7. ANNEX

I.

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Water immersion pretreatment decreases pro-inflammatory cytokine production in cholecystokinin-octapeptide-induced acute pancreatitis in rats: possible role of HSP72

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Heat shock proteins (HSPs) are cytoprotective proteins that are expressed constitutively and/or at elevated levels upon the exposure of cells to stress. The aim of this study was to investigate the potential effects of HSP preinduction by cold- (CWI) or hot-water immersion (HWI) on pro-inflammatory cytokine production (IL-1, IL-6, TNF-α) in cholecystokinin-octapeptide(CCK)-induced acute pancreatitis. Rats were injected with 3 × 75 μg/kg CCK subcutaneously at intervals of 2 h at the peak level of HSP synthesis, as determined by Western blot analysis. The animals were killed by exsanguination through the abdominal aorta 2 h after the last CCK injection. The serum IL-1, IL-6, TNF-α, and amylase levels, the pancreatic weight/body weight ratio, and the pancreatic contents of DNA, protein, amylase, lipase and trypsinogen were measured; biopsy for histology was taken. HWI significantly elevated the HSP72 expression, while CWI significantly increased the HSP60 expression. HWI pretreatment decreased all of the measured serum cytokine levels in this acute pancreatitis model. CWI and HWI pretreatment ameliorated most of the examined laboratory and morphological parameters of CCK-induced pancreatitis. The findings suggest the possible roles of HSP60 and HSP72 in the protection against CCK-induced pancreatitis. HSP72 might also participate in the reduction of pro-inflammatory cytokine synthesis.

Key words: Heat shock proteins, cytokines, water immersion, cholecystokinin-octapeptide, pancreatitis.

1. Introduction

Supramaximal doses of cholecystokinin-octapeptide (CCK) (or its synthetic analogue cerulein) are known to induce a mild form of acute interstitial pancreatitis in rats, characterized by hyperamylasemia, pancreatic oedema, intrapancreatic inflammation and acinar cell injury[1].

The heat shock proteins (HSPs) are a group of highly conserved, ubiquitous and functionally related proteins that play an essential part in cell survival[2,3]. They are involved in the synthesis, folding, transport and translocation of proteins, and the assembly and disassembly of oligomers. HSPs are divided into different families, according to their molecular size (e.g. HSP60 and HSP72)[2,3]. The HSP families have several functional homologues in the different compartments of cells. They are expressed constitutively and/or at elevated levels upon the exposure of cells to a variety of stress conditions in every organ, including the pancreas[4]. The HSPs are

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well known to protect cells against stress[5–10].

Cells subjected to hyper- or hypothermia respond by synthesizing HSPs. Induction of the heat shock response enhances the ability of cells to overcome the effects of further stress[5]. It has been demonstrated that the preinduction of HSP expression has a protective effect against cerulein-induced pancreatitis[7–10]. However, the conclusions drawn from these experiments are somewhat controversial. Otaka *et al.*[8,9] found that the protective effect against this acute pancreatitis model was due to the specific pre-induction of HSP60 (by cold-water immersion (CWI), while Wagner *et al.*[10] attributed it to HSP72 (induced by hyperthermia). Nevertheless, the mechanism of how HSPs protect against CCK-induced pancreatitis remains to be answered.

The aim of the present study was to investigate the potential effects of HSP pre-induction by CWI and hot-water immersion (HWI) on serum pro-inflammatory cytokine levels during CCK-induced acute pancreatitis in rats.

2. Materials and methods

2.1. *Experimental protocol*

2.1.1. *Animals.* Male Wistar rats weighing 250–300 g were used. The animals were kept at a constant room temperature of 25°C with a 12-h light-dark cycle, and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). The rats were fasted for 12 h before the beginning of the experiments. In every group, the rats were anaesthetized with pentobarbital (PB) (44 mg/kg, i.p.) at the starting point of the experiment (t_0). The experiments performed in this study were approved by the Animal Care Committee of the University. The animal experiments were carried out complying with regulations required by Hungary's law.

2.1.2. *CWI and HWI stress.* WI stress was performed according to Otaka *et al.*[9] with some modifications. In group C [CWI] ($n = 24$), the rats were immersed vertically in a 23°C water bath to the depth of the xiphoid process for 6 h. When the animals woke up from the anaesthesia, they were immediately reanaesthetized with 22 mg/kg PB i.p. In group H [HWI] ($n = 24$), the rats were immersed vertically in a 37°C water bath, and the water temperature was then gradually increased to 42°C (during 55 min) and maintained there for 20 min (total 1 h 15 min) (figures 1(*a*) and (*b*)). The rectal temperature of 4–4 animals was monitored during the CWI or HWI every 10 min by a digital thermometer (Omker, Budapest, Hungary) to demonstrate the actual time-body temperature history of the rats.

In order to evaluate the expressions of HSP60, and HSP72 after the CWI or HWI stress, four rats were killed at each time point before (t_0), immediately after (0), or 3, 6, 9 or 12 h after the end of the immersion (figures 1(*a*) and (*b*)). The pancreas was quickly removed, cleaned from fat and lymph nodes, and frozen at −80°C until processing.

2.1.3. *CCK-induced pancreatitis.* Acute pancreatitis was induced near the peak of the HSP synthesis by injecting 75 μg/kg body weight CCK subcutaneously three times at intervals of 2 h. In group CC [CWI + CCK] ($n = 6$), the rats received the CCK immediately after the CWI. In group ∅CC [No CWI + CCK] ($n = 6$), the animals were kept at room temperature and were injected with CCK at $t_0 + 6$, $t_0 + 8$ and $t_0 + 10$ h (figure 1(*c*)). In group HC [HWI + CCK] ($n = 6$), the rats re-

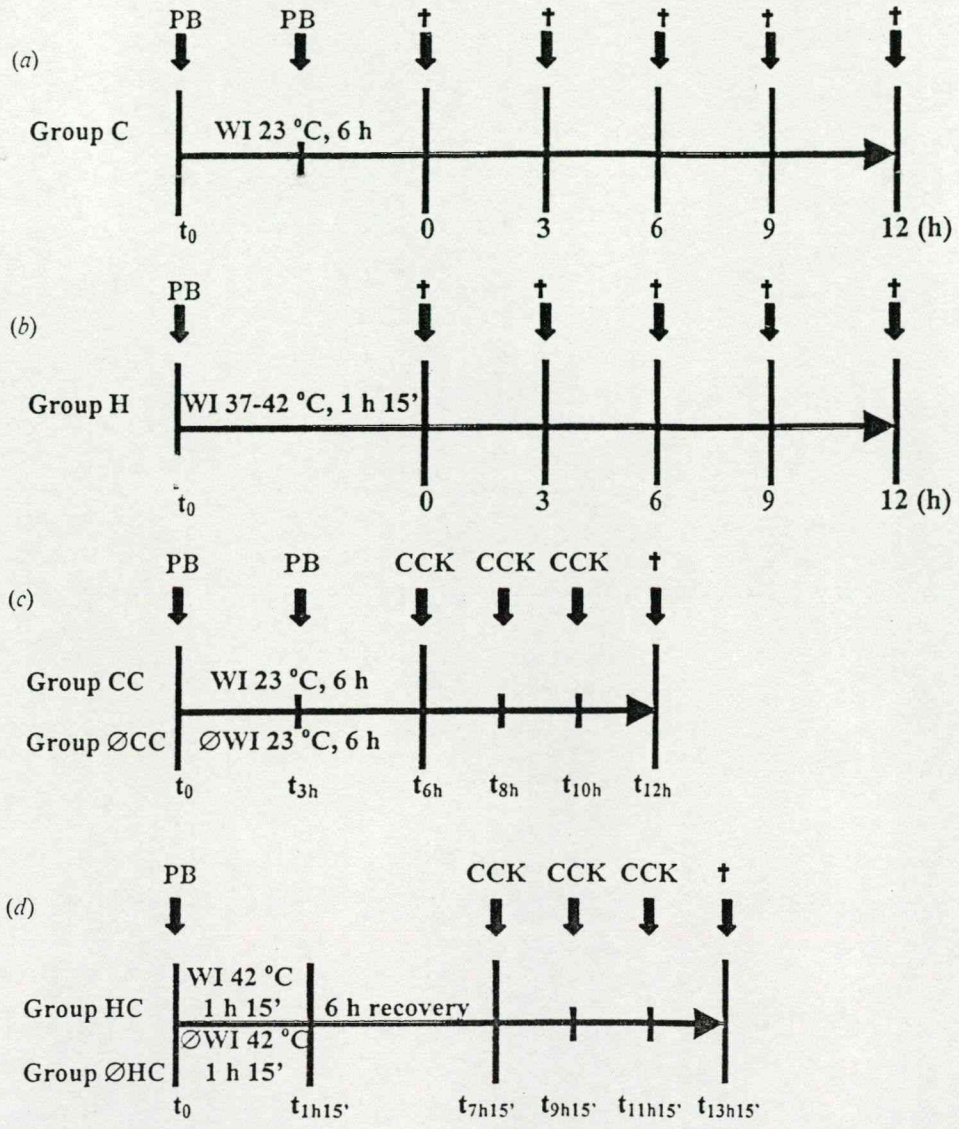


Figure 1. Experimental protocol. In every group, the rats were anaesthetized with 44 mg/kg body weight pentobarbital (PB) intraperitoneally (i.p.) at the starting point of the experiment (t_0). (*a*) Group C: the rats were immersed vertically in a 23°C cold water bath for 6 h. When the animals woke up from the anaesthesia, they were immediately reanaesthetized with 22 mg/kg PB i.p. (*b*) Group H: the rats were immersed vertically in a 37°C water bath, and the water temperature was then gradually increased to 42°C and maintained there for 20 min (total 1 h 15 min). In groups C and H, the rats were sacrificed (†) before (t_0), immediately after (0), or 3, 6, 9 or 12 h after the end of the immersion. (*c*) Group CC: the rats received cholecystokinin-octapeptide (CCK) (75 μg/kg body weight subcutaneously three times at intervals of 2 h) immediately after the cold-water immersion (CWI). Group ØCC: the animals were kept at room temperature and were injected with CCK at $t_0 + 6$, $t_0 + 8$ and $t_0 + 10$ h. (*d*) Group HC: the rats received CCK as mentioned above, following a 6-h recovery period after the hot-water immersion (HWI). Group ØHC: the rodents were given CCK, starting at $t_0 + 7$ h 15 min. In groups CC, ØCC, HC and ØHC, the animals were killed 2 h after the last CCK injection.

ceived CCK as mentioned above, following a 6-h recovery period after the HWI. In group ∅HA [No HWI + CCK] (n = 6), the rodents were given CCK starting at t_0 + 7 h 15 min (figure 1(*d*)). The animals were killed by exsanguination through the abdominal aorta 2 h after the last CCK injection. The pancreas was quickly removed, cleaned from fat and lymph nodes, weighed, and frozen at −80°C until use.

2.2. *Production of HSP60 antibody*

An antibody to HSP60 was produced in rabbits by an intramuscular injection of 1 mg of protein emulsified in Freund's complete adjuvant. Booster shots were given three times in Freund's incomplete adjuvant in a similar manner at 2-week intervals. The rabbit was bled 1 week after the last injection. The antibody was purified by affinity chromatography on a protein A-Sepharose column.

2.3. *Western blotting*

The pancreas was homogenized in a buffer containing 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA and 0.5 mM PMSF, using an Ultra-Turrax homogenizer for 2 min. The homogenates were centrifuged at 20 000 g for 30 min. The supernatants were collected and the protein concentrations were measured by the method of Bradford[11]. Twenty micrograms of protein were loaded per lane. Samples were electrophoresed on an 8% sodium dodecyl sulphate-polyacrylamide gel according to the method of Laemmli[12]. The gels were either stained with Coomassie Brilliant blue (to demonstrate equal loading of proteins for Western blot analysis) or transferred to nitrocellulose membrane for 2.5 h at 30 V. Membranes were blocked in 5% non-fat dry milk for 1 h, and incubated with rabbit anti-HSP60 (1:60 000 dilution) or anti-HSP72 (1:5000 dilution) [a generous gift from István Kurucz, Biorex Laboratories, Veszprém, Hungary, which has been characterized previously[13]] antibody for an additional 1 h at room temperature. The immunoreactive protein was visualized by enhanced chemiluminescence, using a horseradish peroxidase-coupled anti-rabbit immunoglobulin at 1:15 000 dilution (Dako, Denmark). The densities of the bands were quantitated by using an A.A.B. Image Analysis Program (Advanced American Biotechnology, Fullerton, CA). The relative density was calculated as: density at each time point/density before WI stress (control, t_0).

2.4. *Assays*

2.4.1. *Serum amylase activity, and pancreatic contents of amylase, trypsinogen, lipase, DNA and protein.* All plasma samples were centrifuged at 2500 g for 30 min. The serum levels of amylase were determined by a chromogenic method with the Phadebas test[14] (Pharmacia × Upjohn, Uppsala, Sweden). Half of the pancreas was homogenized in a 9-fold excess wt/vol of ice-cold buffer containing 0.02 M Tris-HCl, pH 7.8, 0.15 M NaCl and 0.1% Triton X-100. Enzyme measurements were carried out on the supernatant fractions of the homogenates after centrifugation at 20 000 g for 30 min. Pancreatic amylase activities were determined as described above[14]. Trypsinogen was activated after a 200-fold dilution of the homogenate with 0.02 U enterokinase/μg pancreatic protein (Sigma, St. Louis, MO) in the enzyme buffer containing 80 mM Tris-HCl, pH 8.0, 25 mM $CaCl_2$ and 100 μg/ml bovine serum albumin for 120 min at 37°C[15]. Lipase activities were measured by a pH-stat method[15]. Samples for DNA determination were precipi-

tated with ice-cold 0.8 M perchloric acid, washed in 5% trichloroacetic acid, and then hydrolysed with 0.8 M perchloric acid at 90°C for 10 min[16]. DNA was estimated photometrically with diphenylamine[17]. The protein concentrations in the supernatant fractions of the homogenates were measured by the microbiuret method of Goa[18].

2.4.2. *Serum cytokine concentrations.* TNF-α levels were titrated in a bioassay on the WEHI-164 cell line[19]. IL-6 concentrations were measured via their proliferative action on the IL-6-dependent mouse hybridoma cell line B-9[20]. The activities were calibrated against recombinant TNF (Genzyme, Cambridge, UK) and recombinant IL-6 (Sigma-Aldrich, Munich, Germany). IL-1β concentrations were determined with an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.5. *Histological examination*

2.5.1. *Light microscopy.* A 2–3 mm^3 portion of the pancreas was fixed in an 8% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4 µm thickness and stained with hematoxylin and eosin. The slides were coded and read for the traditional histological markers of pancreatic tissue injury[1] by two independent observers who were blind to the experimental protocol. Semiquantitative grading of interstitial oedema, leukocyte infiltration and adherence, vacuolization, necrosis, and apoptosis of acinar cells (see figure 2), was performed on 8–10 consecutive high-power fields (×400) on a scale of 0–3 or 0–4 (described in more detail in table 1). In addition, basophilic lamellation of the cytoplasm of acinar cells was also graded (see figure 2(*e*)) since a pilot study revealed that besides the traditional markers, areas of basophilic lamellation were more extensive in the more severely damaged pancreata. The score for each graded parameter was averaged and the total pancreatic damage was calculated by adding all the averages together.

2.5.2. *Electron microscopy.* For electron microscopic observations, 1 mm^3 pieces of the pancreas were fixed in 3% phosphate-buffered glutaraldehyde. Tissue blocks were post-fixed in 1% OsO_4, and then rinsed in distilled water, dehydrated in a

Table 1. Histological grading system for the evaluation of cholecystokinin-octapeptide-induced acute pancreatitis in rats. Scoring was performed on 8–10 consecutive high-power fields (400×). The score for each graded parameter was averaged and the total pancreatic damage was calculated by adding all the averages together.

	Scores				
	0	1	2	3	4
Interstitial oedema	0	mild	moderate	severe	–
Leukocyte infiltr. (no. of cells)	0–1	2–5	6–10	>10	–
Leukocyte adh. (no. of cells)	0	1	2–3	>3	–
Vacuolization (% of total acinar cells)	0	1–33	34–66	67–100	–
Necrosis (% of total acinar cells)	0	1–25	26–50	51–75	76–100
Apoptosis (no. of apoptotic bodies)	0–1	2–5	6–10	>10	–
Basoph. lam. (% of total acinar cells)	0	1–33	34–66	67–100	–

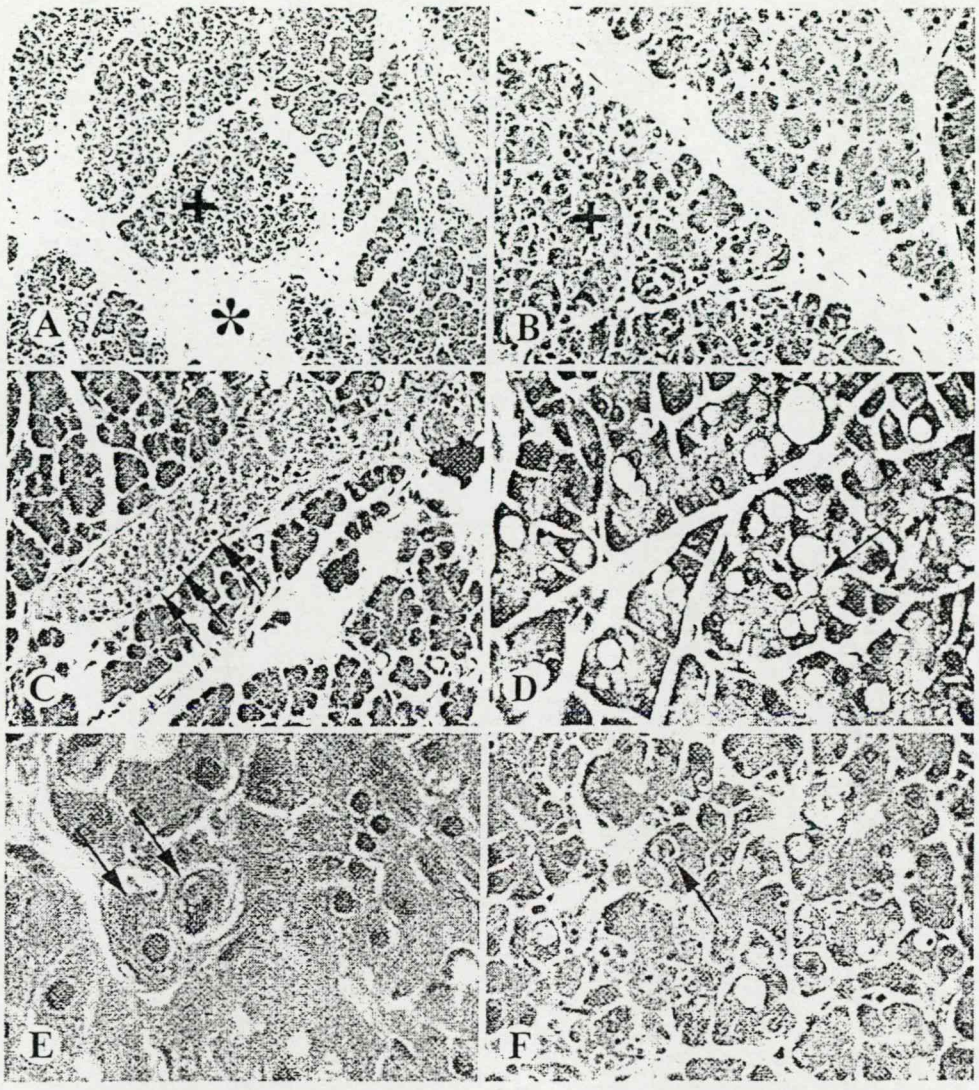


Figure 2. Morphological features of experimental acute pancreatitis in rat. The features include (*a*, *b*) interstitial oedema (asterisk), necrosis of pancreatic acinar cells (plus), (*c*) adherence of leukocytes to the endothelium of venules (arrows), (*d*) vacuolization of acinar cells (arrow), (*e*) basophilic lamellation of the cytoplasm of acinar cells (arrow), and (*f*) apoptosis of acinar cells (arrow) (hematoxilin and eosin, original magnifications were changed during image processing).

graded series of ethanol, and embedded in TAAB Transmit Resin (TAAB, UK). Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined with a Philips electron microscope.

2.6. *Statistical analysis*

Results are expressed as means ± SEM. Experiments were evaluated by using the Student *t*-test when the data consisted of two groups, or by analysis of variance when

three or more groups were compared. Values of $p < 0.05$ were accepted as significant.

3. Results

3.1. *Body temperature of rats during CWI and HWI stress*

The body temperature of rats during CWI and HWI stress is shown in figure 3.

3.2. *Expression of pancreatic HSPs after CWI and HWI stress*

HSP72 could not be detected in the unstressed control, but its expression was significantly increased at 3 h after the HWI and remained elevated until 12 h (figure 4(*a*)). HSP60 is constitutively expressed in the pancreas, HWI did not have a significant effect on its expression (data not shown). The levels of HSP72 after CWI did not differ significantly from the control (data not shown), but the expression of HSP60 increased significantly during CWI. The maximal amount of HSP60 (as compared to the unstressed control) was noted immediately after the end

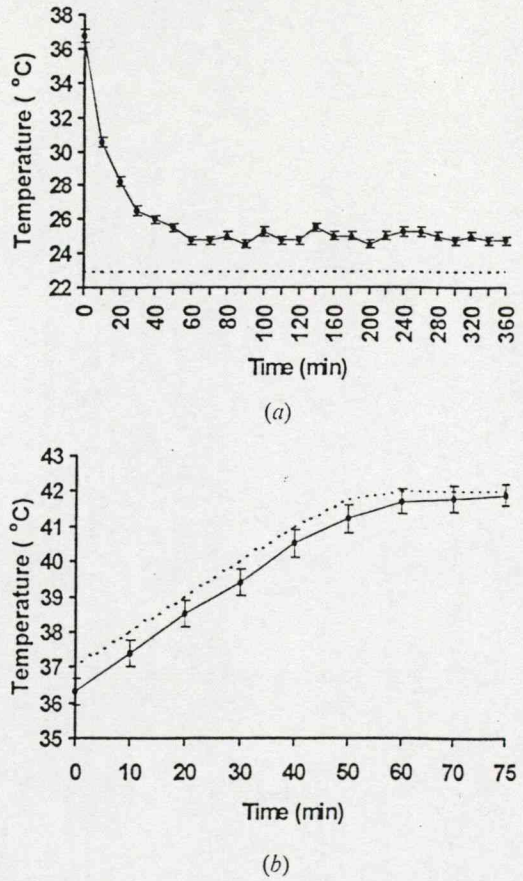


Figure 3. Body temperature of rats during (*a*) CWI and (*b*) HWI stress vs the time after the beginning of the treatments. Values (filled circle) are means ± SEM for four animals at each time point. The dashed lines show the temperature of the water bath.

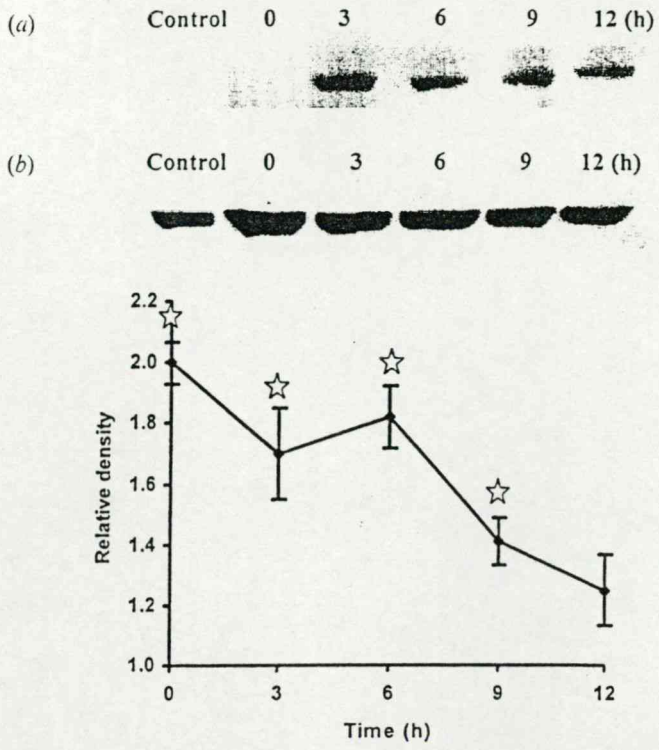


Figure 4. Effect of water immersion stress on pancreatic heat shock protein synthesis. Representative Western immunoblots of protein lysates (20 μg/lane) extracted from pancreata harvested over a time course after the water immersion treatments (0–12 h). The control did not receive any treatment. (*a*) Expression of pancreatic HSP72 after HWI. HSP72 could not be detected in the unstressed control, but its expression was significantly increased at 3 h after the HWI and remained elevated until 12 h. (*b*) Expression of pancreatic HSP60 after CWI. The diagram shows the relative density of the HSP60 bands [density at each time point/density before WI stress (control)] vs the time after the CWI treatment as analysed by densitometry. The maximal amount of HSP60 was noted immediately after the end of the immersion (0), and the levels remained significantly elevated over the next 9 h. Values are means ± SEM for four animals at each time point. * Significant difference ($p < 0.05$) vs the unstressed control group.

Table 2. Effects of cold- and hot-water immersion pretreatment on the histologic parameters in cholecystokinin-octapeptide-induced acute pancreatitis.

	Group ∅CC	Group CC	Group ∅HC	Group HC
Interstitial oedema	1.29 ± 0.15	1.24 ± 0.21	1.17 ± 0.12	0.93 ± 0.16
Leukocyte infiltr.	0.26 ± 0.02	0.29 ± 0.03	0.78 ± 0.15	0.30 ± 0.06*
Leukocyte adh.	0.49 ± 0.05	0.52 ± 0.06	0.40 ± 0.16	0.35 ± 0.05
Vacuolization	1.15 ± 0.12	0.33 ± 0.05*	1.43 ± 0.17	0.95 ± 0.11*
Necrosis (0–4)	0.31 ± 0.20	0.20 ± 0.13	0.35 ± 0.18	0.14 ± 0.12
Apoptosis	0.38 ± 0.04	0.43 ± 0.05	1.03 ± 0.13	1.26 ± 0.17
Basoph. lam.	1.51 ± 0.08	0.90 ± 0.05*	1.83 ± 0.20	1.93 ± 0.29
Total damage	5.07 ± 0.45	3.71 ± 0.53*	6.63 ± 0.82	5.85 ± 0.87

Groups were treated as indicated in figure 1. Data are means ± SEM for 6 animals.
* Significant difference ($p < 0.05$) vs the respective control group.

