Expression of oxidative stress-responsive genes and cytokine genes during caerulein-induced acute pancreatitis

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Fu, Kai, Michael P. Sarras, Jr., Robert C. De Lisle, and Glen K. Andrews. Expression of oxidative stress-responsive genes and cytokine genes during caerulein-induced acute pancreatitis. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G696–G705, 1997.—Oxidative stress and the inflammatory response may play roles in the pathogenesis of acute pancreatitis. Herein, we characterized pancreatic expression of oxidative stress-responsive genes [c-fos, heme oxygenase-1 (HO-1), and metallothionein-I (MT-I)] and cytokine genes [interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α)] during caerulein-induced acute pancreatitis in the mouse. c-fos, HO-1, and MT-I mRNAs were coordinately and rapidly (3–7 h) upregulated, and HO-1 and MT-I protein levels were increased slightly in the pancreas during acute pancreatitis. In addition, IL-1β, IL-6, and TNF-α mRNAs were rapidly (7 h) upregulated in the pancreas, and intrapancreatic IL-1β and IL-6 protein levels rapidly increased (3-fold and 6.4-fold, respectively) during acute pancreatitis. These studies suggest that oxidative stress and inflammation each occur in the pancreas during the early stages of acute pancreatitis. However, under a limited set of experimental conditions, we found that an insult that causes pancreatic oxidative stress (diethylnitrosamine) or one that induces an inflammatory response (bacterial lipopolysaccharide), or a combination of these agents, did not cause the changes characteristic of acute pancreatitis. Therefore, simply inducing oxidative stress and/or inflammation may be insufficient to initiate acute pancreatitis.

c-fos: glutathione; heme oxygenase; inflammation; interleukin; metallothionein; mouse

ACUTE PANCREATITIS IS A COMMON DISEASE characterized by interstitial edema and severe acinar cell damage and hemorrhage, as well as by leukocyte infiltration (46). The morbidity and mortality of this disease remain high even with optimal medical management. Although the major etiologic associations with gallstones and alcohol abuse have long been known, the pathogenesis of this disease remains obscure (40, 44).

It has been suggested that oxidative stress plays an important role in the pathogenesis and development of acute pancreatitis. Studies of ex vivo models of acute pancreatitis suggested that enzymatic free radical scavengers [superoxide dismutase (SOD), catalase] and the xanthine oxidase inhibitor allopurinol diminish pancreatic injury induced by free fatty acid, ischemia, and partial pancreatic duct ligation (39). Studies of in vivo animal models have strengthened this suggestion and further indicate that oxidative stress plays an important role in the early stages of acute pancreatitis (40). During the early stages of acute pancreatitis, lipid peroxides and/or their decomposition products are elevated and glutathione (GSH) is depleted. More recently, the enhanced formation of oxygen radicals and their adducts has been detected early during induction of acute pancreatitis in mice (40). However, whether oxidative stress alone can initiate acute pancreatitis remains to be determined. Oxidative stress-induced gene products, such as metallothionein-I (MT-I), heme oxygenase-1 (HO-1), GSH S-transferase, and quinone reductase, are a part of the general cellular defense system against oxidative damage (2, 10, 23, 42, 47, 49). These oxidative stress-responsive gene products may also play vital roles in the pancreatic adaptive and/or protective response to oxidative stress during acute pancreatitis. Furthermore, c-fos protooncogene is a member of the class of cellular immediate early genes that are rapidly and transiently induced on stimulation of quiescent cells with growth factors or serum (20). In addition, many nonmitogenic signals, including ultraviolet light, oxidants, and antioxidants (26, 32) induce c-fos. Nevertheless, the regulation of these genes during acute pancreatitis has received little attention, and studies of pancreatic expression of these genes have not been reported. We focused herein on the c-fos, MT-I, and HO-1 genes.

