Arginine induced acute pancreatitis alters the actin cytoskeleton and increases heat shock protein expression in rat pancreatic acinar cells

M Tashiro, C Schäfer, H Yao, S A Ernst, J A Williams

Abstract

Arginine induced acute pancreatitis was evaluated as a novel and distinct form of experimental pancreatitis with particular attention to the actin cytoskeleton and expression of heat shock or stress proteins. Arginine induced a dose related necrotising pancreatitis in rats, as shown by histological evaluation, and an increase in serum amylase. Severe pancreatitis induced by 4.5 g/kg arginine was accompanied by dramatic changes in the actin cytoskeleton, as visualised with rhodamine phallodin. Intermediate filaments were also disrupted, as visualised by cytokeratin 8/18 immunocytochemistry. Arginine pancreatitis was accompanied by a stress response with a large increase in the small heat shock protein HSP27, as well as HSP70, peaking at 24 hours and localised to acinar cells. There was a lower increase in HSP60 and HSP90 and no effect on GRP78. HSP27 was also shifted to phosphorylated forms during pancreatitis. A lower dose of arginine (3.0 g/kg) induced less pancreatitis but a larger increase in HSP70 and HSP27 expression and phosphorylation of HSP27. Thus HSP expression can be overwhelmed by severe damage. The present work in conjunction with earlier work on caerulein induced pancreatitis indicates that changes in the actin cytoskeleton are an early component in experimental pancreatitis.

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Keywords: experimental pancreatitis; pancreas; actin cytoskeleton; cytokeratins; heat shock proteins; HSP27; rat

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Acute pancreatitis is an inflammatory process with mild to severe morphological changes over a period of several days to weeks.[1] The pathophysiological process of acute pancreatitis has been divided into three phases: triggering events, early acinar cell events, and later acinar and non-acinar cell events.[2] Although early acinar cell events are thought to be critical to the development of acute pancreatitis, treatment of severe pancreatitis has been limited because the pathophysiological mechanisms are not fully known. Moreover, it is difficult to obtain clinical material from patients to study the early acinar cell events. Thus considerable interest has been directed towards the cell biology and pathophysiology of early acinar cell events in experimental acute pancreatitis.

Several animal models of acute pancreatitis have been developed, including caerulein induced,[3] intraductal infusion of sodium taurocholate induced,[4] closed duodenal loop induced,[5] arginine induced,[6] and pancreatic duct obstruction induced pancreatitis.[7] Among these models, most mechanistic studies have been carried out using caerulein induced pancreatitis because it is rapid, easy to induce, and reproducible. One of the prominent cellular features of caerulein induced pancreatitis is rapid disruption of the actin cytoskeleton with reduction or loss of cell polarity, enlarged acinar lumina, and loss of the actin network surrounding the lumina.[8 9] More detailed studies have been carried out in vitro using isolated pancreatic acini where treatment with supramaximal cholecystokinin (CCK) inhibits secretion and also causes a dramatic blebbing and a decrease in apical filamentous actin.[10–12] Because the small heat shock protein 27 (HSP27) has been shown to be a phosphorylation dependent regulator of actin polymerisation,[13 14] and CCK has been shown to stimulate pancreatic HSP27 phosphorylation both in vivo and in vitro,[15 16] the possibility exists that HSP27 participates in the regulation of the cytoskeleton during caerulein induced acute pancreatitis. Moreover, HSP27 together with other HSPs are induced by cell stress. Thus the amount and phosphorylation of HSP27 may effect the outcome of acute pancreatitis.

To help understand the relevance of cytoskeletal changes and HSP27 in acute pancreatitis, we studied a different model in which L-arginine induces acute necrotising pancreatitis.[6 17 18] This model is non-invasive and has a defined time course following initiation by a single intraperitoneal injection of arginine. Recently it has been used to investigate regeneration following acute necrotising pancreatitis.[19 20] Because the histological pattern of arginine pancreatitis in earlier reports is quite different from caerulein pancreatitis, we investigated whether disruption of the cytoskeleton was a common prominent early feature. We found that this was the case. In addition, arginine induced acute pancreatitis in rats was

Abbreviations used in this paper: BSA, bovine serum albumin; CCK, cholecystokinin; FITC, fluorescein isothiocyanate; DTT, dithiothreitol; HSP, heat shock protein; IEF, isoelectric focusing; MAP, mitogen activated protein; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS, Tris buffered saline.

also accompanied by a stress response which included induction of multiple heat shock proteins and increased phosphorylation of HSP27.

Material and methods

MATERIALS

Monoclonal antimurine HSP27 antibody was provided by Dr MJ Welsh (University of Michigan, Ann Arbor, Michigan, USA) and used as previously described.[16] Polyclonal anticytokeratin 8/18 antibody[21] was provided by Dr MB Omary (Stanford University, Stanford, California, USA). The following materials were purchased: recombinant murine HSP27, polyclonal antihuman HSP27 antibody, polyclonal anti-HSP70 antibody, monoclonal antibodies to HSP70 and HSP60, and the glucose regulated stress protein GRP78 from Stress Gen (Victoria, British Columbia, Canada); monoclonal anti-HSP90 antibody from Transduction Laboratories (Lexington, Kentucky, USA); monoclonal antiactin antibody from Amersham Pharmacia Biotech (Piscataway, New Jersey, USA); dithiothreitol (DTT), aprotinin, leupeptin, and pepstatin from Roche Molecular Biochemicals (Indianapolis, Indiana, USA); rhodamine conjugated phalloidin and BODIPY FL phallacidin from Molecular Probes (Eugene, Oregon, USA); and the fluorescein isothiocyanate (FITC) conjugated antirabbit and Cy3 conjugated antimouse antibodies from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania, USA). All other reagents, including polyclonal anti-α-amylase antibody, were obtained from Sigma Chemical Co. (St Louis, Missouri, USA).

INDUCTION AND EVALUATION OF ACUTE PANCREATITIS

Male Sprague-Dawley rats weighing 160–190 g were used in all experiments. The animals were kept at 22°C on a 12 hour light-dark cycle and fed a standard rat chow. All studies were approved by the University of Michigan Committee on Use and Care of Animals.

