Ascitic fluid and serum from rats with acute pancreatitis injure rat pancreatic tissues and alter the expression of heat shock protein 60

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Abstract Acute pancreatitis (AP) is an inflammatory process in which cytokines and chemokines are involved. After onset, extrapancreatic stimuli can induce the expression of cytokines in pancreatic acinar cells, thereby amplifying this inflammatory loop. To further determine the role and mechanism of irritating agents in the pathogenesis of AP, rat pancreatic tissues were stimulated with ascitic fluid (APa) and serum (APs) from rats with AP or with lipopolysaccharide (LPS). In addition, the alteration of heat shock protein 60 (HSP60) expression was evaluated. Rat pancreas was removed and meticulously snipped to fragments. The snips were cultured for up to 48 h. During this period, the tissue viability as well as amylase and TNF- α levels in the supernatant and the HSP60 expression in the pancreatic tissue before and after stimulation by APa, APs, and LPS were assayed time-dependently. At different time-points during the culture, the viability and the amylase activity in the pancreatic tissue remained largely stable. After stimulation with APa, APs, or LPS for 1 h, the pancreatic tissues showed some damage, and this was followed by a sharp decrease in the viability accompanied

by increased levels of amylase and TNF- α in the culture medium 2 or 4 h after stimulation ($p < 0.05$). In contrast, both the HSP60 mRNA and protein levels had a relatively high expression in the freshly prepared tissue fragments (0 h). As the culturing period was extended, the expression of HSP60 mRNA decreased only slightly; at the same time, the HSP60 protein levels decreased over a prolonged culture time, significantly so from 12 through 48 h ($p < 0.05$). After stimulation with APs, APa, or LPS, both the expression of HSP60 mRNA and protein in the tissue fragments increased slightly at 1 h and decreased significantly thereafter at 2 and 4 h ($p < 0.05$). APa, APs, or LPS induce injuries on isolated pancreatic tissues, accompanied by an altered HSP60 expression pattern in a time-dependent manner.

Keywords Acute pancreatitis · Pancreatic tissue fragments · Heat shock protein 60

Introduction

Acute pancreatitis (AP), especially severe acute pancreatitis (SAP), is a systemic inflammatory disease with high mortality, starting from local inflammatory injuries in the pancreas. As the disease proceeds, the systemic complications rapidly arise, affecting the lungs, liver, kidneys, and other organs (Gravante et al. 2009). Among many theories of the pathogenesis of AP, the research data obtained in the past clearly indicate a central role for trypsinogen activation and self-digestion injury. Hereby, the premature activation of trypsinogen in pancreatic acini is one of the primary early events of AP (VanAcker et al. 2007; Matsukura et al. 2006; Sherwood et al. 2007; Raraty et al.

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2005; Ohmuraya and Yamura 2008). However, the mechanisms of intracellular “trypsinogen activation” remain, thus far, ambiguous.

Heat shock protein 60 (HSP60) is a member of the heat shock protein family and is also known as a molecular chaperone and termed chaperonin 60 (Cpn60). Like other heat shock proteins, the expression of HSP60 increases sharply after the cells are stimulated by a number of factors such as high temperature, hypoxia, cold, infection, hunger, trauma, and poisoning. The increased expression of HSP60 in these stress conditions helps cells to improve tolerance to cellular stress and thereby maintain the stability of the intracellular environment (Bhagat et al. 2000; Nollen and Morimoto 2002; Munro and Pelham 1985). Experiments have confirmed that HSP60 is expressed in pancreatic acinar cells and colocalized with pancreatic enzymes like trypsin and amylase in their secretion pathway (from the endoplasmic reticulum and Golgi apparatus to zymogen granules; Le Gall and Bendayan 1996).

During AP, lipopolysaccharide (LPS) and a variety of chemokines and inflammatory proteins such as TNF- α and kallikrein can be detected in high concentrations in the ascitic fluid (APa) or the serum (APs; Bose et al. 2002; Griesbacher et al. 2002; Guija de Arespacochaga et al. 2006; Ramudo et al. 2005). As the disease proceeds, the pancreas and other organs in the body are exposed to varying degrees of proinflammatory stimuli in the APa and the APs. So far, little is known about the mechanisms and signaling pathways involved in the pathophysiology of AP. In particular, the role of HSP60 in this context is unknown. In this study, we adopted a rat SAP model and collected the APa and APs from the animals and used them to stimulate the isolated and cultured pancreatic tissue pieces. Thereafter, we evaluated the tissue viability and the alteration of HSP60 expression in the tissue to further characterize the role and mechanism of the irritating agents in the pathogenesis of AP.

