Down-regulation of HSP60 expression by RNAi increases lipopolysaccharide- and cerulein-induced damages on isolated rat pancreatic tissues

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Abstract The objective of this study was to investigate the function of heat shock protein 60 (HSP60) on pancreatic tissues by applying HSP60 small interfering RNA (siRNA) to reduce HSP60 expression. Rat pancreas was isolated and pancreatic tissue snips were prepared, cultured, and stimulated with low and high concentrations of cerulein (10^{-11} and 10^{-5} mol/L) or lipopolysaccharide (LPS, 10 and 20 μ g/mL). Before the stimulation and 1 and 4 h after the stimulation, the viability and the level of trypsinogen activation peptide (TAP) in the tissue fragments were determined and the levels of tumor necrosis factor- α (TNF- α) and interleukin 6 (IL-6) in the culture supernatants were measured. Real-time PCR and Western blotting were used to evaluate the HSP60 mRNA and protein expression. After the administration of siRNA to inhibit HSP60 expression in the isolated tissues, these injury parameters were measured and compared. The pancreatic tissues in the control (mock-interfering) group showed a decreased viability to varying degrees after being stimulated with cerulein or LPS, and the levels of TAP, TNF- α , and IL-6 increased significantly ($p < 0.05$) in the tissues and/or in the culture supernatant. The expressions of HSP60 mRNA and protein were raised moderately after stimulating 1 h with low concentrations of cerulein or LPS, but decreased with high concentrations of the toxicants. In particular, the expression of HSP60 protein was reduced significantly ($p < 0.05$) when the tissues were stimulated by the two toxicants for 4 h. In

contrast, the tissue fragments in which HSP60 siRNA was applied showed much lower tissue viability ($p < 0.01$) and higher levels of TNF- α , IL-6, and TAP ($p < 0.01$) in the tissues or culture supernatant after stimulating with the toxicants at the same dose and for the same time duration as compared with those of the control groups ($p < 0.05$). The results indicated that both cerulein and LPS can induce injuries on isolated pancreatic tissues, but the induction effects are dependent on the duration of the stimulation and on the concentrations of the toxicants. HSP60 siRNA reduces HSP60 expression and worsens the cerulein- or LPS-induced injuries on isolated pancreatic tissues, suggesting that HSP60 has a protective effect on pancreatic tissues against these toxicants.

Keywords Isolated rat pancreatic tissue · Heat shock protein 60 · RNA interference · Acute pancreatitis

Introduction

Acute pancreatitis (AP) is an emergent disease commonly seen in clinical practice. Although the pathogenesis of AP remains elusive to our full understanding, scientists are in agreement that AP involves a cascade of events, and numerous reports have suggested that the initial step is the activation of trypsinogen inside the pancreatic acinar cells, resulting in damages evoked by the activated pancreatic enzyme (Chen et al. 2009; Frossard et al. 2008; Leung and Siu 2006). As to the mechanism of the abnormal enzyme activation, a number of theories have been proposed. Raraty et al. (2005) hypothesized that calcium overload ignites zymogen activation, whereas Ohmuraya and Yamura (2008) suggested that cathepsin B, after the lysosomes in the cytoplasm of acinar cells, engulfs zymogens and hydrolyzes the proenzymes, leading to intracellular enzyme

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activation. A newer theory advocates that heat shock protein 60 (HSP60) possesses the function of protecting pancreatic tissues against damages and the malfunctioning or weakened HSP60 under pathological conditions is responsible for the early zymogen activation in AP (Gruden et al. 2009; Ohmuraya and Yamura 2008).

HSP60 is a member of the heat shock protein family, also known as chaperonin 60. This protein, similar to other HSPs, is increased during cellular stress as an adaptive protection strategy (Gruden et al. 2009; Parcellier et al. 2003; Saluja and Dudeja 2008). Over the past decade, investigators (Le Gall and Bendayan 1996; Li et al. 2003) found that HSP60 and the pancreatic enzymes share a common location inside the pancreatic acinar cells, interacting intimately. Moreover, like the distributive characteristics of pancreatic enzymes, HSP60 showed an increasing gradient distribution along the pancreatic secretory pathway from the rough endoplasmic reticulum and Golgi apparatus to zymogen granules in the acinar cells (Le Gall and Bendayan 1996). Lee Sik et al. (2000) found that pre-immersion of cold water could stimulate the high expression of pancreatic HSP60, alleviating rat pancreatic injury induced by cerulein. Our previous experiments on rodents also showed that HSP60 had an initial increase followed by a sharp decrease of expression that was accompanied by tissue damages (Li et al. 2009a). Although these experimental results strongly suggest that HSP60 has a protective effect on pancreatic tissue, more conclusive studies are needed to confirm the molecular chaperone role of HSP60 and to further explore its importance in the pathogenesis of AP.

