Pancreatic response to endotoxin after chronic alcohol exposure: switch from apoptosis to necrosis?

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Fortunato, Franco, Xiaoying Deng, Lawrence K. Gates, Craig J. McClain, Daniel Bimmler, Rolf Graf, and David C. Whitcomb. Pancreatic response to endotoxin after chronic alcohol exposure: switch from apoptosis to necrosis? Am J Physiol Gastrointest Liver Physiol 290: G232–G241, 2006. First published June 23, 2005; doi:10.1152/ajpgi.00040.2005.—Chronic alcohol consumption is known to increase the susceptibility to acute and chronic pancreatitis, and it is likely that a cofactor is required to initiate the progression to alcoholic pancreatitis. The severity and complications of alcoholic and nonalcoholic acute pancreatitis may be influenced by a number of cofactors, including endotoxemia. To explore the effect of a possible cofactor, we used endotoxin [lipopolysaccharide (LPS)] as a tool to induce cellular injury in the alcoholic pancreas. Single, increasing doses of endotoxin were injected in rats fed an alcohol or control diet and killed 24 h after the injection. We examined the mechanism by which LPS exacerbates pancreatic injury in alcohol-fed rats and whether the injury is associated with apoptosis or necrosis. We showed that chronic alcohol exposure alone inhibits apoptosis through the intrinsic pathway and the downstream apoptosis executor caspase-3 compared with the controls. Pancreatic necrosis and inflammation increased after LPS injection in control and alcohol-fed rats in a dose-dependent fashion but with a significantly greater response in the alcohol-fed animals. Caspase activities and TdT-mediated dUTP nick-end labeling positivity were lower in the alcoholic pancreas injected with LPS, whereas the histopathology and inflammation were more severe compared with the control-fed animals. Assessment of a putative indicator of necrosis, the ratio of ADP to ATP, indicated that alcohol exposure accelerates pancreatic necrosis in response to endotoxin. These findings suggest that the pancreas exposed to alcohol is more sensitive to LPS-induced damage because of increased sensitivity to necrotic cell death rather than apoptotic cell death. Similar to the liver, the pancreas is capable of responding to LPS with a more severe response in alcohol-fed animals, favoring pancreatic necrosis rather than apoptosis. We speculate that this mechanism may occur in acute alcoholic pancreatitis patients.

ethanol; lipopolysaccharide; acute pancreatitis

THE PATHOPHYSIOLOGY of acute alcoholic pancreatitis is still not well understood, partly because good animal models are lacking. The lack of models is based on the fact that, in animals, ethanol feeding alone causes mild and variable pathological responses of the pancreas, and only in conjugation with a second hit is the differential cellular response visible. Most recent experimental work has shown that repetitive cerulein injections induce multiple episodes of acute pancreatitis, leading to early fibrosis and calcification, which seem to be exacerbarated by chronic alcohol administration (17). In rodents, chronic alcohol exposure induces mitochondrial damage in pancreatic acinar cells and in hepatocytes. Increased mitochondrial size, damaged cristae, and fragmentation of the inner mitochondrial membrane have been observed in both cell types (26, 42). Abnormal mitochondrial function may contribute to acinar cell injury in alcoholism and may increase the susceptibility to acute pancreatitis.

Increased gut permeability has been described in chronic alcohol users with the effect of bacterial translocation across the mucosal barrier. Bacterial components such as lipopolysaccharide (LPS) may reach the bloodstream and the pancreas and worsen injury in acute pancreatitis (6, 7). LPS, a major cell wall component of gram-negative bacteria, plays a key role in alcoholic liver disease (6), but the role of LPS in acute pancreatitis is less clear. In recent years, considerable evidence has suggested that LPS could be an important cofactor in the pathophysiology of acute pancreatitis (9, 31, 53, 54, 66). We and others have previously shown that LPS directly induces apoptosis in acinar cells and may contribute to the pathophysiology of severe acute pancreatitis (25, 38, 46, 51, 62, 66). Recent clinical work has shown that LPS-binding protein (LBP), a marker for endotoxin, is increased at the onset of acute pancreatitis and continues to be higher in necrotizing patients, whereas in edematous pancreatitis LBP decreases during the first week of hospitalization (54).

One major area of interest in acute and chronic pancreatitis is the cellular response to injury. It appears that the initiation of acute pancreatitis requires cofactors or susceptibility factors that favor the development to pancreatitis. Besides genetic polymorphisms that have been shown to be involved in hereditary pancreatitis, e.g., trypsinogen mutation (65) or SPINK mutation (67), alcohol abuse is one of the most prominent predisposing factors in acute and chronic pancreatitis.

