Effects of pancreatic duct ligation on pancreatic response to bombesin

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Departments of 1Surgery and 2Biochemistry and Molecular Biology, University of Tokyo, Tokyo; 3Department of Surgery, Ichijo-dori Hospital, Ashikawa; 4Department of Surgery, Tokyo Metropolitan Fuchu Hospital, Tokyo; 5Department of Applied Biological Science, Tokyo University of Science, Kashiwa, Japan; and 6Departments of Medicine and Cell Biology, Veterans Affairs Medical Center, Yale University School of Medicine, West Haven, Connecticut

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Otani, Taiichi, Akira Matsukura, Takeshi Takamoto, Yasuji Seyama, Yasuhiro Shimizu, Michiyoshi Shinomiya, Hiroshi Usui, Fred S. Gorelick, and Masatoshi Makuuchi. Effects of pancreatic duct ligation on pancreatic response to bombesin. Am J Physiol Gastrointest Liver Physiol 290: G633–G639, 2006. First published November 17, 2005; doi:10.1152/ajpgi.00377.2005.—To examine mechanisms that might be related to biliary pancreatitis, we examined the effects of pancreatic duct ligation (PDL) with pancreatic stimulation in vivo. PDL alone caused no increase in pancreatic levels of trypsinogen activation peptide (TAP), trypsin, or chymotrypsin and did not initiate pancreatitis. Although bombesin caused zymogen activation within the pancreas, the increases were slight and did not cause pancreatitis. However, the combination of PDL with bombesin resulted in prominent increases in pancreatic TAP, trypsin, chymotrypsin, and the appearance of TAP in acinar cells and caused pancreatitis. Disruption of the apical actin network in the acinar cell was observed when PDL was combined with bombesin but not with PDL or bombesin alone. These studies suggest that when PDL is combined with pancreatic acinar cell stimulation, it can promote zymogen activation, the retention of active enzymes in acinar cells, and the development of acute pancreatitis.

pancreas; pancreatitis; trypsinogen activation peptide; trypsin

ALTHOUGH ACUTE OBSTRUCTION of the pancreatic duct by gallstones is one of the most common causes of acute pancreatitis, the disease mechanism remains unclear. Pathological activation of digestive enzymes within the pancreatic acinar cell plays a central role in the pathogenesis of acute pancreatitis (2, 4, 14). Some studies suggest that zymogen activation must be combined with retention of active enzymes within the acinar cell to cause disease. Supraphysiological concentrations of the cholecystokinin analog caerulein cause zymogen activation, the retention of active enzymes within the acinar cell and acute pancreatitis (5, 8). Thus, although activation of bombesin receptors on the pancreatic acinar cell causes zymogen activation, it does not result in acute pancreatitis (5). A difference in the trafficking of activated enzymes seems to account for the distinct injury responses to the two ligands. Thus, whereas active enzymes are retained in the acinar cell after treatment with supraphysiological concentrations of caerulein, they are secreted after bombesin treatments. Notably, physiologically relevant concentrations of caerulein cause low levels of zymogen activation, but the active enzymes are also secreted, and there is no cell injury (5). The findings of at least two studies have suggested that pancreatic duct obstruction causes reduced secretion from the pancreatic acinar cell (7, 10). Although previous studies have examined the effects of pancreatic duct obstruction, the development of pancreatitis has not been fully examined. We hypothesized that if acute pancreatic duct obstruction was combined with treatments that caused zymogen activation but no pancreatitis, it could lead to retention of active enzymes and acute pancreatitis. In support of this hypothesis, we report that although neither bombesin treatments nor duct obstruction alone cause acute pancreatitis, the combination of the two treatments does result in disease.

MATERIALS AND METHODS

Male Wister rats (150–300 g) were obtained from the Nisseizai Breeding Laboratories (Tokyo, Japan). The experiments were carried out on rats that were fasted overnight with free access to water. The Animal Experimentation Committee of the University of Tokyo approved study protocols. Animals were anesthetized by intraperitoneal injection of 1,000 mg/kg urethane (ICN Biomedicals, Aurora, OH). All other chemicals and supplies were from Sigma Chemical (St. Louis, MO), unless otherwise noted.