This work is an attempt to investigate the protective function of HSP60 on pancreatic tissues using RNA interference (RNAi) technology to silence the HSP60 gene. In particular, we use the siRNA (Chu and Rana 2008; Minois et al. 2010; Wu et al. 2005) against HSP60 in isolated and cultured rat pancreatic tissues to reduce the expression of HSP60, stimulate these tissues with cerulein and lipopolysaccharide (LPS), and observe what ensues and how. Our purpose is to determine if the HSP60 protective function is essential to pancreatic tissue in pathological conditions. Cerulein is a cholecystokinin (CCK) analogue and can evoke, when in overdose, pancreatitis-like changes in vivo (Sidhapuriwala et al. 2009), whereas LPS is a major component of the Gram-negative bacterial cell wall and can trigger endotoxin-induced shock and multiple organ damage, including pancreatic injuries (Li et al. 2010; Jaworek et al. 2008; Vaccaro et al. 2000). Hence, these are the appropriate agents to create the conditions for experimental AP in animals. Since the initial step in the pathogenesis of AP is the activation of trypsinogen inside the pancreatic acinar cells and the pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin 6 (IL-6), universally increase in the serum of AP

patients and experimental animals, we selected trypsinogen activation peptide (TAP), TNF- α , IL-6 and viability of the pancreatic tissues as the experimental indicators. After stimulation by toxicants, the rat pancreatic tissue snips were evaluated for damages at different time points by measuring these injury parameters and by comparing the results of HSP60 interfering and control groups.

Materials and methods

Animals

A total number of 120 healthy and mature Sprague–Dawley (SD) rats (200–250 g each) with equal number of each sex were purchased from the Animal Center of Fudan University, Shanghai, China. Prior to the experiment, the animals were housed under standard conditions with free access to water and lab chow at $25\pm 2^\circ\text{C}$ and $55\pm 5\%$ humidity. The animals' welfare and the experimental procedures on them were approved by the Animal Ethics Committee of Tongji University, Shanghai, China.

Rat pancreatic tissue isolation and culture

Under aseptic conditions, 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, as described previously (Li et al. 2010). The pancreas was then carefully snipped into pieces of <0.5 mm in diameter and resuspended in 6 mL Dulbecco's modified Eagle medium (DMEM; Invitrogen, California, USA) supplemented with 20% fetal bovine serum and 1% of penicillin–streptomycin solution (Invitrogen-Gibco, California, USA). The tissue fragments were distributed evenly in a 24-well Falcon tissue culture plate and cultured at 37°C in a humidified incubator with 5% CO_2 .

Treating the normal pancreatic tissues with LPS or cerulein

In a previous study, Li et al. (2010) showed that the isolated pancreatic tissues were viable for up to 48 h of culture and that tissues at 4 h of culturing were optimal for the stimulating experiments. In this study, different concentrations of cerulein (with final concentrations of 10^{-5} , 10^{-7} , 10^{-10} , and 10^{-11} mol/L) or LPS (with final concentrations of 20 and 10 $\mu\text{g/mL}$) were administered to the isolated tissue snips after 4 h culture, and the responses of the tissue snips to the toxicants were observed. Tissues treated with the same volume of normal medium (DMEM) served as controls. The pancreatic tissues and culture supernatant were harvested before stimulation and 1 or 4 h after

stimulation. The viability and level of TAP of the tissues were determined using 3-[4, 5-dimethylthiazol-2-yl]-2,3-diphenyltetrazolium bromide (MTT) assay kit (KeyGEN Biotechnology Development Ltd., Nanjing, China) and ELISA kit (Sigma, Saint Louis, MO, USA), respectively. The levels of TNF- α and IL-6 in the culture supernatant were measured using an ELISA kit (Sigma) as well. At the same time, the protein content of the tissue in each well was measured with a Bradford protein detection kit (Bio-Rad, California, USA), and the data were presented as the levels per milligram protein in each well to minimize the error caused by uneven distribution of the tissue snips in the wells.

