THE POTENTIAL ROLE OF HEAT SHOCK PROTEINS IN THE PREVENTION OF ACUTE EXPERIMENTAL PANCREATITIS

Ph.D. Thesis

Zoltán Rakonczay, M.D.

First Department of Medicine,
University of Szeged,
Szeged, Hungary

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CONTENTS

ABBREVIATIONS

Arg	-	L-arginine
BiP	-	heavy chain immunoglobulin binding protein
b.w.	-	body weight
BRX-220	-	(+)-/R/-N-[2-hydroxy-3-(1-piperidinyl)-propoxy]-pyridine-1-oxide-3-carboximidoil-chloride (Z)-maleate (1:1)
BU	-	Bergmeyer units
CCK	-	cholecystokinin-octapeptide
CWI	-	cold-water immersion
GPx	-	glutathione peroxidase
GRP	-	glucose-regulated protein
GSH	-	reduced glutathione
HPLC	-	high performance liquid chromatography
HSP(s)	-	heat shock protein(s)
HWI	-	hot-water immersion
IL	-	interleukin
i.p.	-	intraperitoneally
i.v.	-	intravenously
MDA	-	malonyl dialdehyde
NF-κB	-	nuclear factor kappa B
PMSF	-	phenylmethyl sulphonyl fluoride
PB	-	pentobarbital
pw/bw	-	pancreatic weight/body weight ratio
s.c.	-	subcutaneously
SA	-	sodium arsenite
SOD	-	superoxide dismutase
TAP	-	trypsinogen activation peptide
TBA	-	thiobarbituric acid
TC	-	sodium taurocholate
TNF-α	-	tumor necrosis factor-α

LIST OF FULL PAPERS CITED IN THE THESIS

I. **Rakonczay Z. Jr.**, Takács T., Mándi Y., Iványi B., Varga I., Pápai G., Boros I., Lonovics J. Water immersion pretreatment decreases pro-inflammatory cytokine production in cholecystokinin-octapeptide-induced acute pancreatitis in rats: possible role of HSP72. Int J Hyperthermia 2001; **17**:520-535. IF: 0.952

II. **Rakonczay Z. Jr.**, Takács T., Iványi B., Mándi Y., Pápai G., Boros I., Varga I., Jost K., Lonovics J. The effects of hypo- and hyperthermic pretreatment on sodium taurocholate-induced acute pancreatitis in rats. Pancreas 2002; **24**:83-89. IF: 1.648

III. Takács T., **Rakonczay Z. Jr.**, Varga I.S., Iványi B., Mándi Y., Boros I., Lonovics J. The comparative effects of water immersion pretreatment on three different acute pancreatitis models in rats. Biochem Cell Biol 2002; **80**:241-251. IF: 1.937

IV. **Rakonczay Z. Jr.**, Mándi Y., Kaszaki J., Iványi B., Boros I., Lonovics J., Takács T. Induction of HSP72 by sodium arsenite fails to protect against cholecystokinin-octapeptide-induced acute pancreatitis in rats. Digest Dis Sci 2002; accepted. IF: 1.498

V. **Rakonczay Z. Jr.**, Iványi B., Varga I.S., Boros I., Jednákovits A., Németh I., Lonovics J., Takács T. Non-toxic heat-shock protein co-inducer BRX-220 protects against acute pancreatitis in rats. Free Radical Biol Med 2002; accepted. IF: 4.116

LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

1. Takács T., Hegyi P., Jármay K., Czakó L., Góg C., **Rakonczay Z. Jr.**, Németh J., Lonovics J. Cholecystokinin fails to promote pancreatic regeneration in diabetic rats following the induction of experimental pancreatitis. Pharmacol Res 2001; **44**:363-72. IF: 0.805

2. Takács T., Czakó L., Morschl É., László F., Tiszlavicz L., **Rakonczay Z. Jr.**, Lonovics J. The role of nitric oxide in edema formation in L-arginine induced acute pancreatitis. Pancreas 2002; accepted. IF: 1.648

SUMMARY

Background. Heat shock proteins (HSPs) are a group of highly conserved, ubiquitous and functionally related proteins that play an essential part in cell survival. 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. HSPs are also involved in the transport and translocation of different proteins and the biogenesis and degradation of proteins, regulating their structures and functions under normal physiological conditions. HSPs are divided into six families, according to their molecular mass (e.g. HSP60 and HSP72). Cells subjected to a mild, sublethal stress event sufficient to increase the levels of HSPs are able to survive a subsequent more serious stress event. HSP preinduction is known to protect the pancreas against cerulein- and dibutylin dichloride-induced pancreatitis in rats or against choline-deficient ethionine-supplemented diet model pancreatitis in mice.

