Early activation of endoplasmic reticulum stress is associated with arginine-induced acute pancreatitis

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ACUTE PANCREATITIS CAUSES more than 300,000 hospitalizations per year in the United States (25). The pathophysiology of this disease can be divided into three major phases: acinar, local, and systemic events (30). Because of a delay in disease presentation, early acinar events in acute pancreatitis are not fully understood. However, recent studies involving gene profiling have indicated that a complex pattern of altered gene expression occurs early in stressed pancreatic acinar cells (10). Of particular interest was the observation that several of the genes whose expression was altered during the course of acute pancreatitis were known targets of endoplasmic reticulum (ER) stress mechanisms. ER stress and its response mechanisms are central components of a number of important diseases including diabetes mellitus, cystic fibrosis, and Alzheimer disease (1). The pancreas has one of the highest rates of protein synthesis in the body and possesses particularly abundant ER. Furthermore, insults including oxidative stresses and excessive Ca2+ release from ER stores, which are known to activate ER stress (23), are also associated with acute pancreatitis (27, 31). Therefore, it was of interest to determine whether ER stress mechanisms were activated during the course of acute pancreatitis.

Disturbances of ER function lead to a well-identified series of ER stress response mechanisms (7, 20). One of the major reactions of the ER to stress is the unfolded protein response (UPR). The UPR couples the ER protein load with the ER protein folding capacity. It is sensitive to a variety of perturbations, including oxidative stress, Ca2+ imbalance, and mutant proteins that do not fold properly, all of which lead to an accumulation of misfolded proteins (23). Key components of the UPR in mammals include three different ER stress transducers localized to the ER membrane, which are constitutively expressed in all cells and activated during ER stress: PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-required enzyme 1 (IRE1) (7, 20). PERK activation involves autophosphorylation and leads to the subsequent phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) and, consequently, a general decrease in protein synthesis (23). IRE1 mediates the splicing of XBP1 and leads to the expression of BiP (also called GRP78), and protein folding enzymes that help alleviate the stress (28, 35). Activation of IRE1 causes splicing and activation of the transcription factor XBP-1 that is also involved in gene expression of different chaperones and foldases that relieve ER stress (4). However, if the overload of misfolded proteins in the ER is not resolved, then prolonged activation of UPR leads to programmed cell death. This effect is mediated in part by increased expression of the transcription factor C/EBP homologous protein (CHOP, also called GRP78), and protein folding enzymes that help alleviate the stress (28, 35). Activation of IRE1 causes splicing and activation of the transcription factor XBP-1 that is also involved in gene expression of different chaperones and foldases that relieve ER stress (4).

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In the current study, we investigated for the first time the presence and activation of ER stress-related mechanisms in acute pancreatitis using the arginine model. We observed the activation of all major components of the UPR at early time points in the development of the disease simultaneously with pathological alterations. Together, these data indicate that ER stress mechanisms are present in exocrine acini and are activated early in the arginine model of acute pancreatitis.

**METHODS**

*Induction of acute pancreatitis.* Male Wistar rats (Harlan, Indianapolis, IN), weighing 150–200 g, had ad libitum access to regular laboratory chow and water and were housed at 24°C on a 12:12-h light-dark cycle. In each experimental group three or four animals were used. After overnight fasting, rats received a single intraperitoneal injection of 4.0 g/kg body wt L-arginine (Sigma-Aldrich, St. Louis, MO) in saline (pH 4.0) into the left lower abdomen. Rats were euthanized in a CO2 chamber after 1, 4, 8, 24, and 72 h. The local Animal Care and Use Committees (University of Michigan and University of Texas, MDACC) approved all animal experimental protocols. Rats were treated according to the Guiding Principles in the Care and Use of Animals.

*Evaluation of arginine-induced injuries.* Mixed arterial-venous blood and pancreatic tissue were collected and processed as described (14). Serum amylase activity was measured by a Phadebas test (Pharmacia Diagnostics, Uppsala, Sweden). The extent of pancreatic edema was determined by measuring tissue water content (wet weight - dry weight/wet weight × 100 = percent tissue water content).

*Quantification of pancreatic trypsin activity.* Tissue samples were homogenized in MOPS buffer. The homogenate was centrifuged at 3,000 rpm for 5 min, and the supernatant was used for the assay. Trypsin activity was either measured fluorometrically using Boc-Glu-Ala-Arg-AMC-HCl (Bachem Bioscience, King of Prussia, PA) according to the method of Kawabata et al. (13) or chromogenically using Z-Gly-Pro-Arg-p-nitroanilide acetate (Sigma-Aldrich) as a substrate, according to Chen et al. (5), with similar results. Concentrations were calculated using standards generated by purified trypsin (Sigma-Aldrich). Protein concentrations of each sample were determined (Bio-Rad Laboratories, Hercules, CA), and trypsin activity was expressed as nanograms per milligram protein.