Interference of HSP60 expression in rat pancreatic tissues

Design of specific siRNA

We designed three pairs of hairpin oligonucleotide sequences for *HSP60* using the web-based siRNA Target Designer, version 1.51 Software provided by Invitrogen (<http://www.invitrogen.com>). The sequences were submitted for a BLAST search to ensure that there was no existing homologous sequence. All three pairs of hairpin siRNA were synthesized by Invitrogen. These sequences were as follows:

Stealth_58 5'-GAGAAUAUGUUGUCCGCCUG
GACA-3' (sense)
5'-UGUCCAGGCGGACAACAUAUUUCUC-3'
(antisense)
Stealth_687 5'-GAGAGCUGUGAUGUUGGCU
GUUGAU-3' (sense)
5'-AUCAACAGCCAACAUCACAGCUCUC-3'
(antisense)
Stealth_1026 5'-UCAGUCCAUUGUACCUGCU
CUUGAA-3' (sense)
5'-UUCAAGAGCAGGUACAAUGGACUGA-3'
(antisense)

Evaluation of the interfering efficiency in the isolated pancreatic tissues

By following the transfection kit instructions (Invitrogen), siRNA-Lipofectamine™ 2000 mixture was prepared for the HSP60 mRNA interference experiment. The mixture of siRNA-Lipofectamine™ 2000 was added to the cultured pancreatic fragments (100 μ L/well) with a final concentration of 80 nmol/L and served as the interference group. A Negative Universal Control (Invitrogen), whose sequence was not found in rat genome database, was treated with the addition of Lipofectamine™ 2000 to the fragments and was used as negative control, and the group treated with

equivalent DMEM medium served as a blank control. After interference for 24 h, the pancreatic tissues and supernatant were harvested as described above. The interference efficiencies of three pairs of siRNA to HSP60 were detected and compared, and the pair with the strongest interference potency was chosen for subsequent experiments. The survival and functional changes of the pancreatic tissues treated with the chosen pair of siRNA were evaluated.

Treating the HSP60-interfered pancreatic tissues with LPS or cerulein

The pancreatic tissues exposed to the most efficient siRNA Stealth_687 were then treated with LPS or cerulein using the aforementioned method. At 1 and 4 h after the stimulation, the pancreatic tissues and supernatant were harvested and the viability, TAP level in the pancreatic tissues, and the levels of TNF- α and IL-6 in the supernatants were determined using the same methods mentioned above.

HSP60 protein expression in the pancreatic fragments stimulated by LPS and cerulein

HSP60 protein expression in the pancreatic fragments with or without HSP60 siRNA interference was analyzed by Western blotting as described previously (Li et al. 2009a) with a minor modification. The primary antibody was mouse monoclonal anti-HSP60 (Stressgen, Victoria, BC, Canada; product no. SPA-829) diluted 1:1,000. After washing with PBS, 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). A monoclonal β -actin antibody (1:1,000 dilution; Sigma) was used as an internal reference. The grayscale of protein bands was analyzed using the ImageJ analysis system. The relative expression value of HSP60 protein was presented as the ratio of the HSP60 band to that of the corresponding β -actin band.

HSP60 mRNA expression in the pancreatic fragments stimulated by LPS and cerulein

With the similar procedures described previously (Li et al. 2009a), the HSP60 mRNA expression in the pancreas fragments with or without HSP60 siRNA was measured by real-time PCR. Briefly, total RNA was extracted with a Trizol Kit (Invitrogen), and 3 μ g RNA from each 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×10-s incubation, followed by 40 cycles of 95°C×5 s, 60°C×15 s, and 72°C×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 5'-gcaag tcgctcggtca-3', resulting in a 237-bp amplicon fragment; the GAPDH forward primer sequence was 5'-accacagtccatgc catcac-3' and reverse primer 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.