A hallmark of the inflammatory response is the induction of cytokine gene expression (33). Recently, Heath et al. (24) and Norman and colleagues (35, 37) reported a rise in systemic inflammatory cytokine levels during acute pancreatitis. The interleukin-1 (IL-1) receptor is required for maximal progression of acute pancreatitis, and blockade of the cytokine cascade using IL-1 receptor antagonist significantly attenuates the rise in IL-6 and tumor necrosis factor-α (TNF-α) expression and is associated with decreased severity of pancreatitis and reduced intrinsic pancreatic damage (34, 36). In contrast, Tanaka et al. (48) failed to detect a beneficial effect of exogenous IL-1 receptor antagonist on local pancreatic damage, although IL-1 receptor antagonist reduced mortality significantly in deoxycholate retrograde injection-induced pancreatitis in rats. During the early stages of caerulein-induced acute pancreatitis in mice, large increases in intrapancreatic TNF-α and infiltrating macrophages have been reported (37). TNF-α levels were also elevated in rats with acute pancreatitis induced by a retrograde pancreatic ductal infusion of bile. Early treatment with anti-TNF-α antibody may (25, 38) or may not (22) reduce the severity of acute pancreatitis. Heath et al. (24) showed that serum levels of IL-6 from patients with acute pancreatitis correlated with the severity of the disease. These studies suggest that inflammatory cytokines may play important roles...
in the process of acute pancreatitis. Although intrapancreatic cytokine levels during the early stages of acute pancreatitis have been examined recently (35), there have been no systematic studies comparing the activation of cytokine genes with that of oxidative stress-responsive genes in the pancreas proper. Furthermore, whether an acute pancreatic inflammatory response, alone or in combination with oxidative stress, can cause pancreatitis has not been examined.

Herein, we characterized the expression of oxidative stress-responsive and cytokine genes in a mouse model of caerulein-induced acute pancreatitis. We found that oxidative stress-responsive genes and cytokine genes are rapidly upregulated and that antioxidant proteins and inflammatory cytokines rapidly accumulate in the pancreas during caerulein-induced acute pancreatitis. However, we also found that acute induction of pancreatic oxidative stress by diethylmaleate (DEM) injection and/or acute induction of an inflammatory response by lipopolysaccharide (LPS) injection does not cause the changes characteristic of acute pancreatitis.

MATERIALS AND METHODS

Animals. Adult male CD-1 outbred mice (25–30 g) (Charles River Breeding Laboratory, Raleigh, NC) were acclimated for at least 1 wk before use. Animals were maintained on a 12:12-h light-dark cycle and allowed free access to standard rodent chow and tap water. These studies were performed in adherence to the Guide for the Care and Use of Laboratory Animals (DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892) and were approved by the Institutional Animal Care and Use Committee.

Chemicals and reagents. Porcine pancreatic α-amylase was obtained from Boehringer Mannheim (Indianapolis, IN). Caerulein, DEM, and LPS (Escherichia coli serotype 011:B4) were from Sigma Chemical (St. Louis, MO). All other chemicals and solvents were obtained from either Sigma Chemical or Fisher Scientific (Chicago, IL).

Experimental protocols. Acute pancreatitis was induced in CD-1 mice, as described previously (18). Specifically, mice were injected intraperitoneally with 50 μg caerulein/kg body wt for a total of seven hourly injections over a 6-h period. Control or sham-treated mice either received no injections or were injected intraperitoneally with an equal volume of saline on the same injection schedule. All the experiments were initiated between 8:30 AM and 9:30 AM. Animals (3 mice/group) were killed at different time points after the injection of caerulein. Blood was collected by heart puncture and allowed to clot, and serum α-amylase was assayed using the Procion yellow starch method, as reported previously (18). The pancreas was removed and quickly trimmed free of fat in cold phosphate-buffered saline (PBS). A small piece of tissue was weighed and immediately homogenized in 10 vol of 10% trichloroacetic acid (TCA). The supernatant was recovered by centrifugation, and total pancreatic GSH (GSH plus oxidized GSH (GSSG)) content was measured, as previously described (18). For protein extraction and analysis, one piece of pancreas was frozen in liquid nitrogen and stored in −80°C (see below). Another piece was minced and fixed in Bouin’s fixative for histological evaluation and immunohistochemistry or fixed in a standard fixative (4% paraformaldehyde, 1% glutaraldehyde in PBS) for electron microscopy. The remainder of the gland was processed immediately for RNA extraction (see below).

In studies of the effects of DEM, mice were injected intraperitoneally with 5.3 mmol DEM/kg body wt. This dosage has been shown to deplete GSH and induce MT mRNA in various tissues without overt toxicity (6). DEM was dissolved in sesame oil at a concentration of 5.3 mmol/10 ml. Control mice were injected with the same volume (10 ml/kg body wt) of sesame oil. Mice were killed at different time points after DEM injection. Serum and tissue were collected and processed as described above.