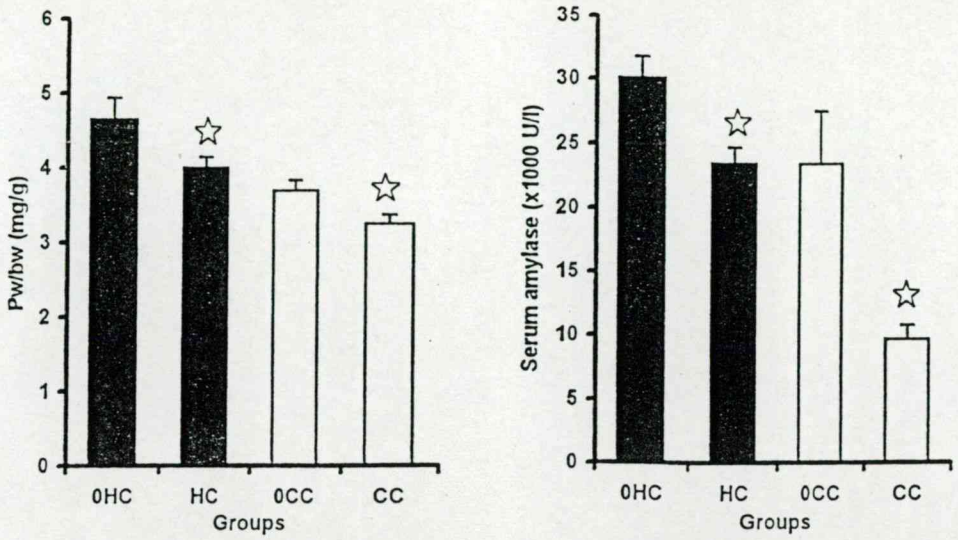


Figure 5. (*a*) Pancreatic weight/body weight ratio (pw/bw), and (*b*) serum amylase levels in groups ∅HC, HC, ∅CC and CC. Groups were treated as indicated in figure 1. Means ± SEM for six animals are shown. * Significant difference ($p < 0.05$) vs the respective control group.

of the immersion, and the levels remained significantly elevated over the next 9 h (figure 4(*b*)).

3.3. *Pancreatic weight/body weight ratio (pw/bw) and serum amylase activity*

The administration of 3 × 75 μg/kg body weight CCK induced the typical laboratory and morphological changes of acute pancreatitis. In group CC, the pw/bw (3.24 ± 0.13 mg/g) and the serum amylase activity (9690 ± 1114 U/l) were significantly decreased vs group ∅CC (3.69 ± 0.15 mg/g and 23 400 ± 4625 U/l, respectively) (figure 5). In group HC, the pw/bw (4.0 ± 0.15 mg/g) and the serum amylase activity (23 330 ± 1412 U/l) were significantly decreased vs group ∅HC (4.65 ± 0.29 mg/g and 30 063 ± 1676 U/l, respectively) (figure 5).

3.4. *Pancreatic contents of DNA, protein, amylase, trypsinogen and lipase*

In group CC, the pancreatic contents of protein (56.3 ± 7.6 mg/pancreas) and DNA (2.18 ± 0.28 mg/pancreas) were significantly decreased vs group ∅CC (84.0 ± 5.16 mg/pancreas and 3.02 ± 0.21 mg/pancreas, respectively) (figures 6(*a*) and (*b*)). The pancreatic contents of amylase, lipase and trypsinogen were significantly decreased in group CC (1008 ± 216 IU/pancreas, 169.1 ± 8.4 IU/pancreas and 3.16 ± 0.60 IU/pancreas, respectively) vs group ∅CC (3612 ± 1007 IU/pancreas, 198.0 ± 15.1 IU/pancreas and 5.52 ± 0.67 IU/pancreas, respectively) (figures 6(*c*) and (*d*)). No significant change was detected in the pancreatic DNA content in group HC (2.47 ± 0.42 mg/pancreas) vs group ∅HC (2.02 ± 0.12 mg/pancreas) (figure 6(*b*)). The pancreatic contents of protein, amylase, lipase and trypsinogen were significantly decreased in group HC (98.9 ± 4.2 mg/pancreas, 6464 ± 519 IU/pancreas, 209.6 ± 26.3 IU/pancreas and 5.09 ± 0.50 IU/

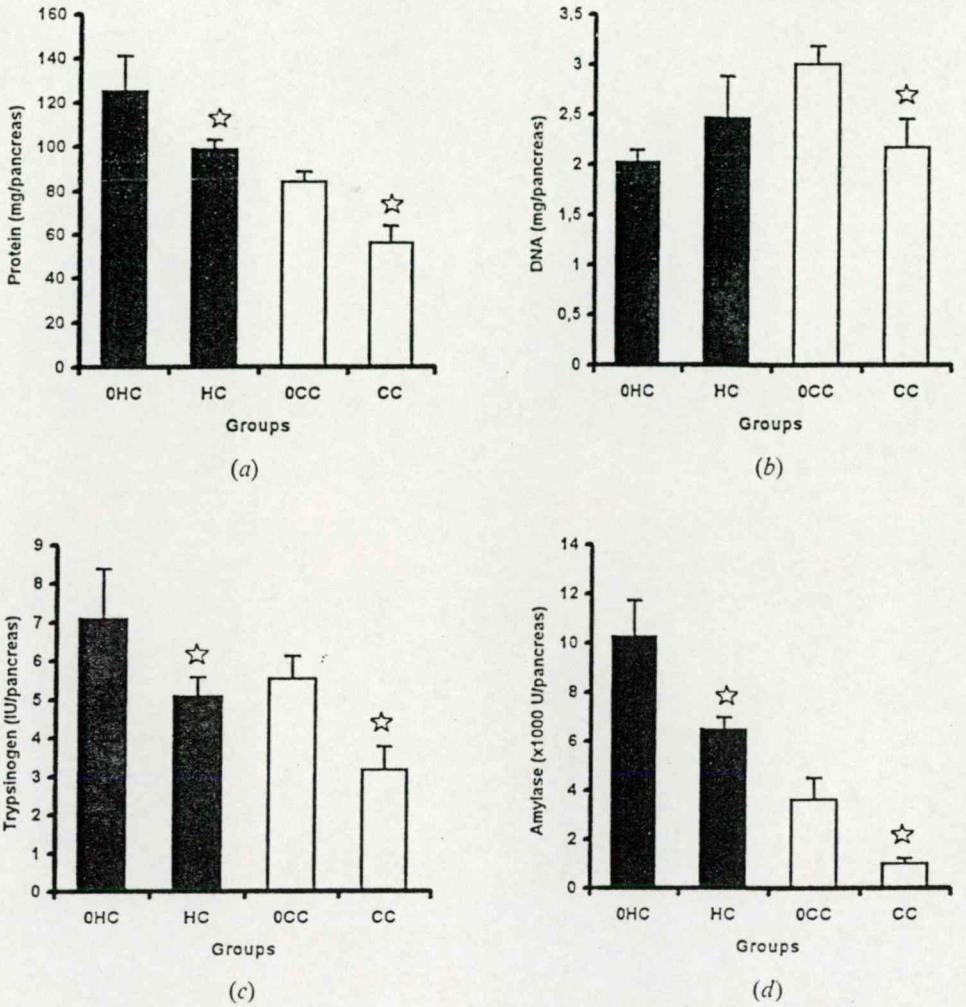


Figure 6. Pancreatic contents of (*a*) protein, (*b*) DNA, (*c*) trypsinogen, and (*d*) amylase in groups ∅HC, HC, ∅CC and CC. Groups were treated as indicated in figure 1. Data are means ± SEM for six animals. * Significant difference ($p < 0.05$) vs the respective control group.

pancreas, respectively) vs group ∅HC (124.9 ± 16.3 mg/pancreas, 10 244 ± 1470 IU/pancreas, 343.3 ± 30.4 IU/pancreas and 7.08 ± 1.30 IU/pancreas, respectively) (figures 6(*a*), (*c*) and (*d*)).

3.5. *Serum cytokine levels*

In group HC, the serum levels of TNF-α (11.3 ± 5.7 U/ml), IL-1 (6.6 ± 3.0 pg/ml) and IL-6 (18.8 ± 10.4 pg/ml) were all significantly decreased vs the corresponding values in group ∅HC (50.0 ± 3.0 U/ml, 38.9 ± 4.6 pg/ml, and 50.0 ± 1.0 pg/ml, respectively) (figure 7). In group CC, the serum TNF-α (76.0 ± 8.0 U/ml) level was significantly elevated vs group ∅CC (not detected) (figure 7(*a*)). No significant changes were observed in the serum IL-1 and IL-6 levels in group CC

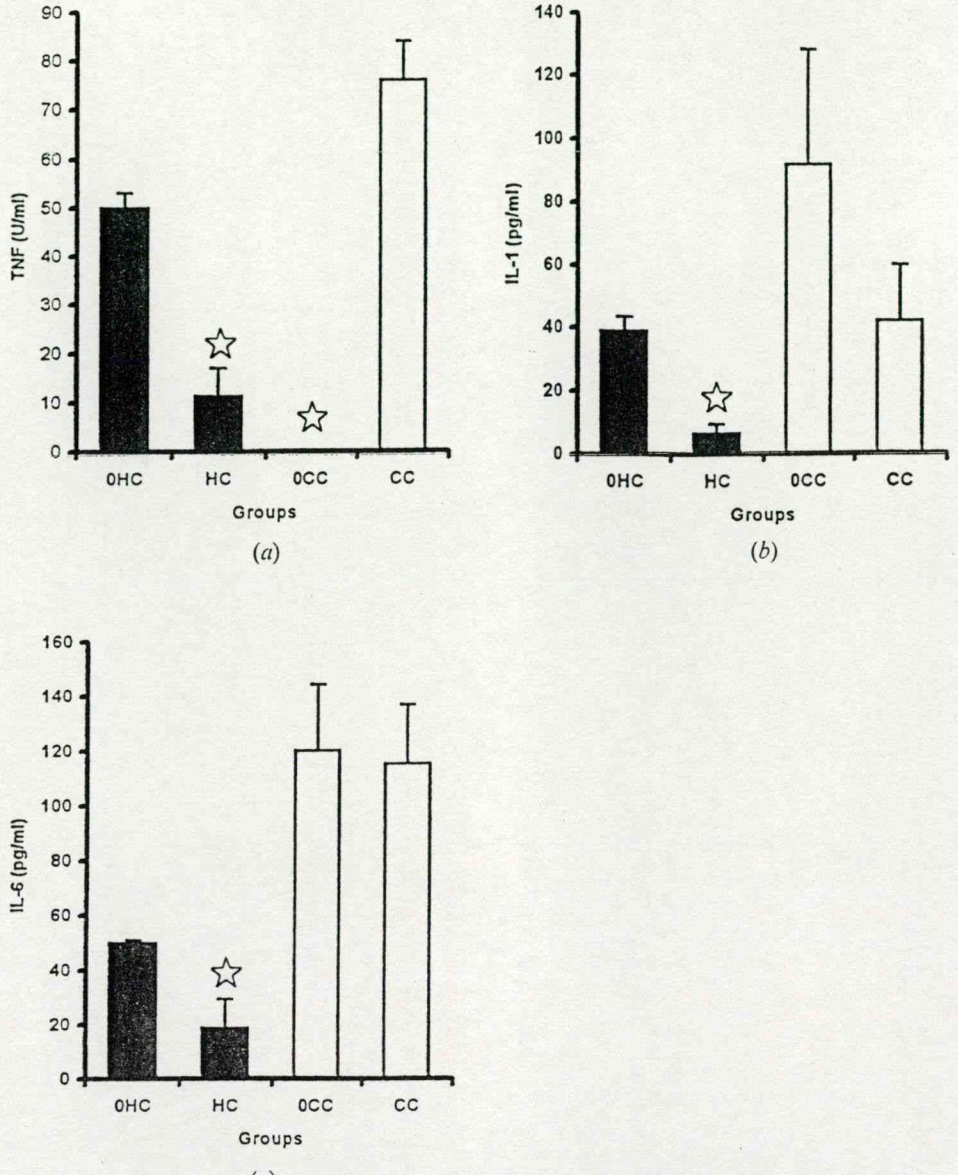
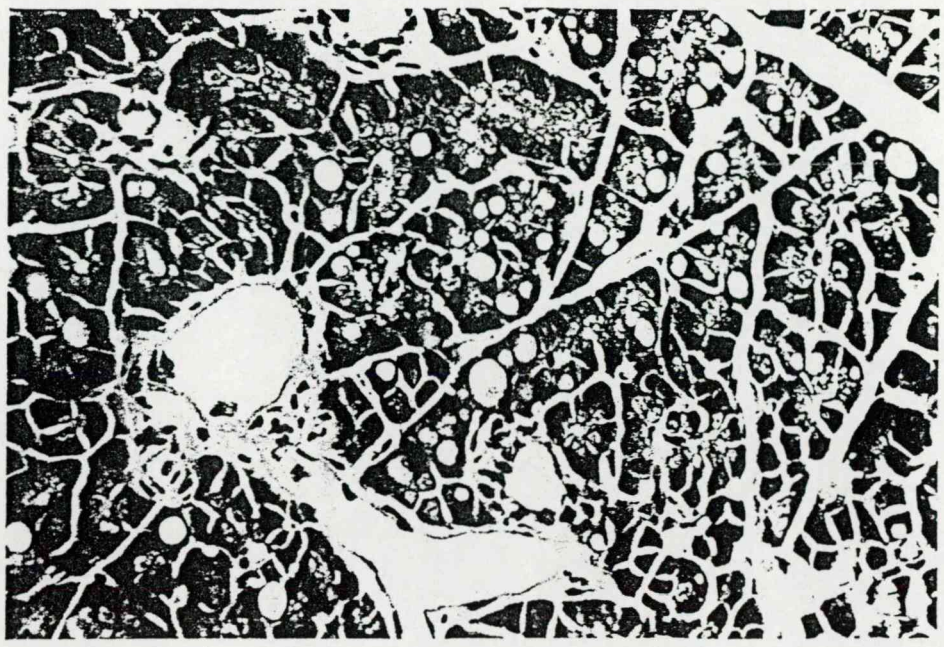


Figure 7. Serum (*a*) TNF-α, (*b*) IL-1, and (*c*) IL-6 levels in groups ∅HC, HC, ∅CC and CC. Groups were treated as indicated in figure 1. Means ± SEM for six animals are shown. * Significant difference ($p < 0.05$) vs the respective control group.

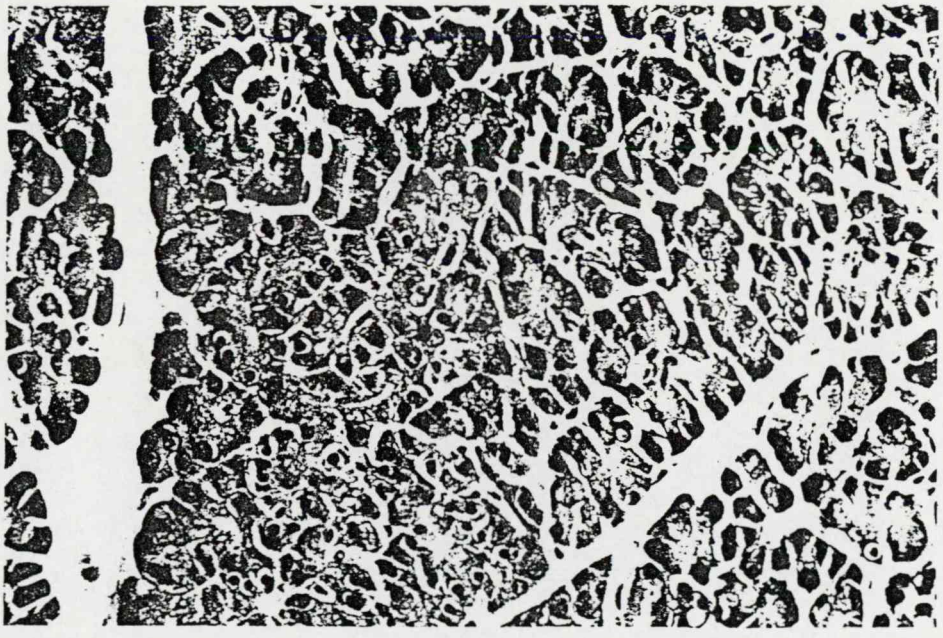
(42.0 ± 17.9 pg/ml and 115.0 ± 21.8 pg/ml, respectively) vs group ∅CC (92.0 ± 36.3 pg/ml and 120.0 ± 24.2 pg/ml, respectively) (figures 7(*b*) and (*c*)).

3.6. *Light microscopy*

In group CC the total damage (3.71 ± 0.53 points) was significantly decreased vs group ∅CC (5.07 ± 0.45 points) (figure 8). No significant alteration was observed in



(*a*)



(*b*)

Figure 8. CWI pretreatment protects against CCK-induced pancreatitis. The pancreata from rats either (*a*) not exposed (Group ∅CC) or (*b*) exposed to CWI (Group CC) 2 h after the last of three s.c. injections of 75 μg/kg body weight of CCK. Pretreatment with CWI (Group CC) greatly reduced the CCK-induced morphological alterations (hematoxylin and eosin, original magnification 400×).

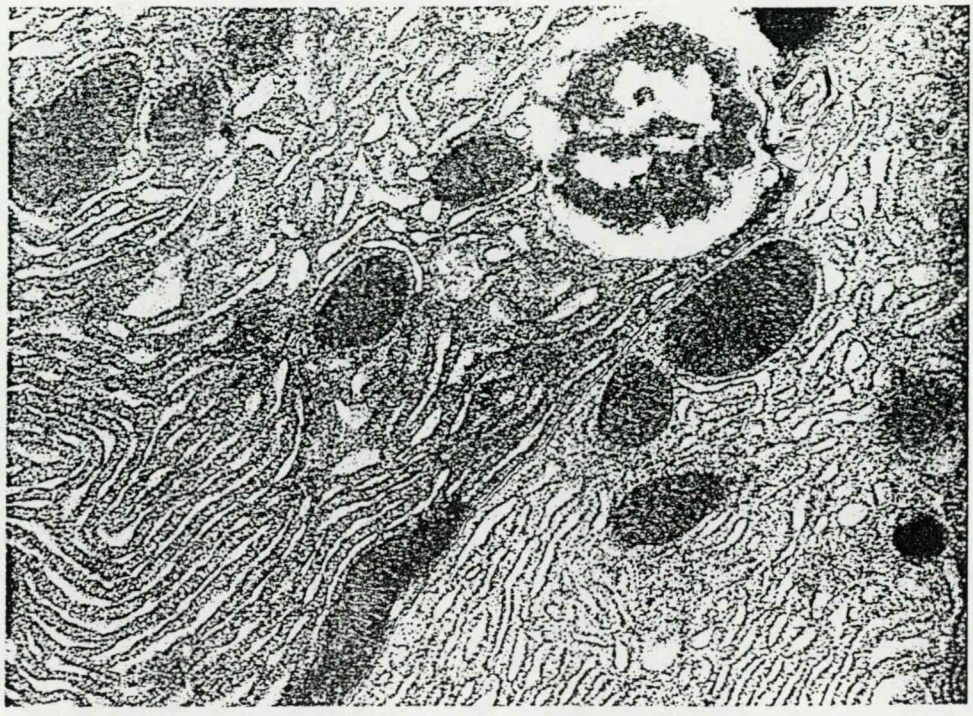


Figure 9. Transmission electron micrograph of the pancreas from a rat 2 h after the last of three s.c. injections of 75 μg/kg body weight of CCK (Group HC). Acinar cells exhibit tightly packed rough endoplasmic reticulum corresponding to the areas of basophilic lamellation of the cytoplasm of the cells (original magnification 4600×).

the total damage between groups HC (5.85 ± 0.87 points) and ∅HC (6.63 ± 0.82 points). The point values for each of the scored parameters are shown in table 1.

3.7. *Electron microscopy*

An electron microscopic study was performed to evaluate the cause of the basophilic lamellation of the cytoplasm of the acinar cells. Figure 9 shows that this was due to tightly packed rough endoplasmic reticulum.

4. Discussion

The members of the major cytoprotective HSPs are constitutively expressed or can be induced in the pancreas[4]. The induction of HSPs are well known to protect the pancreas against stress[7–10]. The study was designed to investigate the dynamics of HSP induction (HSP60 and HSP72) in response to CWI and HWI, and the potential effects of HSP preinduction on pro-inflammatory cytokine production in CCK-induced acute pancreatitis in rats.

In agreement with the findings of Otaka *et al.*[9], the results demonstrate that CWI specifically induces HSP60, while HWI increases the expression of HSP72 in rats. It was found that the levels of HSP60 remained significantly elevated for 9 h after CWI. This is in accordance with the similar findings of Lee *et al.*[7] The quantity of HSP72 was significantly increased at 3 h after HWI, and remained elevated until 12 h. Otaka *et al.*[9] used conscious rats in their experiments and subjected them to a 20-min 42°C

HWI treatment. They also showed a marked elevation of HSP72 synthesis after the HWI treatment, but found the peak of HSP72 expression at 6 h. which could be due to the differences in the experimental protocols.

Pancreatitis was induced near the peak of HSP expression by injecting high doses of CCK subcutaneously. The administration of CCK resulted in the typical laboratory (hyperamylasemia) and morphological changes (interstitial oedema, leukocyte infiltration and acinar cell injury) of acute pancreatitis 2 h after the last CCK injection in rats.

Although HSPs have been implicated as mediators of pancreatic protection, the precise mechanism of their cytoprotective effects remains unknown. The observed protective effects of HSPs are most probably due to their chaperoning activities[3]. The HSPs might also attenuate cellular damage by increasing resistance of the cell to apoptosis[21,22] or necrosis[5], preventing intracellular trypsinogen activation[7,23] and/or decreasing pro-inflammatory cytokine levels[24]. Our findings support these possibilities. It is well known that the pro-inflammatory cytokine levels increase during CCK-induced pancreatitis[25]. Decreased cytokine levels were demonstrated after HWI pretreatment in this acute pancreatitis model. Unexpectedly, CWI pretreatment paradoxically increased the serum TNF-α level in CCK-induced pancreatitis (CWI in itself does not have this effect; unpublished data). In this case, one can speculate that the increased level of this cytokine originates extrapancreatically (mainly from activated macrophages), since the severity of pancreatitis was decreased by CWI pretreatment. However, the circulating levels of TNF-α are not a reliable indicator of the disease severity, since the liver rapidly clears TNF-α before it reaches the general circulation[26]. CWI pretreatment did not influence the serum IL-1 and IL-6 levels in this model of acute pacreatitis (the serum IL-6 levels even increased after CWI without pancreatitis induction; unpublished data).

It was found that both CWI and HWI pretreatment ameliorated most of the examined laboratory parameters of CCK-induced pancreatitis. Moreover, CWI preconditioning significantly improved the morphological picture of the pancreatitis. HWI pretreatment did not influence the histological parameters of the disease.

Previously reported data concerning the protective role of different HSPs against cerulein-induced pancreatitis are conflicting. Wagner *et al.*[9] demonstrated that the expression of HSP70 induced by hyperthermia (with a heat pad and lamp, the core body temperature of the animals was elevated to 42°C and maintained there for 20 min) correlated best with the time course and degree of protection against cerulein-induced pancreatitis. In contrast with these data, Otaka *et al.*[9] found that the specific preinduction of HSP72 (by immersing the rats in a 42.5°C water bath for 20 min) had no preventive effect against cerulein-induced pancreatitis, whereas HSP60 (induced by CWI) did. The beneficial effect of CWI pretreatment and possibly HSP60 against cerulein-induced pancreatitis was also reported by Lee *et al.*[9] In fact, the studies confirm that HSP60 might indeed play a role in the protection (although not through the reduction of pro-inflammatory cytokine levels). In the experiments of Otaka *et al.*[7] and Wagner *et al.*[10], heating the animals did not or just slightly increased the expression of HSP60. Therefore, the possible protective effect of HSP60 could not be excluded by these studies, while the role of HSP72 remained questionable. A possible explanation for the discrepancy between the two types of hyperthermia pretreatment might have been the different stress models used. It was considered that the lack of protection against CCK-induced pancreatitis in the case of HWI pretreatment by Otaka *et al.*[9] was probably due to the inadequate

duration of restraint stress and or the increase of the core body temperature of the rats immersed in the hot-water bath. Therefore, the HSP72 expression did not reach a high enough level to protect the pancreas from acute pancreatitis. In the present study, the duration of the HWI pretreatment was longer than that applied by Otaka *et al.*, because it was hypothesized that the experimental set-up would result in a higher core body temperature and consequently a higher HSP72 synthesis. The results suggest that, besides HSP60, a higher level of HSP72 might also play an important part in protecting the pancreas against CCK-induced damage (at least in part by reducing pro-inflammatory cytokine levels). One must note that HWI pretreatment and possibly even the higher level of HSP72 were not sufficient to decrease the severity of the morphological picture of the disease. This might be due to the different type of pre-conditioning used in these experiments, or the inadequate amount of HSP72 to produce complete protection.

Apart from the main goal, an electron microscopic study was carried out to analyse the basophilic lamellation of the cytoplasm of the acinar cells (accumulation of a degradation product, or something else) in CCK-induced acute pancreatitis. The term 'basophilic lamellation' was actually coined by the pathologist, who observed this phenomenon in the light microscopic sections of the pancreas as a peculiar concentric, basophilic lamellation of the cytoplasm of acinar cells. Electron microscopy revealed that the phenomenon of lamellation corresponded to a tightly packed rough endoplasmic reticulum. This is most probably a compensatory mechanism of the acinar cells to produce proteins and replace damaged structures, so the extent of the change was graded. The development of the changes is beyond the study of this paper, but it's a profitable area for future work.

In conclusion, it was shown that HWI specifically induced the synthesis of HSP72, while CWI specifically elevated the level of HSP60. It was demonstrated that HWI pretreatment, and possibly HSP72, reduces the pro-inflammatory cytokine production in CCK-induced pancreatitis. The findings suggest the possible roles of both HSP72 and HSP60 in the protection against CCK-induced pancreatic damage. A decisive proof of the cytoprotective effect of these HSPs in this acute pancreatitis model would require further studies, in which one would specifically block the expression or function of these proteins.