Because both forms of pancreatitis exhibit variable degrees of apoptosis and necrosis, we tried to determine the cellular response to a cofactor in the alcoholic pancreas. We used LPS as a tool to induce a cellular injury. On the basis of the history of the pancreas, an alcoholic or nonalcoholic response to this trigger should reveal the cellular state of the alcoholic pancreas. We show that alcohol reduces the state of acinar cell apoptosis and hypothesize that alcohol exposure promotes a shift from acinar cell apoptosis to necrosis in response to LPS.

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MATERIALS AND METHODS

Animals and reagents. Male Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). All experiments were approved by the Institutional Animal Care and Use Committee of the Lexington, Kentucky, Veterans Medical Center and performed at the Lexington Veterans Administration. PCR primers were obtained from Integrated DNA Technologies (Coralville, IA), and all other chemicals from Sigma Chemicals (St. Louis, MO).

Alcohol feeding and LPS injection. Rats, weighing 150–175 g, were pair fed a liquid diet (BioServ, Frenchtown, NJ) containing either ethanol (final concentration of 6%, equivalent to 36% of caloric intake) or maltose-dextrin, according to the methods of Lieber and DeCarli (43). After 14 wk of feeding, rats received intravenous injections of various doses of gram-negative bacterial LPS (Escherichia coli, 026-B6; ranging from 0.2, 0.8, 1.5, and 3 mg/kg body wt iv) or vehicle control (sterile saline). After LPS injection (24 h), the rats were anesthetized with pentobarbital sodium (Nembutal, 60 mg/kg body wt ip). Whole pancreata were resected, snap frozen in liquid nitrogen, and stored at −80°C.

Pancreatic histology. Pancreata from rats killed 24 h post-LPS were fixed in 10% formalin, embedded in paraffin, and cut in 4-μm-thick serial sections. Sections were stained with hematoxylin-eosin (H&E; Fig. 1, A and B) and examined by an experienced pancreatologist blinded to the sample identity (X. Deng; see Refs. 14–20, 29, 41, 42, and 61). Sections were examined for parenchymal edema, acinar vacuolization and necrosis, inflammatory cell infiltration, and hemorrhage and then analyzed in 10 randomly selected fields with the aid of the Olympus BX40 microscope camera system. Alterations in average tissue injury were scored on the following scale: 0 = no edema, 1 = interlobular edema, 2 = interlobular and moderate intralobular, 3 = interlobular and severe intralobular edema; neutrophil infiltration: 0 = absent to 3 = high infiltration; necrosis: 0 = no necrosis to 3 = severe necrosis; and vacuoles: 0 = no vacuoles to 3 = maximal vacuoles (Table 1), according to previously described methods (49, 50).

Immunohistochemistry. The paraffin sections were deparaffinized in xylene and hydrated through a graded series of alcohol to tap water. Antigen retrieval was performed by microwave irradiation in citrate buffer for 10 min. Sections were blocked at 4°C overnight with normal goat serum provided in the Vectastain Universal Elite ABC kit (Vector Laboratories) with 1% BSA in histo-Tris buffer (50 mM Tris, pH 7.5, and 150 mM NaCl). After sections were incubated for 2.5 h with anti-heme oxygenase (HO)-1 rabbit polyclonal antibody (Stress...

Fig. 1. Histopathology evaluation of pancreatic tissue sections. Responses of the pancreas from alcohol (EtOH)-fed and pair-fed (PF) rats to lipopolysaccharide (LPS) are shown. Representative rat pancreatic tissue sections were stained with hematoxylin and eosin or with a heme oxygenase (HO)-1 antibody to detect macrophages. Pancreatic tissue was obtained from rats after 14 wk of alcohol (EtOH-fed) or pair-fed control feeding (PF) with subsequent injection of LPS. A: pair-fed control injected with vehicle (saline). B: alcohol-fed rats challenged with a single LPS dose [3 mg/kg body wt (b.w.)] showing interstitial edema, hemorrhage, and some mild necrosis and inflammatory cells. C: HO-1 immunoreactive cells in pancreatic tissue sections from a pair-fed control injected with vehicle (saline). D: HO-1 immunoreactive cells (arrowhead) in a pancreatic tissue section from an alcohol-fed rat challenged with a single LPS dose (3 mg/kg body wt). E: quantitation of the average tissue score obtained from Table 1. Plotted are means ± SE for 4–6 individual animals/group. F: quantitation of macrophages (HO-1-immunoreactive cells). The two LPS dose-response curves are significantly different with P = 0.0086 in the alcohol- and control-fed animals, using two-way ANOVA. Plotted are means ± SE for 4–5 individual animals/group. *P < 0.05 vs. pair-fed control injected with the identical LPS dose, using the t-test.
gen Biotechnologies) diluted 1:1,000 in buffer and washed in buffer three times, they were incubated with diluted biotinylated secondary antibody and ABC reagent for 30 min, consecutively. Finally, the sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride as recommended in the instructions (DAB Substrate Kit; Vector Laboratories) and counterstained with Harris’ hematoxylin (Sigma-Aldrich Fine Chemicals) (Fig. 1, C and D). HO-1-positive cells were examined and analyzed in 20 randomly selected fields with the aid of a Zeiss Axioplan2 Imaging microscope.