In studies of the effects of bacterial endotoxin (LPS), mice were injected intraperitoneally with 100 μg LPS. This dosage of LPS has been shown to induce an inflammatory response, including cytokine and MT gene expression in various tissues without overt toxicity (14). LPS was dissolved by sonication in saline (100 μg/200 μl), and control mice were injected with the same volume (200 μl) of saline alone. Mice were killed at different time points after the injection. Serum and tissue were collected and processed as described above.

In studies of the effects of coadministering DEM and LPS, mice were injected intraperitoneally with 5.3 mmol DEM/kg body wt and then with 100 μg LPS/mouse 30 min later. Control mice were injected with the same volumes of vehicle. Mice were killed at different time points after the LPS injection, and serum and tissue were collected and processed as described above.

RNA isolation and analysis. The pancreas was collected (3 animals/group) and immediately homogenized in 5 M guanidine isothiocyanate, and RNA was prepared as described previously (18). An equal amount of total RNA (2 μg) from each sample was size fractionated by 2.2 M formaldehyde-1.5% agarose gel electrophoresis, transferred by capillary blotting, and ultraviolet-cross-linked to nylon membranes (maximum strength Nytran; Midwest Scientific, St. Louis, MO). Northern blots were prehybridized, hybridized, and washed, as described previously (18). Hybrids were detected by autoradiography using X-ray film (XAR-5, Kodak, Rochester, NY) with intensifying screens at −80°C. In all experiments, duplicated gels were stained with acridine orange to verify integrity and equal RNA loading. After autoradiography, probe was stripped off the membrane by heating at 100°C for 10 min in a large volume of 0.05× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS). Successful removal of the probe was monitored by autoradiography for 24 h. Membranes were rehybridized with each successive probe as above. Hybridization signals were quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

A cDNA clone of mouse α-amylase was generated by reverse transcription polymerase chain reaction using total mouse pancreatic RNA, as described previously (18). Mouse MT-I, rat HO-1, and c-fos cDNAs were inserted into SP6 or pGEM vector (Promega Biotec, Madison, WI) and used as templates for the synthesis of 32P-labeled cRNA probes, as described previously (18). Probes had specific radioactivities of about 30 MBC/μg.

Ribonuclease (RNome) protection assays were performed, as previously described (18), using the RPA II kit (Ambion, Austin, TX) according to the manufacturer’s suggested protocol. Briefly, total pancreatic RNA (20 μg) was hybridized with a freshly prepared 32P-labeled TNF-α riboprobe (5 fmol, 7 × 106 counts/min) at 45°C for 16–18 h. This riboprobe (14) contained 52 nucleotides of vector sequence and 296 nucleotides of TNF α coding region sequence. After hybridization, samples were incubated with RNase A and RNase T1 and the protected riboprobe was precipitated and analyzed on a sequencing gel. The protected fragment was detected by...
autoradiography and quantitated by PhosphorImager analysis.

Protein extraction and quantitation of MT-I, HO-1, IL-1β, and IL-6 levels. The frozen tissue was weighed and homogenized in 10 vol of buffer [10 mM tris(hydroxymethyl)-aminomethane·HCl, pH 7.5, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzamidine]. After centrifugation at 15,000 g for 10 min, the supernatant was recovered and the concentration of total proteins was determined using the Bradford method with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA). Aliquots (40 μl) of the supernatant were dispensed into 1.5 ml Microfuge tubes, frozen, and stored at −80°C. Aliquots from the same group (3 mice/group) were pooled, and 20 μg were subjected to SDS-12% polyacrylamide gel electrophoresis (PAGE). Gels were electroblotted to nitrocellulose membranes, and the membranes were treated with rabbit anti-rat HO-1 (SPA-895 StressGen Biotechnologies, Victoria, BC, Canada; antiseraum, followed by goat-anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoblots were visualized using a Renaissance chemiluminescence reagent (DuPont-NEN, Cambridge, MA).

Intrapancreatic IL-1β and IL-6 concentration were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Genzyme, Cambridge, MA). Each sample was assayed in duplicate.

MT levels were determined by the 109Cd-hemoglobin exchange assay as described (17).

Histological analyses. Bouin’s fixed tissues were dehydrated and embedded for paraffin sections. These sections were stained with hematoxylin and eosin and examined in a blinded fashion by an experienced pathologist who was not aware of the identity of the samples.