Treatments. Intravenous infusions through the femoral vein of 0.9% sodium chloride (control and vehicle), bombesin (5 μg·kg−1·h−1, sham or ligate), or supraphysiological caerulein (5 μg·kg−1·h−1, sham) were administered for 3 h. The 3-h treatment period was selected because in preliminary studies we observed that it caused no pancreatitis after bombesin or duct ligation; it also was an earlier time point than used in previous studies of duct obstruction. Pancreatic duct ligation (PDL) was performed in sedated rats. The extrapancreatic region of the biliopancreatic duct was ligated just adjacent to its entrance into the duodenum. This obstructed both the pancreatic and bile duct. To allow drainage of bile, the bile duct was divided proximally to the obstruction and the drainage was either returned (via a drainage tube placed in the bile duct) or not returned to the duodenum. In preliminary studies we found no difference in unstimulated or caerulein hyperstimulation responses including serum amylase, pancreatic edema, or trypsinogen activation peptide (TAP) levels or histological evidence of pancreatitis between returning and excluding bile from the intestine. Therefore, the studies presented were all performed with bile duct drainage not returned to the small intestine.

Serum amylase level. Serum amylase activities were determined using the Neo amylase test kit (Daichi Chemical Pharmaceutical, Tokyo, Japan). These values are normalized to the control condition.

Pancreatic edema. After treatments, a portion of the pancreas was removed to estimate its water content by calculating the wet-to-dry weight ratio, the ratio of the wet weight of the pancreas to its weight after 7 days of desiccation at 70°C. The values are expressed as a percentage of wet weight.

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Neutrophil infiltration in pancreas. After treatments, the pancreas was removed and cut into ~10-mm³ pieces, fixed in 10% (wt/vol) formaldehyde in phosphate-buffer saline (PBS), and embedded in paraffin. Tissue sections (4-μm thickness) were stained with hematoxylin and eosin and examined using an Olympus BX51 inverted microscope. The number of neutrophils was estimated by counting the number of neutrophils at ×400 magnification in an average of 50 fields with at least 1,000 acinar cells. For each animal, neutrophil numbers were expressed as a percentage of acinar cells.

Electron microscopy. After treatments, the pancreas was removed and cut into ~2-mm³ pieces and fixed in 0.1 M sodium cacodylate containing 3% glutaraldehyde. Samples were then dehydrated and embedded in Epon, postfixed with osmium, and stained with uranyl and lead as described previously (5). Ultrathin sections were examined using a Phillips 300 electron microscope at 60 kV.

TAP immunoreactivity. After treatments, rats were subjected to 5 min of in vivo perfusion fixation through the femoral vein with a buffer containing 0.05% glutaraldehyde and 2% paraformaldehyde as described previously (13). The pancreas was removed, cut into 1-mm³ pieces, and immersed in the same buffer for 2 h at room temperature. After rinsing with a perfusion buffer without fixatives, pancreatic fragments were embedded in OCT compound (Miles, Elkhart, IN) and then snap frozen in liquid nitrogen and isopentane. Approximately 5-μm frozen sections were obtained, placed on alun-gelatin-coated coverslips, and processed for TAP immunoreactivity as described previously (13). Sections were viewed and photographed with an Olympus BX51–34-FL-1 microscope equipped with epifu-rence illumination and barrier filters (Tokyo, Japan) with the use of Kodak Tmax 100 (ASA 100) films at a magnification of ×1,000. Fluorescence micrographs were exposed for 20 s, developed at constant conditions, and digitized using a Nikon Coolscan slide scanner. Images were processed identically using Adobe Photoshop.

TAP activity in pancreas. After treatments, pancreas tissue (0.1–0.3 g) was removed and immersed in 0.2 M Tris·HCl buffer (pH 7.3) containing 20 mmol/l EDTA, boiled for 10 min to denature the remaining proteases, and homogenized in 1-ml conical polypropylene tubes for 30 s. After centrifugation of the samples (1,500 rpm, 10 min, 4°C), the supernatant was stored at −70°C until further assay. TAP activity was measured with a commercial ELISA kit (Biortin International, Dublin, Ireland) and expressed as nanomoles per microgram of pancreatic DNA.