Pancreatitis-associated protein and pancreatic stone protein ELISA. To determine the level of protein expression of pancreatitis-associated protein (PAP)-1 and pancreatic stone protein (PSP/reg), an ELISA analysis was performed. Briefly, basal PSP/reg levels in tissue homogenates were measured by means of a “sandwich” ELISA. Recombinant rat PSP/reg and PAP-1 were used as standards (0.025–5 ng/ml). The development of this ELISA and its standardized application protocol have been described in detail previously (5, 28, 57).

TdT-mediated dUTP nick-end labeling assay. TdT-mediated dUTP nick-end labeling (TUNEL) detection of apoptotic cells was performed in formalin-fixed, paraffin-embedded pancreatic tissue sections as recommended using the ApoTag kit (MP Biomedical, formerly Q-Biogene). Sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride, as recommended by the manufacturer’s instructions (DAB Substrate Kit; Vector Laboratories) and counterstained with Harris’ hematoxylin (Sigma-Aldrich Fine Chemicals) (see Fig. 3). TUNEL-positive nuclei were examined and analyzed (n = 4) in 20 randomly selected fields with the aid of a Zeiss Axioplan2 Imaging microscope.

Caspase-2, -3, -8, and -9 activities. Activities of pancreatic caspase-2, -3, -8, and -9 were measured in pancreatic tissue extracts. Frozen tissue was homogenized at 4°C in a buffer containing 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml each leupeptin, pepstatin, and 1-leucinethiol, and 0.1% Nonidet P-40, pH 7.9, in a final volume of 0.5 ml. The lysate was centrifuged at 15,000 g for 30 min. The supernatant was used for the caspase activity assays. Equal amounts of protein were assayed in duplicates for each sample. Caspase-2, -3, -8, and -9 fluorometric assays are based on the hydrolysis of the peptide substrates Ac-VDVAD-AMC (caspase-2), Ac-DEVAD-AMC (caspase-3), Ac-IETD-AMC (caspase-8), and Ac-LEHD-AMC (caspase-9), resulting in the release of the AMC moiety. Equal aliquots were tested in the presence of inhibitors for caspase-2, -3, -8, and -9 to estimate specific enzyme activity and standardized with 7-amino-4-methyl coumarin for all four caspases. The excitation and emission wavelengths of AMC are 360 and 440 nm, respectively. The concentration of the released AMC was calculated from the calibration curve. It is directly proportional to the actual caspase activity in the pancreatic tissue extract. The assays were performed according to the manufacturer’s instructions (Sigma Chemicals) with one exception: the assay was performed at 37°C and not at room temperature. Fluorescence was measured using a fluorometric spectrometer Synergy HT (BioTek; Winooski). The caspase-9-AMC substrate was purchased from American Peptide (Sunnyvale, CA). The caspase-2-AMC substrate and inhibitor were purchased from A.G. Scientific (San Diego, CA). Results are expressed as the specific caspase substrate cleavage per milligram total protein per minute, after subtraction of the nonspecific product formation (substrate plus inhibitor).

Quantitative ATP assay. The ATP concentration was assessed quantitatively by using the ENLITEN ATP Detection Reagent kit from Promega (Madison, WI). Briefly, pancreatic tissue was homogenized in a Tris buffer (25 mM Tris-HCl and 5 mM EDTA, pH 8.0) containing 10% ATP releasing reagent. ATP concentration was determined in a buffer containing 25 mM HEPES, 10 mM MgCl2, 0.02% sodium azide (pH 7.75), luciferase bioluminescence detection reagent, and an ATP standard, according to the instructions.

ADP-to-ATP ratio. The assay was designed to detect the ADP-to-ATP ratio. The changes in ADP-to-ATP ratios have been used to conveniently differentiate apoptotic from necrotic cell death (8). The assay utilizes bioluminescence detection of ADP and ATP levels for rapid screening of apoptosis, necrosis, growth arrest, and cell proliferation simultaneously in mammalian cells. The ADP-to-ATP ratio was analyzed by the ApoSENSOR ADP/ATP Ratio Assay Kit (Alexis) using the same tissue homogenate as described for the ATP assay, following the instructions of the manufacturer.