Acknowledgements

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The Effects of Hypo- and Hyperthermic Pretreatment on Sodium Taurocholate-Induced Acute Pancreatitis in Rats

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Introduction: Heat shock proteins (HSPs) have indispensable functions in the synthesis, degradation, folding, transport, and translocation of intracellular proteins. HSPs are proteins that help cells to survive stress conditions by repairing damaged proteins.

Aim: To investigate the potential effects of HSP preinduction by cold-water (CWI) or hot-water immersion (HWI) on sodium taurocholate (TC)-induced acute pancreatitis in rats.

Methodology: TC was injected into the common biliopancreatic duct of the animals at the peak level of HSP synthesis, as determined by Western blot analysis. The rats were killed by exsanguination through the abdominal aorta 6 hours after the TC injection. The serum amylase activity, the IL-1, IL-6 and TNF-α levels, the pancreatic weight/body weight ratio, and the pancreatic contents of DNA, protein, amylase, lipase, and trypsinogen were measured, and a biopsy for histology was taken.

Results: HWI significantly elevated HSP72 expression, whereas CWI significantly increased HSP60 expression. It was demonstrated that CWI pretreatment ameliorated the pancreatic edema and the serum amylase level increase, whereas the morphologic damage was more severe in this form of acute pancreatitis. HWI pretreatment did not have any effects on the measured parameters in TC-induced pancreatitis.

Conclusions: The findings suggest a possible role of HSP60, but not HSP72, in the slight protection in the early phase of this necrohemorrhagic pancreatitis model.

Key Words: Heat shock proteins—HSP72—HSP60—Water immersion—Sodium taurocholate—Pancreatitis.

The heat shock proteins (HSPs) are a group of highly conserved proteins that are expressed constitutively and at elevated levels on the exposure of cells to a variety of stress conditions in every organ, including the pancreas (1–3). HSPs not only help cells to survive stress conditions by repairing damaged proteins, but are also involved in the synthesis, degradation, folding, transport, and translocation of proteins (1–3). HSPs are classified according to their molecular mass (e.g., HSP60 and HSP72), their intracellular location, and their functions (1,2). HSP60 is primarily a mitochondrial protein, but it also can be found in the pancreatic zymogen granules (4). The HSP70 family members include the highly stress-inducible HSP72 and the constitutively expressed HSP73 in the cytoplasm, the mitochondrial HSP75, and GRP78 (glucose-regulated protein) in the endoplasmic reticulum (5).

Cells subjected to hypothermia or hyperthermia respond by synthesizing HSPs. The induction of the heat shock response enhances the ability of the cells to overcome the effects of a further stress (6,7). It has been demonstrated that the preinduction of HSP expression has a protective effect against cerulein-induced pancreatitis in rats or against choline-deficient ethionine-supplemented diet model pancreatitis in mice (8–12). The cytoprotective effects of the HSPs on other acute pancreatitis models have not been investigated. Sodium taurocholate (TC) injected into the common biliopancreatic duct of the rat is known to induce acute necrohemorrhagic pancreatitis, characterized by hyperamylasemia, interstitial edema, intrapancreatic inflammation, hemorrhages, and acinar cell injury (13).

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The aim of the current study was to investigate the potential effects of HSP preinduction by cold-water (CWI) and hot-water immersion (HWI) on TC-induced acute pancreatitis in rats.

METHODS

Experimental protocol

Animals

Male Wistar rats weighing 300–350 g were used. The animals were kept at a constant room temperature of 25°C with a 12-hour light–dark cycle and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). The experiments performed in this study were approved by the Animal Care Committee of the University. The rats were fasted for 12 hours before the beginning of the experiments. In every group, the rats were anesthetized with pentobarbital (PB; 44 mg/kg, i.p.) at the starting point of the experiment (t_0).

Cold water immersion and hot water immersion stress

Water immersion stress was performed according to Otaka et al. (9) with some modifications. In group C (CWI; $n = 24$), the rats were immersed vertically in a 23°C water bath to the depth of the xiphoid process for 6 hours. When the animals woke up from the anesthesia, they were immediately reanesthetized with 22 mg/kg PB i.p. In group H (HWI: $n = 24$), the rats were immersed vertically in a 37°C water bath, and the water temperature was then gradually increased to 42°C (during 55 minutes) and maintained there for 20 minutes (total, 1 hour 15 min) (Fig. 1A,B).

To evaluate the expressions of HSP60 and HSP72 after the CWI or HWI stress, four rats were killed at each time point before (t_0), immediately after (0), or 3, 6, 9, or 12 hours after the end of the immersion (Fig. 1A, B). The pancreas was quickly removed, cleaned from fat and lymph nodes, and frozen at −80°C until processing.

TC-induced pancreatitis

Acute pancreatitis was induced near the peak of HSP synthesis by injecting 100 μL/100 g body weight 3% TC into the common biliopancreatic duct under steady manual pressure during a period of 30 seconds as described by Aho et al. (13). All injections were performed by the same investigator to limit technical differences. In group CT (CWI + TC; $n = 6$), the rats received the TC immediately after the CWI. In group θCT (No CWI + TC; $n = 6$), the animals were kept at room temperature and were injected with TC at $t_0 + 6$ hours (Fig. 1C). In group HT (HWI + TC; $n = 6$), the rats received TC as mentioned above, following a 6-hour recovery period after the HWI. In group θHT (No HWI + TC; $n = 6$), the rodents were given TC at $t_0 + 7$ hours 15 minutes (Fig 1D). The animals were killed by exsanguination through the abdominal aorta 6 hours after the TC injection. The pancreas was quickly removed, cleaned from fat and lymph nodes, weighed, and frozen at −80°C until use.

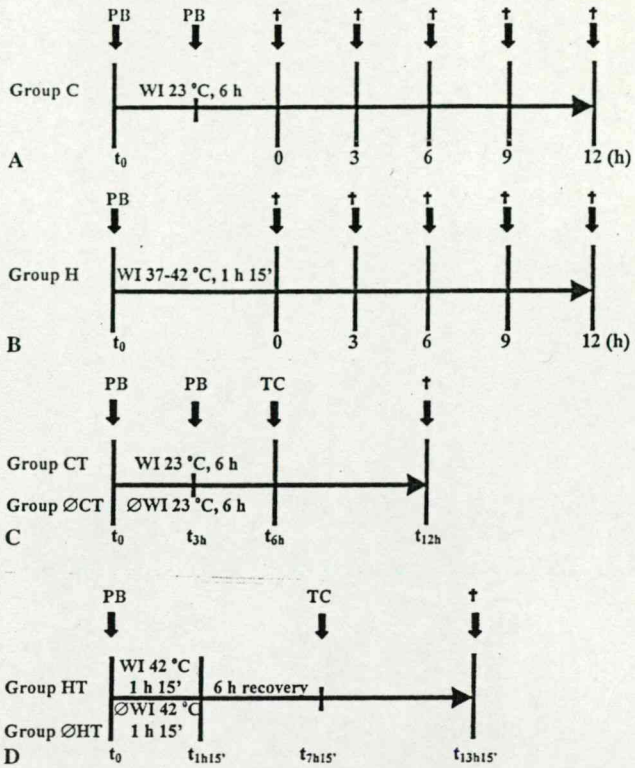

FIG. 1. Experimental protocol. In every group, the rats were anesthetized with 44 mg/kg body weight pentobarbital (PB) intraperitoneally (i.p.) at the starting point of the experiment (t_0). **(A)** Group C: the rats were immersed vertically in a 23°C cold water bath for 6 hours. When the animals woke up from the anesthesia, they were immediately reanesthetized with 22 mg/kg PB i.p. **(B)** Group H: the rats were immersed vertically in a 37°C water bath, and the water temperature was then gradually increased to 42°C and maintained there for 20 minutes (total 1 hour 15 minutes). In groups C and H, the rats were killed before (t_0), immediately after (0), or 3, 6, 9, or 12 hours after the end of the immersion. **(C)** Group CT: 100 μl/100 g body weight 3% sodium taurocholate (TC) was injected into the common biliopancreatic duct under steady manual pressure during a period of 30 seconds as described by Aho et al. (13) immediately after the cold-water immersion (CWI). Group θCT: the animals were kept at room temperature and were injected with TC at $t_0 + 6$ hours. **(D)** Group HT: the rats received TC as mentioned above, following a 6-hour recovery period after the hot-water immersion (HWI). Group θHT: the rodents were given TC at $t_0 + 7$ hours 15 minutes. In groups CT, θCT, HT and θHT, the animals were killed 6 hours after the TC injection.

Production of HSP60 antibody

Antibody to HSP60 was produced in rabbit by an intramuscular injection of 1 mg of protein emulsified in Freund's complete adjuvant. Booster shots were given three times in Freund's incomplete adjuvant in a similar

manner at 2-week intervals. The rabbit was bled 1 week after the last injection. The antibody was purified by affinity chromatography on a protein A Sepharose column.

Western blotting

The pancreas was homogenized in a buffer containing 20 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$, 420 m*M* NaCl, 0.5 m*M* DTT, 0.2 m*M* EDTA, and 0.5 m*M* PMSF, using an Ultra-Turrax homogenizer for 2 minutes. The homogenates were centrifuged at 20,000*g* for 30 minutes. The supernatants were collected, and the protein concentrations were measured by the method of Bradford (14). Twenty micrograms of protein was loaded per lane. Samples were electrophoresed on an 8% sodium dodecyl sulfate–polyacrylamide gel according to the method of Laemmli (15) and transferred to nitrocellulose membrane for 2.5 hours at 30 V. Membranes were blocked in 5% nonfat dry milk for 1 hour and incubated with rabbit anti-HSP60 (1:60,000 dilution) or anti-HSP72 (1:5,000 dilution; a generous gift from István Kurucz, Biorex Laboratories, Veszprém, Hungary, that has been characterized previously (16)) antibody for an additional 1 hour at room temperature. The immunoreactive protein was visualized by enhanced chemiluminescence using a horseradish peroxidase-coupled anti-rabbit immunoglobulin at 1:15,000 dilution (Dako, Denmark). The densities of the bands were quantitated by using an A.A.B. Image Analysis Program (Advanced American Biotechnology, Fullerton, CA, U.S.A.). The relative density was calculated as density at each time point/density before WI stress (t_0).

Assays

Serum amylase activity, and serum cytokine concentrations

All blood samples were centrifuged at 2,500*g* for 30 minutes. The serum levels of amylase were determined by a colorimetric kinetic method (Dialab, Vienna, Austria). TNF-α levels were titrated in a bioassay on the WEHI-164 cell line (17). IL-6 concentrations were measured by their proliferative action on the IL-6-dependent mouse hybridoma cell line B-9 (18). The activities were calibrated against recombinant TNF (Genzyme, Cambridge, UK) and recombinant IL-6 (Sigma-Aldrich, Munich, Germany). IL-1β concentrations were determined with an ELISA kit (R and D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions.

Pancreatic contents of amylase, trypsinogen, lipase, DNA and protein

The pancreas was homogenized in nine volumes of ice-cold buffer containing 0.02 *M* Tris-HCl, pH 7.8, 0.15 *M* NaCl, and 0.1% Triton X-100. Enzyme measurements were carried out on the supernatant fractions of the homogenates after centrifugation at 20,000*g* for 30 minutes. Pancreatic amylase activities were determined as described above. Trypsinogen was activated after a 200-fold dilution of the homogenate with 0.02 U enterokinase/μg pancreatic protein (Sigma, St. Louis, MO, U.S.A.) in the enzyme buffer containing 80 m*M* Tris-HCl, pH 8.0, 25 m*M* $CaCl_2$, and 100 μg/mL bovine serum albumin for 120 minutes at 37°C (19). Lipase activities were measured by a pH-stat method (19). Samples for DNA determination were precipitated with ice-cold 0.8 *M* perchloric acid, washed in 5% trichloroacetic acid, and then hydrolyzed with 0.8 *M* perchloric acid at 90°C for 10 minutes (20). DNA was estimated photometrically with diphenylamine (20). The protein concentrations in the supernatant fractions of the homogenates were measured by the microbiuret method of Goa (21).

Histologic examination

Light microscopy

A 2–3 mm^3 portion of the pancreas head was fixed in an 8% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4-μm thickness and stained with hematoxylin and eosin. The slides were coded and read by two independent observers who were blind to the experimental protocol. Grading of interstitial edema, hemorrhage, hyperemia, necrosis, leukocyte infiltration, and adherence and basophil lamellation of the cytoplasm of acinar cells was performed on 8 to 10 consecutive high-power fields on a scale of 0 to 3 or 0 to 4. The score for each graded parameter was averaged, and the total pancreatic damage was calculated by adding all the averages together.

Statistical analysis

Results are expressed as means ± SEM. Experiments were evaluated by using the Student's *t* test when the data consisted of two groups or by analysis of variance when three or more groups were compared. Values of $p < 0.05$ were accepted as significant.

RESULTS

Expression of pancreatic HSPs after CWI and HWI stress

HSP60 is constitutively expressed in the pancreas, and the level of its expression increases significantly during CWI. The maximal amount of HSP60 (as compared with the unstressed control) was noted immediately after the

immersion, and the levels remained significantly elevated during the next 9 hours (Fig. 2A). HWI did not have a significant effect on the expression of HSP60 (data not shown). HSP72 was present at very low levels in the control, but the expression was significantly increased at 3 hours after HWI and remained elevated until 12 hours (Fig. 2B). The levels of HSP72 after CWI did not differ significantly from the control (data not shown).

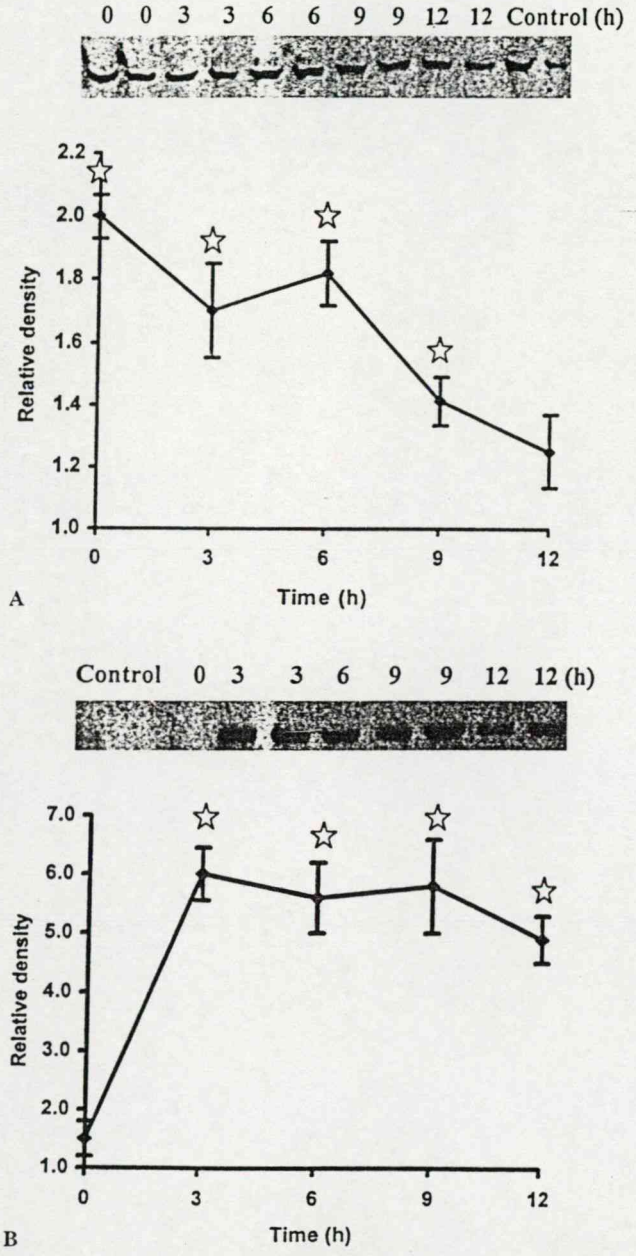

FIG. 2. Expression of pancreatic HSP60 after cold-water immersion (CWI) (**A**), and HSP72 after hot-water immersion (HWI) (**B**). Values are means ± SEM for four animals at each time point. Significant difference ($p < 0.05$) versus the unstressed control group.

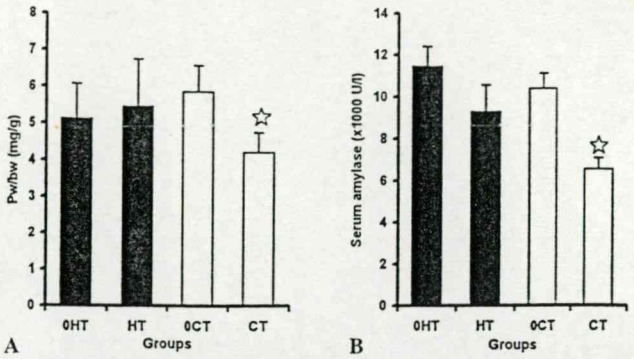

FIG. 3. (**A**) Pancreatic weight/body weight ratio (pw/bw) and (**B**) serum amylase levels in groups θHT, HT, θCT, and CT. Groups were treated as indicated in Fig. 1. Means ± SEM for six animals are shown. Significant difference ($p < 0.05$) versus the respective control group.

Pancreatic weight/body weight ratio (pw/bw) and serum amylase activity

The administration of 3% TC (100 μL/100 g body weight) induced the typical laboratory and morphologic changes of acute pancreatitis. In group CT, pw/bw (4.20 ± 0.28 mg/g) and the serum amylase activity (6,523 ± 536 U/L) were significantly decreased versus group θCT (5.84 ± 0.76 mg/g and 10,360 ± 720 U/L, respectively; Fig. 3). In group HT, pw/bw (5.44 ± 0.35 mg/g) and the serum amylase activity (9,262 ± 1,287 U/L) were not significantly different compared with group θHT (5.12 ± 0.44 mg/g and 11,444 ± 945 U/L, respectively; Fig. 3).

Pancreatic contents of DNA, protein, amylase, trypsinogen, and lipase

In group CT, the pancreatic contents of DNA (32.5 ± 5.2 μg/pancreas), protein (165.1 ± 18.5 mg/pancreas), amylase (10,469 ± 2,443 U/pancreas), lipase (1.98 ± 1.36 IU/pancreas), and trypsinogen (10.5 ± 1.1 IU/pancreas) were not significantly different versus group θCT (28.5 ± 6.8 μg/pancreas, 219.8 ± 35.7 mg/pancreas, 14,071 ± 2,893 U/pancreas, 2.95 ± 1.54 IU/pancreas, and 14.8 ± 2.0 IU/pancreas, respectively; Fig. 4). No significant changes were detected in the pancreatic DNA, protein, amylase, trypsinogen and lipase contents in group HT (29.0 ± 4.9 μg/pancreas, 231.9 ± 11.2 mg/pancreas, 16,307 ± 2,342 IU/pancreas, 15.9 ± 1.4 IU/pancreas and 3.5 ± 2.63 IU/pancreas, respectively) versus group θHT (26.4 ± 2.9 μg/pancreas, 250.3 ± 30.8 mg/pancreas, 16,513 ± 2,961 IU/pancreas, 16.6 ± 2.2 IU/pancreas and 2.38 ± 2.66 IU/pancreas, respectively; Fig. 4).

Serum cytokine levels

In group CT, the serum level of IL-6 (91.7 ± 5.9 pg/mL) was significantly decreased versus that of group θCT (175.0 ± 19.8 pg/mL; Fig. 5C). In group CT, the serum TNF-α (66.7 ± 11.8 U/mL) and IL-1 (101.7 ± 14.8

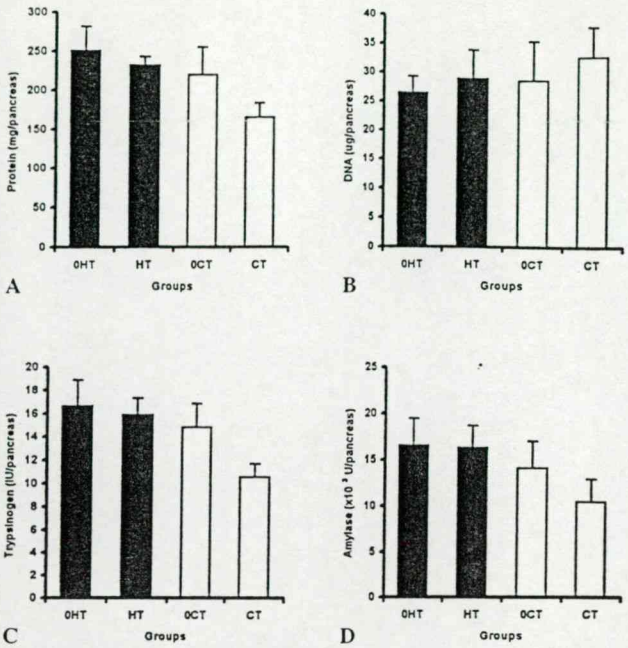

FIG. 4. Pancreatic contents of (**A**) protein, (**B**) DNA, (**C**) trypsinogen, and (**D**) amylase in groups θHT, HT, θCT, and CT. Groups were treated as indicated in Fig. 1. Data are means ± SEM for six animals.

pg/mL) levels were not significantly different versus those of group θCT (91.8 ± 23.8 U/mL and 87.5 ± 6.3 pg/mL, respectively; Fig. 5A, B). No significant changes were observed in the serum TNF-α, IL-1, and IL-6 levels in group HT (not detected, 184.7 ± 84.1 pg/mL, and 8.2 ± 4.6 pg/mL, respectively) versus those of group θHT (not detected, 38.8 ± 11.4 pg/mL and 4.0 ± 4.0 pg/mL, respectively; Fig. 5).

Light microscopy

In group CT, the total damage (8.01 ± 0.29 points) was significantly elevated versus group θCT (6.02 ± 0.83 points; Fig. 6). No significant alteration in the total damage was observed between groups HT (8.15 ± 0.71 points) and θHT (7.87 ± 0.84 points). The point values for each of the scored parameters are shown in Table 1.

DISCUSSION

Cells subjected to a mild, sublethal stress events sufficient to increase the levels of HSPs are able to survive a subsequent more serious stress event (6). HSP preinduction is known to protect the pancreas from cerulein-induced pancreatitis or choline-deficient ethionine-supplemented diet model pancreatitis (8–12). Our study was designed to investigate the dynamics of HSP induction (HSP60 and HSP72) in response to CWI and HWI in the pancreas and the potential effects of HSP preinduction on TC-induced acute pancreatitis in rats. Pancreatitis was induced near the peak of HSP expression by injecting 3% TC into the common biliopancreatic duct.

In agreement with the findings of Otaka et al. (9), our results demonstrate that CWI specifically induces HSP60, whereas HWI increases the expression of HSP72 in rats. Otaka et al. (9) did not check the dynamics of HSP60 expression after CWI. We found that the levels of HSP60 remained significantly elevated for 9 hours after CWI. This is in accordance with the similar findings of Lee et al. (11). The quantity of HSP72 was significantly increased at 3 hours (six times the control level) after HWI and remained elevated until 12 hours. Otaka et al. (9) showed that the peak (three times the control level) in the HSP72 expression was at 6 hours, which could be because the duration of the hyperthermia was shorter in their experiments.

The current study demonstrated that the administration of TC results in the typical laboratory (hyperamylasemia) and morphologic changes (interstitial edema, leukocyte infiltration, hemorrhage, and acinar cell injury) of acute pancreatitis 6 hours after the TC injection in rats.

We found that CWI pretreatment and possibly HSP60 ameliorated the pancreatic edema and the serum amylase level increase in TC-induced pancreatitis 6 hours after

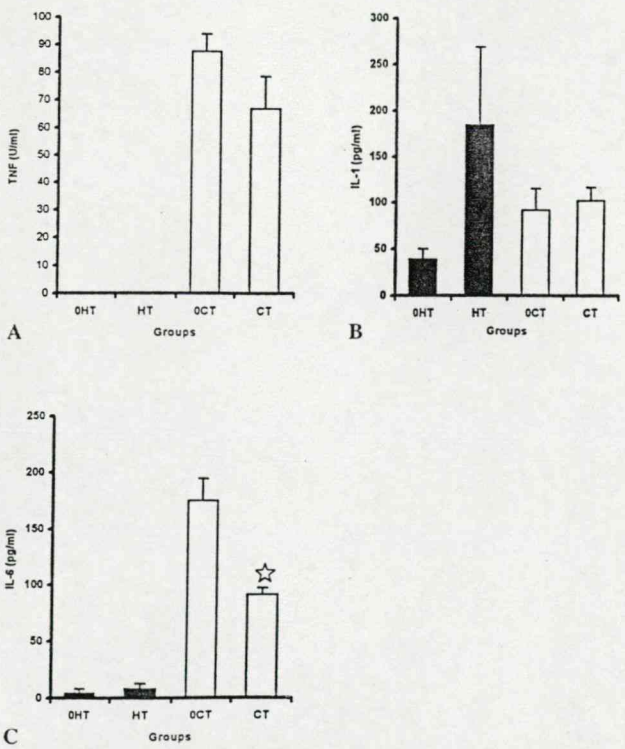

FIG. 5. Serum (**A**) TNF-α, (**B**) IL-1 and (**C**) IL-6 levels in groups θHT, HT, θCT, and CT. Groups were treated as indicated in Fig. 1. Means ± SEM for six animals are shown. *Significant difference ($p < 0.05$) versus the respective control group.