Arginine induced acute pancreatitis was produced in overnight fasted rats. Rats received a single intraperitoneal injection of 2.0–5.0 g/kg body weight L-arginine monohydrochloride in 0.9% sodium chloride (pH 7.0), as previously described in detail.[6] The control group received the same volume of 0.9% sodium chloride. Rats were fed ad libitum after the treatment and killed at specified time points after injection of arginine by decapitation under carbon dioxide anaesthesia. Blood samples of mixed arteriovenous blood were collected from the decapitated body. Serum amylase activity was determined by Phadebas Amylase Test (Pharmacia and Upjohn, Uppsala, Sweden). A portion of the pancreas was fixed overnight at 4°C in phosphate buffered saline (PBS) containing 3.7% formaldehyde (pH 7.4). Tissues were embedded in paraffin, sectioned at the 3 μm setting, and processed for haematoxylin and eosin staining by standard procedures.

IMMUNOFLUORESCENCE STUDIES

Rat pancreata from control and arginine injected rats were fixed on ice for two hours or overnight at 4°C with PBS containing 4% formaldehyde, prepared from paraformaldehyde. Fixed tissue was rinsed, cryoprotected in sucrose, and frozen as previously described.[22 23] Actin was localised in 5 μm thick cryostat sections with rhodamine conjugated phalloidin, as previously described.[16] For double labelling studies, sections were incubated with 1 mg/ml of sodium borohydride in PBS, followed by PBS containing 0.2–0.5% Triton X-100 and 5% normal goat serum. Subsequently, sections were incubated for two hours at room temperature (or overnight at 4°C) with polyclonal antibodies against HSP27, amylase, or cytokeratin 8/18, or monoclonal antibody against HSP70. Primary antibodies were used in the dilution range 1:300 to 1:1000. After rinsing in PBS, sections were incubated with a mixture of Cy3 conjugated second antibody (1:200 dilution) and BODIPY FL phallacidin (1:40 dilution of 200 units/ml stock). Immunofluorescence staining was analysed by epifluorescence or confocal fluorescence microscopy (Noran OZ laser scanning confocal microscope). Digitised images of double labelled cryostat sections were collected as a series of optical sections (1 μm increments in the Z plane) extending throughout the thickness of the section. The distribution of amylase and actin in overlayed images was projected, using Noran Intervision software, as a stack that encompassed the thickness of the cryosection. The distribution of cytokeratin and actin was best visualised in a single optical section overlay at a level in which both cytoskeletal elements were most sharply defined. Digitised images from conventional and confocal microscopy were processed using Adobe Photoshop 5.0 software (Adobe, Mountain View, California, USA).

PREPARATION OF PANCREATIC TISSUE LYSATES FOR ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

Pancreatic tissue was homogenised in ice cold lysis buffer (pH 7.4) containing 50 mM Tris HCl, 5 mM EDTA, 25 mM NaF, 10 mM sodium pyrophosphate, 50 mM β-glycerophosphate, 1 mM phenylmethylsulphonyl fluoride, 0.2 mM Na_3VO_4, 1 mM DTT, 0.2% Triton X-100 (v/v), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Samples were then centrifuged at 3000 rpm for 15 minutes at 4°C. Protein concentrations of pancreatic homogenates were determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, California, USA) using bovine serum albumin (BSA) as a standard. The supernatant was prepared for one dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) gel electrophoresis, as previously reported.[15 16 22] Proteins (30 μg/lane) were then separated by 10% SDS-PAGE or IEF gel electrophoresis[16] using Model 111 Mini IEF Cell (Bio-Rad Laboratories).

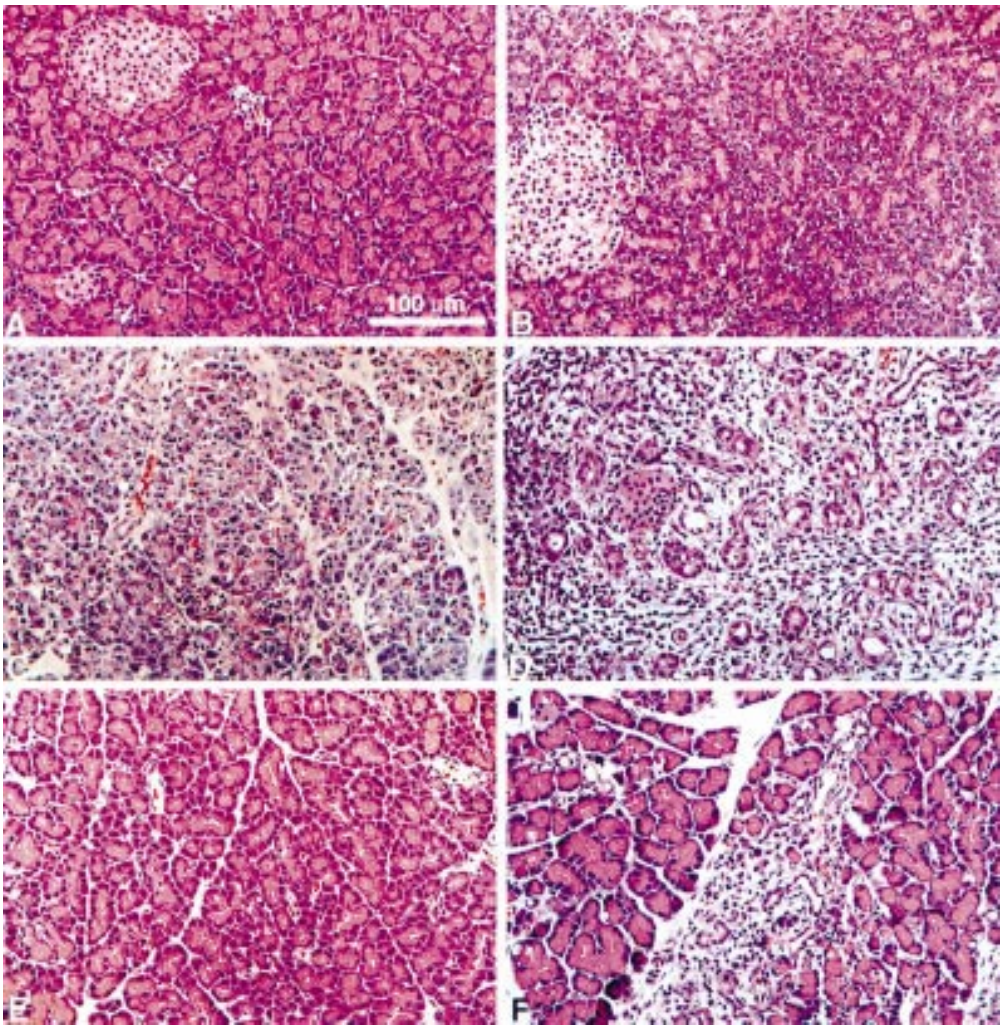

Figure 1 Light micrographs of the pancreas from control and arginine treated rats. Rats were injected with saline (A), 4.5 g/kg arginine (B, C, and D) or with 3.0 g/kg arginine (E and F). After injection with 4.5 g/kg arginine, the pancreas showed interstitial oedema, mild cellular infiltrate, and vacuoles within acinar cells at 12 hours (B), and by 24 hours acinar necrosis involved whole lobules and the acinar architecture was largely disrupted (C). After 72 hours (D), the only remaining acinar remnants were tubular complexes surrounded by fibroblasts and inflammatory cells. Injection of 3.0 g/kg arginine caused less severe pancreatic damage with very mild oedema after 24 hours (E). After 72 hours (F), most of the acinar cells appeared normal but there were small focal areas of fibrosis and inflammation. Haematoxylin and eosin staining.