Statistical analysis. Results are reported as means ± SE for four to six individual animals per group. Statistical significance of variance of the LPS dose response in the pair-fed control and alcohol-fed experimental groups was analyzed by two-way ANOVA. The LPS dose-response curves were considered significantly different when the P value was <0.05. Student’s t-test was applied for each experimental group treated with the identical LPS dose or vehicle in controls or alcohol-fed animals and were considered significant when the P value was <0.05. Statistical calculations were performed using software from GraphPad Prism.

RESULTS

Alcohol plus LPS increased the severity of pancreatic injury in an LPS dose-dependent fashion. To test whether the pancreas is more susceptible to LPS in animals exposed to alcohol, we fed rats with a diet containing alcohol or a control diet containing maltose-dextrin (pair fed). H&E-stained pancreatic sections were prepared from rats fed alcohol or control diet for

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Values are means ± SE; n, no. of rats/group. –, Without; +, with. Parameters were evaluated after 24 h endotoxin [lipopolysaccharide (LPS)] treatment in pair-fed (PF) and alcohol-fed (EtOH) rat pancreata. Data represent the scoring numbers of pancreatic sections by examination for parenchymal edema, acini necrosis and vacuolization, and inflammatory cell infiltration. Data were analyzed randomly in 20 selected fields (see Fig. 1, A and B). Alterations in tissue injury were scored on a scale from 0 to 3 (from no alteration to severe damage), according to previously described methods (50). *P < 0.05 between each LPS dose in control vs. alcohol using Student’s t-test.
14 wk and killed 24 h after a single LPS injection of various concentrations. Sections from four to six rats per group were evaluated by a pancreatic researcher blinded to treatment (X. Deng) using a previously described morphological scoring system (49, 50). As shown in Table 1, alcohol feeding alone for 14 wk induced some pancreatic necrosis. Prior chronic alcohol consumption resulted in increased tissue injury after LPS administration in a dose-dependent fashion; specifically, necrosis, vacuolization, and inflammation were twofold greater than in pair-fed controls. In both groups, LPS dose dependently increased pancreatic necrosis, but the necrosis score increased two times as much in the alcohol-fed animals. Average tissue injury was significantly increased in alcohol-fed rats compared with the controls when rats were injected with the same concentration of LPS (Fig. 1E). Similar pancreatic tissue injury has been recently reported after the first cerulein-induced acute episode in the alcohol-exposed rat pancreas compared with controls (17). These data suggest that chronic alcohol exposure exacerbated pancreatic injury in response to LPS.

Alcohol plus LPS increased the induction of HO-1-positive macrophages in a dose-dependent fashion. In the next step, we investigated whether pancreatic injury seen in the histopathology score is associated with rising immune cell infiltration. The induction of HO-1, an enzyme catalyzing the conversion of heme to biliverdin, is induced by oxidative and nitrosative stress, cytokines, and other factors during inflammation (2). Activated macrophages can be evaluated by using HO-1 immune staining (58, 59). Previous reports have shown that HO-1 activity is increased in macrophages in response to LPS (59). We investigated whether LPS administration showed HO-1-positive infiltrating macrophages and whether an increasing LPS dose induces alterations in the degree of infiltration of inflammatory cells in the alcohol-fed animals. HO-1-positive cells were not detectable in pair-fed control and alcohol-fed animals. Consistent with the pancreatic injury score, inflammatory cell counts increased 24 h after LPS injection in a dose-dependent manner. Inflammation increased significantly in the alcohol-fed animals compared with controls when animals were injected with the same LPS dose.

In the LPS-injected pair-fed animals, the number of positive cells rose from 10 cells per 20 randomized fields (0.2 mg LPS) to 69 and 123 positive cells (1.5–3 mg LPS). LPS administration in the alcohol-fed animals increased HO-1 from 12 (0.2 mg LPS) to 266 positive cells per 20 randomized fields (3 mg LPS), a twofold increase by alcohol exposure (Fig. 1F). These data suggest that alcohol exposure in combination with LPS stimulated pancreatic immune cell infiltration.

LPS dose dependently increased expression of secretory pancreatic stress proteins. During the course of acute or chronic pancreatitis, PAP-1 and PSP/reg, both known indicators for stress and pancreatic disease, are highly induced (22, 34, 48, 64). PAP-1 may play several roles in limiting pancreatic damage during acute pancreatitis. PAP-1 and PSP appear to protect acinar cells against oxidative stress-mediated apoptosis (52) and also have anti-inflammatory effects (64). We investigated whether LPS administration induced the pancreatic acute-phase response and whether an increasing LPS dose induces alterations in the expression of secretory stress protein expression in the alcohol-fed animals. PAP-1 protein levels in pair-fed control and alcohol-fed rats were very low (0.0136 and 0.036 μg/mg total protein, respectively). In alcohol-fed and pair-fed control rats, 24 h after LPS injection, PAP-1 concentration increased in a LPS dose-dependent fashion. There was no difference between the control and alcohol-fed animals when they were treated with equal LPS doses (Fig. 2A).