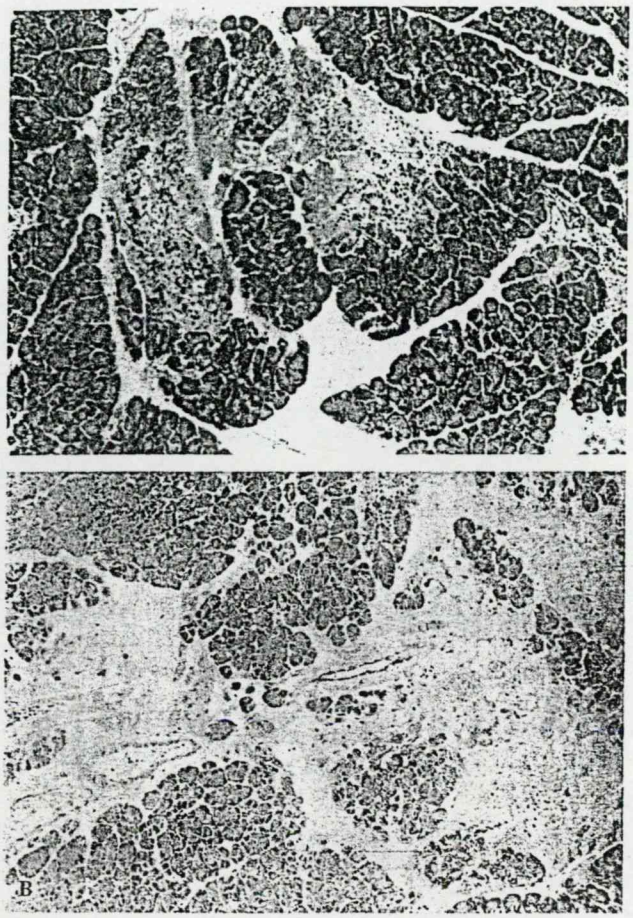

FIG. 6. Histology of the pancreata of rats either not exposed (**A**, Group θCT) or exposed to cold-water immersion (**B**, Group CT) 6 hours after the sodium taurocholate injection. Pretreatment with cold-water immersion (Group CT) significantly increased the TC-induced morphologic damage (hematoxylin and eosin, original magnification ×100).

the induction of the disease. Unexpectedly, CWI preconditioning significantly worsened the morphologic picture of the pancreatitis. This might be a result of the microcirculatory changes caused by CWI (23), which is supported by the fact that the animals in group CT exhibited a greater vascular involvement (hyperemia and hemorrhage) than did those in the control. It is also plausible that, besides or instead of HSP60, hypothermia itself has a protective effect on the pancreas. To investigate this possibility, in a separate set of experiments we increased the body temperature of the rodents to 37°C during a 2.5-hour period after the CWI (results not demonstrated in the report), in the belief that this would not affect the quantity of HSP60. Unfortunately, the reheating process decreased the level of this protein to the basal value. Nevertheless, the reheating abolished the protective effect seen in pw/bw and the serum amylase level, but improved the morphologic parameters (histology) to an insignificant difference as compared with the control. HWI pretreatment did not have any effect on the measured parameters of this severe acute necrohemorrhagic pancreatitis.

Although HSPs have been implicated as mediators of pancreatic protection, the precise mechanism of their cytoprotective effects remains unknown. We could observe only minor protective effects in the early phase of the TC-induced pancreatitis model. These are most probably because of their chaperoning activities (1,2). The HSPs might also attenuate cellular damage by decreasing pro-inflammatory cytokine levels (24) or by preventing intracellular trypsinogen activation (11,25). Our findings support some of these possibilities. It is well known that the pro-inflammatory cytokine levels increase during TC-induced pancreatitis (26). We detected a decreased serum IL-6 level after CWI pretreatment in this acute pancreatitis model. HWI pretreatment did not influence the serum cytokine levels in TC-induced pancreatitis.

It should be emphasized that heat shock (hot or cold) results in a number of stress-induced responses, e.g., metabolic alterations and the synthesis of a variety of proteins besides HSP60 and HSP72, any one of which might have an additional protective role in the prevention

TABLE 1. *Effects of cold- and hot-water immersion pretreatment on the histologic parameters in sodium taurocholate-induced acute pancreatitis*

	Group θHT	Group HT	Group θCT	Group CT
IS edema	2.45 ± 0.22	2.47 ± 0.13	2.27 ± 0.20	1.80 ± 0.15[a]
Hemorrhage	0.32 ± 0.14	0.35 ± 0.12	0.09 ± 0.08	0.42 ± 0.13[a]
Necrosis (0–4)	0.50 ± 0.21	0.50 ± 0.18	0.29 ± 0.16	0.60 ± 0.17
Leukocyte infiltration	2.07 ± 0.21	1.90 ± 0.17	1.09 ± 0.39	1.05 ± 0.22
Leukocyte adherence	0.73 ± 0.13	0.77 ± 0.11	0.57 ± 0.21	0.42 ± 0.17
Basophil lamellation	0.62 ± 0.24	0.43 ± 0.08	0.54 ± 0.08	1.06 ± 0.20[a]
Hyperemia	1.27 ± 0.22	1.33 ± 0.25	1.04 ± 0.15	1.98 ± 0.05[a]
Total damage	7.87 ± 0.84	8.15 ± 0.71	6.02 ± 0.82	7.71 ± 0.29[a]

Groups were treated as indicated in Fig. 1. Data are mean ± SEM for six animals.
[a] $p < 0.05$ versus respective control group.

of a subsequent stress such as pancreatitis. For example, other HSPs and antioxidant enzymes can also take part in the protective effect (27).

In conclusion, in accordance with previous investigators, we showed that HWI specifically induced the synthesis of HSP72, whereas CWI specifically elevated the level of HSP60 in the pancreas. We demonstrated that CWI pretreatment exerts minor protective effects in the early stages of TC-induced pancreatitis. Our findings suggest the possible role of HSP60 in the protection against TC-induced pancreatic damage. A decisive proof of the cytoprotective effect of HSP60 in this acute pancreatitis model would require further studies, in which the expression or function of this protein is specifically blocked. No specific inhibitor of HSP production or function has as yet been developed, however, and no knockout animal model exists.

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III.

Comparative effects of water immersion pretreatment on three different acute pancreatitis models in rats

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Abstract: Cells respond to stress by upregulating the synthesis of cytoprotective heat shock proteins (HSPs) and antioxidant enzymes. The aim of this study was to compare the effects of cold (CWI) or hot water immersion (HWI) stress on three different acute pancreatitis models (cholecystokinin octapeptide (CCK), sodium taurocholate (TC), and L-arginine (Arg)). We examined the levels of pancreatic HSP60, HSP72, and antioxidants after the water immersion stress. Male Wistar rats were injected with CCK, TC, or Arg at the peak level of pancreatic HSP synthesis, as determined by Western blot analysis. HWI significantly elevated HSP72 expression and CWI significantly increased HSP60 expression in the pancreas. Water immersion stress decreased the levels of pancreatic antioxidants. CWI and HWI pretreatment ameliorated most of the examined laboratory and morphological parameters of CCK-induced pancreatitis. CWI pretreatment decreased pancreatic edema and the serum amylase level; however, the morphological damage was more severe in TC-induced acute pancreatitis. Overall, CWI and HWI pretreatment only decreased the serum cytokine concentrations in Arg-induced pancreatitis. CWI and HWI resulted in differential induction of pancreatic HSP60 and HSP72 and the depletion of antioxidants. The findings suggest the possible roles of HSP60 and (or) HSP72 (but not that of the antioxidant enzymes) in the protection against CCK- and TC-induced acute pancreatitis. Unexpectedly, CWI pretreatment was detrimental to the morphological parameters of TC-induced pancreatitis. It was demonstrated that CWI and HWI pretreatment only influenced cytokine synthesis in Arg-induced pancreatitis.

Key words: heat shock proteins, water immersion, cholecystokinin octapeptide, sodium taurocholate, L-arginine, pancreatitis.

Résumé : Les cellules répondent à un stress en augmentant la synthèse de protéines du choc thermique (HSP) et d'enzymes antioxydantes cytoprotectrices. Le but de cette étude était de comparer les effets d'un stress d'immersion dans l'eau froide ou chaude sur trois modèles différents de pancréatite aiguë (octapeptide de la cholécystokinine (CCK-8), taurocholate de sodium (TC), L-arginine (Arg)). Nous avons mesuré la concentration des protéines HSP60 et HSP72 et des enzymes antioxydantes pancréatiques après un stress d'immersion dans l'eau. Des rats Wistar mâles ont reçu une injection de CCK-8, de TC ou d'Arg lorsque le taux de synthèse des protéines HSP pancréatiques, mesurées par transfert Western, était maximal. L'immersion dans l'eau chaude augmente significativement l'expression de la protéine HSP72 dans le pancréas, alors que l'immersion dans l'eau froide augmente significativement l'expression de la protéine HSP60. Le stress d'immersion dans l'eau diminue la concentration des enzymes antioxydantes pancréatiques. Une immersion antérieure dans l'eau froide ou chaude améliore la plupart des paramètres biochimiques et morphologiques de la pancréatite induite par le CCK-8. L'immersion antérieure dans l'eau froide diminue l'oedème pancréatique et la concentration d'amylase sérique dans la pancréatite aiguë induite par le TC, mais elle augmente les altérations morphologiques. Une immersion antérieure dans l'eau froide ou chaude diminue la concentration sérique des cytokines seulement dans la pancréatite induite par l'Arg. En conclusion, une immersion antérieure dans l'eau froide ou chaude entraîne une induction différentielle des protéines HSP60 et HSP72 pancréatiques et une diminution des enzymes antioxydantes. Ces résultats suggèrent que les protéines HSP60 et HSP72 jouent un rôle protecteur contre une pancréatite aiguë induite par le CCK-8 ou le TC, mais non les enzymes antioxydantes. Étonnamment, une immersion antérieure dans l'eau froide entraîne une détérioration des paramètres morphologiques de la pancréatite induite par le

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TC. Enfin, nous montrons qu'une immersion antérieure dans l'eau froide ou chaude influence la synthèse des cytokines seulement dans la pancréatite induite par l'Arg.

Mots clés : protéines du choc thermique, immersion dans l'eau, octapeptide de la cholécystokinine, taurocholate de sodium, L-arginine, pancréatite.

[Traduit par la Rédaction]

Introduction

Heat shock proteins (HSPs) are highly conserved and functionally related proteins that are classified by their approximate molecular weights, such as HSP60 and HSP72 (Lindquist 1986; Welch 1992). The members of the HSP family are expressed constitutively and (or) at elevated levels upon exposure of the cells to a variety of stress conditions in every organ, including the pancreas (Schafer and Williams 2000). HSPs are well known to protect cells against stress (Frossard et al. 2001; Hutter et al. 1994; Lee et al. 2000; Marber et al. 1995; Otaka et al. 1997; Rakonczay et al. 2001; Wagner et al. 1996; Weber et al. 2000). Following stress conditions, many cellular proteins become partially or completely denatured or malfolded. HSPs recognize this, bind to the damaged proteins, and stabilize and refold them, thereby preventing or dissolving otherwise irreversible aggregation. It is important to note that HSPs are also involved in the biogenesis and degradation of proteins, regulating their structures and functions under normal physiological conditions. Moreover, HSPs have a critical role in the transport and translocation of different proteins.

Cells subjected to hyper- or hypothermia respond by synthesizing HSPs. Induction of the heat shock response enhances the ability of the cells to overcome the effects of further stress (Hutter et al. 1994). It has been widely demonstrated that the preinduction of HSP expression has a protective effect against acute interstitial cholecystokinin octapeptide (CCK)- and DBTC-induced pancreatitis (Frossard et al. 2001; Lee et al. 2000; Otaka et al. 1997; Rakonczay et al. 2001; Wagner et al. 1996; Weber et al. 2000). However, the cytoprotective effects of the HSPs on more serious acute pancreatitis models have received relatively little attention (Rakonczay et al. 2002). Sodium taurocholate (TC) injected into the common biliopancreatic duct of the rat also causes severe necrohemorrhagic pancreatitis (Aho et al. 1980). Likewise, it is well known that excessive doses of L-arginine (Arg) induce acute necrotizing pancreatitis (Tani et al. 1990).

The aim of the present study was to compare the effects of cold (CWI) and hot water immersion (HWI) pretreatment and the potential effects of HSP preinduction on three different acute pancreatitis models (CCK, TC, and Arg) in rats. Water immersion stress results in a number of stress-induced responses such as metabolic alterations and the synthesis of a variety of proteins besides HSP60 and HSP72 that may have protective roles in the prevention of a subsequent stress. For example, the antioxidant enzymes catalase and manganese superoxide dismutase can also take part in the protective effect (Kingma et al. 1996; Mizunuma et al. 1984; Yamashita et al. 1998). Therefore (besides HSPs), we also examined the levels of pancreatic antioxidants after the water immersion stress.

Materials and methods

Experimental protocol

Animals

Male Wistar rats weighing 250–350 g were used. The animals were kept at a constant room temperature of 25°C with a 12 h light : 12 h dark cycle and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). The experiments performed in this study were in accordance with the guidelines of *Guide for the Care and Use of Laboratory Animals* and approved by the Animal Care Committee of the University. The rats were fasted for 12 h before the beginning of the experiments. In every group, the rats were anesthetized with pentobarbital (44 mg/kg) intraperitoneally at the starting point of the experiment (t_0).

CWI and HWI stress

Water immersion stress was performed according to Otaka et al. (1997) with some modifications. In group C (CWI) ($n = 24$), the rats were immersed vertically in a 23°C water bath to the depth of the xiphoid process for 6 h. When the animals woke up from the anesthesia, they were immediately reanesthetized with 22 mg pentobarbital/kg intraperitoneally. In group H (HWI) ($n = 24$), the rats were immersed vertically in a 37°C water bath, and the water temperature was then gradually increased to 42°C (during 55 min) and maintained at that temperature for 20 min (total of 1 h 15 min). In order to evaluate the expressions of HSP60 and HSP72 after the CWI or HWI stress, four rats were killed at each time point before (t_0) immediately after (0) or 3, 6, 9, or 12 h after the end of the immersion. The pancreas was quickly removed, cleaned from fat and lymph nodes, and frozen at –80°C until processing. Acute pancreatitis was induced near the peak of HSP synthesis.

CCK-induced pancreatitis

Pancreatitis was induced by injecting 75 μg CCK/kg body weight subcutaneously three times at intervals of 2 h. In group CC (CWI + CCK) ($n = 6$), the rats received CCK immediately after CWI. In group ⌀CC (no CWI + CCK) ($n = 6$), the animals were kept at room temperature and were injected with CCK at $t_0 + 6$, $t_0 + 8$, and $t_0 + 10$ h. In group HC (HWI + CCK) ($n = 6$), the rats received CCK as mentioned above following a 6-h recovery period after HWI. In group ⌀HC (no HWI + CCK) ($n = 6$), the rodents were given CCK starting at $t_0 + 7$ h 15 min.

TC-induced pancreatitis

Three percent TC was injected at 100 µL/100 g body weight into the common biliopancreatic duct under steady manual pressure over a period of 30 s, as described in Aho et al. (1980). All injections were performed by the same investigator to limit technical differences. In group CT (CWI + TC) (n = 6), the rats received the TC immediately after CWI. In group ⌀CT (no CWI + TC) (n = 6), the animals were kept at room temperature and were injected with TC at t_0 + 6 h. In group HT (HWI + TC) (n = 6), the rats received TC as mentioned above following a 6-h recovery period after HWI. In group ⌀HT (no HWI + TC) (n = 6), the rodents were given TC at t_0 + 7 h 15 min.

Arg-induced pancreatitis

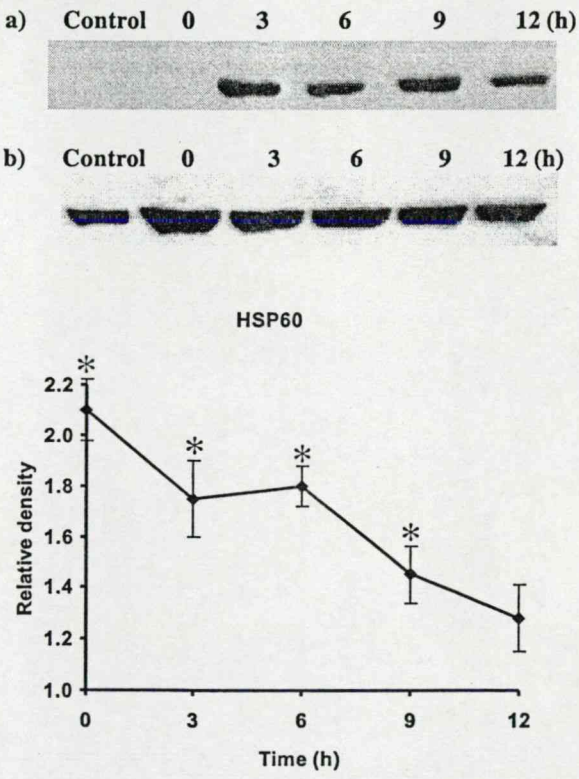
Acute pancreatitis was induced by injecting 230 mg Arg/100 g body weight intraperitoneally twice at an interval of 1 h. In group CA (CWI + Arg) (n = 6), the rats received the Arg immediately after CWI. In group ⌀CA (no CWI + Arg) (n = 6), the animals were kept at room temperature and were injected with Arg at t_0 + 6 and t_0 + 7 h. In group HA (HWI + Arg) (n = 6), the rats received Arg at 6 and 7 h after HWI. In group ⌀HA (no HWI + Arg) (n = 6), the rats were given Arg starting at t_0 + 7 h 15 min.

The animals were killed by exsanguination through the abdominal aorta 2 h after the last CCK injection, 6 h after the TC injection, or 24 h after the second Arg injection. The pancreas was quickly removed, cleaned from fat and lymph nodes, weighed, and frozen at –80°C until use.

Western blotting

A part of the pancreas was homogenized for 2 min in a buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM dithiothreitol, 0.2 mM EDTA, and 0.5 mM phenylmethanesulfonyl fluoride using an Ultra-Turrax homogenizer. The homogenates were centrifuged at 20 000 × g for 30 min. The supernatants were collected and the protein concentrations were measured by the method of Bradford (1976). Twenty micrograms of protein was loaded per lane. Samples were electrophoresed on an 8% sodium dodecyl sulfate – polyacrylamide gel according to the method of Laemmli (1970). The gels were either stained with Coomassie brilliant blue (to demonstrate equal loading of proteins for Western blot analysis) or transferred to nitrocellulose membranes for 2.5 h at 30 V. Membranes were blocked in 5% nonfat dry milk for 1 h and incubated with rabbit anti-HSP60 antibody (produced by ourselves (Rakonczay et al. 2001), 1:60 000 dilution) or anti-HSP72 antibody (1:5000 dilution) (a generous gift from István Kurucz, Biorex Laboratories, Veszprém, Hungary, which has been characterized previously (Kurucz at al. 1999)) for an additional 1 h at room temperature. The immunoreactive protein was visualized by enhanced chemiluminescence using a horseradish peroxidase coupled antirabbit immunoglobulin at 1:15 000 dilution (Dako, Denmark). The densities of the bands were quantitated by using an AAB image analysis program (Advanced American Biotechnology, Fullerton, Calif.). The relative density was calculated as density at each time point / density before WI stress (control, t_0).

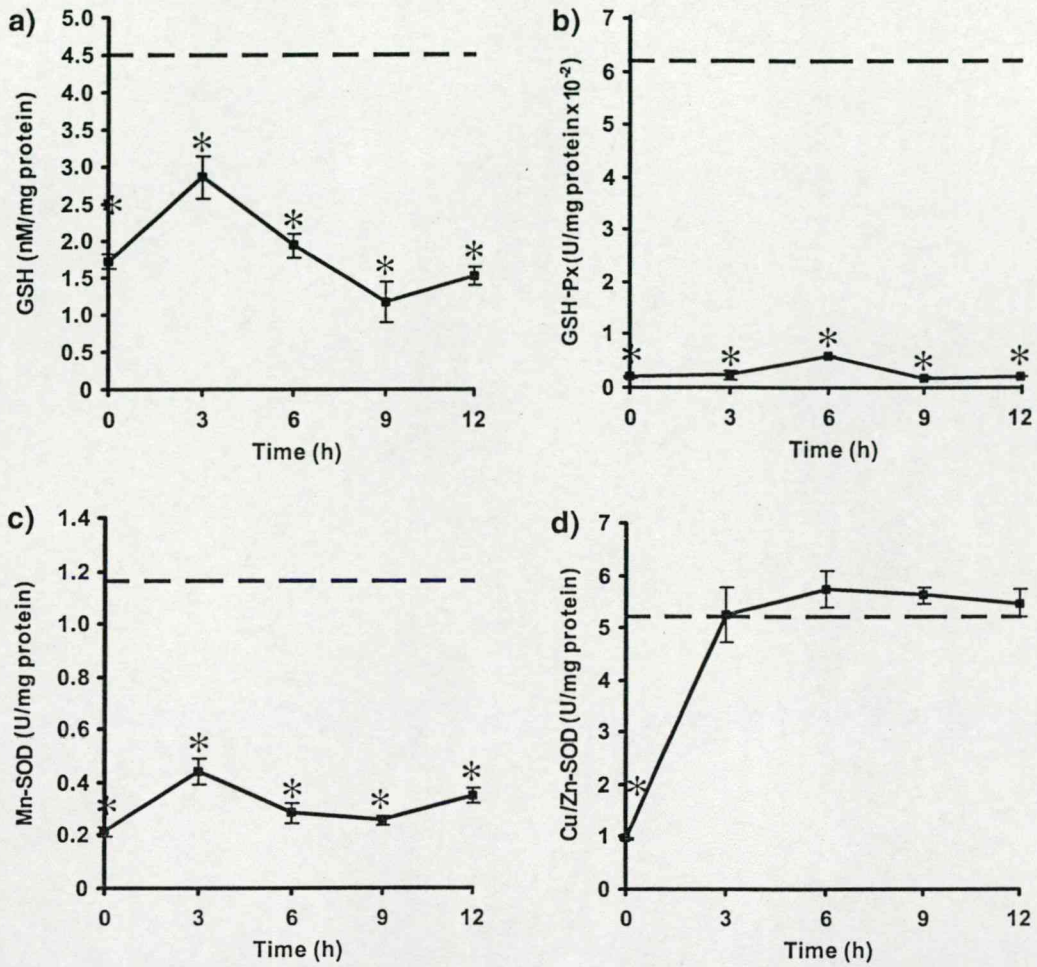
Fig. 1. Effect of water immersion stress on pancreatic HSP synthesis. Representative Western immunoblots of protein lysates (20 µg/lane) extracted from pancreata harvested over a time course after the water immersion treatments (0–12 h). The control did not receive any treatment. (A) Expression of pancreatic HSP72 after HWI. HSP72 could not be detected in the unstressed control, but its expression was significantly increased at 3 h after HWI and remained elevated until 12 h. (B) Expression of pancreatic HSP60 after CWI. The diagram shows the relative density of the HSP60 bands (density at each time point / density before water immersion stress (control)) versus the time after the CWI treatment as analysed by densitometry. The maximal amount of HSP60 was noted immediately after the end of the immersion (0), and the levels remained significantly elevated over the next 9 h. Values are means ± SE for four animals at each time point. *, Significant difference ($p < 0.05$) versus the unstressed control group.



Pancreatic reduced glutathione level and activities of superoxide dismutase, catalase, and glutathione peroxidase

A part of the pancreas was homogenized in fourfold excess (w/v) of ice-cold buffer containing 100 mM K_2HPO_4, 150 mM KCl, and 100 mM EDTA (pH = 7.4). The homogenates were centrifuged at 3000 × g for 10 min, and the supernatants were used for measurements. The protein concentrations of the homogenates were measured by the microbiuret method of Goa (1953). The reduced glutathione (GSH) level was determined spectrophotometrically with Ellman's reagent (Sedlak and Lindsay 1968) and was corrected for the protein content of the tissue. Superoxide

Fig. 2. Effect of CWI on pancreatic antioxidant levels: (A) GSH level and the activities of (B) GPx, (C) Mn-SOD, and (D) Cu/Zn-SOD 0–12 h after the CWI treatment. Values are means ± SE of four animals. The dotted lines represent the level of the unstressed control group. *, Significant difference ($p < 0.05$) versus the control group.



dismutase (SOD) activity was determined on the basis of the inhibition of epinephrine–adrenochrome autooxidation (Misra and Fridovich 1972). Mn-SOD activity was measured by the autooxidation method in the presence of 5×10^{-3} M KCN (Matkovics et al. 1977). Cu/Zn-SOD activity was calculated by subtracting the activity of Mn-SOD from SOD activity. Catalase (CAT) activity was measured spectrophotometrically at 240 nm by the method of Beers and Sizer (1951) and expressed in Bergmeyer units (1 Bergmeyer unit = decomposition of 1 g H_2O_2/min at 25°C). Glutathione peroxidase (GPx) activity was determined according to the chemical method using cumene hydroperoxide and GSH as substrates of GPx (Chiu et al. 1976).

Assays

Pancreatic weight to body weight ratio (PW/BW)

This ratio was utilized to evaluate the degree of pancreatic edema.

Serum amylase activity

All blood samples were centrifuged at 2500 × *g* for 20 min. The serum levels of amylase were determined by a chromogenic method with the Phadebas test (Ceska et al. 1969) (Pharmacia & Upjohn, Uppsala, Sweden).

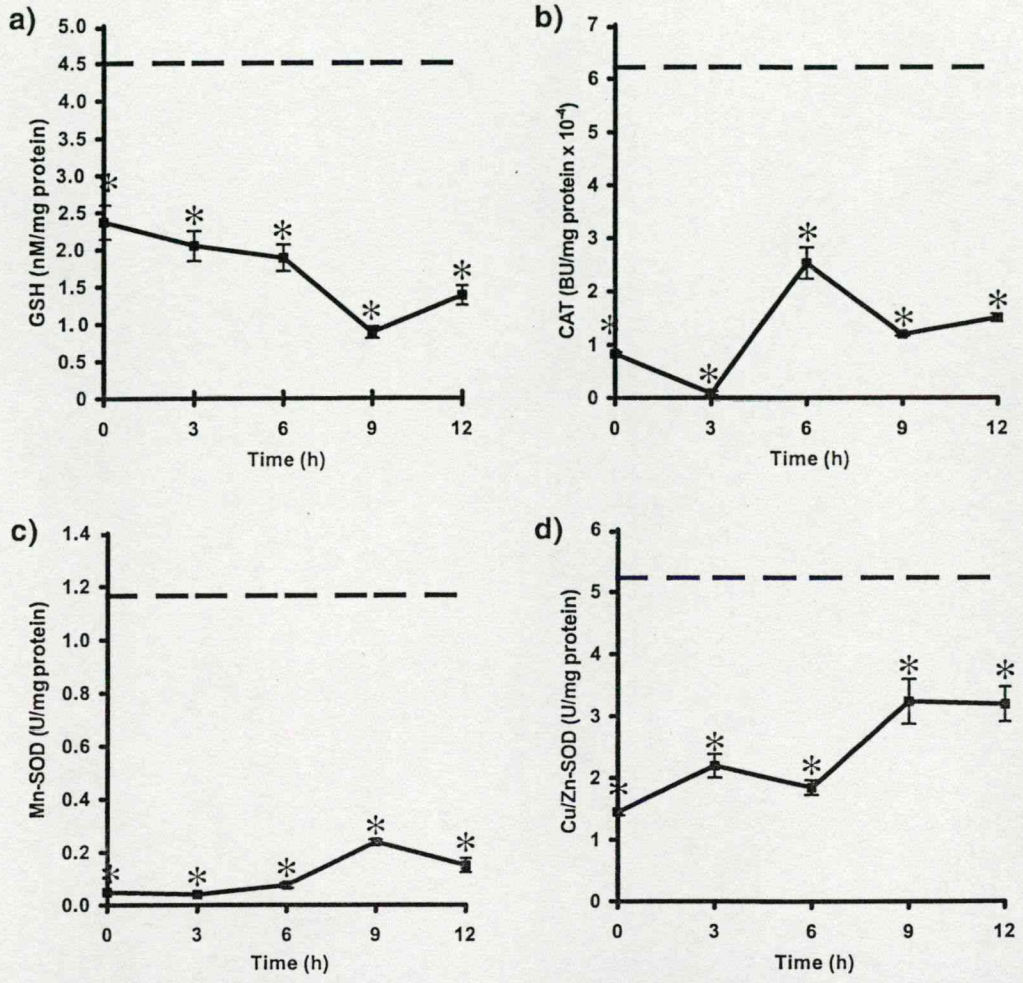
Serum cytokine concentrations

Tumor necrosis factor α (TNF-α) levels were titrated in a bioassay on the WEHI-164 cell line (Espevik and Niessen-Meyer 1986). Interleukin-6 (IL-6) concentrations were measured via their proliferative action on the IL-6-dependent mouse hybridoma cell line B-9 (Arden et al. 1987). The activities were calibrated against recombinant TNF (Genzyme, Cambridge, U.K.) and recombinant IL-6 (Sigma-Aldrich, Munich, Germany). IL-1β concentrations were determined with an ELISA kit (R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions.

