that chronic alcoholism combined with meals containing large amounts of fat, protein and/or calories can initiate the pathologic processes of pancreatitis.

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Abbreviations used in this paper

At	atropine
C	carbachol
CCK	cholecystokinin
CD, C diet	Lieber-Decarli control diet
cer	cerulein
E	ethanol
E diet	Lieber-Decarli ethanol diet
AV	autophagic vacuole

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Figure 1. Dose-response effects of cerulein in the rat exocrine pancreas

In order to determine the maximal dose of cerulein that does not cause pathological damage in the exocrine pancreas of chow fed rats, animals received 4 hourly i.p injection of cerulein at the indicated doses. Rats were sacrificed 1 hour after the last cerulein injection, and levels of lipase in blood and pancreatic edema were analyzed as markers of pancreatic damage. As illustrated in the graphs, cerulein at doses higher than 0.5 $\mu\text{g/kg}$ induces significant increases in blood lipase levels (A) and pancreatic edema (B). Graphs show mean $\pm$ SEM of 4-6 rats per group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared to basal (one way ANOVA followed by Bonferroni t-tests for multiple comparisons versus the control group).

Figure 2. Atropine blocked ethanol-mediated sensitization to cerulein pancreatitis

Rats were pair-fed control (C diet) or ethanol (E diet) Lieber-DeCarli diets for 6 weeks.

Then, rats were treated with maximal doses of cerulein (0.5 $\mu\text{g/kg}$; 4 hourly i.p. injections) and/or atropine (40 $\mu\text{g/kg}$, one i.p. administration 30 min prior the first and third cerulein injections). Rats were sacrificed 1 h after the last cerulein injection. Saline-treated rats (–) were used as controls. As expected, cerulein at 0.5 $\mu\text{g/kg}$ had minor effects on blood lipase levels (A) or trypsin activation (B) in control fed rats. In contrast, in ethanol-fed rats,

cerulein induced marked increases in blood lipase (A) and trypsin activation levels (B) that were significantly blocked by pretreatment with atropine. These data suggest that ethanol-mediated sensitization to cerulein pancreatitis is mediated at least in part by the cholinergic system. Graphs show mean $\pm$ SEM of 8–14 rats per group. Data from treatment groups were analyzed by Kruskal-Wallis ANOVA on ranks followed by Tukey post-hoc tests. No significant differences were found between diet groups in saline-treated rats with or without atropine. Asterisks denote significance between diet groups in cerulein-treated rats:

* $P < 0.05$, *** $P < 0.001$, compared to C diet; # $P < 0.05$ compared to E diet + cerulein without atropine.

Figure 3. Atropine prevented cerulein-induced pathological changes in ethanol-fed rats (A). Representative H&E stained pancreatic sections from rats fed control (C diet) or ethanol (E diet) Lieber-DeCarli diets, and treated with atropine (at) and/or maximal doses of cerulein (cer) as indicated in legend for Fig. 2. Cerulein induced only minor pathological changes in control fed rats treated with or without atropine. In contrast, in ethanol-fed rats cerulein induced marked acinar cell vacuolization, dilation of the ER (visualized at electronic microscopy, not shown), and occasional patchy cellular necrosis (see panels *E diet+cer*). Importantly, pretreatment with atropine dramatically reduced the pathologic changes induced by cerulein in ethanol-fed rats (see panels *E diet+at+cer*). Bars represent 20 μm. (B) The degree of acinar cell vacuolization in H&E stained pancreatic tissue sections was quantified by morphometric analysis and expressed as percentage of acinar area occupied by vacuoles (see Material and Methods section for details). Graph shows mean ±SEM from measurements taken in at least 6 pancreatic tissue sections from 3-5 animals per group. *** $P < 0.001$ compared to C diet; ## $P < 0.01$ compared to E diet + cerulein without atropine (Kruskal-Wallis ANOVA on ranks followed by Tukey post-hoc tests). (C) Representative transmission electron microscopy images showing two examples of vacuoles commonly found in pancreatic acinar cells from ethanol-fed rats treated with cerulein. Some vacuoles exhibit features of autophagic vacuoles (AV) with double membranes and partially or completely digested organelles, while others have single membrane with sparse digested material. Arrowhead in right panel indicates intact membrane structures in an autophagic vacuole. Bars, 2 μm; N, nucleus.

Figure 4. Atropine reduced only partially cerulein-stimulated pancreatic LC3 processing in ethanol-fed rats

To evaluate the accumulation of autophagosomes in pancreas from rats treated with low doses of cerulein, we assessed pancreatic levels of the autophagosome marker LC3. (A) Immunofluorescence images of LC3B (red staining) in control and ethanol-fed rats treated with cerulein and/or atropine. Nuclei were counterstained with DAPI (blue staining). Arrowheads indicate LC3B-stained autophagic compartments. Images are representative of 3 rats analyzed per group. Controls using only secondary antibody were negative (not shown). Bars, 20 μ m. (B) The volume of LC3B dots was assessed by morphometric analysis of the total area of LC3B staining relative to nuclei area, and expressed relative to control-fed rats (C diet). Graph shows results for cerulein treated rats; $n=3$. $*P<0.05$ compared to C diet (one way ANOVA followed by Tukey post-hoc tests). (C) Pancreatic expression levels of LC3-I and LC3-II were analyzed by Western blotting using a specific antibody against LC3B. GAPDH expression was used as internal loading control. Image illustrates a representative immunoblot showing the results from 1-2 animals per group. (D) Graph represents quantification (mean $\pm$ SEM) of the optical density of LC3-II relative to LC3-I. LC3-I and LC3-II levels were first normalized relative to GAPDH. $*P<0.05$ compared to C diet (one way ANOVA followed by Tukey post-hoc tests). As illustrated in the figure, LC3 staining was negligible in saline treated rats fed control (C diet) or ethanol (E diet) diets, but increased significantly in both groups of diet after cerulein (cer) treatment. Atropine alone did not affect total LC3 expression or LC3-II accumulation (not shown), and prevented only partially cerulein effects in ethanol-fed rats.