*Evaluation of pancreatic morphology and immunohistochemistry.* Tissue samples were fixed overnight in fresh 4% formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Four-micrometer sections were prepared and stained with haematoxylin and eosin (H&E) for morphological examination. Active caspase-3 was localized by deparaffinized sections with a rabbit monoclonal antibody to the active fragment of caspase-3 (no. 59565; BD PharMingen, San Diego, CA), using a Vectastain immunoperoxidase ABC kit and Vectastain DAB peroxidase substrate kit (both from Vector Laboratories). Antibody binding was detected by enhanced chemiluminescence (Fierce, Rockford, IL), recorded on X-ray films or with the LI-COR Odyssey infrared imager. Membranes were scanned, recorded digitally, and processed using Quantity One (Bio-Rad Laboratories).

*Statistical analysis.* The Student t-test was used to investigate the difference of each parameter. The results were regarded as significantly different when the P value was <0.05. Values are means ± SE.

**RESULTS**

Arginine treatment induces rapid pancreatitis. Arginine treatment leads to a rapid induction of acute pancreatitis, as previously reported by others (9, 19, 33). In the current study, several biochemical parameters were measured to assess the pancreatitis severity. An early and significant twofold increase in serum amylase after 4 and 8 h was detected in arginine-treated rats (Fig. 1, A.1). These effects were transient and returned to control values after 24 and 72 h. An identical pattern was observed in the measurement of edema with a significant elevation in pancreatic water content at 4 and 8 h after arginine treatment, which was resolved within 24 h (Fig. 1, A.3). In contrast, arginine treatment did not increase trypsin activity in pancreatic tissue before 24 h, where a sevenfold increase in trypsin activity was measured in samples from pancreatitis rats (Fig. 1, A.2). Arginine treatment also induced NFkB activation as has been reported previously (24) (data not shown).

Arginine-induced pancreatitis was further evaluated histologically. Rat pancreata were stained with H&E for morphological examination. None of the control pancreata showed characteristics of acute pancreatitis at any time point (Fig. 1, B.1 and B.2). Arginine-treated animals showed evidence of...
interstitial edema and the accumulation of a huge number of vacuole-like structures distributed throughout the cytoplasm within 4 h (Fig. 1, B.3 and B.4). Evidence of beginning necrosis and infiltration of inflammatory cells was evident 8 h after arginine treatment (Fig. 1, B.5). After 24 h, maximal necrosis was observed. Only scattered acini were intact, and inflammatory cells were observed surrounding the pancreatic lobuli (Fig. 1, B.6). Some recovery was apparent by 72 h after arginine administration as exocrine cells looked more similar to control (Fig. 1, B.7).

Arginine treatment causes rapid induction of ER stress mechanisms. ER stress-related mechanisms were evaluated at various times after arginine treatment. An early marker of ER stress is the activation by autophosphorylation of the ER-resident kinase and stress sensor PERK. Phosphorylation of PERK was increased after 1 h, reached a maximum at 24 h...
(268 ± 11% of control, P < 0.01) after arginine treatment (Fig. 2). The major downstream target of PERK is eIF2α, which controls general protein translation. Phosphorylation of eIF2α, which reduces translation initiation, was apparent 4 h after arginine treatment and remained above control until at least 72 h (Fig. 2C). Basal levels of phosphorylated eIF2α were detected in all saline control animals without any change over time. Western blotting for total eIF2α demonstrated equal protein loading (Fig. 2C, bottom).

Another hallmark of ER stress is the activation of ATF6. ATF6 is localized in the ER membrane under normal conditions but is released and translocated to the nucleus upon ER stress. ATF6 was visualized by Western blotting in two separated cell fractions: cytoplasmic and nuclear. A reduction of cytoplasmic levels and an increase in nuclear levels of ATF6 were observed 4 and 8 h after arginine administration (Fig. 3A). In contrast, in control animals ATF6 was exclusively present in the cytoplasmic fraction at all time points. One of the important ATF6 targets is the ER-resident chaperone BiP. Western blot analysis indicated that BiP levels were elevated in pancreas lysates within 4 h of arginine treatment and remained elevated up to 72 h after pancreatitis induction (Fig. 3B).

The third major UPR mechanism involves activation of the endoribonuclease activity of IRE1 that leads to splicing of XBP-1 into an active transcription factor (sXBP-1). A low level of functional sXBP-1 was present in the pancreas under unstressed conditions. sXBP-1 mRNA levels increased within 4 h after l-arginine administration and stayed elevated for at least 72 h (Fig. 3C). Protein levels of sXBP-1 were also elevated in a time-dependent manner after arginine treatment and were significantly elevated at 8 h (144 ± 6% of control, P < 0.05) and continued to increase at least for 72 h (249 ± 8% of control, P < 0.001) (Fig. 3D).