Similarly, PSP/reg levels were very low in control and alcohol-fed rats (0.144 and 0.036 μg/mg, respectively). In alcohol-fed and pair-fed control rats, 24 h after LPS injection PSP/reg increased also in an LPS dose-dependent fashion but appear to reach a plateau at the highest LPS dose. There was also no difference between the control and alcohol-fed animals when they were treated with equal LPS doses (Fig. 2B). These data suggest that the secretory stress protein expression is very sensitive in response to LPS and reaches a plateau of expression at LPS doses below the lethal dose.

Alcohol exposure reduces TUNEL positivity in response to LPS. We next investigated whether the increased tissue damage, inflammation, and expression of pancreatic secretory stress proteins are accompanied by an increase of pancreatic apoptosis (TUNEL-positive acinar cells). Quantitative apoptosis analysis was determined by an in situ TUNEL assay in pancreatic tissue sections as described previously (11, 25). TUNEL-positive nuclear staining was present only in pancreatic acinar cells, whereas islet or ductal cells were not affected by this experimental setting and did not show signs of apoptosis, as reported earlier (Fig. 3A) (25). Chronic alcohol exposure tended to diminish sixfold TUNEL-positive nuclei in alcohol-fed rats compared with pair-fed (control) animals when rats were injected with equal LPS doses (Fig. 3B). These data suggest that alcohol exposure exacerbated pancreatic injury in response to LPS.
Acinar cells compared with pair-fed rats. LPS at a dose of 3 mg/kg body wt significantly increased 4.4-fold TUNEL positivity in the pair-fed rats, whereas in the alcohol-fed rats LPS increased apoptosis only 2.6-fold. Just missing significance, alcohol exposure tended to decrease apoptosis compared with controls, and LPS-injected animals also showed a tendency to display less apoptotic activity in the alcohol-fed rats (Fig. 3B). These results confirm our previous observation using 1 mg/kg body wt LPS (25) and show that chronic alcohol exposure suppresses pancreatic apoptosis.

Inhibition of caspase-8 activation by chronic ethanol administration. We further investigated whether the elevated pancreatic injury is accompanied by the activation of the initiator caspase-8 and whether caspase-8 activity follows LPS dose dependency in both treatment groups. In pilot experiments, we have shown that single dose of LPS injection (1 mg/kg) activates caspase-8 after 24 h LPS treatment. In the pair-fed control rats 24 h after LPS injection, caspase-8 activity reached 281 and 246 μU/mg at 0.8 and 1.5 mg/kg LPS, respectively. In the alcohol-fed rats injected with equal LPS doses, caspase-8 activity reached only 97 and 103 μU/mg, respectively. This LPS dose-dependent response rate in the alcoholic pancreas was significantly lower compared with pair-fed controls (Fig. 4A). This suggests that alcohol causes a suppression of caspase-8 activation in response to LPS stimulation.

Decreased caspase-9 activity by chronic ethanol administration in response to LPS. We also examined whether increasing LPS doses result in the activation of caspase-9 in a dose-dependent fashion and whether alcohol potentiates caspase-9 activity in response to various LPS doses. Caspase-9 can be activated through the mitochondria apoptosome pathway or intrinsic pathway (10). Similar to the activity of caspase-8, caspase-9 activity was significantly different between the treatment groups in response to various LPS doses. In the pair-fed control rats 24 h after LPS injection, caspase-9 activity reached 281 and 246 μU/mg at 0.8 and 1.5 mg/kg LPS, respectively. In the alcohol-fed rats injected with equal LPS doses, caspase-9 activities were lower and reached only 85 and 96 μU/mg, respectively (Fig. 4B). This suggests that the activation of the intrinsic apoptotic pathway in response to LPS is impaired by chronic alcohol exposure.

Chronic ethanol administration plus LPS accelerate caspase-3 activity. We next investigated whether activation of the extrinsic and intrinsic pathway activates the “downstream” enzyme caspase-3 (10). Caspase-3, when activated through caspase-8 or caspase-9, catalyzes reactions that constitute “points of no return” to the preapoptotic state of the cell (10). Therefore, we quantified caspase-3 activity in pancreatic homogenates from control and alcohol-fed animals 24 h after various doses of LPS application. Caspase-3 activity was significantly different between the treatment groups in the LPS dose-dependent response. Caspase-3 activity reached 2,255 μU/mg at 0.8 mg/kg LPS in the pair-fed animals. In the alcohol-fed rats injected with the same amount of LPS, caspase-3 activity reached only 1,358 μU/mg. However, the reduction was not as pronounced as reported for the other caspases. There was no difference between the groups at the highest LPS dose (Fig. 4C). These data suggest that alcohol interferes with the lower apoptotic response to LPS.