After SDS-PAGE or IEF gel electrophoresis, protein was transferred to a nitrocellulose membrane and western blotting was carried out as previously described.[15 16] Membranes were blocked with 10% fat free dry milk for two hours in Tris buffered saline (TBS) containing 0.15% Tween 20 (TBST buffer, pH 7.6) and incubated with: monoclonal anti-HSP27 (1:1000); polyclonal anti-HSP70 (1:10000); anti-HSP60 (1:1000); anti-GRP78 (1:1000); anti-HSP90 (1:2000); antiactin (1:4000); and antiamylase (1:4000) antibody for one hour in TBST buffer containing 2% BSA. After washing, membranes were incubated with appropriate IgG antibody conjugated with horseradish peroxidase and antibody binding was detected by an enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech). Images were scanned from film with an Agfa Arcus II scanner. Quantification was performed by Multi-Analyst software (Bio-Rad Laboratories).

STATISTICAL ANALYSIS

Results are expressed as mean (SEM). Statistical analysis was carried out by one way ANOVA followed by a post hoc test for multiple group comparisons both using Stat-View software. Differences with $p<0.05$ were considered significant.

Results

HISTOLOGICAL ALTERATIONS AND SERUM LEVEL OF AMYLASE

Intraperitoneal administration of 4.5 g/kg arginine produced acute necrotising pancreatitis after a delay of at least six hours. At 12 hours, interstitial oedema, mild cellular infiltrate, and vacuoles within some acinar cells were observed (fig 1B). By 24 hours, focal acinar necrosis extended to involve almost the entire pancreas and the acinar architecture was partially destroyed (fig 1C). Increased red blood cells were also observed both within and outside blood vessels. At 48 hours, severity of

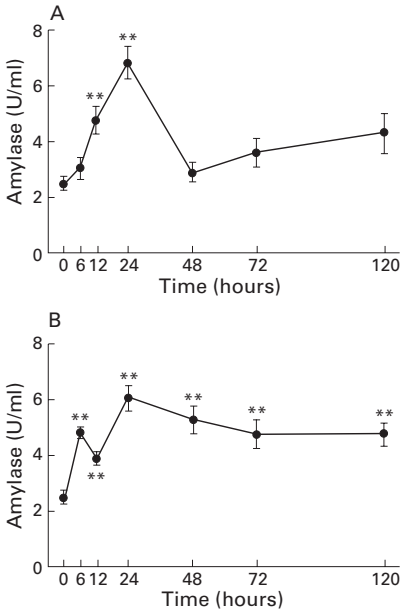

*Figure 2 Time course of serum amylase levels in response to arginine induced acute pancreatitis. Results following administration of 4.5 g/kg arginine (A) or 3.0 g/kg arginine (B). Both show mean (SEM) values from 6–12 animals at each time point. **p<0.01 compared with control (0 hours).*

tissue damage had progressed further (data not shown), and by 72 hours the acinar architecture was markedly disrupted with remaining acinar cells appearing primarily as tubules cut in cross section (fig 1D). Inflammatory cells and fibroblasts were also clearly present, infiltrating the interstitial space. There was little change from 72 to 120 hours (data not shown). A smaller dose of arginine (3.0 g/kg) caused less severe pancreatic damage. At 24 hours, very mild oedema was observed without cellular infiltrate (fig 1E). At 72–120 hours, small areas of the pancreas were disrupted similar to that seen with 4.5 g/kg arginine treatment but the majority of the pancreata contained normal appearing acini (fig 1F).

After injection of 4.5 g/kg arginine, serum amylase activity was significantly increased at 12 hours and peaked at 24 hours when it was increased 2.7-fold (fig 2A). Thereafter, it was decreased at 48 hours but remained slightly elevated until 120 hours. Tissue amylase content, as assessed by western blot analysis, rapidly decreased over 24 hours (most pancreata showed less than 10% of control) and was not detected after 72 hours (data not shown). Injection of 3.0 g/kg arginine also increased serum amylase activity with a significant increase at six hours and a peak at 24 hours (2.4-fold) (fig 2B). Thereafter, serum amylase level gradually decreased but remained elevated until 120 hours. With 3.0 g/kg arginine there was only a small decrease in tissue amylase after 24 hours.

EFFECTS OF ARGININE INJECTION ON THE ACTIN CYTOSKELETON

It is well established that stimulation by caerulein or CCK in vivo and in vitro leads to changes in the acinar cell actin cytoskeleton.[8–12] However, little is known about the effects of arginine induced acute pancreatitis on the actin cytoskeleton in the pancreas. We investigated the actin cytoskeleton in vivo using rhodamine-phalloidin as a probe and epifluorescence microscopy combined with Nomarski images. In the control pancreas obtained from rats which received saline, actin was primarily localised as an intense fluorescent band just beneath the luminal membrane (fig 3A). Sub-basolateral membrane staining appeared as a continuous band but of lesser intensity; appreciable cytoplasmic staining was not present. Figure 3B shows the Nomarski image of the same section shown in fig 3A with granules visible in the apical region of the acinar cells. After injection of 4.5 g/kg arginine, changes were apparent as early as 12 hours, including moderate disorganisation of sub-luminal actin in some acini. Only a few acini showed the sharply delineated sub-luminal actin staining similar to that seen in control pancreas (fig 3C). In addition, there was reduced and discontinuous actin staining of the basolateral membrane. These changes in the actin cytoskeleton were variable, from moderate to very severe, but reproducible in each experiment. Some acini started to round up and acinar cells showed an intense ring of actin staining near the plasma membrane. At 24 hours, disruption of normal acinar organisation became pronounced although poorly organised acini were still present (fig 3E, 3F). The majority of cells were rounded up, with an intense ring of actin staining near the plasma membrane. In some cells, diffuse cytoplasmic actin was present. Consistent with the marked disorganisation of acinar structure after 4.5 g/kg arginine injection, the sharply defined sub-apical actin profiles that characterise acini of control and 12 hours post-arginine tissue (fig 3A–D) were difficult to resolve (fig 3E, 3F). In contrast, after 3.0 g/kg arginine injection, there was no consistent change in actin staining at 12 hours (data not shown). At 24 hours, the overall acinar architecture was intact with sub-luminal actin staining in most acini (fig 3G, 3H) but there was reduced and discontinuous actin staining of the basolateral membrane. Thereafter, much of the pancreas returned to normal by 72–120 hours (data not shown).