Arginine treatment activates ER stress-related apoptotic mechanisms. Next we investigated proapoptotic ER stress pathways that are activated upon prolonged stress. Western blotting for the ER-resident procaspase-12 showed cleavage of this initiator procaspase within 8 h, extending to at least 24 h after arginine administration (Fig. 4A). Immunohistochemical analysis indicated the induction of active caspase-3, which is described as a downstream target of caspase-12, within 24 h after arginine administration (Fig. 4B, left). TUNEL staining of the same specimens revealed a positive signal at 24 h after arginine administration, indicating acinar cell death at this time point (Fig. 4B, left and right). We also analyzed the transcriptional upregulation of CHOP, which is downstream of several ER stress sensors. CHOP mRNA levels were increased in l-arginine-treated rats starting at 4 h with a peak after 24 h (Fig. 4C, top). Western blotting for CHOP showed an increase in protein levels after 8 and 24 h (Fig. 4C, bottom).

**DISCUSSION**

In this study, we have identified activation of ER stress mechanisms as an early event during the development of acute pancreatitis in the arginine model. Activation of all major ER stress transducers were observed within 4 h of arginine treatment, which coincides with the earliest measurements of pancreatitis-related acinar cell disruption in this model. Although the current study was focused on the arginine model, it is likely that these mechanisms are not model specific, as increased expression of ER stress-regulated genes has been previously reported in studies profiling gene expression in caerulein and bile salt injection models of acute pancreatitis (10). Also, we previously reported the activation of a key ER stress mechanism, PERK activation, occurred after caerulein treatment and was responsible for the inhibition of protein synthesis that occurs during acute pancreatitis (26). ER stress mechanisms have previously been found to be of fundamental importance for a number of important diseases (1). Therefore, the observation that these mechanisms are activated in acute pancreatitis may have profound implications to our understanding, preventing, and treating this disease.

ER stress influences cell function at a variety of levels including gene transcription and translation. Activation of the ER-resident kinase PERK is particularly important for effects...
on translation, as one of its major targets is the translation initiation factor eIF2α. Phosphorylation of eIF2α leads to a reduction in the rate of general protein synthesis. We found that PERK was rapidly activated, and eIF2α was phosphorylated within 4 h of arginine treatment. This finding is in accordance with the observation made previously that eIF2α phosphorylation and protein synthesis inhibition occurs in pancreatitis induced by high concentrations of caerulein in mice (26). In addition to PERK, three other mammalian eIF2α kinases have been described (12). Each of these kinases senses stress signals and activates downstream response pathways by regulating cellular translation. These eIF2α kinases include GCN2, which is activated by nutritional stress; HRI, which links protein synthesis to heme availability and is also activated by oxidative and heat stress; and PKR, which is induced by interferon. In our study we focused on the activation of PERK, which occurred early and paralleled the early increase in eIF2α phosphorylation. However, the activation of other kinases, especially at later times, may help explain the observation in the current study of prolonged eIF2α phosphorylation despite a decrease in PERK phosphorylation after 72 h. Further studies will be necessary to evaluate the roles of additional mechanisms.

In contrast to its effects on general protein synthesis, inhibition of eIF2α leads to increased translation of a particular subset of mRNAs that includes important ER stress-related genes and transcription factors (17). Among the transcription factors regulated by PERK phosphorylation of eIF2α is ATF3 (11). We previously reported that ATF3 levels are increased early in pancreatitis induced by either caerulein or intraductal administration of taurocholate (10), and ATF3 levels were also elevated by arginine (unpublished data). Therefore, ER stress influences pancreatic acinar cell function by influencing both translation and transcription of key regulators in distinct experimental models.

After treatment with arginine, we also noted a rapid activation of the other major ER stress molecules ATF6 and IRE1. ATF6, an integral element of the UPR, is released from the ER membrane and translocates to the Golgi apparatus upon dissociation from BiP. Within the Golgi, ATF6 undergoes sequential proteolytic cleavage by the proteases S1P and S2P, yielding a free 50-kDa cytoplasmic domain as an active transcription factor for ER stress target genes (35). IRE1 is a stress sensor that undergoes oligomerization and autophosphorylation of its kinase domain upon the dissociation of BiP. These effects lead to activation of a COOH-terminal endonuclease domain of IRE1, which catalyzes the removal of a small (26 nucleotides) intron from XBP-1 mRNA. The splicing event creates a translational frameshift in XBP-1 mRNA to produce an active translation factor sXBP-1. sXBP-1 binds to the ER stress-specific response element in the promoter region of a variety of molecular chaperones and other ER stress-regulated genes (16). In our study we observed an increase in the constitutive expression level of sXBP-1 after l-arginine treatment, indicating the activation of IRE1. Thus all three major ER stress mechanisms were rapidly activated in the pancreas of rats following arginine treatment.