Materials and methods

Animals

At least 40 healthy and mature Sprague–Dawley (SD) rats (220–250 g each) and 10 BALB/C mice (22–24 g each) of both sexes were purchased, respectively, from the Experimental Animal Center of Fudan University and the Experimental Animal Center of the Chinese Academy of Sciences, Shanghai, China. The animals were housed for 1 week under standard conditions with free access to water and laboratory chow before the experiments. The animals'

welfare and experimental procedures were approved by the Animal Ethics Committee of Tongji University, Shanghai, China.

Induction of mild acute pancreatitis in mice

A mild acute pancreatitis (MAP) model in mice was adopted based on the method as described by Frossard et al. (2002). The mice were fasted with only water access for 12 h prior to the experiment and received four hourly intraperitoneal (i.p.) injections containing a supramaximal dose (50 μ g/kg) of cerulein (Sigma Aldrich, Taufkirchen, Germany) or received comparable injections of 0.9% saline as controls. One hour after the final injection, the animals were killed by decapitation under isoflurane anesthesia and the blood was collected for further analysis. The pancreas was removed immediately, rinsed with normal saline, and then placed into neutral formalin for immunohistochemistry to examine the HSP60 protein expression in the pancreatic tissues.

Induction of severe acute pancreatitis in rats

A SAP model in rats was adopted with a slight modification of the procedure described previously (Li et al. 2003). Briefly, under aseptic and general anesthetic conditions, a laparotomy (~3 cm midline incision) was performed on these rats and the biliopancreatic duct was identified and clamped near the hilus of the liver. The rats in the SAP group received a retrograde injection of 3% sodium deoxycholate (0.1 ml/100 g body weight) into the biliopancreatic duct via the duct opening at the duodenum. Immediately, the injecting needle point was pressed for 1–2 min, the bile duct clamp was removed, and the abdomen was closed up. Rats that underwent the same surgery procedure but without the sodium deoxycholate injection were used as a sham operation control group. Five hours after the surgery, all the rats were killed as described above, and the pancreas, blood, and APa were harvested immediately. The pancreas was placed into neutral formalin for immunohistochemistry to examine the HSP60 protein expression in the pancreatic tissues. The blood and APa were centrifuged and the resulting supernatants (APs and APa) were collected as stimulants for the following experiments *in vitro*.

Immunohistochemistry of HSP60 protein expression in the pancreatic tissues of the rodents with acute pancreatitis

As described previously (Li et al. 2009a), after conventional pretreatment, the slides with sections of rat or mouse pancreatic tissues were incubated overnight at 4°C

in a humidified chamber with the related anti-HSP60 monoclonal antibody (1:1,000 dilution; Stressgen, Victoria, BC, Canada; product no. SPA-829). Biotin-labeled goat antirat or antimouse IgG working fluid 50 μ l (Jinqiao Bio. Ltd., Shanghai, China) was applied onto each slide and incubated at 37°C for 30 min, followed by incubation with a horseradish peroxidase-labeled streptavidin working solution at 37°C for 30 min. Finally, the slides were diaminobenzidine-stained and nuclear restained with hematoxylin. For negative controls, phosphate-buffered saline was used to replace the primary antibody. Image analysis was accomplished using the digital Motic Med 6.0 image analysis system (Motic, Germany).

Preparation and culture of rat pancreatic tissue snips and the evaluation of tissue viability

The excised pancreatic tissue was prepared according to the methods reported earlier (Jaffrey et al. 1999; Bhagat et al. 2000). The pancreas was removed from anesthetized SD rats and rinsed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) saturated with oxygen by bubbling, containing NaCl (130 mM), KCl (5 mM), MgCl₂ (1 mM), CaCl₂ (1 mM), HEPES (10 mM), glucose (15 mM), sodium pyruvate (10 mM), and bovine serum albumin (0.1%). The pancreas was then carefully snipped into pieces of <0.5 mm in diameter and resuspended in 6 ml Dulbecco's modified Eagle medium (Invitrogen, California, USA) supplemented with 20% fetal bovine serum. The pieces were distributed evenly in 24-well Falcon tissue culture plate and cultured for up to 48 h. At different time-points of 0, 12, 24, 36, and 48 h, the snips were harvested and washed with HEPES buffer. The viability of the pancreas tissue snips was determined using 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetrazolium bromide (MTT) assay kit (KeyGEN Biotechnology Development Ltd., Nanjing, China). Amylase activity and the level of TNF- α in the culture supernatant were determined by using amylase detection kit (Jianchen Bioengineering Ltd., Nanjing China) and TNF- α ELISA kit (Sigma, Saint Louis, MO, USA), respectively. The protein concentration of the pancreas snips in each well was measured with a protein detection kit (Bio-Rad, California, USA). The data were presented as levels per milligram of protein in each well in order to minimize the error caused by uneven distribution of the tissue snips in the wells.