Pancreatic contents of amylase, trypsinogen, lipase, DNA, and protein

The pancreas was homogenized in a ninefold excess (w/v) of ice-cold buffer containing 0.02 M Tris–HCl (pH 7.8), 0.15 M NaCl, and 0.1% Triton X-100. Enzyme measurements were carried out on the supernatant fractions of the

Fig. 3. Effect of HWI on pancreatic antioxidant levels: (A) GSH level and the activities of (B) CAT, (C) Mn-SOD, and (D) Cu/Zn-SOD 0–12 h after the HWI treatment. Values are means ± SE of four animals. The dotted lines represent the level of the unstressed control group. *, Significant difference ($p < 0.05$) versus the unstressed control group.



homogenates after centrifugation at 20 000 × *g* for 30 min. Pancreatic amylase activities were determined as described above (Ceska et al. 1969). Trypsinogen was activated after a 200-fold dilution of the homogenate with 0.02 U enterokinase/μg pancreatic protein (Sigma, St. Louis, Mo.) in the enzyme buffer containing 80 mM Tris–HCl (pH 8.0), 25 mM $CaCl_2$, and 100 μg bovine serum albumin/mL for 120 min at 37°C (Nagy et al. 1989). Lipase activities were measured by a pH-stat method (Nagy et al. 1989). Samples for DNA determination were precipitated with ice-cold 0.8 M perchloric acid, washed in 5% trichloroacetic acid, and then hydrolyzed with 0.8 M perchloric acid at 90°C for 10 min (Schneider 1957). DNA was estimated photometrically with diphenylamine (Giles and Myers 1965). The protein concentrations in the supernatant fractions of the homogenates were measured by the method of Goa (1953).

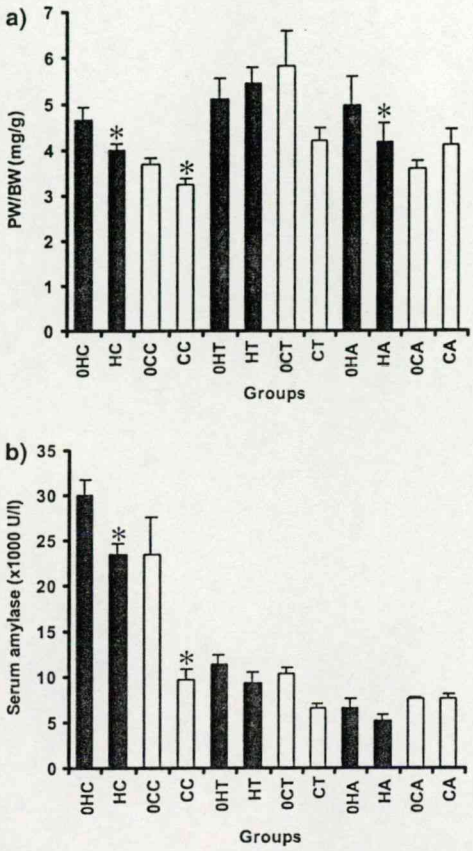
Histological examination

A 2- to 3-mm^3 portion of the pancreas head was fixed in an 8% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4 μm thickness and stained with hematoxylin and eosin. The slides were coded and read for the traditional histological markers of pancreatic tissue injury by two independent observers who were blind to the experimental protocol. Semiquantitative grading of interstitial edema, leukocyte infiltration and adherence, hyperemia, and vacuolization, necrosis, and apoptosis of acinar cells was performed on 8–10 consecutive high-power fields (400×) on a scale of 0–3 or 0–4. Additionally, basophilic lamellation of the cytoplasm of acinar cells was also graded, since a pilot study revealed that, besides the traditional markers, the areas of basophilic lamellation were more extensive in the more severely damaged pancreata. The score for each graded parameter was averaged and the total pancreatic damage was calculated by adding all the averages together. The grading system and basophilic lamellation are described in more detail in one of our previous manuscripts (Rakonczay et al. 2001).

Statistical analysis

Results are expressed as means ± SE. Experiments were evaluated by using the Student *t* test when the data consisted

Fig. 4. (A) PW/BW and (B) serum amylase levels in rats with acute pancreatitis. Groups were treated as described in the text. Values are means ± SE for six animals. *, Significant difference ($p < 0.05$) versus the respective control group.



of two groups or by analysis of variance when three or more groups were compared. Values of $p < 0.05$ were accepted as significant.

Results

Expression of pancreatic HSPs after CWI and HWI stress (Fig. 1)

HSP72 could not be detected in the unstressed control, but its expression was significantly increased at 3 h after HWI and remained elevated until 12 h (Fig. 1A). HSP60 is constitutively expressed in the pancreas; HWI did not have a significant effect on its expression (data not shown). The levels of HSP72 after CWI did not differ significantly from the control (data not shown), but the expression of HSP60 increased significantly during CWI. The maximal amount of HSP60 (as compared with the unstressed control) was noted immediately after the end of the immersion, and the levels remained significantly elevated over the next 9 h (Fig. 1B).

Pancreatic GSH level and activities of GPx, SOD, and CAT after CWI and HWI stress (Figs. 2 and 3)

Both HWI and CWI significantly decreased the pancreatic GSH content and the activities of GPx, Mn-SOD, Cu/Zn-SOD, and CAT immediately after the end of the water immersion treatment (as compared with the unstressed control). Only the activity of Cu/Zn-SOD recovered soon after CWI (3 h). The level of other antioxidants after water immersion remained significantly decreased throughout the examined time period.

PW/BW and serum amylase activity (Fig. 4)

The administration of CCK, TC, or Arg induced the typical laboratory and morphological changes of acute pancreatitis. In group CC, PW/BW (3.24 ± 0.13 mg/g) and serum amylase activity (9690 ± 1114 U/L) were significantly decreased versus group ⌀CC (3.69 ± 0.15 mg/g and 23 400 ± 4625 U/L, respectively). In group HC, PW/BW (4.00 ± 0.15 mg/g) and serum amylase activity (23 330 ± 1412 U/L) were significantly decreased versus group ⌀HC (4.65 ± 0.29 mg/g and 30 063 ± 1676 U/L, respectively). In group CT, PW/BW (4.20 ± 0.28 mg/g) and serum amylase activity (6523 ± 536 U/L) were significantly decreased versus group ⌀CT (5.84 ± 0.76 mg/g and 10 360 ± 720 U/L, respectively).

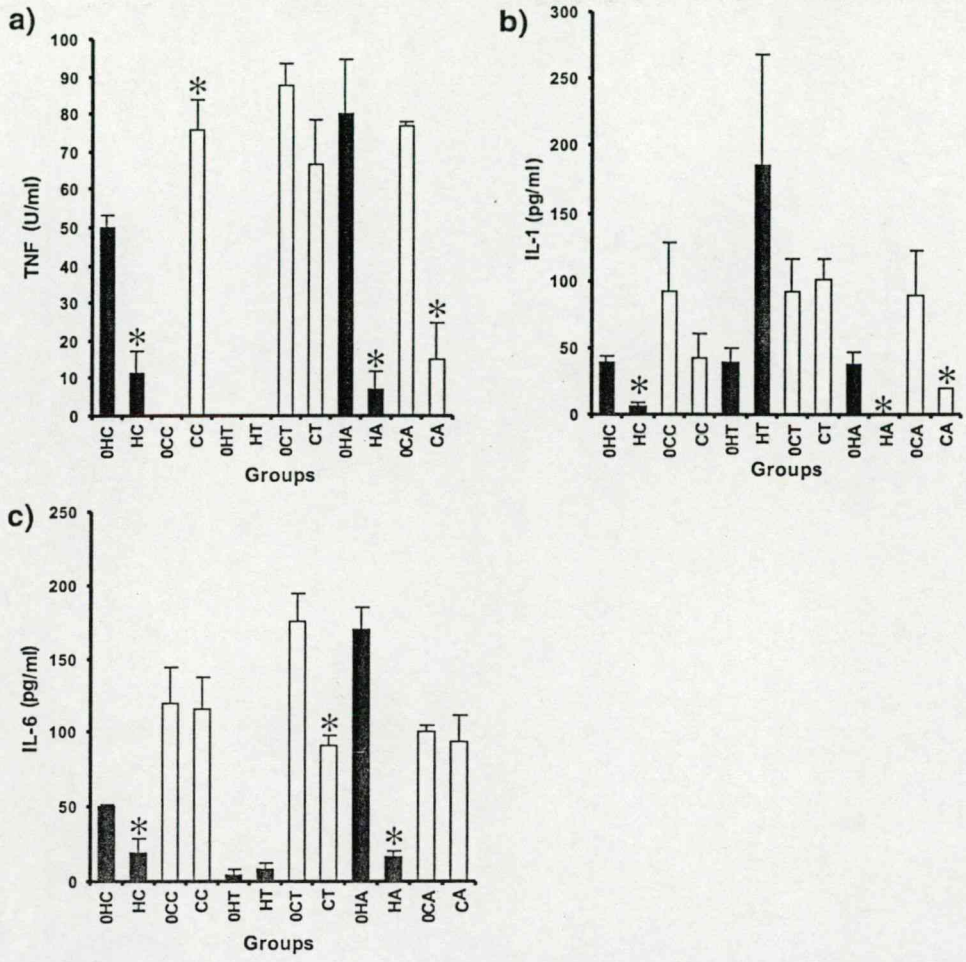
Serum cytokine levels (Fig. 5)

In group HC, the serum levels of TNF-α (11.3 ± 5.7 U/mL), IL-1 (6.6 ± 3.0 pg/mL), and IL-6 (18.8 ± 10.4 pg/mL) were all significantly decreased versus the corresponding values in group ⌀HC (50.0 ± 3.0 U/mL, 38.9 ± 4.6 pg/mL, and 50.0 ± 1.0 pg/mL, respectively). In group CC, the serum TNF-α level (76.0 ± 8.0 U/mL) was significantly elevated versus group ⌀CC (not detected). In group CT, the serum level of IL-6 (91.7 ± 5.9 pg/mL) was significantly decreased versus group ⌀CT (175.0 ± 19.8 pg/mL). In group HA, the serum levels of TNF-α (7.0 ± 4.7 U/mL), IL-1 (not detected), and IL-6 (16.0 ± 4.8 pg/mL) were all significantly decreased versus the corresponding values in group ⌀HA (80.0 ± 14.6 U/mL, 37.6 ± 8.5 pg/mL, and 170.0 ± 14.6 pg/mL, respectively). In group CA, the serum levels of TNF-α (15.0 ± 10.0 U/mL) and IL-1 (18.9 ± 0.7 pg/mL) were significantly decreased versus group ⌀CA (76.7 ± 1.2 U/mL and 88.4 ± 33.2 pg/mL, respectively).

Pancreatic contents of protein, DNA, amylase, trypsinogen, and lipase (Fig. 6)

In group CC, the pancreatic contents of protein (56.3 ± 7.6 mg/pancreas) and DNA (2.18 ± 0.28 mg/pancreas) were significantly decreased versus group ⌀CC (84.0 ± 5.16 and 3.02 ± 0.21 mg/pancreas, respectively). The pancreatic contents of amylase, lipase, and trypsinogen were significantly decreased in group CC (1008 ± 216, 169.1 ± 8.4, and 3.16 ± 0.60 IU/pancreas, respectively) versus group ⌀CC (3612 ± 1007, 198.0 ± 15.1, and 5.52 ± 0.67 IU/pancreas, respectively). The pancreatic contents of protein, amylase, lipase, and trypsinogen were significantly decreased in group HC (98.9 ± 4.2 mg/pancreas and 6464 ± 519, 209.6 ± 26.3, and 5.09 ± 0.50 IU/pancreas, respectively) versus group ⌀HC (124.9 ± 16.3 mg/pancreas and 10 244 ± 1470, 343.3 ± 30.4, and 7.08 ± 1.30 IU/pancreas, respectively). In group HA, the pancreatic content of protein (76.5 ± 13.3 mg/pancreas) was significantly decreased versus group ⌀HA (113.0 ± 10.8 mg/pancreas). The pancreatic contents of amylase and trypsinogen were significantly elevated in group HA (5568 ±

Fig. 5. Serum levels of (A) TNF-α, (B) IL-1, and (C) IL-6 in rats with acute pancreatitis. Groups were treated as described in the text. Values are means ± SE for six animals. *, Significant difference ($p < 0.05$) versus the respective control group.



1719 and 6.7 ± 1.0 IU/pancreas, respectively) versus group ⌀HA (2514 ± 421 and 3.6 ± 1.2 IU/pancreas, respectively).

Light microscopy

In group CC, the total pancreatic damage (3.71 ± 0.53 points) was significantly decreased versus group ⌀CC (5.07 ± 0.45 points). In group CT, the total pancreatic damage (8.01 ± 0.29 points) was significantly elevated versus group ⌀CT (6.02 ± 0.83 points). The point values for each of the scored parameters are shown in Table 1.

Discussion

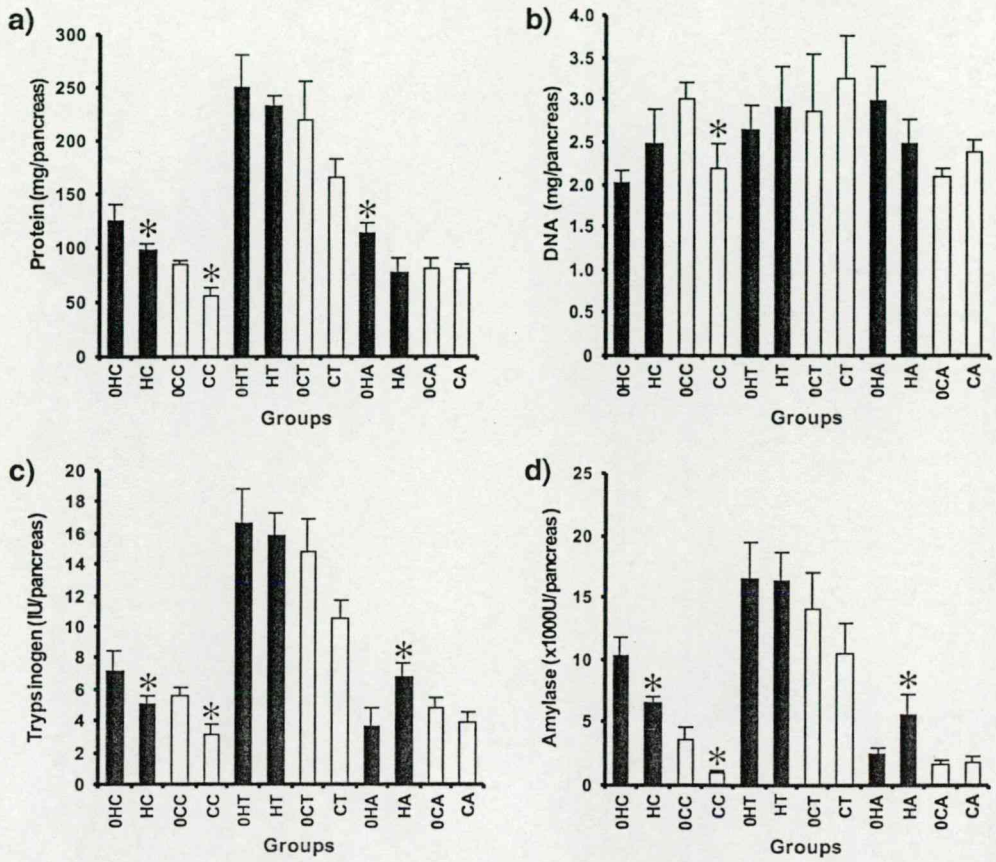
The members of the major cytoprotective HSPs are constitutively expressed or can be induced in the pancreas (Schafer and Williams 2000). Our study was designed to compare the effects of CWI and HWI pretreatment and the potential effects of HSP preinduction (HSP60 and HSP72) on CCK-, TC- and Arg-induced acute pancreatitis. Pancreatitis was induced near the peak of pancreatic HSP expression by administering high doses of CCK or Arg or by the injection of TC into the common biliopancreatic duct.

In agreement with the findings of Otaka et al. (1997), our results demonstrate that CWI specifically induces HSP60 and HWI increases the expression of HSP72 in rats. We found that the levels of HSP60 remained significantly elevated for 9 h after CWI. This is in accordance with what was observed by Lee et al. (2000). The quantity of HSP72 was significantly increased at 3 h after HWI and remained elevated until 12 h. Otaka et al. (1997) reported the HSP72 expression peak at 6 h, which could be due to the differences in the experimental protocols.

Water immersion stress results in a number of stress-induced responses such as the synthesis of a variety of proteins besides HSP60 and HSP72 that may also have protective roles in the prevention of a subsequent stress. For example, the antioxidant enzymes CAT and Mn-SOD were suggested to take part in the protective effect against myocardial ischemia (Kingma et al. 1996; Mizunuma et al. 1984; Yamashita et al. 1998). Therefore, besides HSPs, we also examined the levels of pancreatic antioxidants after water immersion stress. However, we could not detect elevated levels of pancreatic antioxidants.

Previously reported data concerning the protective role of

Fig. 6. Pancreatic contents of (A) protein, (B) DNA, (C) trypsinogen, and (D) amylase in rats with acute pancreatitis. Groups were treated as described in the text. Values are means ± SE for six animals. *, Significant difference ($p < 0.05$) versus the respective control group.



different HSPs against cerulein-induced pancreatitis are conflicting. Wagner et al. (1996) demonstrated that the expression of HSP70 induced by hyperthermia correlated best with the time course and degree of protection against cerulein-induced pancreatitis. Frossard et al. (2001) have also shown that hyperthermia resulted in time-dependent expression of HSP70 within the pancreas associated with a reduction in the severity of acute pancreatitis. In contrast with these data, Otaka et al. (1997) found that the specific preinduction of HSP72 had no preventive effect against cerulein-induced pancreatitis, whereas HSP60 (induced by CWI) did. The beneficial effect of CWI pretreatment and possibly HSP60 against cerulein-induced pancreatitis was also reported by Lee et al. (2000). In fact, our studies confirm that HSP60 might indeed play a role in the protection. In the experiments of Otaka et al. (1997) and Wagner et al. (1996), heating the animals did not increase or just slightly increased the expression of HSP60. Therefore, the possible protective effect of HSP60 could not be excluded by these studies, while the role of HSP72 remained questionable. We considered that the lack of protection against CCK-induced pancreatitis in the case of HWI pretreatment by Otaka et al. (1997) was probably due to the inadequate duration of restraint stress and (or) the increase of the core body temperature of the rats immersed in the hot water bath. Therefore, HSP72 expression did not reach a high enough level to protect the pancreas against acute pancreatitis. In the present study, the duration of the HWI pretreatment was longer than that applied by Otaka et al. (1997) because we hypothesized that our experimental setup would result in a higher core body temperature and consequently a higher HSP72 synthesis. Our results suggest that both HSP60 and HSP72 may play an important part in protecting the pancreas against CCK-induced pancreatic damage. We must note that the HWI pretreatment and possibly even the higher level of HSP72 were not sufficient to decrease the severity of the morphological picture of the disease. This might be due to the different type of preconditioning used in our experiments or the inadequate amount of HSP72 to produce morphological protection.

We found that CWI pretreatment and possibly HSP60 ameliorated the pancreatic edema and the serum amylase level in TC-induced pancreatitis 6 h after the induction of the disease. Unexpectedly, CWI preconditioning significantly worsened the morphological picture of the pancreatitis. This might be due to the microcirculatory changes caused by CWI (Takano et al. 1994), which is supported by the fact that the animals in group CT exhibited a greater vas-

Table 1. Effects of CWI and HWI pretreatment on histologic parameters in CCK-, TC-, and Arg-induced acute pancreatitis.

	Group ⌀HC	Group HC	Group ⌀CC	Group CC
Interstitial edema	1.17±0.12	0.93±0.16	1.29±0.15	1.24±0.21
Leukocyte infiltration	0.78±0.15	0.30±0.06*	0.26±0.02	0.29±0.03
Leukocyte adherence	0.40±0.16	0.35±0.05	0.49±0.05	0.52±0.06
Vacuolization	1.43±0.17	0.95±0.11*	1.15±0.12	0.33±0.05*
Necrosis (0–4)	0.35±0.18	0.14±0.12	0.31±0.20	0.20±0.13
Basophilic lamellation	1.83±0.20	1.93±0.29	1.51±0.08	0.90±0.05*
Apoptosis	1.03±0.13	1.26±0.17	0.38±0.04	0.43±0.05
Total damage	6.63±0.82	5.85±0.87	5.07±0.45	3.71±0.53*
	Group ⌀HT	**Group HT**	**Group ⌀CT**	**Group CT**
Interstitial edema	2.45±0.22	2.47±0.13	2.27±0.20	1.80±0.15*
Leukocyte infiltration	2.07±0.21	1.90±0.17	1.09±0.39	1.05±0.22
Leukocyte adherence	0.73±0.13	0.77±0.11	0.57±0.21	0.42±0.17
Hemorrhage	0.32±0.14	0.35±0.12	0.09±0.08	0.42±0.13*
Necrosis (0–4)	0.50±0.21	0.50±0.18	0.29±0.16	0.60±0.17
Basophilic lamellation	0.62±0.24	0.43±0.08	0.54±0.08	1.06±0.20*
Hyperemia	1.27±0.22	1.33±0.25	1.04 ±0.15	1.98±0.05*
Total damage	7.87±0.84	8.15±0.71	6.02±0.82	7.71±0.29*
	Group ⌀HA	**Group HA**	**Group ⌀CA**	**Group CA**
Interstitial edema	1.23±0.09	1.17±0.45	1.00±0.17	0.86±0.06
Leukocyte infiltration	1.30±0.22	1.01±0.42	0.46±0.15	0.82±0.09*
Leukocyte adherence	0.80±0.26	0.45±0.18	0.74±0.16	0.63±0.18
Vacuolization	0.52±0.08	0.30±0.16	0.52±0.32	0.38±0.17
Necrosis (0–4)	1.12±0.25	0.74±0.40	0.42±0.26	0.57±0.21
Basophilic lamellation	0.65±0.05	0.32±0.15	0.84±0.20	0.39±0.15
Apoptosis	0.64±0.10	0.34±0.19	0.45 ±0.07	1.05±0.41
Total damage	6.36±0.76	4.33±1.26	4.43±1.01	4.70±1.05

Note: Groups were treated as described in the text. Data are means ± SE for six animals. *Significant difference ($p < 0.05$) versus the respective control group.

cular involvement (hyperemia and hemorrhage) than those in the control. It is also plausible that, besides or instead of HSP60, hypothermia itself has a protective effect on the pancreas. To investigate this possibility, in a separate set of experiments, we raised the body temperature of the rodents to 37°C over a 2.5-h period after CWI (results not presented in the paper) in the belief that this would not affect the quantity of HSP60. Unfortunately, the reheating process decreased the level of this protein to the basal value. Nevertheless, the reheating abolished the protective effect seen in PW/BW and the serum amylase level but improved the morphological parameters (histology) to an insignificant difference as compared with the control. HWI pretreatment did not have any effect on the measured parameters of this severe acute necrohemorrhagic pancreatitis.

It is well known that proinflammatory cytokine levels increase during experimental acute pancreatitis (Márton et al. 1998; Takács et al. 1996*a*, 1996*b*). We demonstrated decreases in these cytokine levels after HWI pretreatment in the animals with Arg-induced pancreatitis. Interestingly, even CWI pretreatment decreased the serum IL-1 and TNF-α levels in this acute necrotizing pancreatitis. In this case, we can speculate that the decreased levels of cytokines are due to the reduction of cytokine production in the extrapancreatic origins, since the severity of Arg-induced pancreatitis was not influenced by CWI pretreatment. CWI pretreatment did not influence the serum IL-6 level in this acute pancreatitis model, which closely reflects the severity of acute pancreatitis (Leser et al. 1991). However, we must note that the serum IL-6 level increases after CWI without the induction of pancreatitis (unpublished data).

The differential protective effects of HSPs were called to our attention by several investigators. Cumming et al. (1996) reported that the overexpression of HSP70 protected cardiac cells against subsequent exposure to either thermal or ischemic stress, overexpression of HSP90 produced a protective effect only against thermal stress, while HSP60 had no protective effect. Likely, our experiments suggest that HSP60 and HSP72 may both have differential effects in protecting the pancreas against detrimental stimuli. Their protective actions are not ubiquitous.

In conclusion, we have shown that HWI specifically induces the synthesis of pancreatic HSP72, while CWI specifically elevates the level of HSP60. We demonstrated the differential protective effects of water immersion pretreatment on three acute pancreatitis models. Water immersion pretreatment exerts a definite protective effect in mild pancreatitis, whereas this effect is not seen in more severe pancreatitis models. Our findings suggest the possible roles of HSP60 and HSP72 in the protection against interstitial acute pancreatitis. A decisive proof of the cytoprotective effect of HSPs in this mild acute pancreatitis model would require

further studies in which we would specifically block the expression or function of this protein.