The **aims** of the present investigations summarized in this thesis were to investigate the potential effects of cytoprotective HSP pre-/co-induction by thermal (Annex No. I-III) [cold- (CWI) and hot-water immersion (HWI)] and non-thermal (Annex No. IV-V) [sodium arsenite (SA), BRX-220] methods on three acute pancreatitis models in rats.

Methods. Male Wistar rats were subjected to CWI, HWI and SA treatments and injected with cholecystokinin-octapeptide (CCK), sodium taurocholate (TC), or L-arginine (Arg) at the peak level of pancreatic HSP synthesis, as determined by Western blot analysis. In a separate set of experiments, rats were administered the non-toxic HSP co-inducer pro-drug, BRX-220, orally followed by subcutaneous CCK injections. This whole procedure was repeated for 5 days. The rats were exsanguinated through the abdominal aorta. The pancreas and lungs were quickly removed, cleaned of fat and lymph nodes, and frozen at –70 °C until processing. We determined the serum amylase activity and cytokine concentrations, 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. Lung myeloperoxidase activity, as a marker of tissue leukocyte infiltration, was measured. Histopathological investigation of the pancreas was also performed in all cases.

Results and conclusions. We have shown that HWI and SA specifically induces the synthesis of pancreatic HSP72, while CWI specifically elevates the level of pancreatic HSP60. We demonstrated the differential protective effects of water immersion pretreatment on three acute pancreatitis models. Water immersion pretreatment (and possibly HSP60 and HSP72) exerts a definite protective effect in mild pancreatitis, whereas this was not seen in more severe acute pancreatitis models. On the other hand, the protective effect of hyperthermia seems to be independent of the increased HSP72 synthesis since the non-thermal induction of HSP72 by SA failed to reduce the severity of CCK-induced acute pancreatitis and pancreatitis-associated lung injury. We have found that repeated injections of supramaximal doses of CCK for 5 days paradoxically decrease the levels of pancreatic HSP60 and HSP72. This reduction was ameliorated by the administration of the non-toxic HSP co-inducer, BRX-220. We were the first to demonstrate that a non-toxic HSP-inducer compound administered during the course of the disease can protect against CCK-induced pancreatitis. These cytoprotective effects of the drug are most probably due to the upregulated synthesis of HSPs. Our findings suggest the possible roles of HSP60 and HSP72, in the protection against interstitial acute pancreatitis and the potential therapeutic applications of HSP-inducer drugs in the treatment of acute pancreatitis. Further studies are needed to investigate the promising use of these compounds in humans.

1. INTRODUCTION

Acute pancreatitis is still a disease with an unacceptably high morbidity and mortality (5-10 %,1). The severity of acute pancreatitis ranges from edema to necrosis of the gland (1). The edematous form of the disease occurs in about 80-85% of patients and is self-limited, with recovery in a few days (1,2). In the 15-20% of patients with the most severe form of pancreatitis, hospitalization is prolonged and commonly associated with infection and other complications including multiple organ failure (1,2). Treatment of the disease remains a great challenge. The aims of the present investigations summarized in this thesis were to investigate the potential effects of cytoprotective heat shock protein (HSP) pre-/co-induction by thermal (Annex No. I-III) [cold-water (CWI) and hot-water immersion (HWI)] and non-thermal (Annex No. IV-V) [sodium arsenite (SA), BRX-220] methods, on different acute pancreatitis models in rats. Three experimental acute pancreatitis models which resemble the clinical disease were used. Edematous pancreatitis was induced by subcutaneous injections of cholecystokinin-octapeptide (CCK) (3). Necrohemorrhagic pancreatitis was induced by retrograde infusion of sodium taurocholate (TC) into the pancreatic duct (4). Necrotizing pancreatitis was induced by intraperitoneal injections of L-arginine (Arg) (5-6).