These numerical values were used to estimate the survival and functional status of pancreatic tissues, as well as the base level of TNF- α under experimental conditions during the 48-h culturing process. The data were used as references in the subsequent experiments.

Treatment of the pancreatic tissue snips

In order to avoid the effects of the preparation procedures and depending on the results of the tissue viability evaluation mentioned above, the pancreatic fragments were treated by APa, APs, or LPS (Sigma, Saint Louis, MO, USA) after 4 h of basic culture. Briefly, the medium in the culture wells was aspirated and discarded, and the tissue pellets were resuspended in fresh medium with APs, APa (100 μ l per well), or LPS (100 μ l per well with the final concentration of 10 μ g/ml). The snips in the media without any stimulant were used as controls. At 1, 2, and 4 h after stimulation, the tissue snips and the culture supernatants were harvested to determine the vital and functional status of the pancreatic tissues after stimulation. Amylase activity and the TNF- α level in the supernatant were performed as described above. All samples were measured in triplicate and repeated in at least six rats.

Detection of HSP60 mRNA expression in the pancreatic fragments by RT-PCR

Pancreas fragments were harvested at different basic culture time-points of 0, 12, 24, 36, and 48 h, as well as at 1, 2, or 4 h after stimulation with APs, APa, or LPS. The HSP60 mRNA expression in the pancreas tissue was measured by real-time polymerase chain reaction (PCR) as described previously (Li et al. 2009a). Briefly, total RNA was extracted with a Trizol Kit (Invitrogen, California, USA), and RNA with 3 μ g each from every RNA preparation was used as a template for reverse transcription to cDNA using the reverse transcription system (Promega, Madison, WI, USA), with the addition of primers. Thereafter, the cDNA reaction mixture was applied in real-time PCR using SYBR Primix Ex Taq™ Kit (TaKaRa, Biotechnology Ltd., Dalian, China). The real-time PCR reaction was performed in a Quantitative PCR Instrument (Rotor Gene™, USA) with a "hot start" program: 95°C $\times$ 10 s incubation, followed by 40 cycles of 95°C $\times$ 5 s, 60°C $\times$ 15 s, and 72°C $\times$ 15 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included in each reaction as an internal standard. The HSP60 forward primer sequence was 5'-ggctatcgctactggt-3' and reverse primer was 5'-gcaagtcgctcggtca-3', resulting in a 237-bp amplicon fragment; the GAPDH forward primer sequence was 5'-accacagtccatgccatcac-3' and reverse primer was 5'-tccaccacctgtgctgta-3', resulting in a 452-bp amplicon fragment. The relative expression of HSP60 mRNA was presented as the percentage of normal control with the individual ratio of the HSP60 amplified cycle threshold (CT) value versus the respective GAPDH-amplified CT value. All samples were run in triplicate, averaged, and repeated in at least three rats.

Fig. 1 The expression of HSP60 in pancreatic tissue in mouse with MAP and rats with SAP (paraffin sections were stained as described in the “Materials and methods” section; amplification $\times 400$). In mice, MAP was induced by four hourly i.p. injections of cerulein, while SAP was induced in rats by retrograde injections of deoxycholate sodium into the biliopancreatic duct as described in the “Materials and methods” section. The pancreatic tissues from the animals with MAP or SAP showed varying degrees of edema, necrosis, and HSP60 staining (brown yellow). The experiments were performed in at least six animals

Detection of HSP60 protein expression in the pancreatic fragments by Western blotting