Statistical analysis

All samples in the experiment were measured in triplicate and averaged and repeated in at least four rats unless otherwise stated. All data are presented as the means ± SEM and statistical analysis was performed using the SPSS package, version 13.0 for Windows. First, datum quality was verified using the Levene test for equality of variances. If the samples collectively accorded with the requirement of equality of variances, data would be analyzed using the ANOVA test. In case of a variance inequality, the Kruskal–Wallis test was used for analysis. Statistical significance was assessed as $p < 0.05$ or $p < 0.01$.

Results

Expressions of HSP60 protein and mRNA in rat pancreas fragments after stimulation with cerulein

As shown in Fig. 1a, after stimulation by cerulein with different concentrations (from 10^{-11} to 10^{-5} mol/L) for different time durations (1 and 4 h), the expression of HSP60 protein in the pancreatic fragments decreased slightly after 1 h of stimulation ($p > 0.05$) and then reduced significantly in the 10^{-5} and 10^{-7} mol/L groups 4 h after stimulation ($p < 0.05$; Fig. 1a, b). The relative expression of HSP60 protein in the tissues stimulated with the lowest and highest concentrations of cerulein (10^{-11} mol/L Cer-L and 10^{-5} mol/L Cer-H) showed the most prominent change; hence, these concentrations of Cer-L and Cer-H were chosen for subsequent stimulation experiments.

After stimulating by Cer-L and Cer-H for 1 or 4 h, the expression of HSP60 mRNA in the pancreatic fragments was measured. The data demonstrated that in the Cer-L group, the HSP60 mRNA expression increased significantly at 4 h ($p < 0.05$) compared to those that received 1-h stimulation (Fig. 1c). In the Cer-H group, however, the expression of HSP60 mRNA increased sharply in both subgroups that received 1- or 4-h stimulation as compared to the controls ($p < 0.01$; Fig. 1c).

Expressions of HSP60 protein and mRNA in rat pancreas fragments after stimulation with LPS

The expression of HSP60 protein in pancreatic fragments showed no obvious change after 1-h stimulation at low or high concentrations of LPS (10 µg/mL LPS-L and 20 µg/mL LPS-H), but decreased significantly with prolonged stimulation (4 h) compared to the controls ($p < 0.05$; Fig. 2a, b).

The expression of HSP60 mRNA in pancreatic fragments was essentially unchanged after stimulation with LPS-L for 1 h, but decreased significantly after 4 h stimulation as compared with the controls ($p < 0.05$; Fig. 2c). For tissue snips stimulated with LPS-H, HSP60 mRNA expression decreased sharply after 1-h stimulation, followed by an even more significant decrease as the stimulation extended to 4 h ($p < 0.01$; Fig. 2c).

The interfering efficiency on HSP60 in the isolated and cultured pancreatic tissues

After RNA interference, the expressions of HSP60 mRNA and protein in the pancreatic tissues were measured to determine the interfering efficiency of the three pairs of siRNA. The results demonstrated that the interference efficiency on HSP60 mRNA was 19.8% for stealth_58, 62.1% for stealth_687, and 35.5% for stealth_1026, respectively (Table 1). As to HSP60 protein expression, stealth_687 also showed the highest interference efficiency which reached 36.4% (Table 1). So stealth_687 was chosen as the only siRNA for the subsequent interference experiments.

To exclude possible side effects of RNA interference on the pancreatic tissues, some related parameters were examined as well. The results shown in Table 2 demonstrated that the viability of the tissue fragments and the levels of amylase and TNF-α in the culture supernatant essentially remained unchanged in the group that HSP60 was interfered compared with that in the negative control ($p > 0.05$). Compared to the blank control, the results also showed that Lipofectamine TM 2000 had no apparent toxic effect on the pancreatic tissues in current experimental conditions as shown in Table 2.