The mechanisms involved in the induction of ER stress by arginine are not completely understood. However, it is known that arginine treatment causes oxidative stress in the pancreas (6, 34), and oxidative imbalance induces ER stress (29).
Reduction of oxidative stress has previously been found to reduce the severity of acute pancreatitis, but the mechanisms that explain the role of oxidative stress in acute pancreatitis have not been clear (27). The current results suggest that ER stress may be a common consequence of oxidative stress leading to changes in gene expression and activation of the cellular processes that result in acute pancreatitis. This hypothesis is supported by experiments using antioxidants that reduce both ER stress and the severity of acute pancreatitis (unpublished observations). However, it also remains possible that ER stress and the severity of pancreatitis are independently influenced by oxidative imbalance. Furthermore, research will be necessary to fully understand the relationship between oxidative stress, ER stress, and severity of acute pancreatitis.

Based on what is known from other systems, it seems likely that under normal conditions ER stress mechanisms would play primarily a protective role by induction of chaperones, antioxidants, and other protective molecules. Simultaneous increases in both protective and proinflammatory and proapoptotic genes have previously been noted in profiling studies of acute pancreatitis. The figure shows how arginine treatment activates the apoptotic mechanisms caspase-12, caspase-3, and CHOP. Rats were treated with saline or l-arginine and killed after 1, 4, 8, 24, and 72 h. A: Western blotting using an antibody for caspase-12 revealed a loss of the pro-caspase-12 band at a molecular mass of 50 kDa, indicating cleavage of the initiator procaspase within 8 h of arginine treatment. Starting at 8 h after arginine administration, a lower molecular mass band of 20 kDa appeared, corresponding to the cleaved activated caspase-12. B: arginine administration also induced active effector caspase-3 by 24 h, as indicated by immunohistochemical analysis with an antibody specific for active caspase-3 (left). Sections of the same specimens were stained for TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labeling (TUNEL) and are displayed on the right. TUNEL staining was observed within 24 h after arginine administration, indicating an acinar cell undergoing apoptosis. C: CHOP mRNA levels were elevated within 4 h of arginine administration with a peak observed after 24 h. RT-PCR of ribosomal 18S RNA was used to indicate equal sample loading. The bottom shows CHOP protein levels. Arginine treatment increased CHOP protein levels after 8 and 24 h. The anti-actin Western blot underneath was used as loading control.
endoplasmic reticulum stress in acute pancreatitis.

In the current study we observed a rapid and dramatic increase in the level of BiP, an ER-specific member of the heat shock HSP70 family of molecular chaperones. It has long been observed that heat shock proteins are induced during acute pancreatitis, but the significance of this observation linking acute pancreatitis to ER stress has not previously been noted. More importantly, previous induction and overexpression of heat shock proteins has been found to prevent or reduce the severity of subsequent pancreatitis (3, 32). The explanation for this effect of HSPs has not been completely understood. HSPs have previously been found to reduce or prevent ER stress-related cell damage and apoptosis in other systems (2). Our data suggest that increased HSPs may reduce subsequent ER stress and thereby reduce the alterations in cytokine and chemokine gene expression that lead to many of the systemic aspects of the disease. Further studies will be necessary to investigate this suggestion.

Severe or prolonged ER stress leads to cell damage that plays a critical role in several important diseases including diabetes, α1-antitrypsin deficiency, and Alzheimer disease (1). In the current study we show that specific ER stress-related apoptotic pathways are activated after arginine treatment. The ER-resident procaspase-12 is an ER stress-specific proapoptotic molecule (22). Procasapase-12 gets cleaved and can sequentially lead to the activation of the downstream effector caspase-3. We observed that procaspase-12 and the downstream caspase-3 are activated after arginine treatment and confirmed acinar cell death with a positive TUNEL staining. Another ER stress-related proapoptotic mechanism involves induction of the transcription factor CHOP, which is regulated by all three ER stress sensors PERK, IRE1, and ATF6. We observed an increase of CHOP mRNA and protein levels after arginine administration. These data support the conclusion that arginine treatment leads to the activation of ER stress mechanisms and further indicate that this stress is severe and prolonged. The specific role of apoptosis in acute pancreatitis is currently under discussion, but these data suggest that ER stress-regulated mechanisms are likely involved in the apoptotosis observed in this model of the disease. In conclusion, we have demonstrated for the first time that ER stress signaling pathways are activated early in the course of acute pancreatitis and correlate with the severity of the disease using the arginine model. Based on the significance of ER stress mechanisms in several other diseases, these mechanisms are likely to also be salient in acute pancreatitis, and understanding their role in this disease will have important implications for the design of future treatments.

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