HSPs were first discovered 40 years ago by Ritossa (7). The HSPs are a group of highly conserved, ubiquitous and functionally related proteins that play an essential part in cell survival (8,9). Cells subjected stress respond by synthesising HSPs. The induction of the heat shock response enhances the ability of the cells to overcome the effects of the stress (9). 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. The HSPs are well known to protect cells against stress (9-11). HSPs are also necessary during normal physiological conditions since they are involved in the synthesis, degradation, folding, transport and translocation of proteins, and the assembly and disassembly of oligomers (9). HSPs are divided into different families, according to their molecular size (e.g. HSP60 and HSP70) (12). The HSP families have several functional homologs in the different compartments of cells. HSP60 is primarily a mitochondrial protein, but it can also be found in the pancreatic zymogen granules (13). The HSP70 family of HSPs is perhaps the most well studied. The HSP70 family members include the highly stress-inducible HSP72 and the constitutively expressed HSP73 in the cytoplasm, the mitochondrial HSP75, and the GRP78

(glucose-regulated protein) in the endoplasmic reticulum which is also referred to as heavy chain immunoglobulin binding protein (BiP) (12). HSPs 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 (14).

In addition to causing pancreatitis, dibutylin dichloride, L-arginine, and supramaximally stimulating doses of cerulein act as stressors and have been shown to induce expression of HSPs in the pancreas (16,23,26-28). In contrast, Strowski *et al.* demonstrated that cerulein pancreatitis in itself increases mRNA but paradoxically reduces protein levels of rat pancreatic HSPs (29). These observations even suggest that the low/high levels of pancreatic HSPs might be involved in the development/amelioration of cerulein-induced pancreatitis. For the above-mentioned reasons, it may be speculated that the administration of HSP-inducer compounds during 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.

It has been demonstrated that the preinduction of HSP expression has a protective effect against cerulein-and dibutylin dichloride-induced pancreatitis in rats or against choline-deficient ethionine-supplemented diet model pancreatitis in mice (15-23). However, the conclusions drawn from these experiments are somewhat controversial. Lee *et al.* (19) and Otaka *et al.* (20) found that the protective effect against this acute pancreatitis model was due to the specific preinduction of pancreatic HSP60 (by CWI), while Wagner *et al.* (21) and Frossard *et al.* (17) attributed it to pancreatic HSP72 (induced by hyperthermia, HWI). Convincing evidence of the protective effects of pancreatic HSP72 against cerulein-induced pancreatitis was provided by Bhagat *et al.* just recently (16). Nevertheless, the mechanism of how HSPs protect against CCK-induced pancreatitis remains to be answered. The cytoprotective effects of the HSPs on other (more serious) acute pancreatitis models have not been investigated.

Water immersion stress results in a number of stress-induced responses, like metabolic alterations and the synthesis of a variety of proteins besides HSP60 and HSP72, which may have protective roles in the prevention of a subsequent stress. For example, the antioxidant enzymes catalase and manganese superoxide dismutase (SOD) can also take part in the protective effect (24,25). Therefore, (besides HSPs) we also examined the levels of pancreatic antioxidants after the water immersion stress. To bypass the confounding effects of the changes in the body temperature, rats were administered SA (IV) and BRX-220 (V).

2. MATERIALS AND METHODS

2.1. Animals.

Male Wistar rats weighing 250-350 g were used. The animals were housed in standard cages 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 randomly assigned to control or experimental groups and were fasted overnight before the induction of acute pancreatitis. The experiments performed in this study were approved by the Animal Care Committee of the University of Szeged.

2.2. 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 overexpressing rat HSP60.

2.3. Induction of HSPs.

2.3.1. Cold- and hot-water immersion stress.

The rats were anesthetized with pentobarbital (PB) (44 mg/kg) intraperitoneally (i.p.). Water immersion stress was performed according to Otaka *et al.* (20) 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). The rectal temperature of 4-4 animals was monitored during the CWI or HWI every 10 minutes 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, 4 rats were killed at each time point before (t_o), immediately after (0), or 3, 6, 9 or 12 h after the end of the immersion. The pancreas was quickly removed, cleaned of fat and lymph nodes, and frozen at –70 °C until processing.