EFFECTS OF ARGININE INJECTION ON THE DISTRIBUTION OF AMYLASE AND CYTOKERATIN 8/18

To further characterise structural changes present at the early stages of arginine induced acute necrotising pancreatitis, we colocalised amylase as a marker for secretory granules and cytokeratin 8/18 to visualise the intermediate filament component of the cytoskeleton with actin. Sections were evaluated by confocal microscopy. The changes in actin in response to the high dose of arginine at 24 hours were essentially similar to those shown in fig 3. Amylase was localised in control tissue to numerous secretory granules that filled the apical pole of the cell just beneath the actin rich terminal web which surrounds the lumen (fig 4A). Twenty four hours after injection of 4.5

g/kg arginine, only a few small clusters of amylase containing secretory granules remained without any specific cellular localisation (fig 4B). Cytokeratin 8/18 was present in control cells within the terminal web where it colocalised with actin, and as filamentous strands extending from the terminal web region to the basal aspects of the acinar cells (fig 4C). Ducts and centroacinar cells contained abundant cytokeratin 8/18 and actin. Twenty four hours after injection of 4.5 g/kg arginine, the intermediate filaments containing cytokeratin 8/18 were disrupted and replaced by a few focal deposits or small aggregates (fig 4D). Pancreatic sections from rats treated with 3.0 g/kg arginine were also evaluated but showed inconsistent changes with some acini showing some alteration of actin, cytokeratin 8/18, and granule polarity, but with most acini appearing normal (data not shown).

HSP27 EXPRESSION AFTER L-ARGININE INJECTION

Because HSP27 is believed in model cells to regulate the actin cytoskeleton in a phosphorylation dependent manner, we investigated whether arginine pancreatitis induced HSP27 expression. After injection of 4.5 g/kg arginine, HSP27 protein was increased at six hours, peaked at 24 hours where there was a 6.4-fold increase (SEM 1.0), and remained elevated at 120 hours (fig 5A). Administration of 3.0 g/kg arginine which led to less pancreatic damage induced a larger but more transient increase in HSP27 expression. HSP27 expression peaked at 24 hours with a 32.9-fold (SEM 1.0) increase, and then decreased but remained higher at 120 hours than control levels (fig 5B). For both doses there were animals that showed no increase at six, 72, and 120 hours. However, these were included in the average and range as we had no obvious justification to eliminate these animals. Because of a possible non-linear response in the western blotting, we analysed the response at 24 hours simultaneously with standards from 0.1 to 100 ng of recombinant murine HSP27. By this comparison, the basal amount of HSP27 in the rat pancreas was calculated as 16 (1) pg/μg protein and the increase was 12-fold after 4.5 g/kg arginine and 38-fold after 3.0 g/kg arginine, confirming the relative changes compared with control in fig 5. We also evaluated HSP27 protein expression in other tissues, including the liver, kidney, and lung, and these tissues showed no increase in response to either dose of arginine at 24 hours.

Immunohistochemistry revealed that HSP27 protein was expressed at a low level in normal pancreas, primarily in small blood vessels (fig 6A), compatible with a previous report of constitutive expression in vascular endothelial cells.[24] After injection of 3.0 g/kg arginine, HSP27 was highly expressed in the cytoplasm of many but not all pancreatic acinar cells at 24 hours (fig 6B). Some areas showed little or no staining. Morphologically, acinar structure was well preserved throughout the tissue sections. HSP27 was also expressed in the cytoplasm of acinar cells after injection of 4.5 g/kg arginine. In this case, only a few strongly expressing acinar cells were generally present, scattered