Chronic ethanol administration plus LPS accelerates caspase-2 activity. Our results on caspase-9 led us to investigate whether activation of the intrinsic pathway is associated with the activation of “upstream” enzyme caspase-2. Several reports have suggested that caspase-2 is able to permeabilize mitochondrial membrane and promote the release of cytochrome c (24, 44). Caspase-2 activity was significantly different between the treatments groups in the LPS dose-dependent response, with an activity rate similar to caspase-3. In the pair-fed control rats 24 h after LPS injection, caspase-2 activity reached 1,234 μU/mg at 0.8 mg/kg LPS with no further increase at the higher LPS dose. In alcohol-fed rats injected with an equal LPS dose, caspase-2 reached only 718 μU/mg. This LPS dose-dependent response rate in the alcoholic pancreas was significantly lower compared with pair-fed controls (Fig. 4D). In pilot experiments using a RNase protection assay, we have found that caspase-2 mRNA expression was highly reduced after chronic alcohol exposure (data not shown). These data suggest that alcohol causes a suppression of caspase-2 activation in response to LPS stimulation and may contribute to the reduction of caspase-9 activation through the intrinsic pathway.
ATP concentration depletion is more severe in alcohol-fed rats in response to LPS. We next investigated whether the difference in the apoptotic rate in the alcohol- and control-fed animals is associated with an alteration in the cellular nucleotide levels. Apoptosis is an active self-destructive cell death process and requires a concentration of ATP ([ATP]) (4). Decreased levels of [ATP] have been recognized in apoptotic cells, whereas more pronounced [ATP] depletion has been associated with necrosis rather than apoptosis (4, 33). In alcohol-fed rats, decreased [ATP] levels were more pronounced, ranging from 47 to 6 nM/mg protein (from 100 to 13%) in response to rising LPS doses. In pair-fed control animals, decreased [ATP] levels were less severe, ranging from 20 to 7.7 nM/mg protein (from 100 to 38%) in response to increasing LPS doses (Fig. 5A). The [ATP] determined in this investigation was very similar to those reported by others. An [ATP] depletion over 85% has been strongly implicated with necrosis (32, 39, 40). To test whether the drop in [ATP] was different, data were normalized to values of saline-injected alcohol-fed or pair-fed animals. As shown in Fig. 5B, a loss of ~60% [ATP] was determined in control rats injected with 3 mg LPS, whereas the alcohol-fed group exhibited a loss of >85% pancreatic [ATP]. This result indicates a severe metabolic change in the alcoholic pancreas in response to LPS.

Ado-to-ATP ratio is more pronounced in alcohol-fed rats in response to LPS. Because the [ATP] level alone appears to be insufficient to determine whether acinar cells undergo apoptosis or necrosis in response to an LPS stimulus in alcohol-fed rats, we further investigated the ratio of cellular ADP to ATP. A moderate increase in the ADP-to-ATP ratio indicates apoptosis, whereas a higher ADP-to-ATP ratio points toward necrosis (8). In our experimental settings, the ADP-to-ATP ratio was significantly lower in the pair-fed group compared with the alcohol-fed rats when injected with the same LPS dose. The ratio of ADP to ATP in the alcohol-fed rats was consistently higher over the whole range of LPS dose responses, suggesting that pancreatic acinar cells were forced to a necrotic form of cell death (Fig. 5C).

DISCUSSION

In this report, we demonstrated in a rat model that LPS treatment causes dose-dependent pancreatic injury and infiltration of inflammatory cells, an effect exacerbated by preceding chronic alcohol exposure in the rat pancreas (Fig. 1, E and F). Furthermore, we showed that alcohol reduces several cellular markers of apoptosis after a proapoptotic stimulus (LPS). The increase in inflammation combined with a reduction in apoptosis led us to postulate a switch from controlled cell death to uncontrolled cell death, e.g., necrosis. The latter is supported by the change in energy status.