Fig. 1 Effect of different concentrations of cerulein on the expression of HSP60 protein and mRNA in rat pancreas fragments (mean $\pm$ SE, $n = 4$). **a** Western blot profiling of HSP60 protein expression. **b** Relative expression value of HSP60 protein expression, presented as the grayscale of the HSP60 band versus that of the corresponding β -actin band. **c** Relative expression value of HSP60 mRNA, calculated as the individual ratio of the HSP60-amplified CT value against the respective GAPDH-amplified CT value. The final concentration of cerulein was 10^{-11} mol/L (Cer-L) or 10^{-5} mol/L (Cer-H). Data were presented as percentage of normal control, with * $p < 0.05$ and ** $p < 0.01$ when compared to the control group

Expressions of HSP60 protein and mRNA in rat pancreas fragments treated with cerulein and LPS after HSP60 RNA interference

After interfering with siRNA stealth_687, the pancreatic tissues were treated with LPS or cerulein. As shown in Fig. 3a, the expression of HSP60 mRNA in group LPS-L and LPS-H decreased significantly after 1-h stimulation and reduced to even lower levels with the 4-h stimulation compared with the negative control ($p < 0.01$; Fig. 3a). HSP60 mRNA expression also decreased after 1-h stimulation with Cer-L ($p < 0.05$; Fig. 3a), but showed a tendency to increase after 4 h of stimulation, albeit without statistical significance. In the group Cer-H, the expression of HSP60

mRNA increased slightly after 1 h and significantly after 4 h of stimulation ($p < 0.05$; Fig. 3a).

The expression of HSP60 protein in the pancreatic tissues exposed to siRNA interference was also evaluated after stimulating with LPS or cerulein. The results revealed that HSP60 protein expression reduced sharply after 1-h stimulation and further decreased after 4-h of stimulation compared with the negative control ($p < 0.05$; Fig. 3b, c).

The data demonstrated that after the stimulation of LPS or cerulein, HSP60 expression in the pancreatic tissues changed in a similar pattern with or without siRNA interference, but more drastic changes were observed in those treated with siRNA (Figs. 1, 2, and 3).

Fig. 2 Effect of different concentrations of LPS on the expression of HSP60 protein and mRNA in rat pancreas fragments (mean $\pm$ SE, $n = 4$). **a** Western blot profiling of HSP60 protein expression. **b** Relative expression value of HSP60 protein, presented as the grayscale of the HSP60 band versus that of the corresponding β -actin band. **c** Relative expression value of HSP60 mRNA, calculated as the individual ratio of the HSP60-amplified CT value versus the respective GAPDH-amplified CT value. LPS concentration was 10 μ g/mL (LPS-L) or 20 μ g/mL (LPS-H). Data were presented as percentage of normal control, with * $p < 0.05$ and ** $p < 0.01$ when compared to the control

Effects of HSP60 siRNA on the viability of rat pancreas tissue fragments treated with LPS

In the pancreas fragments in which no HSP60 siRNA was applied, treatment with LPS-L or LPS-H led to a decline in the tissues' viability 1 h after stimulation, and an even more significant decrease was observed after 4-h stimulation ($p < 0.05$; Fig. 4a, b). In the tissues treated with HSP60 siRNA LPS-L or LPS-H, a much lower viability change was observed both after 1 h ($p < 0.05$) or 4 h ($p < 0.01$) of stimulation, and the difference was significant when compared with that in the control group ($p < 0.05$; Fig. 4a, b).

Effects of HSP60 siRNA on the viability of rat pancreas fragments treated with cerulein

As shown in Fig. 5, the viability of the tissue fragments in the negative control group showed no obvious change 1 h after stimulation with Cer-L or Cer-H, then decreased markedly after 4 h of stimulation compared with those in the blank control group ($p < 0.05$; Fig. 5a, b). Conversely, the viability of the tissue fragments in the RNA interference group reduced significantly both 1 or 4 h after stimulation with Cer-L or Cer-H ($p < 0.05$; Fig. 5a, b).

Effects of HSP60 siRNA on the TAP level of rat pancreas fragments treated with LPS and cerulein

Before and after interference in the expression of HSP60, the TAP level in the pancreatic fragments treated with LPS (20 μ g/mL) or cerulein (10^{-5} mol/L) was measured and the results presented in Fig. 6a, b. After 1 h of stimulation, TAP level in the pancreatic tissues increased, especially in the group Cer-H ($p < 0.05$; Fig. 6b). With prolonged stimulation, these two toxicants induced a much higher level of TAP after 4 h of stimulation compared to those in the control groups ($p < 0.05$). In the pancreatic fragments exposed to stealth_687, TAP level in LPS or Cer groups

Table 1 Expressions of HSP60 mRNA and protein in rat pancreas fragments treated with three stealth siRNA (mean $\pm$ SE, $n = 4$)