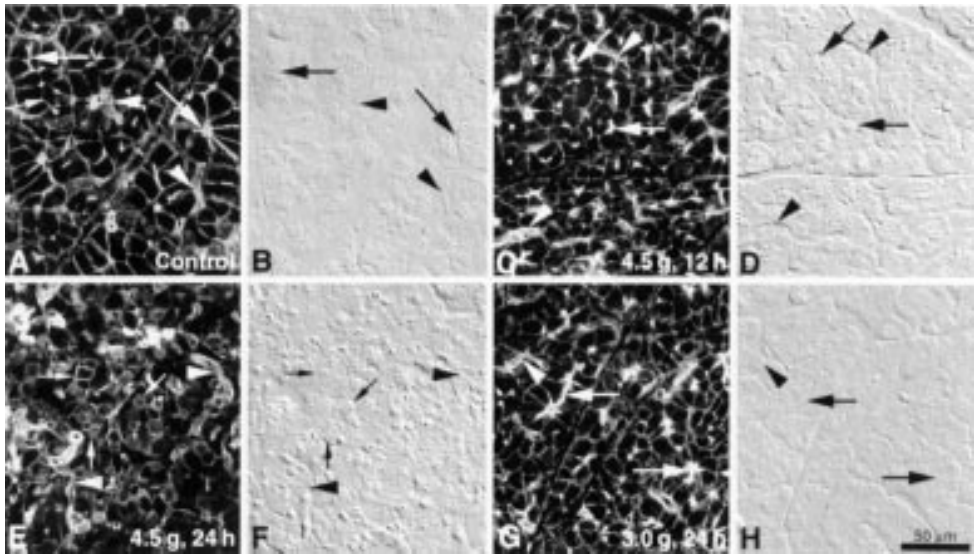

Figure 3 Effects of arginine injection on filamentous actin visualised with phalloidin in pancreatic acinar cells. Rats were injected with either 4.5 or 3.0 g/kg arginine, and pancreata were subsequently processed as described in material and methods. Paired Nomarski images were collected to provide bright field morphological detail. In control pancreatic acini (A, B), actin was present as sharply demarcated bands in the terminal web region just beneath the acinar lumen (large arrows) and subjacent to the lateral and basal plasma membranes. Actin was also present in duct and centroacinar cells (arrowheads). At 12 hours following injection with 4.5 g/kg arginine (C, D), moderate disorganisation/disruption of the normal actin staining pattern was apparent in many acini. Actin was less sharply defined along the basolateral membranes and often appeared discontinuous. Subluminal actin was present as in controls (large arrows) but the staining was often less sharply delineated. Acinar organisation appeared similar to controls (compare B and D). By 24 hours (E, F), the normal pattern of actin distribution was severely disrupted. Readily identifiable sub-luminal actin was no longer apparent, and acinar cells in poorly organised or fragmented acini were often rounded up and these cells generally expressed actin staining continuously along the cell periphery (small arrows in E). Diffuse cytoplasmic fluorescence was also present to varying degrees. Strong actin staining remained in the duct/centroacinar cells (arrowheads). In contrast, at 24 hours after injection with 3.0 g/kg arginine (G, H), acini retained their polarised state, and actin organisation was only moderately pertubed as was the case with the higher dose at 12 hours (compare C and G). Images are representative of at least four individual animals in each group.

among cells with a low level of expression or without detectable HSP27 (fig 6C). However, a few foci of relatively well organised acini with prominent apical granules were found in some sections of pancreas from 4.5 g/kg arginine treated animals. Acinar cells in these areas strongly expressed HSP27 whereas the adjacent areas composed of disrupted acini or single cells showed little if any HSP27 staining (fig 6D). Morphological preservation was poor in these extended areas (see fig 3). Thus in all conditions examined there was heterogeneity both between different regions of the pancreas and sometimes between cells within the same acinus. In addition, there appeared to be a correlation between retention of relatively normal acinar morphology and HSP27 expression in acini from arginine treated animals (fig 6B, 6D) although at the high dose disorganised acini and apparently necrotic acinar cells predominated (fig 6C).

EXPRESSION OF HSP70, HSP65, GRP78, HSP90, AND ACTIN 24 HOURS AFTER L-ARGININE INJECTION

To determine the specificity of the HSP27 response, we evaluated expression of other HSP proteins as well as total actin at 24 hours after arginine injection. Arginine administration induced an increase in HSP70 protein expression in response to 4.5 g/kg arginine at 24 hours whereas injection of 3.0 g/kg arginine induced a much larger increase in HSP70 protein expression, parallel with the increase seen in HSP27 (fig 7). It was not possible to quantify the fold increase because we could not consistently detect HSP70 expression in control pancreas. Immunohistochemistry showed