Alcohol exposure alone induces pancreatic necrosis, and the increased necrosis score was twofold higher in alcohol-fed animals in response to LPS (Table 1). Inflammatory cells, predominantly infiltrating monocytes/macrophages, apparently migrate in the pancreatic tissue from the interstitium 24 h after LPS injection (Fig. 1D). Because HO-1 is not a specific macrophage marker, we cannot rule out that some of the pancreatic endothelial cells become HO-1 positive in response to LPS. However, our data suggest that the pancreas exposed to alcohol and injected with LPS appears to be in an ongoing state of inflammation. In a model of alcoholic pancreatitis, using multiple injections of cerulein, similar pancreatic injury has been reported in alcohol-fed rats after the first episode of cerulean injections (17).

Unfortunately, there are no adequate quantitative methods available to determine the rate of necrosis in vivo. Blood lactate dehydrogenase has been implicated as a marker for...
pancreatic injury (60). However, this marker represents a systemic response in which several organs, e.g., the lung and liver, may respond differently to alcohol or alcohol in combination with LPS. A quantitative marker for granulocyte infiltration (37), myeloperoxidase (MPO) activity, showed no difference (data not shown), suggesting that we missed the peak time point of granulocyte infiltration 24 h after LPS injection. This is likely, because in cerulein-induced pancreatitis, MPO activity only transiently increased during the first few hours, after which MPO activity may be lost or degraded by granulocytes.

Previous investigations have demonstrated that alcohol induces acinar mitochondrial damage (42). Surprisingly, our investigation showed that alcohol administration alone inhibits the mitochondria-associated apoptotic pathway, whereas the receptor-mediated apoptotic pathway was not affected (Fig. 4, A-C). Caspase activities showed a significantly lower activation rate in alcohol-fed rats in an LPS dose-dependent fashion compared with pair-fed control rats (Fig. 4, A-C). We confirmed our observation by using a TUNEL assay, which demonstrated a clear trend: chronic alcohol exposure reduced pancreatic apoptosis, and the reduction appeared to be maintained in response to a proapoptotic stimulus, such as LPS (Fig. 3B). This observation is supported by our previous report (25).

Our earlier study indicated that chronic alcohol administration alone decreased caspase-3 activity compared with the pair-fed controls (25). Several lines of additional evidence, such as RNase protection assay and competitive RT-PCR, have confirmed that chronic alcohol exposure significantly suppresses mRNA levels of caspase-3 (data not shown). Consistent with this investigation, we confirmed that alcohol intake significantly reduces at least caspase-3 and caspase-9 compared with controls without LPS injection, suggesting that the intrinsic and executioner apoptotic pathway is reduced, whereas the extrinsic, caspase-8-mediated pathway is not affected by alcohol. This result suggests that alcohol exposure impairs the intrinsic apoptotic pathway in a similar way to the liver (12, 13).

We confirmed our observations by measuring activated caspase-2 activity, reported to act upstream from the intrinsic mitochondria pathway (24, 44, 45, 63). Caspase-2 activity was significantly lower in alcohol-fed rats compared with pair-fed control rats when they were stimulated with LPS (Fig. 4D). Our pilot RNase protection assay confirmed that chronic alcohol exposure significantly reduces mRNA expression of caspase-2 (data not shown). These data suggest that caspase-2-reduced activation by alcohol may be linked with the diminished caspase-9 activity. Presumably, the suppression of apoptosis in alcohol-fed rats makes the pancreas more susceptible to deleterious agents, resulting in enhanced necrosis rather than apoptosis. A similar observation has been reported by Koteish and colleagues (36); in their experiments, they showed that endotoxin potentiates alcoholic liver injury. In necrotizing acute pancreatitis, necrosis is the predominant form of cell death, greatly contributing to the severity of pancreatitis (4). Because clinical work has shown that LPS plays an important role in acute necrotizing pancreatitis, our findings indicate that chronic alcohol consumption predisposes to necrotizing pancreatitis by inhibiting apoptosis and promoting necrosis.

Investigation into the mechanisms leading to necrotic cell death has been difficult to accomplish in experimental models. Besides careful morphological tissue examination, there is only

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**Fig. 5. Nucleotide levels.** The determination of pancreatic ATP concentration ([ATP]) content and the ADP-to-ATP ratio in alcohol- and control-fed animals in response to LPS are shown. **A:** [ATP] content in pancreata of alcohol- and control-fed animals in response to LPS. #The two LPS dose-response curves are significantly different (p = 0.015) in the alcohol- and pair-fed animals, using two-way ANOVA. *P < 0.05 vs. pair-fed control animals injected with the identical LPS dose, using the t-test. **B:** Relative loss of [ATP] in %normalized to vehicle injection in the alcohol- and control-fed animals as 100% ATP. Plotted are loss of [ATP] in %. #The two LPS dose-response curves are significantly different (P = 0.0072) in the alcohol- and pair-fed animals, using two-way ANOVA. *P < 0.05 vs. alcohol-fed animals injected with the identical LPS dose, using the t-test. **C:** Ratio of ADP to ATP in alcohol- and control-fed animals in response to a rising LPS dose. #The two LPS dose-response curves are significantly different (P = 0.0002) in the alcohol- and pair-fed animals, using two-way ANOVA. *P < 0.05 vs. pair-fed control animals injected with an equal LPS dose, using the Students’ t-test.