Fixed tissues (4% paraformaldehyde, 1.6% glutaraldehyde in PBS) for electron microscopic examination were embedded in Embed 812 (Electron Microscopy Scientific, Fort Washington, PA), and thin sections were stained with lead citrate and uranyl acetate. The tissue was examined by electron microscopy at the Electron Microscopy Research Laboratory.

Statistical analysis. Statistical significance was determined through using analysis of variance and Student’s t-test. Differences were considered significant at P < 0.05. All values are given as means ± SE. Deviation bars in the figures indicate SE, whereas the absence of such bars indicates that SE was too small to indicate.

RESULTS

Oxidative stress-responsive genes are coordinately and rapidly upregulated in the pancreas during caerulein-induced acute pancreatitis. Induction of acute pancreatitis by repeated intraperitoneal injections of a supramaximal dose of caerulein resulted in a 10-fold increase in serum α-amylase activity (P < 0.01) and a 60% reduction in total GSH content in the pancreas by 9 h after initiation of these injections (P < 0.05) (18). Histological evaluation of the pancreas revealed vacuolization by 3 h and marked inflammation and necrosis by 9 h (Fig. 1). These results establish that acute pancreatitis was induced in these mice.

Pancreatic c-fos, HO-1, MT-I, and α-amylase mRNA levels during acute pancreatitis were determined by Northern blot analysis as shown in Fig. 2A. c-Fos mRNA was barely detectable in the control pancreas but was rapidly and dramatically increased ≥30-fold during the early period (3–7 h) of caerulein-induced acute pancreatitis. Thereafter c-fos mRNA returned to near control levels. HO-1 mRNA levels were low in the control pancreas but increased by 3 h and reached a peak (~9-fold above control levels) at 7 h. HO-1 mRNA returned to the control levels by 72 h. MT-I mRNA was readily detectable in the control pancreas, and levels of this mRNA were elevated by 3 h and were sevenfold above control levels by 7 h. MT-I mRNA returned to control levels by 72 h. In sharp contrast, pancreatic α-amylase mRNA levels did not change appreciably during caerulein-induced acute pancreatitis.

In addition, we noted that hepatic HO-1 and MT-I mRNAs were not affected (<2-fold) during caerulein-induced acute pancreatitis (Fig. 2B), which is consistent with the pancreas-specific damage that occurs in this experimental model.

Antioxidant proteins accumulate in the pancreas during caerulein induced acute pancreatitis. Despite the seven- to ninefold induction of MT-I and HO-1 mRNAs in the pancreas, only modest increases in the proteins were noted during caerulein-induced acute pancreatitis. HO-1 protein was detected in the control pancreata through the use of Western blots analysis, and levels increased approximately three- to fourfold by 7 h and remained elevated for 24 h (Fig. 3A). MT protein levels, as determined by the Cd-heme exchange assay, were high in the control pancreas relative to other major organs (11). During caerulein-induced acute pancreatitis, MT-I mRNA levels increased only 1.7-fold (P < 0.05) by 7 h, and this level was maintained for 24 h (Fig. 3B).

Immunolocalization of HO-1 protein in the pancreas revealed acinar cell and islet cell immunostaining. The apparent level of immunoreactive HO-1 in the acinar cells increased substantially during caerulein-induced acute pancreatitis (data not shown). Our previous studies have localized MT to the acinar cells of the mouse pancreas (15).

Cytokine genes are rapidly upregulated in the pancreas during caerulein-induced acute pancreatitis. During caerulein-induced acute pancreatitis, pancreatic IL-1β, IL-6, and TNF-α mRNA levels were examined by Northern analysis or RNase protection assays (Fig. 4). IL-1β and IL-6 mRNAs were at or below the limits of detection by Northern blotting, using total RNA from control pancreas. However, these mRNAs were upregulated and reached peak levels at ~7 h after initiation of caerulein injections (Fig. 4A). TNF-α mRNA was consistently below the detection limits in the control pancreas, as determined using a sensitive RNase protection assay. However, levels of this mRNA were elevated and also peaked by 7 h of acute pancreatitis (Fig. 4B). In contrast to these results, hepatic IL-1β, IL-6, and TNF-α mRNAs remained unchanged during caerulein-induced acute pancreatitis (data not shown).