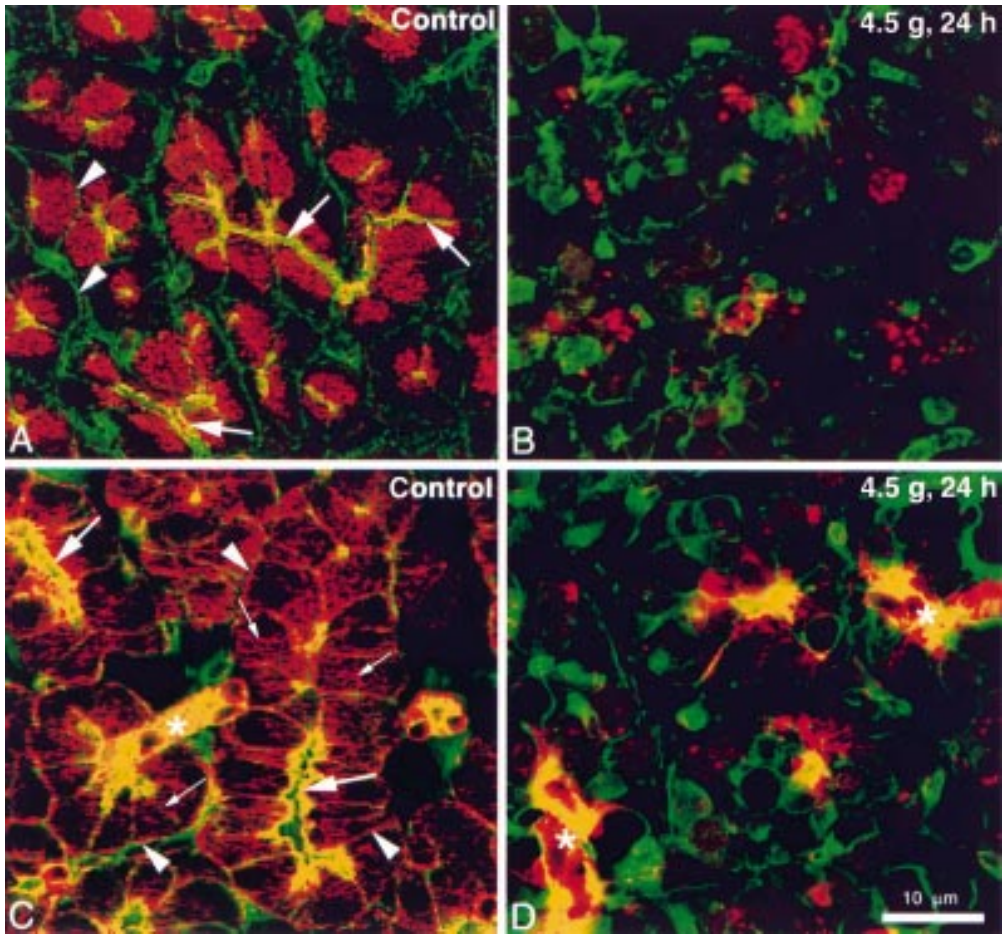

Figure 4 Effects of arginine injection on the distribution of amylase, cytokeratin, and actin. Cryostat sections from control animals (A, C) and in animals 24 hours post-injection (4.5 g/kg arginine) (B, D) were double labelled with BODIPY FL labelled phallacidin, and either antiamylase or anticytokeratin 8/18 antisera, followed by Cy3 conjugated second antibody. Immunofluorescence was monitored by confocal microscopy, and digitised images of FITC and Cy3 channels were superimposed. In control tissue (A), amylase (red fluorescence) is present in the secretory granules that fill the apical cytoplasm of acinar cells beneath the actin rich terminal web and lumen (arrows). Actin (green fluorescence) is also associated with basolateral membranes (arrowheads). Cytokeratin distribution (red fluorescence) in control acinar cells (C) is also present within the terminal web (large arrows) where it partially overlaps actin (yellow fluorescence) and along basolateral membranes (arrowheads). A thin band of cytokeratin free actin is present immediately beneath the luminal membrane (green fluorescence). In addition, filamentous cytokeratin strands extend in a rich network from the terminal region towards the basal aspects of the acinar cells (small arrows). Small ducts (asterisk) and their centroacinar cell termini express actin and abundant cytokeratin which overlap in their distribution. After 24 hours, the highly polarised distribution of actin, amylase, and cytokeratin in acinar cells is severely disrupted (B, D). Only a few small clusters of amylase containing secretory granules remain (B), and filamentous cytokeratin is lost, being replaced by a few focal deposits or small aggregates (D). Ducts and centroacinar cells (asterisk) retain actin and cytokeratin staining but in a markedly disorganised form. (A) and (B) are images reconstituted from complete Z series (1 µm steps) using Noran Intervision software. (C) and (D) are single optical sections taken at the focal plane in the Z series where both actin and cytokeratin filaments were defined most sharply.

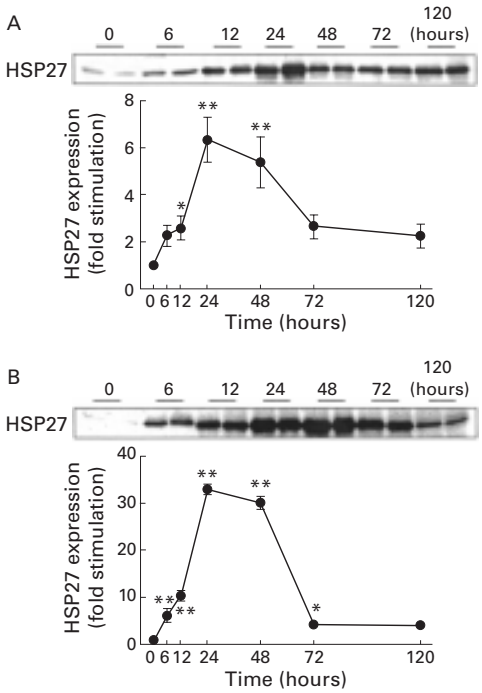

*Figure 5 Time course of heat shock protein 27 (HSP27) expression in response to arginine induced acute pancreatitis. Representative western blots of pancreatic protein lysates from two rats following sodium dodecyl sulphate-polyacrylamide gel electrophoresis (30 µg/lane) are shown for each time point. Results following injection of 4.5 g/kg arginine (A) or 3.0 g/kg arginine (B). Both graphs show mean (SEM) values from 6–12 animals at each time point. In (A), the range of sample fold increases was six hours (0.6–4.6), 12 hours (0.3–5.1), 24 hours (2.5–10.7), 48 hours (1.0–9.0), 72 hours (0.5–6.5), and 120 hours (0.3–3.8). In (B), the range of fold increases was six hours (0.9–17.0), 12 hours (5.1–17.6), 24 hours (28.1–36.0), 48 hours (24.5–32.5), 72 hours (0.9–6.8), and 120 hours (2.0—6.4). *p<0.05; **p<0.01 compared with control (0 hours).*

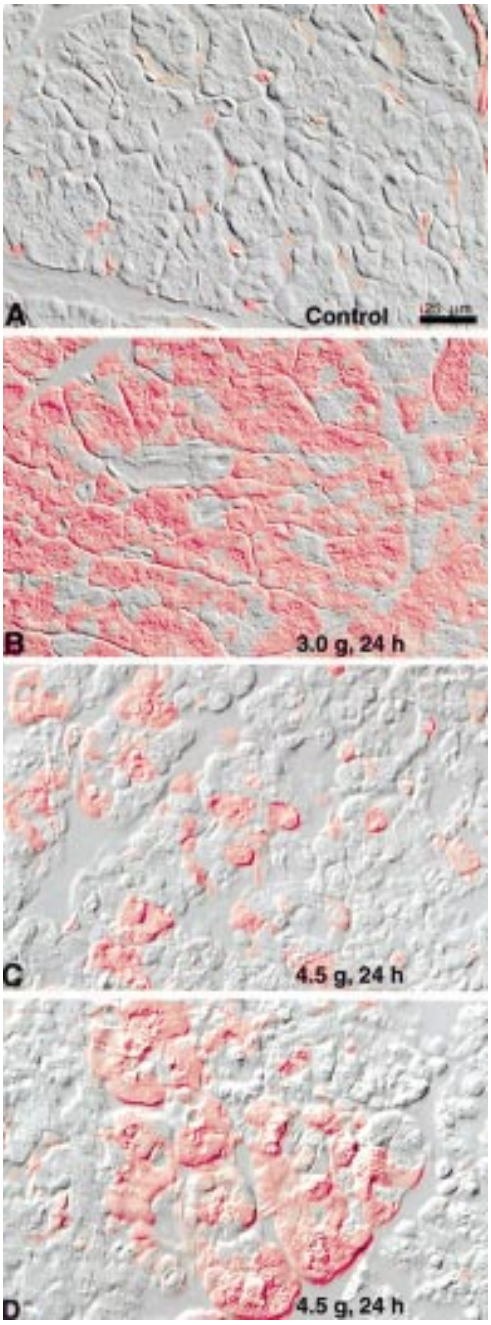

Figure 6 Localisation of heat shock protein 27 (HSP27) in the pancreas of control rats and 24 hours after 3.0 g/kg or 4.5 g/kg arginine injection. Epifluorescence images of HSP27 (coloured red) are overlaid on corresponding Nomarski images. In control rats (A), fluorescent HSP27 staining was limited to small blood vessels. After 3.0 g/kg arginine treatment (B), acinar cells were strongly labelled in a patchy manner. Ducts and blood vessels in the areas strongly expressing HSP27 in acinar cells showed little staining. After 4.5 g/kg arginine treatment (C, D), fewer acinar cells expressed HSP strongly whereas most showed weak or no expression. Acinar structure was compromised at the higher arginine dose, and oedematous areas were present (C). In some areas, foci of well organised acini were sometimes present (D). These acini expressed HSP27 whereas adjacent areas showed little HSP27 staining and poorly organised or disrupted acini. Images are representative of at least three individual animals in each group.

a pattern of induced HSP70 expression in acinar cells similar to HSP27 (data not shown). HSP90 was increased to a lesser extent, being 190 (17)% for 4.5 g/kg arginine and 475 (63)% for 3.0 g/kg arginine (both $p<0.01$). In contrast with other HSPs, HSP60 showed a larger effect at 4.5 g/kg arginine (676 (110)%, $p<0.05$) whereas 3.0 g/kg arginine induced only a modest increase in this HSP (198 (56)%; NS) (fig 7). In addition to the HSP we also evaluated GRP78 (also known as BiP), an endoplasmic reticulum luminal stress protein and total actin. GRP78 levels were 128 (19)% and 140 (17)% of control after 4.5 and 3.0 g/kg arginine, indicating that stress did not involve an unfolded protein response in the endoplasmic reticulum lumen.[25] Actin levels were 191 (30)% and 198 (32)% of control 24 hours after 4.5 and 3.0 g/kg arginine ($p<0.05$). Thus arginine administration resulted in a large increase in HSP27 and HSP70 and a more moderate increase in HSP60 and HSP90.