HSP60 protein expression in the pancreatic fragments was measured by Western blotting as described previously (Li et al. 2009a, c) with a minor modification. At different basic culture time-points of 0, 12, 24, 36, or 48 h and at 1 or 4 h after the stimulation with APs, APa, or LPS, the fragments were harvested for examination of HSP60 protein expression. The primary antibody is monoclonal HSP60 (Stressgen, Victoria, BC, Canada; product no. SPA-829) in a 1:1,000 dilution. After washing, the nitrocellulose membranes (Whatman GmbH, Dassel, Germany) was incubated with 1:5,000 IR Dye800-conjugated affinity-purified antimouse IgG (H&L, Rockland, MA, USA; product no. 610-132-121) at room temperature for 1 h, washed with TBS, and visualized with the Odyssey

Infrared Imaging System (LI-COR, USA). For internal reference, a monoclonal β -actin antibody (1:1,000 dilution; Sigma, Saint Louis, MO, USA) was used. The Image J analysis system was applied for analysis of the gray scale of the protein bands. The relative expression levels of HSP60 were expressed as percentages of the normal control, compared with the individual gray scale of HSP60 bands versus that of the corresponding β -actin bands. All samples were run in triplicate and repeated in at least three rats.

Statistical analysis

All data are presented as the mean $\pm$ standard error (SE) and were evaluated using analysis of variance (SPSS 13.0 software), unless stated otherwise. Statistical significance was assessed as $p < 0.05$ (significant) or $p < 0.01$ (very significant).

Table 1 Changes of amylase, TNF- α , and MTT of the cultured pancreatic tissue snips at different time-points (mean $\pm$ SE, $n=6$)

	Amylase (U/mg protein)	TNF- α (pg/mg protein)	Viability (MTT) (OD/mg protein)
0 h	1,558.697 $\pm$ 89.696	165.8802 $\pm$ 9.434	1.6896 $\pm$ 0.121
12 h	1,647.276 $\pm$ 105.912	166.5624 $\pm$ 10.184	1.9601 $\pm$ 0.092
24 h	1,549.812 $\pm$ 89.512	168.2742 $\pm$ 10.486	1.6646 $\pm$ 0.192
36 h	1,505.430 $\pm$ 96.421	158.9667 $\pm$ 9.197	1.8017 $\pm$ 0.052
48 h	1,635.026 $\pm$ 91.636	164.7269 $\pm$ 8.625	1.9396 $\pm$ 0.100

All the examinations for amylase activity and TNF- α levels in the culture supernatant fluid and the viability of the pancreatic tissues were performed according to the instruction of related kits as described above. All data were expressed as the levels of per milligram of protein of the pancreatic tissues

Results

Expression of HSP60 protein in the pancreatic tissue of rodents with acute pancreatitis by immunohistochemistry

In the normal pancreatic tissues from mice or rats, almost no obvious positive staining of HSP60 was detectable (Fig. 1, mouse control and rat control). Edema is one of the major changes detectable in the pancreas of mice with MAP. The morphologic changes coincide with the high expression of HSP60, which is distributed inside the pancreatic acinar cells (Fig. 1, mouse MAP). In the SAP model of rats, necrosis and hemorrhage is detectable and HSP60 is mainly expressed around the injured zone (Fig. 1, rat SAP). These results indicate an enhanced expression of HSP60 in the pancreatic tissues of rodents after the induction of AP.

Viability and basic assessments for the rat pancreatic tissue snips during the 48-h culture

As shown in Table 1, the viability of the isolated and cultured normal rat pancreatic fragments and the basic levels of amylase and TNF- α in the supernatant were measured during culture at the time-points of 0, 12, 24, 36, or 48 h. The results indicate that there is no clear difference among the corresponding parameters throughout the culturing time periods ($p>0.05$), suggesting that the function and viability of the isolated pancreatic tissues remained stable and intact during the 48-h culture.

Effect of LPS, APs, or APa on the viability of rat pancreas fragments and on the amylase and TNF- α levels in the culture supernatant

The cultured pancreatic rat fragments were incubated for 4 h and then stimulated with 100 μ l APa, APs, or LPS with a final concentration of 10 μ g/ml for another 1, 2, and 4 h, respectively. The viability of the pancreatic tissue was slightly lower at 1 h after stimulation and then decreased significantly with the long incubation at the 2- or 4-h time-points, compared with the controls ($p<0.05$; Fig. 2a). The amylase activity and TNF- α levels in the culture supernatants remained essentially unchanged from the control at 1 h after stimulation with APa, APs, or LPS, but increased significantly with longer incubation periods (2 or 4 h), compared to the controls ($p<0.05$; Fig. 2b, c).