Trypsin and chymotrypsin activities in pancreas. Trypsin activity was measured fluorimetrically using Boc-Gln-Ala-Arg-MCA (Peptide Institute, Osaka, Japan) as the substrate. Briefly, excised pancreatic tissues were homogenized in 5 mmol/l MOPS buffer, pH 8.0, containing 250 mmol/l sucrose and 1 mmol/l MgSO₄ (pH 7.0), and then 100 μg of the homogenates were added to a 24-well plate containing 350 μg of assay buffer [50 mmol/l Tris (pH 8.0), 150 mmol/l NaCl, 1 mmol/l CaCl₂, and 0.1% bovine serum albumin]. After 50 μg of the substrate were added to each well, the fluorescence was monitored with excitation at 380 nm and emission at 440 nm. The trypsin activity was calculated as the slope of fluorescence emission expressed per milligram of DNA in the homogenates. Chymotrypsin activity was measured following the same method as trypsin activity by using Suc-Ala-Ala-Pho-Phe-MCA (Peptide Institute) as the substrate. The trypsin and chymotrypsin activities were expressed as the relative increase over controls in each experiment. DNA was measured using Hoechst dye 33258.

F-actin distribution. After treatments, the pancreas was fixed in 0.05% glutaraldehyde-2% paraformaldehyde for 2 h at room temperature. Frozen sections were labeled with TRITC-phalloidin and Hoechst dye 33258.

Data analysis. Data are presented as means ± SE. Differences between the groups were compared using nonparametric analysis of variance (Kruskal-Wallis test), followed by the Mann-Whitney U-test. A 5% probability of type I experimental error (P < 0.05) was considered statistically significant; n = 3–6 in all experimental groups.

RESULTS

Serum amylase level. Serum amylase levels 3 h after either PDL or bombesin stimulation alone were similar to those for untreated controls (Fig. 1). However, serum amylase levels after bombesin stimulation combined with PDL increased about threefold over control conditions and were comparable to that caused by caerulein hyperstimulation.

Pancreatic edema. The wet-to-dry weight ratios 3 h after PDL or bombesin stimulation alone were similar to the untreated control value of 3.2 (Fig. 2). However, when bombesin stimulation was combined with PDL, pancreatic edema increased significantly and was similar to that caused by caeru-lein hyperstimulation (value of 7.4). The results for water content from unstimulated and caerulein-hyperstimulated samples are consistent with those reported in the literature (un-stimulated, 72–74% water content; caerulein hyperstimulated, 86–89%). Although edema has been reported to occur after PDL in other studies, the study periods were all greater than the 3 h used in this study (9, 10).

Pancreatic neutrophil infiltration. Virtually no neutrophil infiltration was seen after 3 h with PDL or bombesin stimulation alone or in unstimulated controls (Fig. 3). However, after bombesin stimulation combined with PDL, leukocyte infiltration increased over control conditions and was comparable to that caused by caerulein hyperstimulation.

Pancreatic acinar cell vacuoles. Transmission electron micros-copy was used to evaluate the acinar cell vacuole formation that is typical of acute pancreatitis. Vacuoles were uncommon in control and PDL rats (Fig. 4, A and C). However, numerous small vacuoles were seen after caerulein hyperstimulation (Fig. 4B). Vacuoles that formed after bombesin stimulation (Fig. 4, D and E) were larger and less numerous than those observed after caerulein hyperstimulation. Many of these appeared to contain other organelles, suggesting that they were autophagic. Compared with bombesin stimulation alone (Fig. 4D), large vacuoles were still prominent but small vacuoles were more common after bombesin stimulation with PDL (Fig. 4E).
Together, the findings of edema, inflammation, and vacuole formation suggest that although neither PDL nor bombesin alone cause pancreatitis, the combination of both treatments leads to a disease of similar severity to that observed with caerulein hyperstimulation. Because the pathological activation of digestive zymogens within the pancreatic acinar cell is a hallmark of acute pancreatitis, we next examined this parameter.