IL-1β and IL-6 rapidly accumulate in the pancreas during caerulein-induced acute pancreatitis. Previous studies demonstrated that TNF-α levels increase during caerulein-induced acute pancreatitis (37). Pancreatic IL-1β and IL-6 concentrations were further exam-
Gene Regulation in Caerulein-Induced Acute Pancreatitis

Fig. 1. Histological evaluation of pancreatic damage during caerulein-induced acute pancreatitis. Acute pancreatitis was induced by repeated hourly injections of caerulein (50 µg/kg body wt) over a 6-h period. Representative high power (×520) photomicrographs of pancreas from a normal mouse (A) and from a caerulein-injected mouse killed 9 h after initiation of caerulein injections (B) are shown. Electron micrographs (×17,200) showing pancreatic acinar cells from a normal animal (C) and from a mouse after 3 hourly caerulein injections (D).

Pancreatic IL-1β was detectable in control mice, and levels of this cytokine increased significantly (>3-fold) during the early period of acute pancreatitis (P < 0.01) but returned to control levels by 24 h (Fig. 5A). IL-6 was more abundant than IL-1β in the control pancreas. Levels of this cytokine increased rapidly (6.4-fold by 3 h) during the early period of caerulein-induced acute pancreatitis (P < 0.01) and then returned to control levels by 24 h (Fig. 5B).

Injection of DEM and/or LPS does not cause acute pancreatitis. Because oxidative stress and inflammation appear to be involved in caerulein-induced acute pancreatitis, we sought to determine if all oxidative stresses and/or inflammation-inducing agents can cause the initiation of acute pancreatitis.

To examine the effects of oxidative stress on the pancreas, we injected mice with DEM, which causes oxidative stress and depletion of GSH (6, 9). DEM caused the depletion of pancreatic GSH (Fig. 6A) and rapid induction of HO-1 and MT-I mRNAs (Fig. 6B). Pancreatic HO-1 mRNA levels increased more than fourfold above control by 3 h and returned to control levels by 72 h (Fig. 6B). Pancreatic MT-I mRNA levels increased by 3 h, reached a peak (about 15-fold above control) by 5 h, and returned to control levels by 72 h (Fig. 6B). The depletion of GSH and upregulation of HO-1 and MT-I mRNAs establish that oxidative stress was induced in the pancreas. However, DEM administration did not cause characteristic morphological alterations in the pancreas (Fig. 7A) or hyperamylasemia (Fig. 6A) associated with pancreatitis.

To examine the effects of inflammation on the pancreas, we injected mice with LPS. LPS administration had little effect on pancreatic GSH levels (Fig. 8A), but pancreatic IL-1β and IL-6 mRNA levels were rapidly upregulated and peaked at 1 and 3 h, respectively (Fig. 8B). However, LPS did not cause hyperamylasemia (Fig. 8A) associated with pancreatitis.

Because oxidative stress and inflammatory responses occur concurrently during caerulein-induced acute pancreatitis, we examined the effects of coinjection of DEM and LPS on the mouse pancreas. Coinjection of DEM and LPS resulted in the depletion of pancreatic GSH (Fig. 9), but it did not cause morphological alterations in the pancreas (Fig. 7B) or hyperamylasemia (Fig. 9) associated with pancreatitis. Thus, under this limited set of experimental conditions, oxidative...
how the tissue responds to this insult, we sought to determine the role of oxidative stress in this model of acute pancreatitis.

HO-1 catalyzes the first and rate-limiting step in the conversion of prooxidant heme to biliverdin, which is subsequently converted to an antioxidant, bilirubin, by the action of biliverdin reductase (31). Expression of the HO-1 gene is induced not only by heme but also by a variety of nonheme inducers, including agents that cause oxidative stress (4, 50). The magnitude of HO-1 induction by oxidative stress and the wide distribution of this enzyme in different tissues, coupled with the biological activities of the catalytic byproducts, carbon monoxide, iron, and bilirubin, strongly suggest that HO-1 induction is a part of a general response to oxidant stress and that this enzyme plays an important role in maintaining cellular homeostasis in response to oxidative stress (see Ref. 10 for review). MTs are small cysteine-rich heavy metal-binding proteins that can efficiently scavenge hydroxyl radicals (2, 49). Furthermore, yeast and mammalian MTs can functionally substitute for SOD mutation in protecting yeast from oxidative stress (47). Mammalian cells that constitutively overexpress the MT gene are resistant to the toxic effects of tert-butylhydroperoxide (42). Furthermore, oxidative stress leads to transcriptional activa-