	HSP60mRNA expression	Interfering efficiency of HSP60 mRNA (%)	HSP60 protein expression	Interfering efficiency of HSP60 protein (%)
Negative control	4.130 $\pm$ 2.127	0	1.759 $\pm$ 0.378	0
Stealth_58	3.981 $\pm$ 2.738	19.809	1.413 $\pm$ 0.295	14.395
Stealth_687	1.892 $\pm$ 1.497*	62.099	1.119 $\pm$ 0.100*	36.390
Stealth_1026	3.086 $\pm$ 2.452	35.502	1.267 $\pm$ 0.258	27.947

Relative expression value of HSP mRNA in rat pancreas fragments was presented as the copy numbers of HSP60 mRNA versus those of the corresponding GAPDH. The interference efficiency of HSP60 mRNA = $(1 - \text{stealth siRNA group/negative control group}) \times 100\%$. Relative expression value of HSP60 protein was presented as the grayscale of the HSP60 band versus that of the corresponding β -actin band, and the interference efficiency of HSP60 protein = $(1 - \text{stealth siRNA group/negative control group}) \times 100\%$

* $P < 0.05$ (when compared with the negative control)

Table 2 Changes of the injury parameters after the interference in expression of HSP60 by stealth_687 siRNA in the pancreatic tissues snips (mean $\pm$ SE, $n = 6$)

	Viability of pancreatic tissues (OD/mg protein)	Amylase (U/mg protein)	TNF- α (pg/mg protein)
Stealth_687	1.755 $\pm$ 0.056	1,649.742 $\pm$ 107.920	164.452 $\pm$ 8.847
Negative control	1.820 $\pm$ 0.098	1,635.026 $\pm$ 91.639	162.997 $\pm$ 7.552
Blank control	1.783 $\pm$ 0.048	1,184.810 $\pm$ 212.217	138.521 $\pm$ 16.618

Stealth_687: rat pancreatic tissues treated with Lipofectamine™ 2000 mixture containing Stealth_687 siRNA. Negative control: rat pancreatic tissues treated with equivalent Lipofectamine™2000 mixture without siRNA (mock interference). Blank control: rat pancreatic tissues treated with equivalent DMEM medium only. All the experimental procedures were described in “Materials and methods”

Fig. 3 Effects of LPS and cerulein on HSP60 mRNA and protein expression in rat pancreas fragments after interfering HSP60 expression with siRNA (mean $\pm$ SE, $n = 4$). **a** Relative expression value of HSP60 mRNA, calculated as the individual ratio of the HSP60-amplified CT value against the respective GAPDH-amplified CT value. **b** Western blot profiling of HSP60 protein expression. **c** Relative expression value of HSP60 protein expression, presented as the grayscale of the HSP60 band versus that of the corresponding β -actin band. Data were expressed as percentage of negative control (N.C.), as described in “Materials and methods,” with $*p < 0.05$ and $**p < 0.01$ when compared to N.C.

increased markedly after 1 h of stimulation, and the effect was even more significant after 4 h of stimulation ($p < 0.01$; Fig. 6a, b).

Effects of HSP60 siRNA on the level of cytokines in the culture supernatant of rat pancreas fragments treated with LPS and cerulein

The levels of TNF- α and IL-6 in the culture supernatant of the pancreatic fragments with or without HSP60 RNA interfer-

Fig. 4 Effects of HSP60 RNAi on the viability of pancreatic fragments stimulated with LPS (mean $\pm$ SE, $n = 6$). **a** LPS-L group (10 μ g/mL). **b** LPS-H group (20 μ g/mL). Data are expressed as percentage of the control without stimulants. $*p < 0.05$ and $**p < 0.01$ when compared with the control group; $\#p < 0.05$ when compared with samples treated with LPS only

Fig. 5 Effects of HSP60 RNAi on the viability of pancreatic fragments stimulated with cerulein (mean $\pm$ SE, $n = 6$). **a** Cer-L group (10^{-11} mol/L). **b** Cer-H group (10^{-5} mol/L). Data are expressed as percentage of the control. * $p < 0.05$ and ** $p < 0.01$ when compared with the control; # $p < 0.05$ when compared with those treated with cerulein only

ence were measured after stimulation with LPS-H (20 μ g/mL) or Cer-H (10^{-5} mol/L). Without RNA interference, the toxicants induced a similar tendency for the cytokines: a slightly higher level after 1 h and a significant increase after 4 h of stimulation when compared with the controls ($p < 0.05$; Fig. 7). For the pancreatic tissues with RNA interference, the levels of TNF- α and IL-6 in the supernatant were elevated higher 1 h after stimulation of LPS-H or Cer-H, and the cytokine levels were even higher after 4 h of stimulation ($p < 0.01$; Fig. 7) compared to those in the control group.