2.3.2. Sodium arsenite injection.

Twenty-eight rats were injected i.p. with progressive doses of SA (2 to 14 mg/kg body weight [b.w.]) [Merck, Darmstadt, Germany] to investigate the SA dose response. The control animals (n=4) received physiological saline injection i.p. The animals were killed by exsanguination through the abdominal aorta 12 h after the injections. The pancreas and lungs were quickly isolated and frozen at –70 °C until the 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 b.w. of SA i.p. and were killed at different time-points after the injection (3-48 h). The control animals received a saline injection i.p. and were killed after 12 h. The pancreas and lungs were processed for HSP determinations.

2.3.3. Administration of BRX-220.

Rats were administered 20 mg/kg BRX-220 {(+)-/R/-N-[2-hydroxy-3-(1-piperidinyl)-propoxy]-pyridine-1-oxide-3-carboximidoil-chloride (Z)-maleate (1:1), BIOREX Research and Development Co., Veszprém-Szabadságpuszta, Hungary} intragastrically.

2.4. Induction of acute pancreatitis.

Acute pancreatitis was induced near the peak of the HSP synthesis by the following methods:

CCK-induced pancreatitis. Edematous pancreatitis was induced by injecting 75 μg/kg body weight (b.w.) CCK (synthetized by Penke *et al.*, Dept. of Medical Chemistry, University of Szeged) subcutaneously (s.c.) three times at intervals of 2 h.

TC-induced pancreatitis. 100 μl/100 g b.w. 3% TC (Sigma-Aldrich, St. Louis, MO, USA) was injected into the common biliopancreatic duct under steady manual pressure over a

period of 30 seconds as described by Aho *et al.* (4). All injections were performed by the same investigator to limit technical differences.

Arg-induced pancreatitis. Necrotizing acute pancreatitis was induced by injecting 230 mg/100 g b.w. Arg (Sigma-Aldrich, St. Louis, MO, USA) i.p. twice at an interval of 1 h.

2.5. Experimental protocol.

2.5.1. The effects of cold- and hot-water immersion pretreatment on experimental acute pancreatitis models (I-III).

In every group, the rats were anesthetized with PB (44 mg/kg) i.p. at the starting point of the experiment (t_o).

CCK-induced pancreatitis (Fig. 1). 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_o + 6, t_o + 8 and t_o + 10 h. In group HC [HWI + CCK] (n=6), the rats received CCK as mentioned above, following a 6-h recovery period after the HWI. In group ∅HC [No HWI + CCK] (n=6), the rodents were given CCK starting at t_o + 7 h 15 min.