Expressions of HSP60 mRNA in rat pancreas fragments during basic culture and after stimulation with APs, APa, or LPS

The expression of HSP60 mRNA in the rat pancreas fragments was measured during the culture at the time-

Fig. 2 Effects of LPS, APs, or APa on the viability of the pancreatic tissue (a) and on the amylase (b) and TNF- α levels (c) in the culture supernatant (mean $\pm$ SE, $n=6$). Data are expressed as percentage of controls without stimulants. LPS was used in the final concentration of 10 μ g/ml, while APs or APa were used as 100 μ l per reaction. * $p<0.05$ when compared to the control group

points of 0, 12, 24, 36, and 48 h. The semiquantified data revealed that the freshly prepared pancreatic snips (0 h) had a relatively high expression of HSP60 mRNA. During the culturing period, the expression of HSP60 mRNA gradually decreased from median 8.427 at baseline to 7.956 at 12 h, 7.493 at 24 h, 7.336 at 36 h, and 7.862 at 48 h, but without any statistical significance ($p>0.05$).

In order to avoid the effects of the preparation procedures, as mentioned above, the pancreatic fragments were treated by these stimulants after 4 h of basic culture. In contrast, when the rat pancreas fragments were incubated with APs, APa, or LPS, the expression of HSP60 mRNA increased initially at 1 h, followed by a sharp and significant decrease as the incubation period elongated to 2 and 4 h ($p < 0.05$; Fig. 3).

Expressions of HSP60 protein in rat pancreas fragments during basic culture and after stimulation by LPS, APs, or APa

The protein expression of HSP60 in rat pancreas fragments was analyzed using pancreas lysates from fragments prepared at baseline and at 4, 12, 24, 36, and 48 h by Western blotting. Similar to the expression of HSP60 at the mRNA level, the HSP60 protein expression was higher in the freshly prepared fragments (0 h) and decreased as the

culturing time increased. A significant reduction in HSP60 protein was observed after 12 h of culture and beyond ($*p < 0.05$ or $**p < 0.01$; Fig. 4).

From the results shown in Figs. 2 and 3, it is obvious that the effects of APa are comparable to APs. Therefore, after 4 h of basic culture, the pancreatic fragments were treated by LPS (10 $\mu\text{g/ml}$) or APa (100 μl per well). The results demonstrated that the expression of HSP60 protein in pancreatic fragments increased slightly after 1 h stimulation, but decreased after 4 h of stimulation with LPS or APa, respectively (Fig. 5a, b).

Our data above revealed that LPS, APs, and APa have a significant impact on pancreatic tissues, which reduces the viability of rat pancreatic fragments but increases the levels of amylase and TNF- α in the culture supernatant. In addition, these stimulants initially raise the expression of HSP60 mRNA and protein levels in rat pancreas tissue fragments, while after longer stimulation (> 2 h), a significant decrease was observed.

Fig. 3 Effects of APs, APa, or LPS on HSP60 mRNA expression of rat pancreas fragments (mean $\pm$ SE; $n=3$). **a** Original recording for amplification curve of HSP60 mRNA expression in rat pancreatic fragments. **b** Relative expression of HSP60 mRNA was calculated as the individual ratio of the HSP60-amplified CT value against the respective GAPDH-amplified CT value. The data were expressed as percentage of control as described in the “Materials and methods” section. $*p < 0.05$ when compared to the controls

Fig. 4 Effects on HSP60 protein expression in rat pancreas fragments during culturing (mean $\pm$ SE, $n=3$). **a** Western blot profiling of HSP60 protein expression in rat pancreatic fragments at different time-points of culture. **b** Quantitative analysis of the relative expression of HSP60 in rat pancreatic fragments, expressed as the gray scale of HSP60 band versus that of the corresponding β -actin band. The data are expressed as percentage of control (0 h). * $p<0.05$, ** $p<0.01$ when compared to the control (0 h)