Discussion

In recent years, studies confirmed the expression of HSPs, especially HSP60, in pancreatic tissues and the close correlation of HSP60 and pancreatic enzymes along the secretory pathway in the acinar cells (Chu and Rana 2008; Lee Sik et al. 2000; Li et al. 2009a; Mofidi et al. 2006). The functions of HSP60 may include the regulation for folding, assembly, and degradation of many proteins, including

enzymes. Investigators further found that AP was frequently accompanied by alterations of HSP60 and HSP25 (Schafer and Williams 2000; Schafer et al. 2005; Li et al. 2009b). Another important discovery was accomplished by Lee Sik et al. (2000) who found that pre-immersion of rats with cold water could induce a high expression of HSP60 in rat pancreatic tissues and inhibited cerulein-induced pancreatitis in rats. On the other hand, low HSP60 expression may result in a weakened role of the molecular chaperone and an imbalance between pancreatic enzymes and HSPs, prompting the occurrence and progression of AP. To further confirm HSP60 protective activity on pancreatic tissues, we studied the liaison of HSP alteration and the corresponding changes in isolated rat pancreatic tissues in response to injury-causing toxicants.

Jaffrey and Norman firstly pioneered using pancreatic tissue snips in related studies (Jaffrey et al. 1999), and they found that the isolated tissue snips could remain viable and retain almost all activity for up to 48 h, and within 24 h, these tissues could still have dose-dependent amylase secretory responses to the incremental concentrations of cerulein, suggesting that the affinity of CCK-A receptors of the isolated tissues to pancreatic acinar cells had not

Fig. 6 Effects of HSP60 RNAi on the TAP level of pancreatic fragments stimulated with LPS and cerulein (mean $\pm$ SE, $n = 6$). **a** LPS-H group (20 μ g/mL). **b** Cer-H group (10^{-5} mol/L). Data are expressed as percentage of the control. * $p < 0.05$ and ** $p < 0.01$ when compared with the control; # $p < 0.05$ when compared with those treated with LPS or cerulein only

Fig. 7 Effects of HSP60 RNAi on TNF- α and IL-6 level in the culture supernatant of pancreatic fragments stimulated with LPS or cerulein (mean $\pm$ SE, $n = 5$). **a, b** Effect of LPS-H (20 μ g/mL) on the levels of cytokines. **c, d** Effect of cerulean-H (10⁻⁵ mol/L) on the

levels of cytokines. Data are expressed as percentage of the control. * $p < 0.05$ and ** $p < 0.01$ when compared with the control; # $p < 0.05$ when compared with samples treated with LPS or cerulein only

changed much during this culturing course. The secretory response of the freshly cultured pancreatic tissue to the stimulation of high-dose cerulein was compatible to that of the freshly cultured acinar cells and similar to the *in vivo* reactions of the pancreas after stimulation by super-physiologic doses of cerulein including the co-localization of lysosomal hydrolase, the intracellular activation of trypsinogen, and the subsequent acinar cell injury (Bhagat et al. 2000, 2008; Jaworek et al. 2008). We have recently found that throughout the 48 h of *in vitro* culturing, the fragments of isolated rat pancreas retain the viability and the function of secreting amylase (Li et al. 2010), confirming the feasibility of using the pancreatic tissue snips in *in vitro* experiments.

In this study, we also examined if the cultured pancreatic tissue snips were suitable for RNA interference experiments. As shown in Table 2, the levels of amylase and TNF- α in the culture supernatant remained essentially unchanged after siRNA was transfected. Moreover, the transfection agent Lipofectamine TM 2000 caused no apparent toxic effect on the pancreatic tissues under the indicated experimental conditions, justifying the use of pancreatic tissue snips in the RNA-interfering experiments.