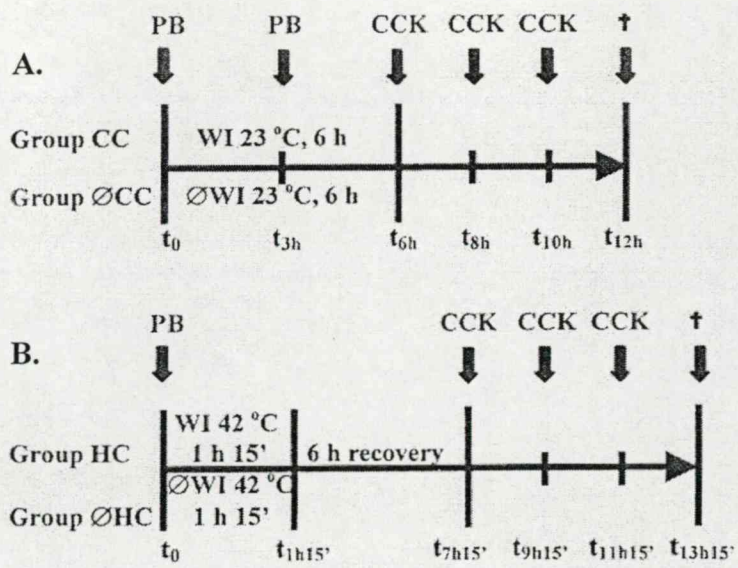

Fig. 1. Experimental protocol – CCK-induced pancreatitis. In every group, the rats were anesthetized with 44 mg/kg body weight (b.w.) pentobarbital (PB) intraperitoneally (i.p.) at the starting point of the experiment (t_o). **A.** Group CC: the rats received 75 μg/kg b.w. cholecystokinin-octapeptide (CCK) subcutaneously three times at intervals of 2 h immediately after the cold-water immersion (CWI). Group ∅CC: the animals were kept at room temperature and were injected with CCK at t_o + 6, t_o + 8 and t_o + 10 h. **B.** Group HC: the rats received CCK as mentioned above, following a 6-h recovery period after the hot-water immersion (HWI). Group ∅HC: the rodents were given CCK, starting at t_o + 7 h 15 min. The animals were killed (+) 2 h after the last CCK injection.

TC-induced pancreatitis (Fig. 2). In group CT [CWI + TC] (n = 6), the rats received the TC immediately after the CWI. In group ∅CT [No CWI + TC] (n = 6), the animals were kept at room temperature and were injected with TC at t_o + 6 h. In group HT [HWI + TC]

(n=6), the rats received TC as mentioned above, following a 6-h recovery period after the HWI. In group ØHT [No HWI + TC] (n=6), the rodents were given TC at t_o + 7 h 15 min.

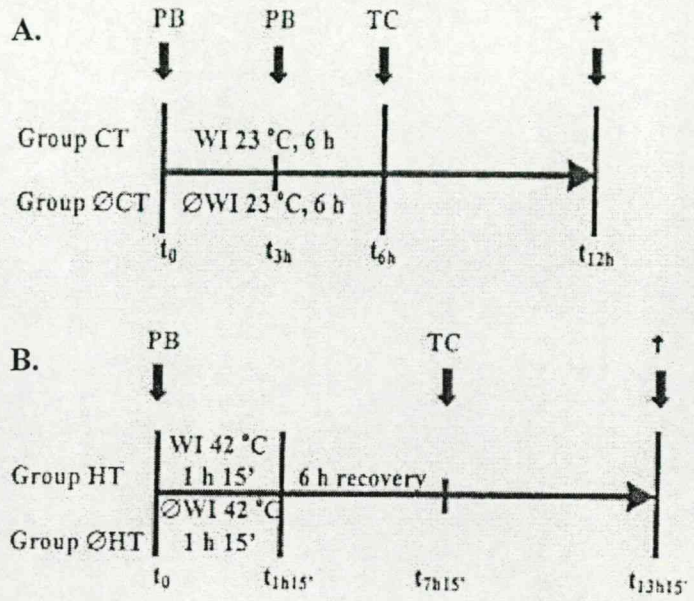

Fig. 2. Experimental protocol – TC-induced pancreatitis. In every group, the rats were anesthetized with 44 mg/kg b.w. PB i.p. at the starting point of the experiment (t_o). **A.** Group CT: 100 µl/100 g b.w. 3% sodium taurocholate (TC) was injected into the common biliopancreatic duct under steady manual pressure over a period of 30 seconds as described by Aho et al. (4) immediately after the CWI. Group ØCT: the animals were kept at room temperature and were injected with TC at t_o + 6 h. **B.** Group HT: the rats received TC as mentioned above, following a 6-h recovery period after the HWI. Group ØHT: the rodents were given TC at t_o + 7 h 15 min. The animals were killed (+) 6 h after the TC injection.

Arg-induced pancreatitis (Fig. 3). In group CA [CWI + Arg] (n = 6), the rats received the Arg immediately after the CWI. In group ØCA [No CWI + Arg] (n = 6), the animals were kept at room temperature and were injected with Arg at t_o + 6 and t_o + 7 h. In group HA [HWI + Arg] (n=6), the rats received Arg at 6 and 7 h after the HWI. In group ØHA [No HWI + Arg] (n=6), the rats were given Arg starting at t_o + 7 h 15 min.