Pancreatic TAP immunoreactivity. Virtually no TAP immunofluorescence was observed in PDL rats and controls (Fig. 5, A and C). Most TAP immunoreactivity was found in small vesicles in the supranuclear region and in vacuoles after caerulein hyperstimulation (Fig. 5B). A small amount of TAP immunoreactivity was occasionally found in the periphery of vacuoles after bombesin stimulation alone (Fig. 5D). However, the appearance of TAP immunoreactivity within acinar cells was much more prominent after bombesin stimulation with PDL. Thus TAP was found in a greater number of vacuoles, and it often filled the vacuole (Fig. 5E). Although the number of TAP-immunoreactive structures increased after caerulein hyperstimulation or when bombesin stimulation was combined with PDL, vacuoles observed after caerulein hyperstimulation...
were smaller and more numerous than those associated with bombesin treatment. These studies suggested that when PDL is superimposed on bombesin treatment, there is an increase in TAP generated within acinar cells. To quantify the levels of zymogen activation under various treatment conditions, we performed additional assays.

Pancreatic TAP levels. TAP generation in pancreatic homogenates 3 h after PDL was similar to that in untreated controls (Fig. 6). TAP values 3 h after bombesin stimulation (5 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)) alone were slightly higher than under control conditions but were not significantly different. However, pancreatic TAP 3 h after bombesin stimulation combined with PDL increased about 10-fold over control conditions and was comparable to 3 h of caerulein hyperstimulation (5 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)). These findings support the conclusion that trypsinogen activation, as reflected by TAP generation, is dramatically enhanced when PDL is combined with bombesin treatment. Because TAP is an indirect measure of trypsinogen activation, we next performed direct assays of enzyme activities.

Trypsin and chymotrypsin activities in pancreas. Trypsin activity, normalized to the control conditions, 3 h after PDL or bombesin stimulation (5 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)) alone was similar to that in untreated controls (Fig. 7A). However, trypsin activity 3 h after bombesin stimulation combined with PDL increased about twofold over control conditions and was comparable to 3 h of caerulein hyperstimulation (5 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)). Similarly, chymotrypsin activity, normalized to the control conditions, 3 h after PDL or bombesin stimulation (5 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)) alone was equivalent to that in untreated controls (Fig. 7B). However, chymotrypsin activity 3 h after bombesin stimulation combined with PDL increased about 2.5-fold over control conditions and was comparable to 3 h of caerulein hyperstimulation (5 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)). These studies demonstrate that whereas PDL alone does not affect zymogen activation, it may amplify the pancreatic zymogen activation caused by bombesin alone. However, because active enzymes generated by bombesin alone may be secreted into the duct and the small intestine, it is possible that the levels of activation with bombesin alone are underestimated. Nonetheless, the finding that TAP accumulates in acinar cells only after bombesin treatment is combined with PDL suggests that there is more active enzyme retained in the acinar cell with PDL.
**F-actin distribution.** F-actin fluorescence was normally distributed with linear labeling at the apical membrane of acinar and duct cells in PDL rats and controls (Fig. 8, A and C). After caerulein hyperstimulation, labeling of the apical actin network was decreased, clumped, and discontinuous, and basolateral labeling increased (Fig. 8B) (19, 20). After bombesin stimulation alone, some clumping of the apical actin network was observed, but continuity was maintained. Some increased labeling of the basolateral membrane was observed after bombesin treatment (Fig. 8D). Bombesin stimulation with PDL caused changes similar to those caused by bombesin alone (Fig. 8C), but interruption of the apical labeling similar to that caused by caerulein hyperstimulation was observed (Fig. 8E).

**DISCUSSION**

The pathogenesis of biliary pancreatitis remains unclear. It appears that even transient obstruction can cause acute pancreatitis in humans. However, it is likely that only a minority of patients that pass gallstones through the common channel that drains the pancreatic and bile ducts develop pancreatitis. Thus other factors, such as pancreatic stimulation, might increase the risk of developing disease. In that context, the present study reports several key observations. Although neither short-term PDL nor bombesin treatment caused acute pancreatitis, a...
combination of the two treatments caused disease. Furthermore, the combined treatments caused a prominent increase in active proteases within the pancreas that was not observed with either treatment alone but equaled that caused by caerulein hyperstimulation. Finally, the observation that prominent TAP accumulation was detected in acinar cells when PDL was combined with bombesin, but not with either treatment alone, supports the conclusion that proteases are being both activated and retained in the pancreatic acinar cell.