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one well-accepted biochemical approach in vivo that is able to distinguish between apoptotic and necrotic cell death, using the relative cellular level of ADP to ATP. Thus, to assess tissue necrosis quantitatively, the changes in [ATP] and the ADP-to-ATP ratio are the only biochemical markers. Consistent with previous reports, our data show not only a more pronounced ATP depletion but also a significantly higher ADP-to-ATP ratio in alcohol-fed rats in an LPS dose-dependent fashion (4, 8, 33).

These results confirm that the energy charge of the pancreas is changed after alcohol exposure. In view of the current literature on the requirement of ATP to execute apoptosis, a dramatic loss of ATP might result in the interruption of apoptosis and initiation of necrosis, possibly with the consequence of increasing the subsequent inflammatory reaction.

Apoptosis has been associated with little inflammation, whereas necrosis is known to be a strong inflammatory inducer. Our data suggest that chronic alcohol exposure promotes necrotic cell death and accelerates neuroinflammation in response to a second hit. [ATP] depletion is known to inhibit the induction steps of the apoptotic pathway, e.g., the apoptosis formation of the intrinsic pathway (23, 32). It has been suggested that such depletion in [ATP] levels promotes a switch from apoptotic to necrotic cell death and that a depletion by >80% led to necrosis (Fig. 5B) (32). Consistent with this observation, our data show a depletion of ~80% at the highest LPS dose (3 mg/kg) in the alcohol-fed animals. In contrast, in the pair-fed control animals, the ATP depletion reached only 60% at the highest LPS dose, which might be insufficient for the necrosis induction (Fig. 5, A–C). A recent report by Whitcomb and colleagues demonstrated that, after chronic alcohol exposure, mitochondria were injured and an ATP synthase subunit was upregulated, suggesting mitochondrial reparation (42). These morphological and functional data might provide an explanation for the lower ATP levels in the alcohol-exposed rat pancreas (42). Such mitochondria may make the acinar cells more vulnerable to LPS-induced damage, which could lead to the more pronounced ATP depletion and the switch to more necrosis.

One potentially important defense mechanism involves the induction of acute secretory stress proteins PAP-1 and PSP/reg. These polypeptides are upregulated during acute and chronic pancreatitis (21, 28, 30, 47) and, specifically, by LPS, which appears to be increased in some alcoholics (56). We examined the effect of alcohol on PAP-1 and PSP/reg expression and the response to an LPS challenge using acute secretory stress and LPS (3 mg/kg) in alcohol-fed animals. In contrast, in the pair-fed control animals, the ATP depletion reached only 60% at the highest LPS dose, which might be insufficient for the necrosis induction (Fig. 5, A–C). These morphological and functional data might provide an explanation for the lower ATP levels in the alcohol-exposed rat pancreas (42). Such mitochondria may make the acinar cells more vulnerable to LPS-induced damage, which could lead to the more pronounced ATP depletion and the switch to more necrosis.

One potentially important defense mechanism involves the induction of acute secretory stress proteins PAP-1 and PSP/reg. These polypeptides are upregulated during acute and chronic pancreatitis (21, 28, 30, 47) and, specifically, by LPS, which appears to be increased in some alcoholics (56). We examined the effect of alcohol on PAP-1 and PSP/reg expression and the response to an LPS challenge using acute secretory stress proteins as a surrogate marker of the inflammatory response in the pancreas and as potential protective mechanisms that might be altered by chronic alcohol ingestion. In the current report, we demonstrated that both PAP-1 and PSP/reg were highly increased in an LPS dose-dependent fashion, with no difference between the two treatment groups (Fig. 2, A and B). The maximal pancreatic tissue levels of PAP and PSP/reg were very similar to those in other experimental models of acute pancreatitis (28). This supports the concept that these proteins are highly expressed as a consequence of pancreatic injury.

In summary, in this report, we showed that LPS induces dose-dependent pancreatic injury. The reduced ATP content is consistent with the diminished caspase activities, in particular caspase-2 and caspase-3, as well as the TUNEL positivity in the alcohol-fed rats in response to LPS. These findings suggest that the pancreas exposed to alcohol is more sensitive to LPS-induced damage, resulting in an increase in necrotic cell death. These findings would predict greater susceptibility to pancreatic necrosis in alcoholic patients.

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