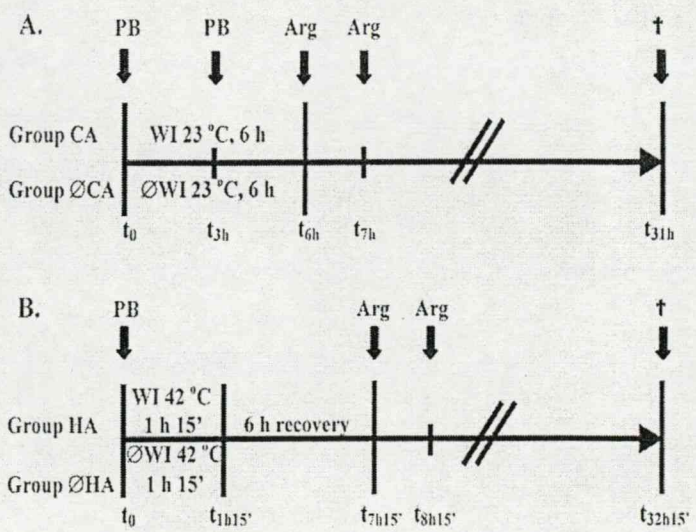

Fig. 3. Experimental protocol – Arg-induced pancreatitis. In every group, the rats were anesthetized with 44 mg/kg b.w. PB i.p. at the starting point of the experiment (t_o). **A.** Group CA: the rats received L-arginine (Arg) (2 x 230 mg/100 g b.w. at a 1-h interval i.p.) immediately after the CWI. Group ØCA: the animals were kept at room temperature and were injected with Arg at t_o + 6 and t_o + 7 h. **B.** Group HA: the rats received Arg at 6 and 7 h after the HWI. Group ØHA: the rats were given Arg, starting at t_o + 7 h 15 min. The animals were killed (+) 24 h after the second Arg injection.

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 –70 °C until use.

2.5.2. The effect of sodium arsenite pretreatment on CCK-induced acute pancreatitis (Fig. 4, IV).

In groups AC-2 (n=6) and AC-6 (n=6) [SA + CCK], the rats were injected with 10 mg/kg of SA i.p., and then received CCK s.c., starting at 9 h after the SA injection. In groups SC-2 (n=6) and SC-6 (n=6) [saline + CCK], the animals were injected with physiological saline i.p. instead of SA, and the 3 x 75 μg/kg b.w. CCK was administered after 9 h (Fig. 1/B). The animals were killed by exsanguination through the abdominal aorta 2 or 6 h 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 –70 °C until use.

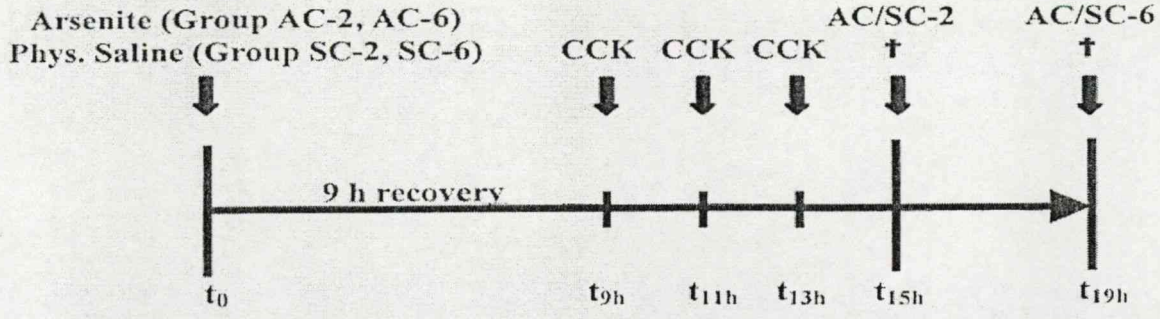

Fig. 4. Experimental protocol. In groups AC-2 (n=6) and AC-6 (n=6) [SA + CCK], the rats were injected with 10 mg/kg of sodium arsenite (SA) i.p. at the beginning of the experiment (t_0), and then received 75 μg/kg b.w. CCK s.c. three times at intervals of 2 h, starting at 9 h (t_9) after the SA injection. In groups SC-2 (n=6) and SC-6 (n=6) [saline + CCK], the animals were injected with physiological saline i.p. instead of SA, and the 3 x 75 μg/kg b.w. CCK was administered after 9 h. The rats were sacrificed (†) 2 or 6 h after the last CCK injection as indicated by the number after the dash in the group names.