Fig. 2. Northern blot detection of c-fos, heme oxygenase-1 (HO-1), and metallothionein-I (MT-I) transcripts in pancreas and liver during caerulein-induced acute pancreatitis. Acute pancreatitis was induced by injections of caerulein as described in Fig. 1 legend. Pancreatic (A) and liver (B) total RNA was prepared from saline-treated control animals (time 0) and from caerulein-treated animals at indicated times after initiation of caerulein injections. Total RNA was subjected to formaldehyde-agarose gel electrophoresis and blotted onto a nylon membrane, and the membrane was hybridized with 32P-labeled c-fos riboprobe. Hybrids were detected by autoradiography and quantitated by PhosphorImager analysis. The membrane was then stripped of probe and successively rehybridized with HO-1 and MT-I riboprobes. The membrane with the pancreas samples was also hybridized with α-amylase riboprobe, whereas with the liver samples was hybridized with Cu,Zn-SOD riboprobe to ensure equal loading and transfer. This experiment was repeated twice with identical results.

Fig. 3. Detection of pancreatic HO-1 and MT protein during caerulein-induced acute pancreatitis. Acute pancreatitis was induced as described in Fig. 1 legend. A: HO-1 was detected by Western blotting. Pancreatic soluble proteins (20 μg) were subjected to SDS-polyacrylamide gel electrophoresis and then electroblotted to a nitrocellulose membrane. Recombinant HO-1 protein (1 ng) was included as a control (C). Membranes were treated with rabbit anti-rat HO-1 antiserum, followed by goat-anti-rabbit immunoglobulin G conjugated with horseradish peroxidase. ImmunobLOTS were visualized by chemiluminescence. B: MT was quantitated using the Cd-heme exchange assay. Results are means ± SE (n = 3). *P < 0.05.
Fig. 4. Detection of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) transcripts in pancreas during caerulein-induced acute pancreatitis. Acute pancreatitis was induced as described in Fig. 1 legend. Pancreatic total RNA was prepared from saline-treated control animals (time 0) and caerulein-treated animals at different times after initiation of the caerulein injections. A: total RNA was subjected to Northern blot hybridization as described in Fig. 2 legend. Total RNA (0.05 μg) isolated from lipopolysaccharide (LPS)-treated macrophage cells (Mφ) was included as positive control. The membrane was also hybridized with β-amylase riboprobe to ensure equal loading and transfer. This experiment was repeated twice with identical results. B: total pancreatic RNA or the same amount of E. coli rRNA was hybridized with antisense mouse TNF-α riboprobe as described in MATERIALS AND METHODS. Samples were then treated with RNases and analyzed on a sequencing gel. Co and Ci refer to E. coli rRNA samples not treated or treated with RNases, respectively. Total RNA (0.1 μg) isolated from LPS-treated macrophage cells (Mφ) was also included as a positive control; nt, nucleotide.

Fig. 5. Quantitation of pancreatic IL-1β and IL-6 by ELISA. Acute pancreatitis was induced as described in Fig. 1 legend. Pancreatic soluble proteins were prepared from saline-treated control animals (time 0) and from caerulein-treated animals at different times after initiation of caerulein injections. Concentrations of IL-1β (A) and IL-6 (B) were measured as described in MATERIALS AND METHODS using commercially available ELISA kits. Results are expressed as means ± SE (n = 3). *P < 0.01.

Furthermore, these findings demonstrate that the pancreas is competent to respond to oxidative stress by changing the pattern of gene expression and induction of antioxidants, which could then play an important protective role(s) against oxidative stress-induced pancreatic damage.

The c-fos protooncogene is rapidly and transiently induced by a variety of oxidants and antioxidants (26, 32). c-Fos is a transcription factor that heterodimerizes with a member of the Jun family and binds to DNA in a complex called AP-1. Interestingly, many of the genes induced by oxidative stress contain AP-1 binding sites in their promoters. This is the case from yeast to mammals (7, 27), suggesting that one conserved and pivotal function of AP-1 transcription factors is the maintenance of intracellular redox homeostasis. Rapid induction of c-fos during acute pancreatitis, as we show here, further supports the notion that oxidative stress occurs rapidly during caerulein-induced acute pancreatitis.