TIME COURSE OF HSP27 PHOSPHORYLATION AFTER L-ARGININE INJECTION

Recently we demonstrated that HSP27 exists as three isoforms (unphosphorylated, monophosphorylated, and diphosphorylated) in rat pancreatic acinar cells.[15 16] Furthermore, we also demonstrated that HSP27 phosphorylation is stimulated by CCK in vivo and in vitro[15] and by osmotic stress in vitro.[16] In the current

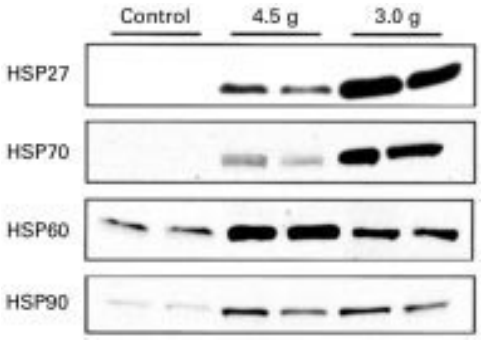

Figure 7 Analysis of heat shock proteins HSP27, HSP70, HSP60, and HSP90 expression at 24 hours after injection of 4.5 g/kg or 3.0 g/kg arginine or saline. Representative western blots of pancreatic protein lysates (30 μg/lane) from two rats following sodium dodecyl sulphate-polyacrylamide gel electrophoresis are shown for each dose of arginine (3.0 and 4.5 g).

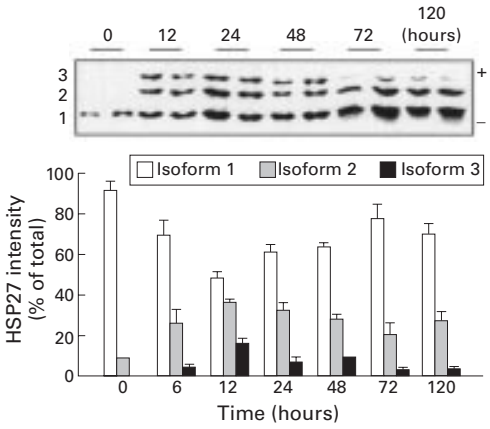

Figure 8 Time course of heat shock protein 27 (HSP27) protein phosphorylation in response to 4.5 g/kg arginine induced acute pancreatitis. Representative western blot analysis of pancreatic protein lysates from two rats following isoelectric focusing gel electrophoresis are shown for each time point. Isoform 1, unphosphorylated; isoform 2, monophosphorylated; and isoform 3, diphosphorylated. Both show mean (SEM) values for each isoform as a percentage of the total, obtained from 6–12 animals per time point.

work, evaluation of the pancreatic homogenates from control rats revealed that HSP27 was primarily unphosphorylated (91.3 (4.5)% of total) and only a small fraction existed as monophosphorylated isoforms (8.8 (4.5)% of total) (fig 8). After injection of 4.5 g/kg arginine, IEF showed an acidic shift of the HSP27 isoforms, indicating increased phosphorylation. This was seen at six hours and was maximal at 12 hours when the amount of the unphosphorylated form was reduced to 48.1 (3.3)% and the mono- and diphosphorylated forms peaked at 36.0 (1.8)% and 15.9 (2.6)%, respectively (all $p<0.01$). After 24 hours, both the mono- and diphosphorylated isoforms gradually decreased but more than 30% were still phosphorylated at 120 hours. Injection of 3.0 g/kg arginine induced a higher ratio of phosphorylated:unphosphorylated protein (data not shown).

Discussion

In the present study we demonstrated that arginine induced acute pancreatitis resulted in early disruption of the cytoskeleton with dramatic changes in both actin and intermediate filament organisation without a loss of total actin. Arginine pancreatitis was also accompanied by an increase in multiple stress heat shock proteins with large increases in HSP27 and HSP70 which were localised to acinar cells. We also observed an increase in HSP27 phosphorylation.

The histological alterations observed in response to 4.5 g/kg arginine were very similar to previous reports in which acute pancreatitis was induced by 5.0 g/kg[6] or 4.5 g/kg[19] arginine in male Wistar rats, while 2.5 g/kg arginine was reported to cause less severe pancreatic damage.[6] Our results with 3.0 g/kg arginine also showed that general acinar structure was preserved at 24 hours, and that the majority of the pancreas contained normal appearing acini at 72 hours. Thus the extent of arginine induced pancreatitis depended on its dose. Arginine induced acute pancreatitis has not been studied in as much detail as other models but appears moderate to profound in severity and has the advantage of being easy to induce and with a well timed starting point. In our study, similar to the results of Tani and colleagues,[6] serum amylase levels peaked at 24 hours and then declined. The relatively low and short lived increase in serum amylase levels can be readily explained by the rapid loss of tissue amylase that occurs over the 72 hour time period after arginine administration.[6] Injection of 3.0 g/kg arginine, which does not lead to the profound loss of tissue amylase, is therefore able to induce a more prolonged increase in serum amylase levels.

The mechanism by which arginine induces pancreatitis is not well understood. Because excessive doses of lysine, another basic amino acid, also lead to pancreatic damage[26] the mechanism may be related to inhibition of protein synthesis. Arginine inhibits the growth of some tumours and this has been related to inhibition of ornithine decarboxylase.[27 28] Nitric oxide produced from arginine could also be involved although the parallel effects of the other amino acids make this less likely. Other studies have shown increased lipid peroxidation indicative of oxidative stress[29 30] and increased plasma levels of cytokines such as tumour necrosis factor α and interleukin 6[31] following arginine induced pancreatitis. Endogenous CCK was suggested to play a possible role in the development of arginine induced acute pancreatitis[19 32] but another study showed that acute pancreatitis was not prevented by a specific CCK-A receptor antagonist.[33] Arginine does not appear to affect any organs other than the pancreas[18] and we found no increase in HSP27 protein expression in the liver, the kidneys, or the lungs.