Discussion

In this report, we adopted the mouse MAP model induced by i.p. injection of cerulein, an analog of cholecystokinin, and the rat SAP model induced by retrograde injection of sodium deoxycholate into the biliopancreatic duct to investigate the HSP60 expression and its relationship during the development of AP. In particular, our study focused on the impact of APa and APs, which contain proinflammatory cytokines, on the expression of HSP60. In both models, an increased HSP60 in the pancreas was detectable. These data are in line with earlier observations on elevated levels of HSP60 in pancreatic tissue after the induction of AP (Li et al. 2009a), whether in a mild or severe form in rodents. The immunohistochemical analysis revealed the expression of HSP60 mainly in the cytoplasm of acinar cells from mice with MAP and in the seriously inflamed tissues of rats with SAP. Data from recent published studies implied that a high expression of HSP60 might offer protection to cells in stress. For instance, Lee et al. found that the prestimulation by cold water immersion could specifically induce the synthesis of HSP60 in pancreatic cells, and this high expression of HSP60 inhibited cerulein-induced rat pancreatitis in a dose-dependent manner (Lee et al. 2000). To explain the protective mechanism, it has been proposed that HSP60

arrests the transit of lysosomal cathepsin B to the rich parts of digestive zymogen granules. Thus, the colocalization of lysosomal cathepsin B with digestive enzymes is inhibited and thereby the premature activation of trypsinogen in pancreatic cells is prevented (Rakonczay et al. 2002). Our earlier study showed that HSP60 expression exhibited an initial increase followed by a sharp decrease in SAP, accompanied by severe damage to pancreatic tissues (Li et al. 2008). These results suggest that the low expression of HSP60 in these stressed acinar cells may reduce its molecular chaperoning effect in preventing and clearing the premature activation of trypsinogen inside the cells, which may contribute to the self-digestion of pancreatic tissues and promote the infiltration of inflammatory cells. Hence, we propose that the imbalance between trypsin activation and the molecular chaperone activity in the pancreatic acinar cells is one of the critical mechanisms leading to the onset of inflammation and is part of the early intracellular events of AP. Recently, we investigated whether the regulation of the p38/MAPKAP kinase 2 (MK2) signaling pathway is important for HSP60 regulation using MK2-gene-deleted mice. Our results demonstrated that, within a certain time period after the induction of AP, the expression of HSP60 increased remarkably in MK2-gene-knockout mice and in wild-type mice alike, and

Fig. 5 Effect of LPS or APa on HSP60 protein expression in rat pancreas fragments (mean $\pm$ SE, $n=3$). **a** Western blot profiling of HSP60 protein expression in rat pancreatic fragments after treatment with LPS or APa. **b** Relative expression value of HSP60 protein expression in the fragments, presented as the gray scale of the HSP60 band versus that of the corresponding β -actin band. Data were expressed as percentage of control with * $p<0.05$ when compared to the controls

there was no significant difference between the two types of mice regarding HSP60 expression. However, MK2-gene-knockout mice emerged with less pathological changes in the pancreatic tissue accompanied by mild systemic inflammation, sharply different to severely suffered (locally and systemically) wild-type mice. Clearly, the p38/MK2 pathway plays an important role in the regulation of cytokines and small heat shock proteins like HSP27. Nevertheless, these data indicate that, for the regulation of HSP60, other pathways, as yet unidentified, are involved and more conclusive experimental results are needed to further characterize them.

In essence, pancreatitis is like an internal injury or burn, hence the inner connections between different injury models. For example, in a comprehensive review, Mallouk et al. (1999) summarized how heat shock protein 70 (HSP70) and ATP coordinated in cell homeostasis under particular stress conditions—in ATP depletion models—showing that HSP70 mediated protection against ATP depletion. More recently, Duan et al. (2006) found an altered proteomic expression pattern in a rat hypermetabolic model induced by burn sepsis and identified 15 different proteins, including HSP27, HSP60, and HSP70, consistent with a protective role for HSP70 and/or other HSPs against proteolysis. This report, in agreement with these disease/injury models, demonstrates that HSP60 expression varies under different pathologic conditions in a manner consistent with a protective role.

In the present study, we adopted procedures from Jaffrey's laboratory (Jaffrey et al. 1999; Bhagat et al. 2000) for preparing rat pancreatic tissue snips for in vitro stimulation. At different time-points during basic culture of tissue snips, we examined cell viability, as well as pancreatic amylase and TNF- α levels in the supernatant. All these parameters remained stable during 48 h of culture, while the HSP60 mRNA and protein expression exhibited an initial increase (0 h), possibly due to the stress of sample preparation, followed by decreased level up to 4 h of culture. After 4 h, the HSP60 mRNA level reached a steady phase, while the protein level continued to decrease up to 48 h. These results indicate that it is necessary to consider the stressful effect of the preparation of isolated tissues for HSPs studies. In general, mRNA expression is an indicator of protein synthesis, but in many cases, mRNA level changes may not reflect that of proteins due to decreased synthesis and increased degradation of proteins (Duan et al. 2006). Hence, the discrepancy between the expression of HSP60 mRNA and protein suggests that posttranscriptional regulation is much more sensitive to stress and irritations. In order to eliminate the HSP alteration that resulted from the stress of sample preparation, we performed our stimulating experiments in vitro 4 h after the tissue preparation, by which time the HSP60 expression had stabilized.