Acknowledgements

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IV.

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Induction of HSP72 by Sodium Arsenite Fails to Protect Against Cholecystokinin–Octapeptide-Induced Acute Pancreatitis in Rats

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A number of investigators have demonstrated that the preinduction of heat-shock protein (HSP) expression (particularly HSP60 and HSP72) by hyper- or hypothermia may have a protective effect against cerulein-induced acute pancreatitis. The aim of the present study was to induce HSPs in the pancreas and lungs by thermal (hot-water immersion, HWI) and nonthermal methods (injection of sodium arsenite intraperitoneally) and to investigate the potential effects of HSP preinduction on cholecystokinin-octapeptide (CCK) induced acute pancreatitis and pancreatitis-associated lung injury in rats. The dose–response and time–effect curves observed following HWI and sodium arsenite treatments were evaluated. Animals were injected with 3 × 75 μg/kg CCK subcutaneously at intervals of 2 hr at the peak level of HSP synthesis, as determined by Western blot analysis. The rats were killed by exsanguination through the abdominal aorta 2 or 6 hr after the last CCK injection. HWI and the injection of sodium arsenite significantly elevated the expression of HSP72 in the pancreas and lungs, whereas they did not influence the levels of HSP60. Overall, HWI pretreatment had a protective effect against CCK-induced pancreatitis and pancreatitis-associated lung injury. In contrast, the nonthermal preinduction of HSP72 by sodium arsenite did not result in any beneficial effects on the measured parameters of the disease. The findings suggest that the preinduction of HSP72 is not sufficient to protect against CCK-induced acute pancreatitis and pancreatitis-associated lung injury or that the beneficial effect of hyperthermia may not be exclusively related to HSP72 expression.

KEY WORDS: heat-shock proteins; HSP72; hot-water immersion; sodium arsenite; cholecystokinin–octapeptide; pancreatitis.

Cells subjected to stress respond by synthesizing heat-shock proteins (HSPs) (1). The HSPs are a group of highly conserved, ubiquitous, and functionally related proteins that play an essential part in cell survival (1, 2). They are involved in the synthesis, folding, transport, and translocation of proteins, and the assembly

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and disassembly of oligomers. HSPs are divided into different families, according to their molecular mass (eg, HSP60 and HSP72) (1, 2). The HSP families have several functional homologs in the different compartments of cells. HSP60 is mainly a mitochondrial protein, but it can also be found in the pancreatic zymogen granules (3). HSP72 is located in the cytoplasm of cells. They are expressed constitutively (eg, HSP60) and/or at elevated levels (eg, HSP72) upon exposure of the cells to a variety of stress conditions (1). Induction of the heat-shock response enhances the ability of cells to overcome the effects of further stress (2). It has been demonstrated that the preinduction of HSP expression (particularly HSP60 and HSP72) by hyper- or hypothermia may have a protective effect against cerulein-induced acute pancreatitis (4–8). However, Kruger et al have established that, although hyperthermia can directly abolish the earliest initiating event involved in the onset of pancreatitis (the premature and intracellular activation of digestive zymogens), this is independent of the increased pancreatic HSP synthesis (9). Changes in the body temperature of animals have diverse effects on the organism in addition to the induction of HSPs (10, 11). It is possible that the protective effect of heating (4–8) is not due merely to HSP synthesis, but rather to nonspecific effects such as inhibition of NF-κB binding activity (5) or hormonal responses (10–12).

The aim of the present study was to investigate whether hot-water immersion (HWI) and sodium arsenite (SA) can induce HSP60 and HSP72 in the pancreas and lungs and to evaluate the potential effects of HSP preinduction on CCK-induced acute pancreatitis and pancreatitis-associated lung injury in rats. SA itself does not influence the body temperature of the animals (13); the confounding effects of hyper- or hypothermia that accompanies HSP induction could therefore be bypassed.

MATERIALS AND METHODS

Experimental Protocol

Animals. Male Wistar rats weighing 250–300 g were used. The animals were kept at a constant room temperature of 25°C with a 12-hr light–dark cycle, and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). The rats were fasted for 12 hr before the induction of acute pancreatitis. The experiments performed in this study were approved by the Animal Care Committee of the University.

HWI Stress. HWI stress was performed according to Otaka et al (7) with some modifications. The rats were anesthetized with pentobarbital (PB) (44 mg/kg, intraperitoneally) at the starting point of the experiment (t_0). The animals were then immersed vertically in a 37°C water bath, and the water temperature was then gradually increased to 42°C (during 55 min) and maintained there for 20 min (total 1 hr 15 min). In order to evaluate the expressions of HSP60 and HSP72 after the HWI stress, four rats were killed at each time point before (t_0), immediately after (0), or 3, 6, 9, or 12 hr after the end of the immersion. The pancreas was quickly removed, cleaned of fat and lymph nodes, and frozen at −80°C until processing.

HSP Time Course and Dose–Response After SA Injection. Twenty-eight rats were injected intraperitoneally with progressive doses of SA (2–14 mg/kg body weight) (Merck, Darmstadt, Germany) to investigate the SA dose–response. The control animals ($N = 4$) received physiological saline injection intraperitoneally. The animals were killed by exsanguination through the abdominal aorta 12 hr after the injections. The pancreas and lungs were quickly isolated and frozen at −80°C until western blot analysis was performed. In order to evaluate the time-course response of the expressions of HSP60 and HSP72, the least toxic SA dose producing a high amount of HSP was chosen. A group of 32 animals received 10 mg/kg body wt of SA intraperitoneally and were killed at different time points after the injection (3–48 hr). The control animals received a saline injection intraperitoneally and were killed after 12 hr. The pancreas and lungs were processed for HSP determinations.

CCK-Induced Pancreatitis. Acute pancreatitis was induced near the peak of the HSP synthesis by injecting 75 μg/kg body wt CCK subcutaneously three times at intervals of 2 hr. In group HC (HWI + CCK; $N = 6$), the rats were subjected to HWI and after a 6-hr recovery period they received CCK as mentioned before. In group ØHC (No HWI + CCK; $N = 6$), the rodents were anesthetised by PB, were kept at room temperature for 7 hr 15 min, and then they were then given CCK injections (Figure 1A). In groups AC-2 ($N = 6$) and AC-6 ($N = 6$) (SA + CCK), the rats were injected with 10 mg/kg of SA intraperitoneally, and then received CCK subcutaneously, starting at 9 hr after the SA injection. In groups SC-2 ($N = 6$) and SC-6 ($N = 6$) (saline + CCK), the animals were injected with physiological saline intraperitoneally instead of SA, and the 3 × 75 μg/kg body wt CCK was administered after 9 hr (Figure 1B). The animals were killed by exsanguination through the abdominal aorta 2 or 6 hr after the last CCK injection, as indicated by the number after the dash in the group names. The pancreas and lungs were quickly removed, cleaned of fat and lymph nodes, weighed, and frozen at −80°C until use.

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Western Blotting

Samples of the pancreas and lungs were homogenized in a fourfold excess (w/v) of ice-cold buffer containing 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 4 mM benzamidine, 5 mM iodoacetamide, 1.5 mM PMSF, and 100 IU/ml aprotinin, using an Ultra-Turrax homogenizer for 2 min. The homogenates were centrifuged at 20,000*g* for 20 min. The supernatants were collected and the protein concentrations were measured by the microbiuret method of Goa (14). Fifty micrograms of protein was loaded per lane. Samples were elec-

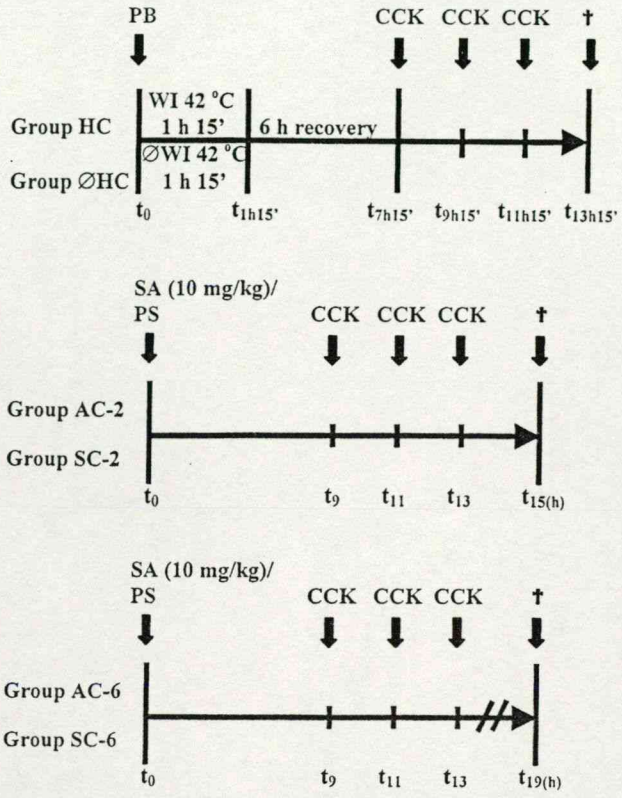

Fig 1. Experimental protocol. (A) In group HC and ØHC, the rats were anesthetized with 44 mg/kg body wt pentobarbital (PB) intraperitoneally at the starting point of the experiment (t_0). The rats in group HC were immersed vertically in a 37°C water bath, and the water temperature was then gradually increased to 42°C and maintained there for 20 min (total 1 hr 15 min). At 6 hr after the hot-water immersion (HWI), the rats received 75 μg/kg body wt cholecystokinin–octapeptide (CCK) subcutaneously three times at intervals of 2 hr. In group ØHC, the rodents were given CCK injections, starting at t_0 + 7 hr 15 min. The rats were killed (†) 2 hr after the last CCK treatment. (B) In groups AC-2 (N = 6) and-AC-6 (N = 6) (SA + CCK), the rats were injected with 10 mg/kg of sodium arsenite (SA) intraperitoneally at the beginning of the experiment (t_0), and then received 75 μg/kg body wt CCK subcutaneously three times at intervals of 2 hr, starting at 9 hr (t_9) after the SA injection. In groups SC-2 (N = 6) and SC-6 (N = 6) (saline + CCK), the animals were injected with physiological saline intraperitoneally instead of SA, and the 3 × 75 μg/kg body wt CCK was administered after 9 hr. The rats were killed (†) 2 or 6 hr after the last CCK injection as indicated by the number after the dash in the group names.

trophoresed on an 8% sodium dodecylsulfate–polyacrylamide gel according to the method of Laemmli (15). The gels were either stained with Coomassie brilliant blue (to demonstrate equal loading of proteins for western blot analysis) or transferred to a nitrocellulose membrane for 2.5 hr at 30 V. Membranes were blocked in 5% nonfat dry milk for 1 hr, and incubated with rabbit anti-HSP60 [produced by ourselves (16), 1:60,000 dilution] or anti-HSP72 (1:5000 dilution) [a generous gift from István Kurucz, Biorex Laboratories, Veszprém, Hungary, which has been characterized previously (17)] antibody for an additional 1 hr at room temperature. The immunoreactive protein was visualized by enhanced chemiluminescence, using horseradish peroxidase-coupled anti-rabbit immunoglobulin at 1:10,000 dilution (Dako, Glostrap, Denmark).

Assays

Pancreatic Weight/Body Weight Ratio. The pw/bw ratio was utilized to evaluate the degree of pancreatic edema.

Serum Amylase Activity. All blood samples were centrifuged at 2500*g* for 20 min. The serum levels of amylase were determined by a colorimetric kinetic method (Dialab, Vienna, Austria).

Serum Cytokine Concentrations. IL-1β concentrations were determined with an ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions. IL-6 concentrations were measured via their proliferative action on the IL-6-dependent mouse hybridoma cell line B-9 (18). The activities were calibrated against recombinant TNF (Genzyme, Cambridge, UK) and recombinant IL-6 (Sigma-Aldrich, Munich, Germany).

Pancreatic Contents of Amylase, Trypsinogen, Lipase, DNA, and Protein. The pancreas was homogenized in a ninefold excess (w/v) of ice-cold buffer containing 0.02 M Tris HCl, pH 7.8, 0.15 M NaCl, and 0.1% Triton X-100. Enzyme measurements were carried out on the supernatant fractions of the homogenates after centrifugation at 20,000*g* for 30 min. Pancreatic amylase activities were determined as described above. Trypsinogen was activated after a 200-fold dilution of the homogenate with 0.02 units enterokinase/μg pancreatic protein (Sigma, St. Louis, Minneapolis, USA) in the enzyme buffer containing 80 mM Tris HCl, pH 8.0, 25 mM $CaCl_2$, and 100 μg/ml bovine serum albumin for 120 min at 37°C (19). Lipase activities were measured by a pH-stat method (19). Samples for DNA determination were precipitated with ice-cold 0.8 M perchloric acid, washed in 5% trichloroacetic acid, and then hydrolyzed with 0.8 M perchloric acid at 90°C for 10 min (20). DNA was estimated photometrically with diphenylamine (21). The protein concentrations in the supernatant fractions of the homogenates were measured by the method of Goa (14).

Lung Myeloperoxidase Activity. Lung myeloperoxidase activity, as a marker of tissue leukocyte infiltration, was determined by the method of Kuebler et al (22).

Histological Examination

Light Microscopy. A 2 to 3-mm^3 portion of the pancreas was fixed in an 8% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4 μm thickness and stained with hematoxylin and eosin. The slides were coded and read for the traditional histological markers of pancreatic tissue injury by two independent observers who were blind to the experimental protocol. Semiquantitative grading of interstitial edema, leukocyte infiltration, hyperemia, and vacuolization, necrosis, and apoptosis of acinar cells was performed on 8–10 consecutive high-power fields (×400) on a scale of 0–3 or 0–4. Additionally, basophilic lamellation of the cytoplasm of acinar cells was also graded, since a pilot study revealed that, besides the traditional markers, the areas of basophilic lamellation were more extensive in the more severely damaged pancreata. The score for each graded parameter was

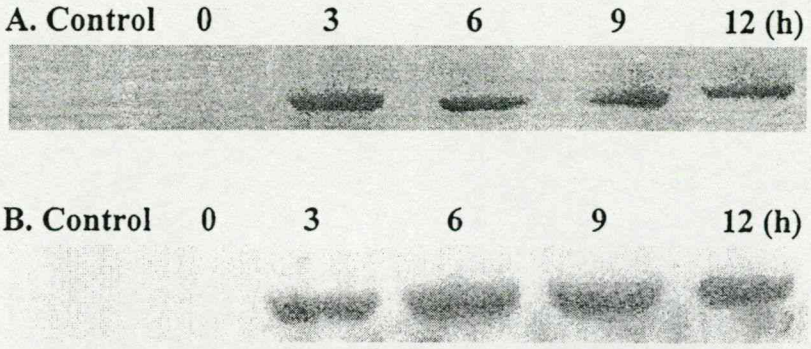

Fig 2. Effect of water immersion stress on pancreatic heat shock protein synthesis. Representative western immunoblots of protein lysates (20 μg/lane) extracted from the (A) pancreas and (B) lungs harvested over a time course after the water immersion treatments (0–12 hr) showing the expression of HSP72. The control did not receive any treatment. HSP72 could not be detected in the unstressed control, but its expression was significantly increased at 3 hr after the HWI and remained elevated until 12 hr in both the pancreas and lungs.

averaged, and the total pancreatic damage was calculated by adding all the averages together. The grading system and basophilic lamellation are described in more detail in one of our previous manuscripts (16).

Statistical Analysis

Results are expressed as means ± SEM. Experiments were evaluated by using the Student's *t* test when the data consisted of two groups, or by analysis of variance when three or more groups were compared. Values of $P < 0.05$ were accepted as significant.

RESULTS

Response of HSP Expression to HWI and Various Doses of SA Injection. HSP72 could not be detected in the unstressed control, but its expression was significantly increased at 3 hr after the HWI and remained elevated until 12 hr in the pancreas and lungs (Figure 2). The progressive doses of SA produced an increased expression of HSP72 in both of the examined organs (Figure 3). A 25% mortality rate was observed in the group of animals that received 14 mg/kg body wt of SA. Moreover, most of the animals in this group appeared lethargic and anorectic. No mortality occurred at lower doses of SA. The time course of HSP72 expression after SA treatment was obtained by using the 10 mg/kg body wt dose. HSP72 was already significantly increased at 3 hr, peaked at 9–24 hr after the SA injection, and remained elevated until 48 hr in both organs (Figure 4). HSP60 is constitutively expressed in the pancreas and lungs. HWI and SA did not have a significant effect on its expression (results not shown).

The pw/bw Ratio and Serum Amylase Activity. The administration of 3 × 75 μg/kg body wt. CCK induced the typical laboratory and morphological changes of experimental acute pancreatitis. In group HC, the pw/bw (4.05 ± 0.27 mg/g) and the serum amylase activity (12,428 ± 1134 units/liter) were significantly decreased versus group ØHC (4.89 ± 0.28 mg/g and 18,251 ± 1993 units/liter, respectively). In group AC-2, the serum amylase activity (10,515 ± 1539 units/liter was significantly decreased versus group

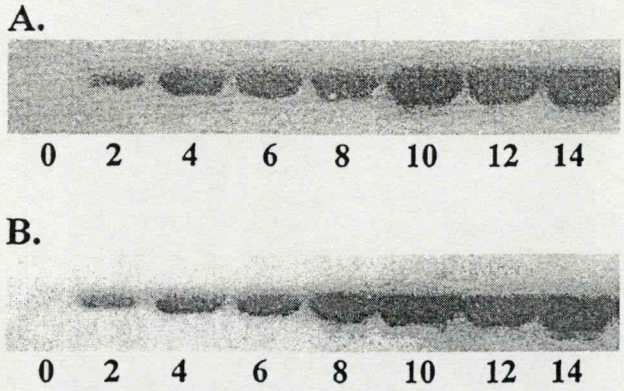

Fig 3. Effects of progressive doses of SA injection on the synthesis of HSPs in the pancreas and lungs. The figure depicts representative western immunoblot analysis of protein lysates (50 μg/lane) from the (A) pancreat and (B) lungs of rats, showing the expression of HSP72 after the injection of progressive doses of SA. Twenty-eight animals were injected intraperitoneally with progressive doses of SA (2–14 mg/kg body wt) (Merck, Darmstadt, Germany); the control rats (0, $N = 4$) received a physiological saline injection intraperitoneally. The rats were killed 12 hr after the injection, and the pancreas and lungs were quickly isolated and frozen at −80°C for western blot analysis. HSP72 could not be detected in the control group, but progressive doses of SA produced an increased expression of HSP72 in the pancreas and lungs. HSP60 was constitutively expressed in the pancreas and lungs, SA did not have a significant effect on its expression. A 25% mortality rate was seen in the group of animals that received 14 mg/kg body wt of SA.

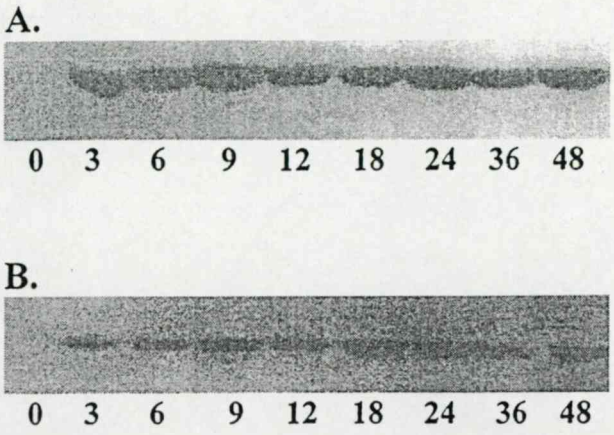

Fig 4. Effects of SA injection on the synthesis of HSPs in the pancreas and lungs as a function of time. Representative western immunoblot analysis of protein lysates (50 μg/lane) from the (A) pancreat and (B) lungs of rats, showing the expression of HSP72 as a function of time after SA injection. A group of 32 animals were injected with 10 mg/kg body wt of SA intraperitoneally and were killed at different time-points after the injection (3–48 hr). The control animals (0; $N = 4$) received a physiological saline injection intraperitoneally and were killed after 12 hr. HSP72 could not be detected in the control group, but its expression was already significantly increased at 3 hr, peaked at 9–24 hr after the SA injection, and remained elevated until 48 hr in both organs. HSP60 was constitutively expressed in the pancreas and lungs; SA did not have a significant effect on its expression (results not shown).

SC-2 (16,150 ± 1633 units/liter). Otherwise, SA pretreatment had no effect on pw/bw and serum amylase activity (Figure 5).

Serum Cytokine Levels. In group HC, the serum levels of IL-1 (5.3 ± 2.1 pg/ml) and IL-6 (28.0 ± 8.6 pg/ml) were all significantly decreased versus the corresponding values in group ØHC (34.8 ± 7.2 pg/ml, and 60.0 ± 10.0 pg/ml, respectively). No significant changes were observed in the serum IL-1 and IL-6 levels in the SA-pretreated groups versus the respective controls (Figure 6). F6

Pancreatic Contents of DNA, Protein, Amylase, Trypsinogen, and Lipase. The pancreatic contents of protein were significantly decreased in group HC (85 ± 6 mg/pancreas) versus group ØHC (115 ± 11 mg/pancreas). The pancreatic contents of amylase, trypsinogen, and lipase in group HC (6280 ± 690 IU/pancreas, 7.98 ± 0.91 IU/pancreas, 310 ± 29 IU/pancreas, respectively) were significantly elevated versus group ØHC (4,120 ± 450 IU/pancreas, 5.30 ± 0.65 IU/pancreas, 220 ± 24 IU/pancreas, respectively). In group AC-6, the pancreatic contents of protein (95 ± 13 mg/pancreas), amylase (4692 ± 1008 IU/pancreas), and trypsinogen (7.6 ± 1.2 IU/pancreas) were significantly decreased versus group SC-6 (126 ± 7 mg/pancreas, 8369 ± 2020 IU/pancreas and 11.1 ± 0.9 IU/pancreas, respectively) (Figure 7). F7

Lung Myeloperoxidase Activity. In group HC, the lung myeloperoxidase activity (25.2 ± 4.7 U/mg protein/min) was significantly decreased versus group ØHC (45.1 ± 9.6 Units/mg protein/min). No signifi-

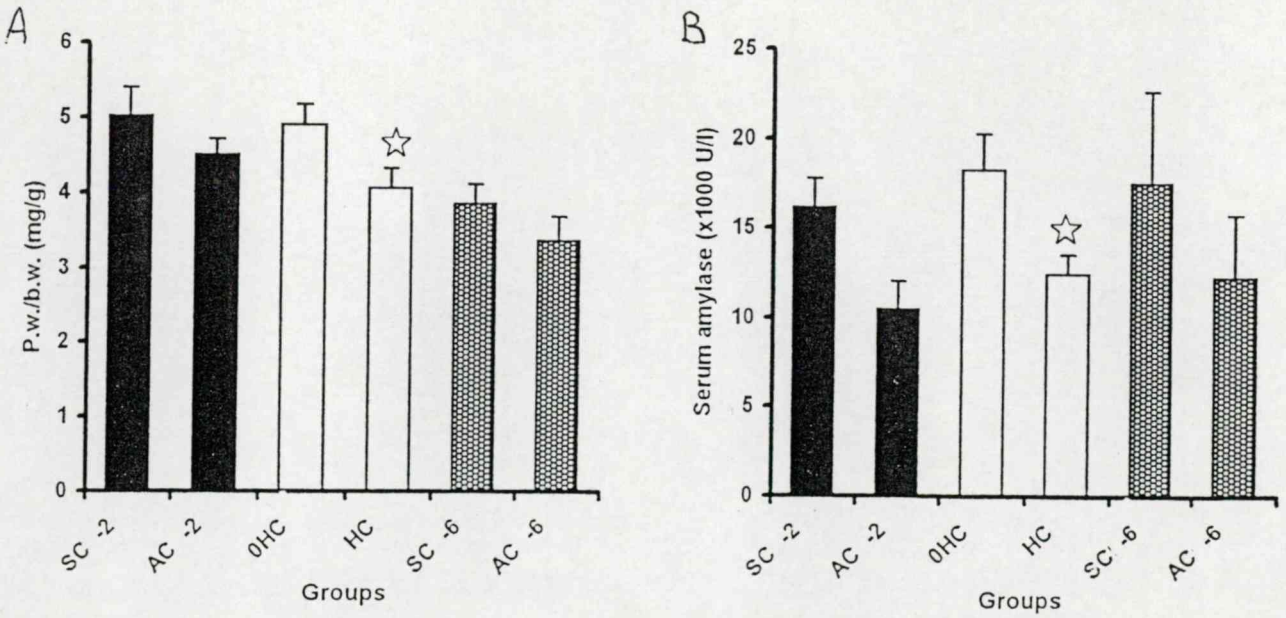

Fig 5. (A) The pancreatic weight/body weight ratio (pw/bw) and (B) serum amylase levels in groups ØHC, HC, SC-2, AC-2, SC-6, and AC-6. The groups were treated as indicated in Figure 1. Means ± SEM for six animals are shown. ☆Significant difference ($P < 0.05$) versus the respective control group.

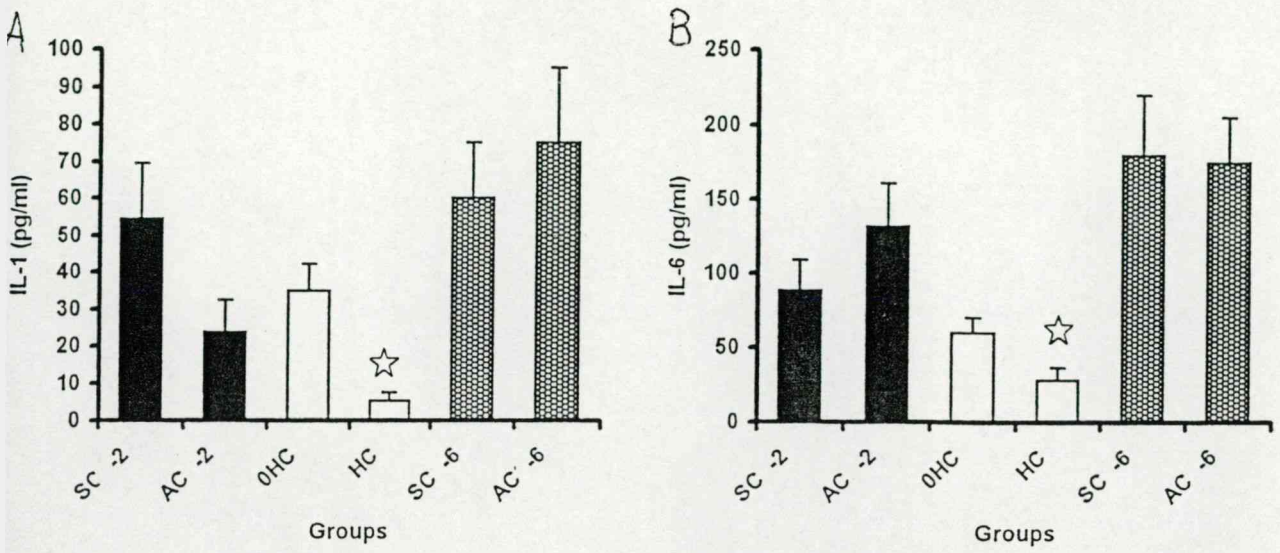

Fig 6. Serum (A) IL-1 and (B) IL-6 levels in groups ØHC, HC, SC-2, AC-2, SC-6, and AC-6. Groups were treated as indicated in Figure 1. Means ± SEM for six animals are shown. ☆Significant difference ($P < 0.05$) vs the respective control group.

cant change in lung myeloperoxidase activity was detected between the SA-treated and the control groups (Figure 8).