The concomitant production of various soluble mediators, including cytokines, is central to the inflammatory response (33). It has been suggested that inflammation...
that intrapancreatic IL-1β, IL-6, and TNF-α mRNA and proteins are rapidly and coordinately increased during the early stages of acute pancreatitis. Consistent with our data, Norman et al. (35) recently reported that caerulein-induced pancreatitis in mice was associated with a rapid increase of IL-1, IL-6, and TNF-α in the serum and within the pancreas itself. Furthermore, rapid activation of cytokine genes occurred in the pancreas but not the liver, as we show here, which indicates that this inflammatory response is localized to the site of injury during the early stages of acute pancreatitis. Moreover, the concentration of intrapancreatic cytokine proteins increased substantially during the early stages of acute pancreatitis, whereas the synthesis of MT-I and HO-1 in the pancreatic acinar cells was compromised. These findings are consistent with the notion that activated pancreatic macrophages are the major source of increased pancreatic cytokines, whereas HO-1 and MT-I genes are activated in pancreatic acinar cells (15). These cytokines and others (e.g., IL-8, platelet-activating factor) would be expected to act locally to aggravate the pancreatic damage and both locally and systemically to increase capillary permeability and promote leukocyte adhesion and extravasation during acute pancreatitis (29). The recruitment of neutrophils and subsequent release of neutrophil elastase occurs in the inflamed pancreas. Circulating levels of neutrophil elastase correlate with the severity of acute pancreatitis (21). Furthermore, neutrophils (19) have been suggested as a possible source of reactive oxygen species produced in experimentally induced acute pancreatitis. Blocking the activation of cytokines via either IL-1 receptor antagonist or anti-TNF antibody may (25, 34, 36, 38) or may not (22, 48) provide protection to the pancreas and ameliorate the pancreatic damage during acute pancreatitis, but this remains controversial. Moreover, IL-10, a potent anti-inflammatory cytokine, prevents the activation of macrophages and their release of cytokines. IL-10 treatment prevented acinar necrosis during caerulein-induced acute pancreatitis in mice (51). More recently, Kusske et al. (28) showed that IL-10 treatment de-

Fig. 6. Evaluation of pancreatic damage, glutathione (GSH) levels, and oxidative stress-responsive gene expression after diethylmaleate (DEM) injection. Animals were injected with DEM and killed at different times after injection. Serum and tissues were collected and processed as described in MATERIALS AND METHODS. Vehicle-treated animals (time 0) served as control. A: serum α-amylase activity and pancreatic total GSH (GSH + oxidized GSH (GSSG)) content were also measured as described in MATERIALS AND METHODS. Results are means ± SE (n = 3). B: total RNA was analyzed by Northern blot hybridization as described in Fig. 2 legend. This experiment was repeated twice with identical results.

plays a role in acute pancreatitis (29). Recent studies (24, 35, 37) suggest that, during acute pancreatitis, activated pancreatic macrophages release inflammatory cytokines (IL-1, IL-6, and TNF-α) in response to pancreatic damage. In this study, we demonstrated

Fig. 7. Histological evaluation of pancreatic damage after DEM injection or after DEM and LPS coinjection. Mice were injected with DEM (5.3 mmol/kg) or DEM + LPS (100 µg/mouse), and the pancreas was collected for histological examinations. A: electron micrograph (×17,200) of pancreatic cells 3 h after DEM injection. B: representative high power (×520) photomicrograph of pancreas 9 h after coinjecting DEM and LPS.
Fig. 8. Evaluation of pancreatic damage, GSH levels, and cytokine gene expression after LPS injection. Animals were injected with LPS and killed at different times after injection. Serum and tissue were collected and processed as described in MATERIALS AND METHODS. Saline-treated animals (time 0) served as control. A: serum α-amylase activity and pancreatic total GSH (GSH + GSSG) content were measured as described in MATERIALS AND METHODS. Results are means ± SE (n = 3). B: Northern blot detection of pancreatic IL-1β and IL-6 transcripts during caerulein-induced acute pancreatitis (C) and after LPS (L) treatment. Acute pancreatitis was induced by caerulein as described in Fig. 1 legend. Pancreatic total RNA was prepared at different times after initiation of caerulein or LPS injections. Total RNA was subjected to Northern blot hybridization as described in Fig. 2 legend. Total RNA (0.05 μg) isolated from LPS-treated macrophage cells was included as a positive control. The membrane was also hybridized with an α-amylase riboprobe to ensure equal loading and transfiltering. This experiment was repeated twice with identical results.