From clinical practice and laboratory research, it is known that AP, especially SAP, is frequently complicated by APa. Both APa and APs contain large quantities of toxic substances including cytokines and inflammatory macrophages (Andican et al. 2005; Ikei et al. 1998; Ramudo et al. 2005; Algul et al. 2002). In this pathological situation, the inflamed pancreatic tissue is soaked in the APa and perfused with blood containing the irritating toxic substances. Our recent investigations reported that $[Ca^{2+}]$ is significantly increased in pancreatic acinar cells bathed in APs and APa, while the cell viability decreased sharply. The effect of APa, comparable to LPS, was more potent than that of APs, suggesting that APa contained more harmful ingredients or that these ingredients were in higher concentrations (Li et al. 2009b). In the current study, we used again APa, APs, and LPS to stimulate isolated pancreatic tissue snips. This treatment induced a higher expression of both HSP60 mRNA and protein at 1 h after stimulation. Subsequently, the expression of HSP60 mRNA and protein decreased following 2 or 4 h stimulation and was accompanied by decreased tissue viability. These results offer further evidence that SAP APa, APs, as well as LPS injure pancreatic tissues, resulting in a vicious cycle in the development of AP. In addition, as the disease progressed, the expression of HSP60 decreased in the pancreatic tissue and its important role as a molecular chaperone diminished. Nevertheless, the exact significance of the HSP60 alteration and its causality with pancreatic damage remains to be further elucidated.

In summary, our data indicate that APa and APs from rats with AP have an impact on the inflammatory process of pancreatitis and alter the expression of HSP60 in animal models. These results suggest a clue for studying the pathogenesis of this potentially fatal disease by exploring the molecular mechanism of chaperonin HSP60.

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References

- Algul H, Tando Y, Schneider G, Weidenbach H, Adler G, Schmid RMP (2002) Acute experimental pancreatitis and NF-kappaB/Rel activation. *Pancreatol* 2:503–509
- Andican G, Gelisgen R, Unal E, Tortum OB, Dervisoglu S, Karahasanoglu T, Burcak G (2005) Oxidative stress and nitric oxide in rats with alcohol-induced acute pancreatitis. *World J Gastroenterol* 11:2340–2345
- Bhagat L, Singh VP, Hietaranta AJ, Agrawal S, Steer ML, Saluja AK (2000) Heat shock protein 70 prevents secretagogue-induced cell injury in the pancreas by preventing intracellular trypsinogen activation. *J Clin Invest* 106:81–89
- Bose SM, Verma GR, Mazumdar A, Giridhar M, Ganguly NK (2002) Significance of serum endotoxin and antiendotoxin antibody