Figure 5. Atropine blocked ethanol-mediated sensitization to acinar cell death induced by cerulein

Rats fed ethanol or control diets for 6 weeks were treated with atropine and/or maximal doses of cerulein as indicated in the legend for Fig. 2. As expected, 0.5 $\mu\text{g/kg}$ cerulein did not induce acinar cell necrosis (panel A) or alter pancreatic caspase 3 activity (panel B) in control fed rats. However, in ethanol-fed rats this dose of cerulein induced a 10-fold increase in acinar cell necrosis and a 3-fold increase in pancreatic caspase 3 activity compared to saline-treated rats. As observed for other pancreatitis parameters, atropine effectively blocked cerulein effects on acinar cell necrosis and caspase 3 activity in ethanol-fed rats. Graphs show mean $\pm$ SEM, $n=4-5$ rats per group. * $P < 0.05$, *** $P < 0.001$, compared to control diet; # $P < 0.05$, ## $P < 0.01$ compared to Ethanol diet + cerulein without atropine (one way ANOVA followed by Tukey post-hoc tests).

Figure 6. Subdiaphragmatic bilateral vagotomy did not block ethanol-mediated sensitization to cerulein pancreatitis

Rats fed ethanol (E diet) or control (C diet) Lieber-DeCarli diets for 6 weeks were subjected to subdiaphragmatic bilateral vagotomy or sham surgery, then treated with maximal doses of cerulein (cer, 0.5 μ g/kg; 4 hourly i.p. injections). Ethanol feeding enhanced in sham-operated rats the pathologic effects of cerulein on pancreas, as assessed by measurements of (A) blood lipase levels and (B) trypsin activity. Although vagotomy reduced by approximately 30% the increases in blood lipase levels and trypsin activation induced by cerulein, it was less effective than atropine in reversing cerulein effects in ethanol-fed rats (see Fig. 2 for comparisons). Graphs show mean $\pm$ SEM, n=5-8 rats per group. Data from treatment groups were analyzed by one way ANOVA followed by Tukey post-hoc tests. No significant differences were found between diet groups in saline-treated rats with or without vagotomy. Asterisks denote significance between diet groups in cerulein-treated rats: * $P<0.05$, ** $P<0.01$, compared to control diet. (C) Pancreatic histological damage, assessed in H&E staining of pancreatic sections, was comparable in sham-operated and vagotomized rats fed ethanol diets (see lower panels), suggesting that vagal cholinergic pathways mediate only partially the sensitizing effects of ethanol to cerulein pancreatitis. Of note, in our experimental settings, vagotomy alone had no pathological effects on pancreas of saline-treated control or ethanol fed rats (not shown). Bars, 20 μ m.

Figure 7. Chronic ethanol exposure decreased pancreatic acetylcholinesterase activity
 (A) Acetylcholinesterase (AChE) activity was analyzed in pancreatic tissue homogenates using the Ellman method. Graph shows the mean $\pm$ SEM from 4-5 rats per group. Data was analyzed by three way ANOVA followed by Tukey post-hoc tests; Factors: diet ($P=0.013$, $*p<0.05$), cerulein or saline treatment ($P=0.093$, ns), atropine treatment ($P=0.087$, ns), no interaction between factors). (B) Expression levels of muscarinic receptor subtypes 1 (mAChR M1) and 3 (mAChR M3) in pancreatic homogenates from control and ethanol fed rats were analyzed by Western blotting. Pancreatic expression of ERK $\frac{1}{2}$ is used as an internal loading control. Each blot represents an individual rat; immunoblot shows results from three animals per diet group. As illustrated in the figure, chronic ethanol feeding reduces up to 30% pancreatic AChE activity compared to controls, without affecting expression levels of muscarinic receptors M1 and M3.

Figure 8.

Dispersed pancreatic acini were isolated from untreated rats and preincubated for 3 h with and without 100 mM ethanol. Cells were then stimulated for 30 min (for trypsin measurements) or 2 h (for analysis of vacuolization) with the muscarinic agonist carbachol at the indicated concentrations. (A) Carbachol at concentrations higher than 10 μM significantly increased intracellular trypsin activation. Ethanol treatment did not significantly alter the increase in trypsin activation elicited by high doses of carbachol, but it had synergistic effects with carbachol at 1 (see graph) and 5 μM (not shown), doses that by themselves are not pathological and elicit maximal digestive enzyme secretion *in vitro* (see ref. Thrower et al, 2009). Graph shows mean±SEM of 5-8 independent experiments. *P<0.05 compared to 1 μM carbachol alone (t-test). (B) Acinar cell vacuolization was assessed in toluidine blue stained acinar cells. Pictures are representative of cells left untreated (a) or treated with ethanol (b), 50 μM carbachol (c), or a combination of ethanol and 50 μM carbachol (d). As illustrated in panel d, only carbachol-stimulated cells treated with ethanol display significant vacuole accumulation (see arrows). Bars, 20 μm. (C) Pancreatic protein levels of LC3-II were used as an index of autophagosome accumulation. Picture shows representative immunoblots for LC3B protein expression in acinar cells stimulated with 1 or 50 μM carbachol (C) in the presence or absence of 100 mM ethanol (E). ERK ½ expression was used as internal loading control. (D) Graph represents quantification (mean±SEM) of the optical densities of LC3-II relative to LC3-I immunoblots, both normalized first to those of ERK ½. Data in (C) and (D) are representative of two independent experiments (statistics were not performed because of the small sample size).