Light Microscopy. No significant alteration was observed between the groups as concerns the total pancreatic damage (Figure 9). The point values for each of the scored parameters are shown in Table 1.

DISCUSSION

The members of the major cytoprotective HSPs are constitutively expressed or can be induced in the pancreas (23). The induction of HSPs by thermal methods has been shown to protect the pancreas against cerulein-induced acute pancreatitis (4–8). However, HSP expression can also be induced by various nonthermal methods, including the use of oxygen-derived free radicals, endotoxins, and SA; in this way the confounding effects of hyperthermia are bypassed (24). Our study was designed to investigate the *in vivo* dynamics of HSP induction (HSP60 and HSP72) in the pancreas and lungs in response to HWI or SA and to compare the potential effects of HSP preinduction on CCK-induced acute pancreatitis and pancreatitis-associated lung injury in rats.

In agreement with the findings of Otaka et al (7), our results demonstrate that HWI increases the expression of HSP72 in the pancreas of rats. Intravenous administration of SA is known to increase the level of HSP72 in the kidney, heart, and liver of rabbits (25). Our results are in accordance with those of Ribeiro et al (13), who demonstrated that the injection of SA specifically induces HSP72 in the lungs of rats. Ribeiro et al used a dose of 6 mg/kg intravenously to induce HSP72 (13). In their hands, a dose of 10 mg/kg produced signs of acute poisoning in the animals (this appeared at a higher dose in our experiments). However, the route of SA administration was different. Moreover, our study revealed that HSP72 was also induced in the pancreas of the animals. The levels of HSP60 were unchanged after HWI or SA treatment in both of the examined organs. Similarly, Wijeweera et al showed that SA treatment does not influence HSP60 expression (whereas the level of HSP72 increases) in precision-cut rat lung slices (26).

Acute pancreatitis was induced near the peak of HSP expression in the pancreas (6 hr after the HWI treatment or 9 hr after the SA injection) by administering high doses of CCK subcutaneously. The administration of CCK resulted in the typical laboratory (hyperamylasemia) and morphological changes (interstitial edema, leukocyte infiltration and acinar cell injury) of acute pancreatitis 2 or 6 hr after the last CCK injection (27). HWI pretreatment ameliorated most of the examined laboratory and some morphological parameters of CCK-induced pancreatitis. In contrast, we did not find such an effect after SA pretreatment. Morover, the pancreatic enzyme contents were more depleted in one of the SA-pretreated groups (AC-6), which indicates a more severe pancreatitis. The lung injury was assessed via myeloperoxidase activity. HWI pretreatment decreased leukocyte infiltration into the lungs, whereas we did not see

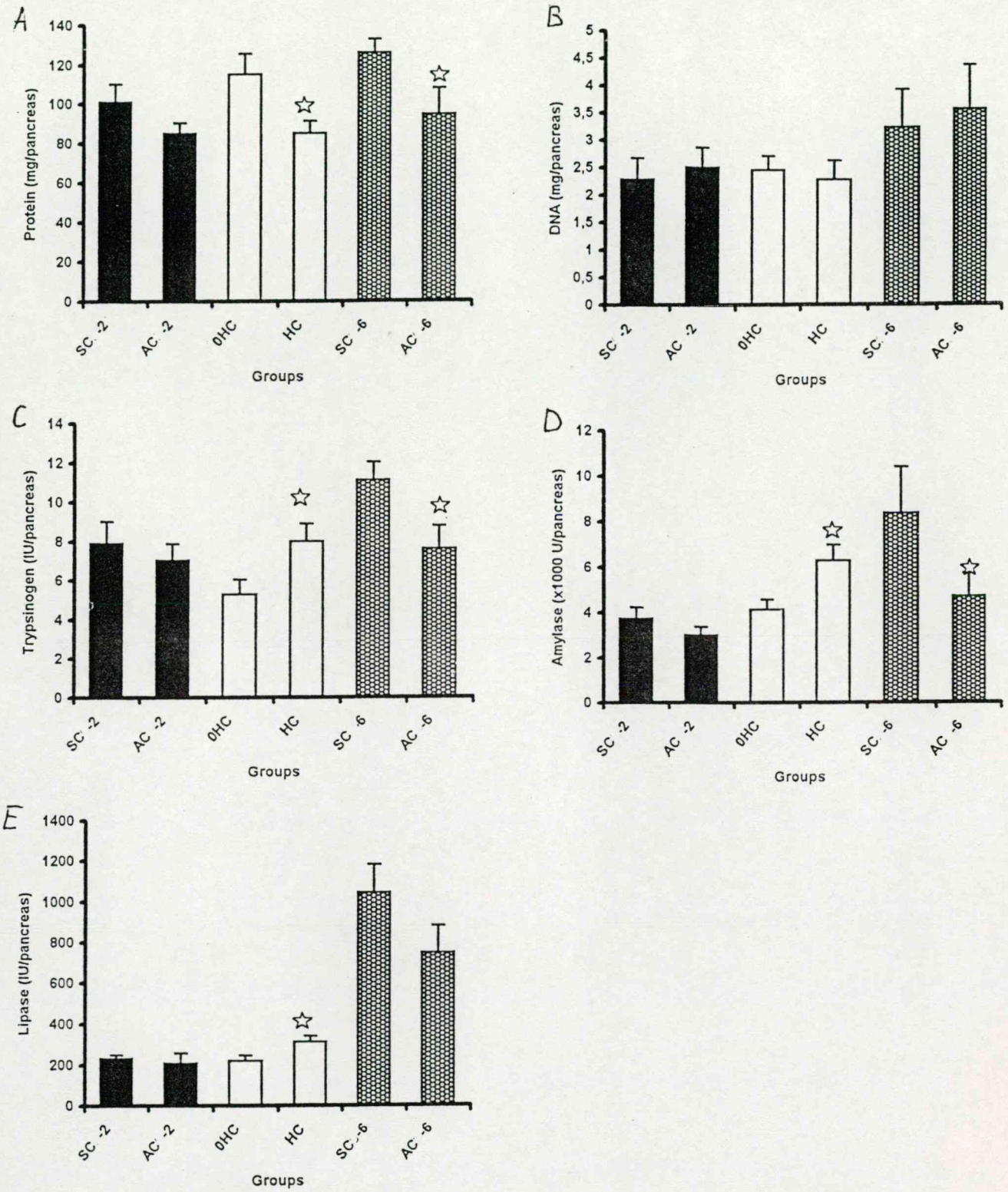

Fig 7. Pancreatic contents of (A) protein, (B) DNA, (C) trypsinogen, (D) amylase, and (E) lipase in groups. ØHC, HC, SC-2, AC-2, SC-6, and AC-6. Groups were treated as indicated in Figure 1. Data are means ± SEM for six animals. ☆Significant difference ($P < 0.05$) versus the respective control group.

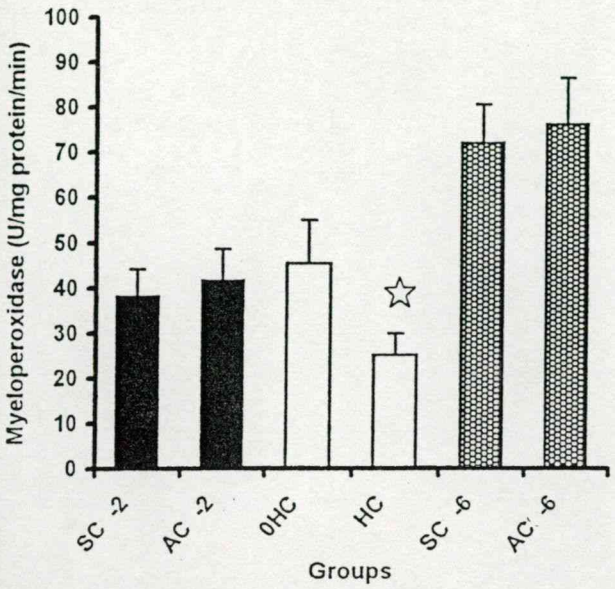

Fig 8. Lung myeloperoxidase activities in groups ØHC, HC, SC-2, AC-2, SC-6, and AC-6. Groups were treated as indicated in Figure 1. Data are means ± SEM for six animals. ☆Significant difference ($P < 0.05$) versus the respective control group.

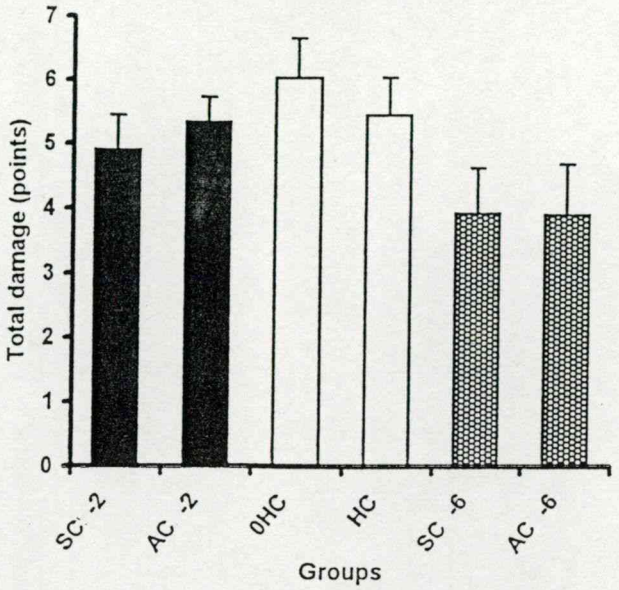

Fig 9. Total morphological pancreatic damage as assessed by histological evaluation in groups ØHC, HC, SC-2, AC-2, SC-6, and AC-6. of The interstitial edema, leukocyte infiltration, hyperemia, and vacuolization, necrosis, apoptosis, and basophilic lamellation of acinar cells were assessed by semiquantitative grading in 8–10 consecutive high-power fields (×400) on a scale of 0–3 or 0–4. The score for each graded parameter was averaged and the total pancreatic damage was calculated by adding all the averages together. Groups were treated as indicated in Figure 1. Data are means ± SEM for six animals.

such an effect after SA treatment, despite the induction of HSP72.

Previously reported data concerning the protective roles of different HSPs (particularly HSP60 and HSP72) against cerulein-induced pancreatitis are somewhat conflicting. Although HSPs have been implicated as mediators of pancreatic protection, these proteins were almost always induced by thermal methods (4–8). Kruger et al found that, although hyperthermia can directly abolish the premature and intracellular activation of digestive zymogens in cerulein-induced pancreatitis, this is independent of the synthesis of pancreatic HSPs (9). Therefore, it is possible that the protective effect of heating or cooling is due not merely to increased HSP synthesis, but also to nonspecific effects such as inhibition of NF-κB binding activity (5) or hormonal release (10, 11). *In vitro* studies have shown that culture stress-induced HSP72 can prevent the intraacinar cell activation of trypsinogen caused by cerulein in freshly prepared rat pancreas segments (28). Wagner et al (4) demonstrated that the expression of HSP70 induced by hyperthermia (the core body temperature of the animals was elevated to 42°C with a heat pad and lamp and maintained there for 20 min) correlated best with the time course and degree of protection against cerulein-induced pancreatitis. Similarly, Frossard et al (5) showed that hyperthermia (provoked by 42°C hot-water immersion) resulted in a time-dependent

TABLE 1. EFFECTS OF SODIUM ARSENITE PRETREATMENT ON HISTOLOGIC PARAMETERS IN CHOLECYSTOKININ–OCTAPEPTIDE-INDUCED ACUTE PANCREATITIS*

	Group SC-2	*Group AC-2*	*Group ØHC*	*Group HC*	*Group SC-6*	*Group AC-6*
Interstitial edema	1.40 ± 0.15	1.08 ± 0.16	1.20 ± 0.13	1.02 ± 0.09	1.13 ± 0.16	1.19 ± 0.24
Leukocyte infiltr.	0.56 ± 0.11	0.48 ± 0.09	0.70 ± 0.16	0.22 ± 0.05*	0.68 ± 0.38	0.37 ± 0.20
Hyperemia	0.27 ± 0.07	0.38 ± 0.10	0.39 ± 0.11	0.45 ± 0.08	0.70 ± 0.16	0.60 ± 0.13
Vacuolization	0.88 ± 0.14	0.92 ± 0.12	1.11 ± 0.19	0.70 ± 0.08*	0.32 ± 0.14	0.35 ± 0.07
Necrosis (0–4)	0.42 ± 0.07	0.48 ± 0.08	0.39 ± 0.08	0.25 ± 0.05	0.45 ± 0.06	0.42 ± 0.09
Apoptosis	0.73 ± 0.15	0.98 ± 0.19	0.90 ± 0.15	0.95 ± 0.16	0.34 ± 0.05	0.24 ± 0.04
Basoph. lam.	0.96 ± 0.19	1.35 ± 0.12	1.38 ± 0.18	1.32 ± 0.13	0.30 ± 0.12	0.73 ± 0.27
Total damage	4.90 ± 0.56	5.34 ± 0.38	6.03 ± 0.63	5.45 ± 0.58	3.93 ± 0.70	3.90 ± 0.78

*Groups were treated as indicated in Figure 1. Data are means ± SEM for six animals.

expression of HSP70 within the pancreas, which was associated with a reduction in the severity of cerulein-induced pancreatitis. In contrast with these data, Otaka et al (7) found that the specific preinduction of HSP72 by hot-water immersion (42°C, 20 min) did not have a preventive effect against cerulein-induced pancreatitis, whereas the preinduction of HSP60 (induced by immersion in 23°C water for 6 hr) did. In fact, our previous study demonstrated the beneficial effects of cold- and hot-water immersion pretreatments in CCK-induced acute pancreatitis (16). The protective effects of cold-water immersion pretreatment, and possibly HSP60, against cerulein-induced pancreatitis were also reported by Lee et al (8). Unfortunately, the present study does not confirm that increased HSP60 synthesis can play a role in the protection, since it was not influenced by HWI or SA injection. Although a simple up-regulation of HSP72 is clearly not sufficient for protection, our results do not completely rule out the protective effect of HSP72 in CCK-induced pancreatitis after hyperthermia, since the elevation of body temperature could result in conformational changes and posttranslational modifications of the HSPs which could account for the protective effect (29). Taken together, the possible protective effect of HSP60 in CCK-induced acute pancreatitis is not excluded by our experiments, while the role of HSP72 remains questionable.

In conclusion, we have revealed that HWI or an intraperitoneal injection of SA specifically and dose-dependently induces the synthesis of HSP72 in the pancreas and lungs of rats. We demonstrated that HWI pretreatment ameliorates CCK-induced pancreatitis and pancreatitis-associated lung injury. This protective effect of hyperthermia seems to be independent of the increased HSP72 synthesis since the nonthermal induction of HSP72 failed to reduce the severity of CCK-induced acute pancreatitis and pancreatitis-associated lung injury. Nevertheless, the protective nature of hyperthermia in this experimental acute pancreatitis model warrants further investigation.

ACKNOWLEDGMENTS

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7. ANNEX

V.

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Original Contribution

NONTOXIC HEAT SHOCK PROTEIN COINDUCER BRX-220 PROTECTS AGAINST ACUTE PANCREATITIS IN RATS

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Abstract—Background: Nontoxic heat shock protein (HSP) inducer compounds open up promising therapeutic possibilities by activating one of the natural and highly conserved defense mechanisms of the organism. Aims: In the present experiments, we examined the effects of a HSP coinducer drug-candidate, BRX-220, on the cholecystokinin-octapeptide (CCK)-induced acute pancreatitis in rats. Methods: Male Wistar rats weighing 240 to 270 g were divided into two groups. In group B, 20 mg/kg BRX-220 was administered orally, followed by 75 μg/kg CCK subcutaneously three times, after 1, 3, and 5 h. This whole procedure was repeated for 5 d. The aminals in group ∅B received physiological saline orally instead of BRX-220, but otherwise the protocol was the same as in group B. The rats were exsanguinated through the abdominal aorta 12 h after the last administration of CCK. We determined the serum amylase activity, the plasma trypsinogen activation peptide concentration, the pancreatic weight/body weight ratio, the DNA and total protein contents of the pancreas, the levels of pancreatic HSP60 and HSP72, the activities of pancreatic amylase, lipase, trypsinogen, and free radical scavenger enzymes (superoxide dismutase, catalase, and glutathione peroxidase), the degree of lipid peroxidation, protein oxidation, and the reduced glutathione level. Histopathological investigation of the pancreas was also performed in all cases. Results: Repeated CCK treatment resulted in the typical laboratory and morphological changes of experimentally induced pancreatitis. The pancreatic levels of HSP60 and HSP72 were significantly increased in the animals treated with BRX-220. In group B, the plasma trypsinogen activation peptide concentration, the pancreatic total protein content and the amylase and trypsinogen activities were also significantly higher vs. group ∅B. The lipid peroxidation, protein oxidation, and the activity of Cu/Zn-superoxide dismutase were significantly decreased in group B vs. group ∅B, whereas the glutathione peroxidase activity was increased. The morphological damage in group B was significantly lower than that in group ∅B. Conclusion: The HSP coinducer BRX-220, administered for 5 d, has a protective effect against CCK-induced acute pancreatitis. © 2002 Elsevier Science Inc.

Keywords—Heat shock protein, Coinducer, BRX-220, Cholecystokinin-octapeptide, Pancreatitis, Free radicals

INTRODUCTION

Cells respond to heat shock or other stresses with the rapid synthesis of heat-shock proteins (HSPs) [1]. The induction of the heat shock response enhances the ability of the cells to overcome the effects of the stress [2]. Following the development of stress conditions, many cellular proteins become partially or completely denatured or malfolded. HSPs recognize this, bind to the damaged proteins, and stabilize and refold them, thereby preventing or dissolving otherwise irreversible aggregation. HSPs are also necessary during normal physiological conditions since they are involved in the synthesis, degradation, folding, transport, and translocation of proteins [2]. HSPs have been classified into six families according to their molecular mass (e.g., HSP60 and HSP72). It has been shown that the pre-induction of HSP expression has a protective effect

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against cerulein-induced pancreatitis in rats or choline-deficient ethionine-supplemented diet model pancreatitis in mice [3–9]. Strowski et al. demonstrated that cerulein pancreatitis in itself increases mRNA but paradoxically reduces protein levels of rat pancreatic HSPs [10]. These observations even suggest that the low levels of pancreatic HSPs might be involved in the development of cerulein-induced pancreatitis. For the above-mentioned reasons, it may be speculated that the administration of HSP-inducer compounds during cholecystokinin-octapeptide(CCK)-induced pancreatitis should ameliorate the severity of the disease. However, the main problem regarding HSP induction is that the HSPs are mostly induced by harmful conditions. The real challenge is to upregulate HSP synthesis without any toxic side effects.

The aim of the present study was to investigate the potential effects of a nontoxic HSP coinducer drug-candidate, BRX-220 {(+)-*R*/-N-[2-hydroxy-3-(1-piperidinyl)-propoxy]-pyridine-1-oxide-3-carboximidoil-chloride (Z)-maleate (1:1)}, on CCK-induced acute pancreatitis in rats. BRX-220 is a structural relative of bimoclomol, which can increase the production of HSPs only in the presence of a stress condition [11]. In our case, this stress condition was pancreatitis.

MATERIALS AND METHODS

Experimental protocol

Male Wistar rats weighing 240–270 g were used. The animals were kept at a constant room temperature of 25°C with a 12 h light-dark cycle, and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). The rats were fasted 18 h before the end of the experiment. In group B ($n = 6$), 20 mg/kg BRX-220 was administered intragastrically, followed by 75 μg/kg CCK subcutaneously three times, after 1, 3, and 5 h. This whole procedure was repeated for 5 d. The animals in group ØB ($n = 6$) received physiological saline intragastrically instead of BRX-220, but otherwise the protocol was the same as in group B. The animals were sacrificed by exsanguination through the abdominal aorta 12 h after the last CCK injection. Three untreated rats (Ø) were killed for HSP60 and 72 determinations. The pancreas was quickly removed, cleaned from fat and lymph nodes, weighed, and frozen at −70°C until use. The experiments performed in this study were approved by the Animal Care Committee of the University and comply with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Production of HSP60 antibody

Antibody against HSP60 was produced in rabbit by an intramuscular injection of 1 mg of protein emulsified in Freund's complete adjuvant. Booster shots were given three times in Freund's incomplete adjuvant in a similar manner at 2 week intervals. The rabbit was bled 1 week after the last injection. The antibody was purified by affinity chromatography on a protein A-Sepharose column. The specificity of the antibody was checked on rat pancreas homogenates and on bacteria over-expressing rat HSP60.

Western blotting

A part of the pancreas was homogenized in 9 vol of ice-cold buffer containing 0.02 M Tris-HCl, pH 7.8, 0.15 M NaCl 0.1% Triton X-100, 1 mM PMSF, 4 mM benzamidine, 5 mM iodoacetamide, and 100 IU/ml aprotinin using an Ultra-Turrax homogenizer for 2.5 min. The homogenates were centrifuged at 20,000 × *g* for 30 min. The supernatants were collected and the protein concentrations were measured by the method of Goa [12]. Twenty micrograms of protein was loaded per lane. Samples were electrophoresed on an 8% sodium dodecylsulfate-polyacrylamide gel according to the method of Laemmli [13], and transferred to nitrocellulose membrane for 2.5 h at 30 V or stained with Coomassie Brilliant blue (to check equal loading of proteins for Western blot analysis). Membranes were blocked in 5% nonfat dry milk for 1 h, and incubated with rabbit anti-HSP60 (1:10,000 dilution) or anti-HSP72 (1:2,500 dilution) (a generous gift from István Kurucz, Biorex Laboratories, Veszprém, Hungary, which has been characterized previously [14]) antibody for 1 or 3 h, respectively, at room temperature. The immunoreactive protein was visualized by enhanced chemiluminescence, using horseradish peroxidase-coupled anti-rabbit immunoglobulin at 1:10,000 dilution (Dako, Glostrup, Denmark). The densities of the bands were quantitated by using an A.A.B. Image Analysis Program (Advanced American Biotechnology, Fullerton, CA, USA).

Assays

Pancreatic weight/body weight ratio (pw/bw). Pancreatic weight was divided by the body weight of the rat.

Serum amylase activity and plasma trypsinogen activation peptide concentration. All blood samples were centrifuged at 2500 × *g* for 30 min. The serum levels of amylase were determined by a colorimetric kinetic method (Dialab, Vienna, Austria). Plasma

trypsinogen activation peptide (TAP) concentrations were determined with an ELISA kit (Biotrin, Dublin, Ireland) according to the manufacturer's instructions.

Pancreatic contents of amylase, trypsinogen, lipase, DNA, and protein. A part of the pancreas was homogenized in the previously mentioned homogenizing buffer not containing any protease inhibitors. Enzyme measurements were carried out on the supernatant fractions of the pancreatic homogenates. Pancreatic amylase activities were determined as described above. Trypsinogen was activated after a 200-fold dilution of the homogenate with 0.02 U enterokinase/μg pancreatic protein (Sigma, St. Louis, MO, USA) in the enzyme buffer containing 80 mM Tris-HCl, pH 8.0, 25 mM $CaCl_2$, and 100 μg/ml bovine serum albumin for 120 min at 37°C [15]. Lipase activities were measured by a pH-stat method [15]. Samples for DNA determination were precipitated with ice-cold 0.8 M perchloric acid, washed in 5% trichloroacetic acid, and then hydrolyzed with 0.8 M perchloric acid at 90°C for 10 min [16]. DNA was estimated photometrically with diphenylamine [17]. The protein concentrations in the supernatant fractions of the homogenates were measured as mentioned before [12].

Pancreatic lipid peroxidation, carbonyl protein, and reduced glutathione levels, and the activities of superoxide dismutase, catalase, and glutathione peroxidase. The remaining part of the pancreas was homogenized, the homogenates centrifuged at 3000 × *g* for 10 min, and the supernatants were used for measurements. Lipid peroxides can undergo metal-catalyzed or enzyme-catalyzed decomposition to form multiple products, including malondialdehyde (MDA). The pancreatic MDA level was measured according to the MDA/TBA-high performance liquid chromatographic (HPLC) method of Wong et al. [18], and was corrected for the protein content of the tissue [12]. This HPLC assay is more specific, sensitive, and reproducible than spectrophotometric techniques [18]. The concentration of protein carbonyls was determined by the 2,4-dinitrophenylhydrazine reaction according to the method of Levine et al. [19]. Carbonyl protein content was calculated by using the absorption coefficient of 22,000 $M^{-1}cm^{-1}$ at 370 nm for aliphatic hydrazones and expressed as nmol carbonyl/mg protein. Reduced glutathione (GSH) level was determined spectrophotometrically with Ellman's reagent [20]. Superoxide dismutase (SOD) activity was determined on the basis of the inhibition of epinephrine-adrenochrome autooxidation [21]. Mn-SOD activity was measured by the auto-oxidation method in the presence of 5×10^{-3} M KCN [22]. Cu/Zn-SOD activity was calculated by subtracting the activity of Mn-SOD from SOD activity. Catalase activity was measured spectrophotometrically at 240 nm by the method of Beers and Sizer [23] and expressed in Bergmeyer units (BU) (1 BU = decomposition of 1 g H_2O_2/min at 25°C). Gluthathione peroxidase (GPx) activity was determined according to the chemical method, using cumene hydroperoxide and GSH as substrates of GPx [24].