- levels in predicting the severity of acute pancreatitis. *Surg Today* 32:602–607
- Duan X, Berthiaume F, Yarmush D, Yarmush ML (2006) Proteomic analysis of altered protein expression in skeletal muscle of rats in a hypermetabolic state induced by burn sepsis. *Biochem J* 397:149–158
- Frossard JL, Bhagat L, Lee HS, Hietaranta AJ, Singh VP, Song AM, Steer ML, Saluja AK (2002) Both thermal and non-thermal stress protect against caerulein induced pancreatitis and prevent trypsinogen activation in the pancreas. *Gut* 50:78–83
- Gravante G, Garcea G, Ong SL, Metcalfe MS, Berry DP, Lloyd DM, Dennison AR (2009) Prediction of mortality in acute pancreatitis: a systematic review of the published evidence. *Pancreatolgy* 9:601–614
- Griesbacher T, Rainer I, Tiran B, Evans DM (2002) Involvement of tissue kallikrein but not plasma kallikrein in the development of symptoms mediated by endogenous kinins in acute pancreatitis in rats. *Br J Pharmacol* 137:692–700
- Guija de Arespacochaga A, Hittmair KM, Schwendenwein I (2006) Comparison of lipase activity in peritoneal fluid of dogs with different pathologies—a complementary diagnostic tool in acute pancreatitis? *J Vet Med A Physiol Pathol Clin Med* 53: 119–122
- Ikei S, Ogawa M, Yamaguchi Y (1998) Blood concentrations of polymorphonuclear leucocyte elastase and interleukin-6 are indicators for the occurrence of multiple organ failures at the early stage of acute pancreatitis. *J Gastroenterol Hepatol* 13: 1274–1283
- Jaffrey C, Eichenbaum D, Denham DW, Norman J (1999) A novel pancreatic model: the snip method of pancreatic isolation for in vitro study. *Pancreas* 19:377–381
- Le Gall IM, Bendayan M (1996) Possible association of chaperonin 60 with secretory proteins in pancreatic acinar cells. *J Histochem Cytochem* 44:743–749
- Lee HS, Bhagat L, Frossard JL, Hietaranta A, Singh VP, Steer ML, Saluja AK (2000) Water immersion stress induces heat shock protein 60 expression and protects against pancreatitis in rats. *Gastroenterology* 31:220–229
- Li YY, Gingras D, Londono I, Bendayan M (2003) Expression differences in mitochondrial and secretory chaperonin 60 (Cpn60) in pancreatic acinar cells. *Cell Stress Chaperones* 8:287–294
- Li K, Li YY, Li XJ, Li YN, Lu XY, Chen CJ (2008) Expression and potential role of chaperonin 60 in experimental acute pancreatitis. *Chin J Pathophysiol* 24:1804–1810
- Li XL, Li K, Li YY, Feng Y, Gong Q, Li YN, Li XJ, Chen CJ (2009a) Alteration of Cpn60 expression in pancreatic tissue of rats with acute pancreatitis. *Cell Stress Chaperones* 14:199–204
- Li YY, Lu XY, Li XJ, Li YN, Li K, Chen CJ (2009b) Intervention of pyrrolidine dithiocarbamate and tetrandrine on cellular calcium overload of pancreatic acinar cells induced by serum and ascitic fluid from rats with acute pancreatitis. *J Gastroenterol Hepatol* 24:155–165
- Li YY, Ochs S, Gao ZR, Malo A, Chen CJ, Lv S, Gallmeier E, Goke B, Schafer C (2009c) Regulation of Hsp60 and the role of MK2 in a new model of severe experimental pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 297:G981–G989
- Mallouk Y, Vayssier-Taussat M, Bonventre JV, Polla BS (1999) Heat shock protein 70 and ATP as partners in cell homeostasis. *Int J Mol Med* 4:463–474
- Matsukura A, Otani T, Takamoto T, Usui H, Goto Y, Makuuchi M (2006) Intracellular activation of trypsinogen in rat pancreatic acini after supramaximal secretagogue stimulation: cysteine protease and serine protease activity. *Pancreas* 32:197–204
- Munro S, Pelham H (1985) What turns on heat-shockgenes? *Nature* 317:477–478
- Nollen EA, Morimoto RI (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing ‘heat shock’ proteins. *J Cell Sci* 115(Pt 14):2809–2816
- Ohmuraya M, Yamura K (2008) Autophagy and acute pancreatitis: a novel autophagy theory for trypsinogen activation. *Autophagy* 4:1060–1062
- Rakonczay Z Jr, Takacs T, Ivanyi B, Mandi Y, Papai G, Boros I, Varga IS, Jost K, Lonovics J (2002) The effects of hypo- and hyperthermic pretreatment on sodium taurocholate-induced acute pancreatitis in rats. *Pancreas* 24:83–89
- Ramudo L, Manso MA, Dedio I (2005) Biliary pancreatitis-associated ascitic fluid activates the production of tumor necrosis factor- α in acinar cells. *Crit Care Med* 33:143–148
- Raraty MG, Murphy JA, Mcloughlin E, Smith D, Criddle D, Sutton R (2005) Mechanisms of acinar cell injury in acute pancreatitis. *Scand J Surg* 94:89–96
- Sherwood MW, Prior IA, Voronina SG, Barrow SL, Woodsmith JD, Gerasimenko OV, Petersen OH, Tepikin AV (2007) Activation of trypsinogen in large endocytic vacuoles of pancreatic acinar cells. *Proc Natl Acad Sci USA* 104:5674–5679
- VanAcker GJ, Weiss E, Steer ML, Perides G (2007) Cause-effect relationships between zymogen activation and other early events in secretagogue-induced acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 292:1738–1746