Although previous studies have examined the effects of PDL in the rat, the effects of superimposing secretagogues on zymogen activation and other parameters of acute pancreatitis have not been systematically evaluated. Short-term (1–6 h) PDL in the rat has been found to cause pancreatic edema (11). Similar to our study, when secretin (another agent that does not cause pancreatitis) was superimposed on obstruction, edema increased. However, this and other studies have not examined other parameters of acute pancreatitis such as protease activation or inflammation after PDL in rats (10, 15, 16). Mooren et al. (10) observed acinar cell dysfunction after PDL in the rat and mouse. Thus PDL induced decreases in both secretion and peak cytosolic Ca\(^{2+}\) acinar cell responses to acetylcholine. Because zymogen activation depends on pathological increases in cytosolic Ca\(^{2+}\) in response to ligands, the study by Mooren et al. raised the possibility that PDL might even reduce zymogen activation. In this context, our study suggests that the acinar cell can respond with pathological zymogen activation even after PDL. This study confirms that when PDL is combined with an agent that does not cause pancreatitis, the combination causes pancreatic edema (11). In addition, it demonstrates that bombesin with PDL causes pancreatitis.

Previous studies have suggested that both zymogen activation and the retention of active enzymes within the acinar cell are needed to initiate acute pancreatitis (1, 5). The observation that there is prominent accumulation of TAP within the acinar cell only when PDL was combined with bombesin is consistent with the hypothesis that PDL inhibits acinar cell secretion. Previous studies have suggested that acinar cell secretion from zymogen granules is inhibited by pancreatic duct obstruction (6, 9). Because zymogens appear to be activated in and secreted from nonzymogen granule compartments (6, 13), it was unclear whether they would exhibit the same inhibitory response to PDL. Preliminary studies suggest that secretion from zymogen granules and activated zymogen may arise from distinct compartments that are selectively regulated by different second messengers (Gorelick FS, unpublished observation). Our observation that TAP accumulates in acinar cells only when bombesin stimulation is combined with PDL supports the conclusion that secretion of activated enzymes is inhibited. Although the mechanism of the inhibited secretion remains unclear, the response could be related to the disordered Ca\(^{2+}\) signaling observed by Mooren et al. (10) with PDL or derangements in the apical actin cytoskeleton observed in this study and by others (3, 12).

The disruption of the apical cytoskeleton in pancreatic acinar and duct cells observed with PDL and bombesin might have other consequences that are relevant to pancreatitis. This actin network regulates paracellular permeability; its disruption could allow the free movement of active enzymes from the duct lumen into the interstitial space (3). Thus, even if the high levels of pancreatic enzymes observed after bombesin with PDL compared with bombesin are secreted from the acinar cell, they could cause disease by entering the intracellular space.

Although the changes elicited with bombesin with PDL and caerulein hyperstimulation were similar, they were not identical. The levels of zymogen activation tended to be greater after caerulein treatment, but the effects on edema, inflammation, and the actin cytoskeleton were similar in the two models. However, in the PDL plus bombesin model, TAP immunoreactivity was restricted to large vacuoles, whereas in the caerulein model, it was found in smaller vacuoles diffusely distributed throughout the cytoplasm. Ultrastructural differences also were appreciated; although an increased number of autophagic vacuoles were observed after both treatments, they appeared to be larger and fewer in number after bombesin plus PDL than after caerulein hyperstimulation. Whether these differences represent changes in distinct vesicle populations or divergent histories, such as a preference for autologous fusion after bombesin treatments, remains unclear.

In summary, we find that two treatments that do not cause acute pancreatitis, short-term PDL and bombesin, when combined cause acute pancreatitis. We find that the combined treatments caused a dramatic increase in pancreatic zymogen activation and appear to cause retention of active enzymes within the acinar cell. One possible conclusion from these findings is that short-term PDL alone may not be sufficient to initiate acute pancreatitis but must be combined with factors that stimulate acinar cell zymogen activation to cause disease.

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