Fig. 9. Evaluation of pancreatic damage and GSH levels after co-injection of DEM and LPS. Mice were injected with DEM (5.3 mmol/kg) plus LPS (100 μg/mouse) and killed at different times after injection. Serum and tissues were collected and processed as described in MATERIALS AND METHODS. Vehicle-treated animals (time 0) served as control. Serum α-amylase activity and pancreatic total GSH (GSH + GSSG) content were measured as described in MATERIALS AND METHODS. Results are means ± SE (n = 3).

to induce oxidative stress (6, 9). DEM decreases GSH levels in various organs by enzymatic conjugation with reduced GSH catalyzed by GSH transferase (9). During the period of oxidative stress induced by DEM administration, lipid peroxidation, liver necrosis, decreases in vitamin E levels, and increases in the oxidized form of vitamin C have been observed (30). In the present study, a single maximally tolerated dosage of DEM (6) did not cause acute pancreatitis. However, we noted that, relative to caerulein-induced acute pancreatitis (18), DEM caused a more profound and transient depletion of pancreatic GSH, and the kinetics of induction of HO-1 and MT-I mRNAs were comparable but not identical. Thus the oxidative stress induced by a single injection of DEM differs from that which occurs during caerulein-induced acute pancreatitis. Future studies using different DEM regimens or different oxidative stress-inducing agents should provide more information regarding a cause-effect relationship between oxidative stress and acute pancreatitis. However, our data suggest that oxidative stress per se may be insufficient to initiate acute pancreatitis. Moreover, LPS injection, which induced the activation of tissue macrophages and the upregulation of several cytokine genes (16), did not cause changes characteristic of acute pancreatitis. A similar result was reported for rats injected with LPS (1). However, we also noted that the kinetics of LPS induction of pancreatic cytokine genes differed from that found after caerulein injections. Thus, under this limited set of experimental conditions, an inflammatory response per se is also insufficient to initiate acute pancreatitis. Furthermore, the present study showed that coadministration of DEM and LPS did not cause acute pancreatitis. Therefore, our studies provide evidence that oxidative stress and inflammation each occur in the pancreas during the early stages of acute pancreatitis, but simply inducing oxidative stress and/or inflammation in the pancreas may be insufficient to initiate this disease process.

Acute pancreatitis has been suggested to involve a block in secretion and subsequent fusion of zymogen increased inflammation and prevented death in a mouse model of hemorrhagic acute pancreatitis, even when given after acute pancreatitis had been established.

We had hoped that studying the temporal regulation of cytokine and oxidative stress-responsive genes during the early stages of acute pancreatitis might indirectly provide clues as to the source or mechanism of generation of oxidative stress and the roles of inflammatory cytokines during acute pancreatitis. However, our data demonstrate that cytokine genes and oxidative stress-responsive genes are activated rapidly and concurrently after the initiation of caerulein injections. Nevertheless, these studies and others suggest that both oxidative stress and inflammation play important roles during the pathogenesis of acute pancreatitis.

We also examined, under a limited set of experimental conditions, whether oxidative stress and/or inflammation can initiate acute pancreatitis. DEM was used
granules with lysosomes or missorting of the contents of these organelles by processes that are not well understood (43). This failure to segregate lysosomal and secretory enzymes results in the inappropriate accumulation and oxidative stress, follows that can exacerbate the focal pancreatic injury and even lead to multiple organ failure and death.

In summary, the present study demonstrates that oxidative stress-responsive genes, including c-fos, HO-1, and MT-I, are coordinately and rapidly upregulated and antioxidant proteins, HO-1 and MT-I, are coordinately and rapidly upregulated in the pancreas during caerulein-induced acute pancreatitis. In addition, pancreatic cytokine genes, including IL-1β, IL-6, and TNF-α, are rapidly upregulated in the pancreas, and intrapancrnetic IL-1β and IL-6 rapidly accumulate during caerulein-induced acute pancreatitis. Nevertheless, under a limited set of experimental conditions, oxidative stress and an inflammatory response, alone or concurrently, were insufficient to initiate acute pancreatitis.

We are indebted to Jim Geiser and Elleen Roach for excellent technical assistance. We also thank Dr. T. Tsumita, Dept. of Pathology, University of Kansas Medical Center, for assistance with evaluation of histological samples.

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-50181 (G. K. Andrews), DK-47840 (M. P. Sarras, Jr.) and DK-46594 (R. C. DeLisle). Address for reprint requests: G. K. Andrews, Dept. of Biochemistry and Molecular Biology, BKF 2034, Univ. of Kansas Medical Center, 39th and Rainbow Blvd., Kansas City, KS 66160-7421.

Received 9 January 1997; accepted in final form 9 June 1997.

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