The outcomes of this research demonstrated that the stimulation of normal pancreatic tissues by either LPS or cerulein had caused pancreatic tissue injury with manifestations of decreased viability and increased trypsin activity, which was represented by TAP, a small peptide cleaved during the activation of trypsinogen and usually used as an index of trypsin activity in basic or clinical research (Foitzik et al. 1994; Schmidt et al. 1992), and by elevated levels of the pro-inflammatory cytokines like TNF- α and IL-6 in the tissue or the culture supernatants. In addition, the hierarchy of severity was dependent upon the duration of irritation and concentration of toxicants. Meanwhile, the changes of the injury parameters in the pancreatic tissues were accompanied by the changes of HSP60 expression: the lower concentrations of toxicants generated less irritation, producing a raised HSP60 protein expression in a short time period. As time goes on, 4 h after stimulation by either LPS or cerulein, the expression of HSP60 protein declined. The results also suggested that some post-transcriptional modification might be involved, such as deficient protein synthesis or increased protein degradation for HSP60, because both low and high concentrations of cerulein induced an increased expression

of HSP60 mRNA, but decreased expression of HSP60 protein. Some reports presented evidence of distinction between the expression of HSPs mRNA levels and of protein levels in the AP pancreatic tissues, with an elevated mRNA but decreased protein expression level (Li et al. 2009a, b; Strowski et al. 1997).

As a molecular chaperone, HSP60 is a cyto-protecting factor and is able to increase its expression to antagonize cell-injuring factors. Some investigators have suggested that the failure of cells to induce HSP proteins, when potent irritation occurs, might contribute to the progression of pancreatitis since the reduced HSP60 only occurred when the toxicant doses sufficient for inducing pancreatitis were used (Bonior et al. 2005, 2007; Strowski et al. 1997; Yu et al. 2009). Although these experiments support the notion that a high expression of pancreatic HSP60 has a protective function on pancreatic tissues, solid and more specific evidences are needed to pinpoint this action by increasing or blocking the expression of HSP60. Therefore, in this study, we used the chemically synthesized siRNA in the isolated rat pancreatic tissue to reduce the expression of HSP60, and we found that the HSP60 mRNA and protein expression could be partially blocked. After stimulating the pancreatic tissue fragments with cerulein or LPS, HSP60 expression had significantly reduced in the RNA-interfering group, and the injuries to the tissue were clear; meanwhile, trypsin activity in the pancreatic tissues and the pro-inflammatory cytokines IL-6 and TNF- α levels in the culture supernatant were significantly higher in the interfering group. Interestingly, the same toxicant caused more severe damage in the HSP60 interference group than that in the non-interference group at all time points, implying that HSP60 decrease indeed aggravated the injuries induced by the toxicants on pancreatic tissues, possibly due to enhanced tissue sensitivity and responsiveness to the damaging factors. In rodent models of severe acute pancreatitis, as demonstrated in our previous study (Li and Bendayan 2005), the decreased HSP60 expression in pancreatic tissue was invariably accompanied by high levels of corresponding trypsin and pancreatic lipase, as well as grave tissue lesions. After the experimental pancreatitis was induced, the expression of HSP60 was increased initially and then decreased rapidly, while severe tissue damages occurred (Li et al. 2009a). Thus, it is reasonable to propose that the imbalance between trypsin and its molecular chaperones may account for one of the mechanisms that facilitate the occurrence and progression of AP. These results are in agreement with the findings of the present study that when HSP60 expression is blocked, trypsinogen is more likely to be activated and injuries in pancreatic tissues are more severe after stimulating by toxicants such as cerulein or LPS, further confirming that HSP60 possesses a protective function on pancreatic tissues.

Based on our results in this study, we conclude that HSP60 siRNA can reduce the expression of HSP60 in isolated pancreatic tissues, and the lower HSP60 expression accelerates and increases the injuries of pancreatic tissues caused by toxicants LPS and cerulein and, moreover, that this effect is dependent upon time duration of irritation and concentration of toxicants, confirming the protective function of HSP60 on pancreatic tissues. Thus, the outcomes of this study provide experimental evidence for the hypothesis that the malfunction of HSP60 plays an important role in the pathogenesis of acute pancreatitis.

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