Histological examination

A 2–3 mm^3 portion of the pancreas head was fixed in an 8% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4 μm thickness and stained with hematoxylin and eosin. The slides were coded and read by two independent observers who were blind to the experimental protocol. Semiquantitative grading of interstitial edema, leukocyte infiltration, hyperemia, and vacuolization, necrosis, and apoptosis of acinar cells, was performed on 8–10 consecutive high-power fields (×400) on a scale of 0–3 or 0–4. Additionally, basophilic lamellation of the cytoplasm of acinar cells was also graded since a pilot study revealed that, besides the traditional markers, the areas of basophilic lamellation were more extensive in the more severely damaged pancreata. The score for each graded parameter was averaged and the total pancreatic damage was calculated by adding all the averages together. The grading system and basophilic lamellation are decribed in more detail in one of our previous manuscripts [8].

Statistical analysis

Results are expressed as means ± SEM. Experiments were evaluated by using the Student's *t*-test when the data consisted of two groups, or by analysis of variance when three or more groups were compared. Values of $p < .05$ were accepted as significant.

RESULTS

Specificity of the HSP60 antibody

The specificity of the HSP60 antibody is shown in Fig. 1. Only the 60 kD band was stained. F1

Expressions of pancreatic HSPs

The expressions of pancreatic HSP60 and HSP72 were significantly decreased in the animals with pancreatitis (B, ØB) vs. the untreated animals (Ø, not receiving

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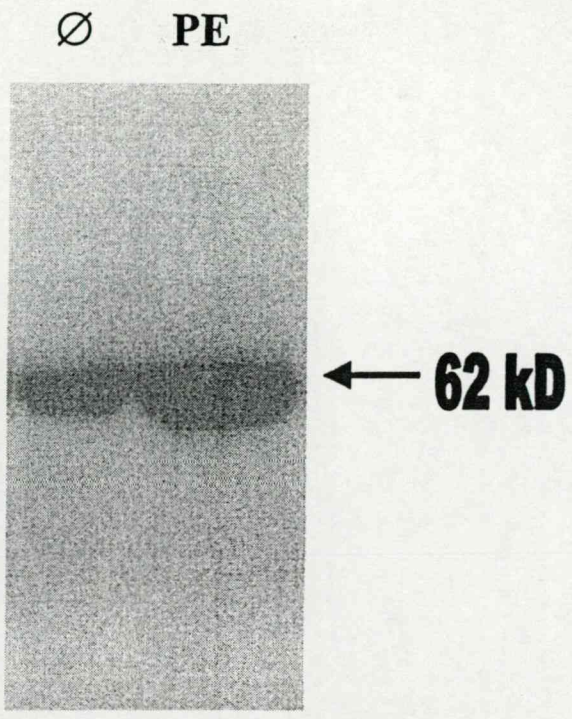

Fig. 1. Specificity of the home-made HSP60 antibody. The figure shows a picture of a Western blot in which a 60 kD protein band is stained both in the pancreas homogenate of an untreated rat (∅) and in the protein extract (PE) of bacteria overexpressing rat HSP60. 20 micrograms of protein was loaded per lane. Samples were separated on an 8% sodium dodecylsulfate-polyacrylamide gel according to the method of Laemmli [13], and transferred to nitrocellulose membrane for 2.5 h at 30 V. The nitrocellulose membrane was blocked in 5% nonfat dry milk for 1 h, and was incubated with rabbit anti-HSP60 (1:10.000 dilution) for 1 h. The immunoreactive protein was visualized by enhanced chemiluminescence, using a horseradish peroxidase-coupled anti-rabbit immunoglobulin at 1:10,000 dilution (Dako, Glostrup, Denmark).

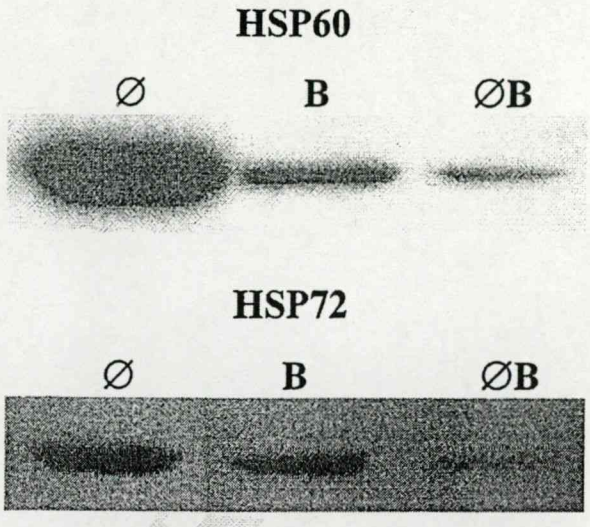

Fig. 2. Western blot analysis of pancreatic HSP60 and HSP72 expression. Representative Western blots of protein (20 μg/lane) extracted from the pancreata of untreated rats (∅), and rats treated (B) or not treated (∅B) with the HSP coinducer BRX-220 (20 mg/kg) during CCK-induced acute pancreatitis are shown (see the detailed description in the Experimental protocol). Supramaximal doses of CCK (B, ∅B) reduce the pancreatic levels of HSP60 and HSP72 vs. animals not receiving any CCK (∅). Rats treated with BRX-220 (B) are shown to have higher levels of HSP60 and HSP72 vs rats receiving physiological saline (∅B) during CCK-induced acute pancreatitis.

F2 BRX-220, and CCK) (Fig. 2). In group B, HSP60 (1.8 ×) and HSP72 (2.9 ×) were significantly increased vs. group ∅B (Fig. 2).

Pancreatic weight/body weight ratio (pw/bw), serum amylase activity, and plasma TAP concentration

In group B, pw/bw (2.16 ± 0.05 mg/g) and the serum amylase activity (1447 ± 108 IU/l) were not significantly different vs. group ∅B (1.89 ± 0.15 mg/g and F3 1317 ± 142 IU/l, respectively) (Fig. 3A, B). In group B, the plasma TAP concentration (39 ± 6 nM/ml) was significantly decreased vs. group ∅B (20 ± 5 nM/ml).

Pancreatic contents of DNA, protein, amylase, trypsinogen, and lipase

In group B, the pancreatic contents of protein (38.0 ± 4.1 mg/pancreas), amylase (932 ± 138 IU/pancreas) and trypsinogen (2.99 ± 0.16 IU/pancreas) were significantly increased vs. group ∅B (21.0 ± 1.5 mg/pancreas, 482 ± 109 IU/pancreas, and 1.88 ± 0.23 IU/pancreas, respectively) (Fig. 4). No significant changes were detected in F4 the pancreatic contents of DNA and lipase in group B (1.69 ± 0.25 mg/pancreas, 8.45 ± 9.45 IU/pancreas, respectively) vs. group ∅B (1.47 ± 0.31 mg/pancreas, 8.98 ± 6.44 IU/pancreas).

Pancreatic lipid peroxidation, protein carbonyl, and reduced glutathione levels, and the activities of superoxide dismutase, catalase, and glutathione peroxidase

In group B, the pancreatic MDA level and protein carbonyl content were significantly decreased (0.19 ± 0.01 nM/mg protein, 8.36 ± 0.49 nM/mg protein, respectively) vs. group ∅B (0.35 ± 0.04 nM/mg protein, 6.75 ± 0.38) (Fig. 5A, B). The activity of pancreatic GPx F5 was significantly increased in group B (2.07 ± 0.17 U/mg protein × 10^{-3}) vs. group ∅B (not detected) (Fig. 5C). In group B, the activity of pancreatic Cu/Zn-SOD (4.90 ± 0.59 U/mg protein) was significantly decreased vs. group ∅B (7.13 ± 0.56 U/mg protein) (Fig 5D). No significant alterations were observed in the pancreatic GSH level (1.00 ± 0.13 μM/mg protein × 10^{-2}), or the activities of Mn-SOD (not detected) and catalase (0.41 ±

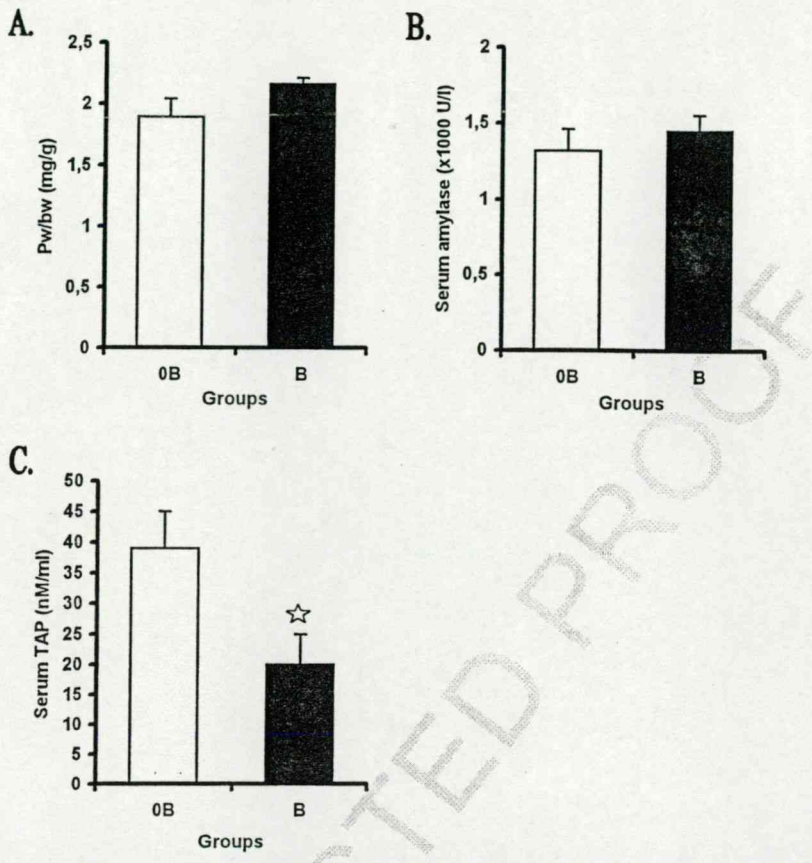

Fig. 3. (A) Pancreatic weight/body weight ratio (pw/bw), (B) serum amylase, and (C) plasma trypsinogen activation peptide (TAP) levels in groups ∅B and B. Groups were treated as indicated in the Experimental protocol. Means ± SEM for 6 animals are shown. ☆Significant difference ($p < .05$) vs. the control group.

0.06 U/mg protein × 10^{-3}) in group B vs. group ∅B (0.86 ± 0.06 μM/mg protein × $10^{\times 2}$, not detected, 0.54 ± 0.07 U/mg protein × 10^{-3}, respectively).

Histological examination

The administration of repeated injections of CCK resulted in the typical picture of an acute interstitial pancreatitis (interstitial edema, vacuolization, leukocyte infiltration, and acinar cell injury of the pancreas). In group B, the total morphological damage (4.75 ± 0.17 points) was significantly decreased vs. group ∅B (6.17 ± 0.53 points) (Fig. 6). The point values for each of the scored parameters are shown in Table 1. BRX-220 treatment significantly ameliorated the pancreatic leukocyte infiltration and adherence and the vacuolization, necrosis and apoptosis of the acinar cells.

F6 T1

DISCUSSION

HSPs play a universal role in the maintenance of cellular homeostasis. They are expressed constitutively and/or at elevated levels upon the exposure of cells to a variety of stress conditions in every organ, including the pancreas [1,2,25]. The HSPs are involved in the synthesis, degradation, folding, transport, and translocation of proteins [1,2]. Whereas many diseases result in increased levels of HSPs, Strowski et al. demonstrated that cerulein-induced pancreatitis reduces the levels of pancreatic HSPs [10]. This observation even suggests that the low levels of pancreatic HSPs might be involved in the development of cerulein-induced pancreatitis. Moreover, an increasing body of evidence from experimental animal studies has documented an essential role of HSPs in the prevention of acute pancreatitis. HSP preinduction is known to protect the pancreas from cerulein-induced pancreatitis in rats or choline-deficient ethionine-supplemented diet model pancreatitis in mice [3–9]. However, these investigators induced HSPs by thermal methods before the onset of acute pancreatitis, which does not mimic the clinical reality. Our study was designed to investigate the potential effects of a nontoxic HSP coinducer (induction of

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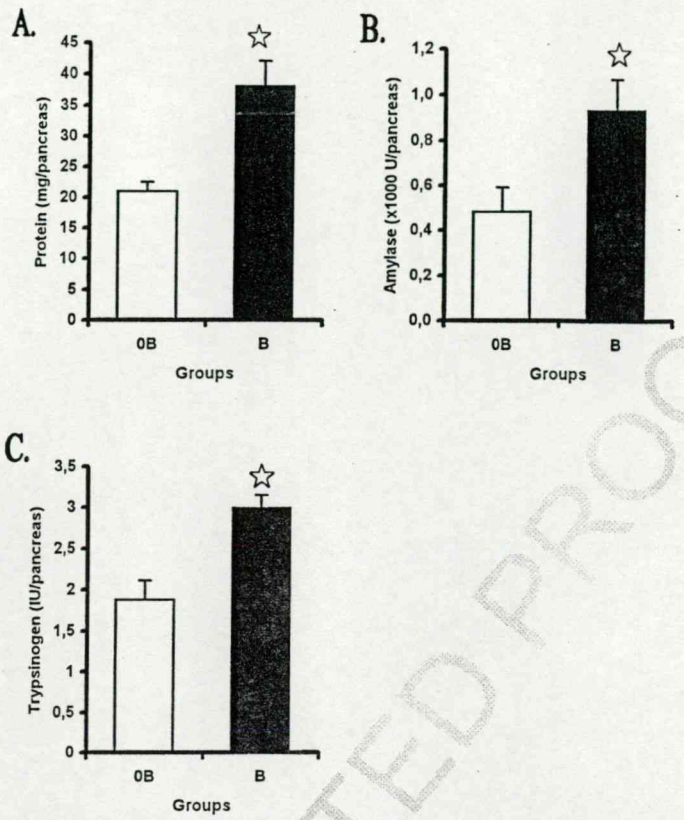

Fig. 4. Pancreatic contents of (A) protein, (B) amylase, and (C) trypsinogen in groups ØB and B. Groups were treated as indicated in the Experimental protocol. Data are means ± SEM for 6 animals. ☆Significant difference ($p < .05$) vs. the control group.

HSPs during the course of the disease) drug-candidate, BRX-220, on CCK-induced acute pancreatitis in rats. BRX-220 is a structural relative of bimoclomol, which has been shown to have a wide range of beneficial properties in experimental models of ischemic diseases and diabetic complications, particularly retinopathy, neuropathy, and angiopathy [11]. BRX-220 exerts its beneficial effects over a longer time period, and we therefore chose to administer the drug and CCK for 5 d.

In accordance with Strowski et al., we have shown that supramaximal doses of CCK reduce the levels of HSP60 and HSP72. However, this decrease was ameliorated by the administration of the HSP coinducer, BRX-220. This nontoxic hydroxylamine derivative upregulated the expression of pancreatic HSP60 and HSP72 about 2-fold. Therefore, BRX-220 acted against the effect of CCK-induced pancreatitis to decrease the levels of these HSPs, and clearly increased the protection against the disease as discussed below. Since we only examined the quantities of the most widely investigated HSP60 and HSP72, we can't exclude that other HSPs are induced and contribute to the protective effects of BRX-220.

Repeated supramaximal doses of CCK stimulation for 5 d are known to induce a prolongation of the morphological (decreased pw/bw, intrapancreatic inflammation, and acinar cell injury) and biochemical changes (decreased pancreatic protein, DNA, and GSH contents; decreased pancreatic amylase, lipase, trypsinogen, and free radical scavenger enzyme activities; increased pancreatic lipid peroxidation and protein oxidation; increased plasma TAP concentration) of CCK-induced acute interstitial pancreatits in rats [26,27]. We have found that BRX-220 treatment ameliorated many of these laboratory (plasma TAP concentration; pancreatic lipid peroxidation and protein oxidation; pancreatic GPx, trypsinogen, and amylase activity; total protein content) and morphological changes (vacuolization, necrosis, and apoptosis of acinar cells, and intrapanreatic inflammation). Though not significantly, there was a tendency of BRX-220 to hinder the decrease in pw/bw caused by the 5 d CCK injections ($p = .08$). For the above-mentioned reasons,

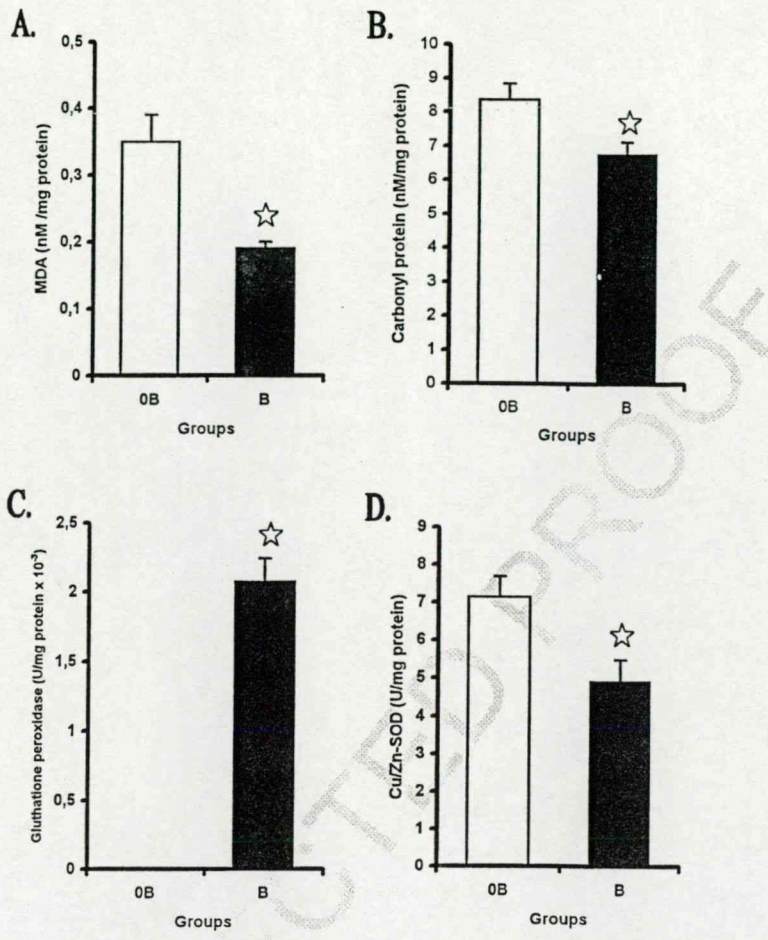

Fig. 5. Pancreatic (A) malondialdehyde levels, (B) protein carbonyl content, and (C) glutathione peroxidase, and (D) Cu/Zn-superoxide dismutase activities in groups ØB and B. Groups were treated as indicated in the Experimental protocol. Means ± SEM for 6 animals are shown. ☆Significant difference ($p < .05$) vs. the control group.

we believe that these cytoprotective effects are due to the beneficial effects of HSPs. Absolutely decisive proof of the protective effects of HSPs in this acute pancreatitis model would require the blockade of the expression or function of these proteins. Unfortunately, there are as yet no specific inhibitors of this kind in vivo.

Although HSPs have been implicated as mediators of pancreatic protection, the precise mechanism of their cytoprotective effects remains to be completely elucidated. Accumulating evidence suggests that HSPs are most likely to attenuate cellular damage by their chaperoning activities [1], by increasing the resistance of the cells to apoptosis [28,29] or necrosis [30], by decreasing pro-inflammatory cytokine levels [31], by their antioxidant effects [32], and/or by preventing intracellular trypsinogen activation [13,33]. Our findings support many of these possibilities.

Water immersion stress is known to increase the level of HSP60 [3,4]. Previous investigations have shown, however, that injections of cerulein combined with water-immersion stress increase the severity of the disease (although these investigators were dealing with the effects of stress on pancreatitis, and not HSPs) [34]. An explanation for this in respect of the HSPs was offered by Otaka et al., who proposed that, when stress was applied after cerulein injection, the acinar cells had already been damaged by cerulein and HSP60 could not be synthesized in the cells [4]. Furakawa et al. claim that pancreatitis is exacerbated due to the decreased blood flow to the pancreas and microcirculatory disturbances, which lead to the activation of zymogen proteases in the pancreas [35]. Therefore, the above-mentioned data are not in contradiction with ours, since BRX-220 has no such side effects. Moreover, this calls attention to the fact that

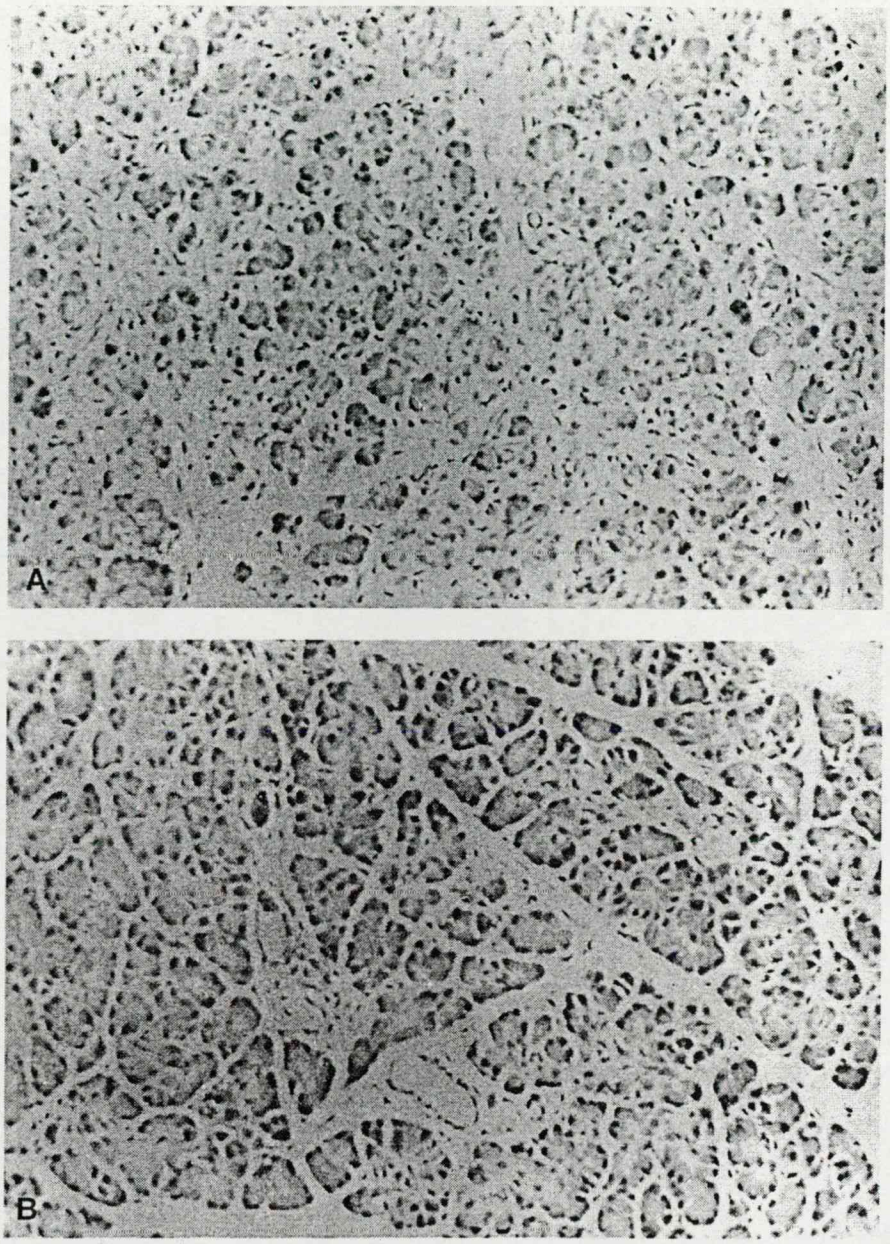

Fig. 6. Heat shock protein coinducer BRX-220 protects against CCK-induced acute pancreatitis. The figure shows the histological pictures of pancreata from rats either (A) not treated (Group ØB) or (B) treated with BRX-220 (Group B) 12 h after the last injection of 75 μg/kg body weight of CCK. Treatment with BRX-220 (Group B) significantly reduced the CCK-induced morphological alterations (intrapancreatic inflammation, vacuolization, necrosis, and apoptosis of acinar cells) (hematoxylin and eosin, original magnification 200×).

the coinduction of HSPs should be achieved only by a method that does not have harmful effects on the disease to be treated. Nontoxic HSP-inducer drugs could be one convenient way to approach this problem.

In conclusion, we have demonstrated that the HSP coinducer BRX-220, administered for 5 d, has a protective effect against CCK-induced acute pancreatitis. In fact, our study was the first to demonstrate that a nontoxic HSP-inducer administered during the course of the disease can also protect against CCK-induced pancreatitis. These cytoprotective effects are most probably due to the increased synthesis of HSPs. Our findings suggest the potential therapeutic applications of HSP-inducer drugs in the treatment of acute pancreatitis. Further studies are

Table 1. Effects of BRX-220 on the Histologic Parameters in Cholecystokinin-octapeptide-induced Acute Pancreatitis

	Group ØB	Group B
IS edema	1.44 ± 0.20	1.46 ± 0.17
Leukocyte infiltration	2.29 ± 0.20	1.83 ± 0.12*
Leukocyte adherence	0.17 ± 0.03	0.06 ± 0.01*
Vacuolization	0.44 ± 0.07	0.10 ± 0.01*
Necrosis (0–4)	0.29 ± 0.03	0.19 ± 0.03*
Apoptosis	0.79 ± 0.09	0.42 ± 0.03*
Basophilic lamellation	0.15 ± 0.03	0.13 ± 0.01
Hyperemia	0.60 ± 0.08	0.56 ± 0.05
Total damage	6.17 ± 0.53	4.75 ± 0.17*

Groups were treated as indicated in the Experimental protocol. Data are means ± SEM for 6 animals.
* Significant difference ($p < .05$) vs. the control group.

needed to investigate the promising use of these compounds in humans.

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ABBREVIATIONS

BU—Bergmeyer units
CCK—cholecystokinin-octapeptide
GPx—glutathione peroxidase
GSH—reduced glutathione
HPLC—high performance liquid chromatography
HSP—heat shock protein
MDA—malonyl dialdehyde
pw/bw—pancreatic weight/body weight ratio
SOD—superoxide dismutase
TAP—trypsinogen activation peptide
TBA—thiobarbituric acid