It is well known that stimulation by caerulein or high dose CCK results in oedematous pancreatitis in rats and a more severe necrotising pancreatitis in mice. Associated with these changes are reduction or loss of cell polarity, an enlarged acinar lumen, and loss of actin network surrounding the acinar lumina. Pancreatic acinar cells contain all three major

cytoskeletal components: microfilaments, intermediate filaments, and microtubles. Previous studies have shown that CCK or caerulein stimulation resulted in loss of filamentous actin, as judged by phalloidin staining.[8 16] More detailed studies have shown that CCK causes a dramatic basolateral blebbing and a decrease in apical filamentous actin in in vitro studies of isolated pancreatic acini,[10–12] and that basolateral blebbing is a reversible event, involving activation of myosin as well as redistribution of actin.[12] Other reports have shown that acute pancreatitis induced by pancreatic duct ligation resulted in decreased apical microfilaments,[34] and that caerulein induced acute pancreatitis caused disassembly of microtubules and microfilaments.[9] However, little is known about changes in other models of pancreatitis. Modulation of the actin network changes the mechanical properties of the cell that are essential for functions such as locomotion and cytokinesis[35] and these changes are also thought to play a role in the final fusion events before the exocytosis of zymogen granule content takes place.[9] Arginine administration resulted in changes of the actin cytoskeleton, including reduced actin staining under the luminal membrane and increased cytoplasmic staining. Arginine administration also induced disruption of the intermediate filaments which are composed of cytokeratins 8 and 18 in acinar cells. The keratin intermediate filament network however is not essential in protection against pancreatitis.[36] Western blotting showed that total actin was increased twofold at 24 hours. Our data are in contrast with a previous report in which caerulein induced pancreatitis resulted in proteolysis of actin.[9] This difference most likely reflects the fact that arginine induced pancreatitis is a distinct model and further study may be required.

Heat shock proteins (HSPs) are a highly conserved group of proteins induced by a variety of stresses including heat, toxins, heavy metals, and free radicals. They are distinguished by their molecular weight with HSP27 included in the low molecular weight group. Hyperthermia was shown to induce HSP70 mRNA in the pancreas resulting in increased protein both in vivo and in vitro.[37 38] In our studies however rectal temperature did not increase after arginine injection (unpublished data). Pancreatic HSP60 has been reported to be weakly induced or not at all by hyperthermia but rather to be induced by water immersion stress.[39] Expression of HSP70 and HSP60 has also been evaluated in caerulein induced acute pancreatitis in rats.[38 40] Both HSP70 mRNA and protein were upregulated in one study.[40] However, another study showed that HSP70 mRNA was increased but HSP70 and HSP60 protein levels were decreased[38] in this model of acute pancreatitis.

In arginine induced acute pancreatitis, 3.0 g/kg arginine induced a much larger HSP27 response than 4.5 g/kg. Whereas acinar organisation was well preserved at the lower dose and HSP27 expression was widespread, morphology was severely compromised at the higher dose, and HSP27 expression was restricted to widely scattered cells and to a few foci of relatively intact acini. However, the appearance of HSP27 expression in well organised acini following low dose and, in some tissue areas, after high dose arginine treatment, suggests a possible protective effect of HSP27 although elevated levels of other HSPs may also be involved. It is likely that the reduced amount of HSP27 (and HSP70) following 4.5 g/kg arginine is due to protein degradation because of the greater extent of acinar necrosis as has been proposed for caerulein induced pancreatitis[37 38] although decreased gene expression may also participate.

HSP27, similar to other HSPs, is induced by stress, and overexpression of HSP27 is known to confer resistance to heat and other stresses.[41] HSP27 differs from other HSPs in that it modulates actin dynamics following HSP27 phosphorylation and because its phosphorylation is regulated by a distinct signal transduction pathway, which includes p38 mitogen activated protein (MAP) kinase and MAP kinase activated protein kinase 2/3.[16] A previous study suggested that regulation of actin and preservation of glutathione together accounted for the protective effects of HSP27.[42] The cytoskeleton is a filamentous network of F-actin, microtubules, and intermediate filaments. Previous studies indicate that HSP27 phosphorylation abolishes actin polymerisation inhibiting activity[14 43] and its phosphorylation results in changes in the actin cytoskeleton.[44 45] Therefore, HSP27 mediated stabilisation of microfilaments is thought to be responsible for an increased survival of cells recovering from cell stress.[42–45] Although a recent study revealed increased HSP27 phosphorylation induced by CCK in rat pancreatic acinar cells in vivo and in vitro,[15] the total amount of protein was not evaluated. Thus little is known about the role of HSP27 and changes in the actin cytoskeleton in acute pancreatitis.

Not only was HSP27 protein expression in the pancreas increased but also its phosphorylation. This result is similar to previous observations that CCK or osmotic stress induced phosphorylation of HSP27.[15 16] Furthermore, parallel with the increase in HSP27, arginine administration also induced a large increase in HSP70 but had a smaller effect on other stress proteins, HSP90, HSP60, and GRP78. Moreover, overexpression of human wild-type HSP27 and its phosphorylated form has recently been shown to protect the cell architecture against supramaximal concentrations of CCK in Chinese hamster ovary cells stably transfected with the CCK-A receptor.[46] In another cell culture, model HSP70 has also been reported to protect the integrity of the actin cytoskeleton against oxidant induced damage.[47] Therefore, increased HSP27 protein expression and its phosphorylation may contribute, in conjunction with other stress proteins such as HSP70, to the maintenance of cellular integrity in the face of cell stress.

In summary, we demonstrated that changes in the cytoskeleton occurred in pancreatic acinar cells following arginine induced acute pancreatitis in rats, including disruption of the

actin cytoskeleton as well as the intermediate filaments. These results suggest that cytoskeletal alterations are a common feature of acute pancreatitis in rats. We also showed that arginine induced pancreatitis was accompanied by an increase in stress proteins, especially HSP27 and HSP70, and that phosphorylation of HSP27 was enhanced. Since HSPs are known to protect against different stresses, their role as a potential protective factor deserves further study.

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