2.5.3. The effect of BRX-220 on CCK-induced acute pancreatitis (V).

In group B (n=6), 20 mg/kg BRX-220 (dissolved in physiological saline) was administered intragastrically, followed by 75 μg/kg CCK s.c. three times, after 1, 3 and 5 h. This whole procedure was repeated for 5 days. The aminals 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 (∅) 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.

2.6. Assays.

Pancreatic weight/body weight ratio (pw/bw). Pancreatic weight was divided by the body weight of the rat. This ratio was utilized to evaluate the degree of pancreatic edema.

Serum amylase activity and plasma trypsinogen activation peptide concentration. All blood samples were centrifuged at 2500g 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.

Serum cytokine concentrations. TNF-α levels were titrated in a bioassay on the WEHI-164 cell line (30). IL-6 concentrations were measured via their proliferative action on the IL-6-dependent mouse hybridoma cell line B-9 (31). 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.

Pancreatic contents of amylase, trypsinogen, lipase, DNA and protein. 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. 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 (32). Lipase activities were measured by a pH-stat method (32). 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 (33). DNA was estimated photometrically with diphenylamine (34). The protein concentrations in the supernatant fractions of the homogenates were measured by the method of Goa (35).

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 3000g for 10 min, and the supernatants were used for measurements. Lipid peroxides can undergo metal- or enzyme-catalyzed decomposition to form multiple products, including malondialdehyde (MDA). The pancreatic MDA level was measured after the reaction with thiobarbituric acid, according to the method of Placer *et al.* (36) or according to the MDA/TBA-high performance liquid chromatographic (HPLC) method of Wong *et al.* (37), and was corrected for the protein content of the tissue (35). The HPLC assay is more specific, sensitive and reproducible than spectrophotometric techniques (37). The concentration of protein carbonyls was determined by the 2,4-dinitrophenylhydrazine reaction according to the method of Levine *et al.* (38). 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 (39). Superoxide dismutase (SOD) activity was determined on the basis of the inhibition of epinephrine-adrenochrome autooxidation (40). Mn-SOD activity was measured by the autooxidation method in the presence of 5 x 10^{-3} M KCN (41). Cu/Zn-SOD activity was calculated by substracting the activity of Mn-SOD from SOD activity. Catalase activity was measured spectrophotometrically at 240 nm by the method of Beers and Sizer (42) 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 (43).

Western blotting. A part of the pancreas was homogenized in the previously mentioned homogenizing buffer supplemented with the protease inhibitors 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 20,000g for 30 min. The supernatants were collected and the protein concentrations were measured as mentioned before (35). Twenty micrograms of protein was loaded per lane. Samples were electrophoresed on an 8% sodium dodecylsulfate-polyacrylamide gel according to the method of Laemmli (44), 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 [45]) antibody for 1 h at room temperature. The immunoreactive protein was visualized by enhanced chemiluminescence, using horseradish peroxidase-coupled anti-rabbit

immunoglobulin at 1:10,000 dilution (Dako, Denmark). The densities of the bands were quantitated by using an A.A.B. Image Analysis Program (Advanced American Biotechnology, Fullerton, CA).

Lung myeloperoxidase activity. Lung myeloperoxidase activity, as a marker of tissue leukocyte infiltration, was determined by the method of Kuebler *et al.* (46).

2.7. Histological examination.

2.7.1. 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 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 (Fig. 5), was performed on 8-10 consecutive high-power fields (x400) 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 (Fig. 5/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.

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	–

2.7.2. Electron microscopy.

For electron microscopic observations, 1 mm^3 pieces of the pancreas were fixed in 3% phosphate-buffered glutaraldehyde. Tissue blocks were postfixed in 1% OsO_4, and then rinsed in distilled water, dehydrated in a graded series of ethanol, and embedded in TAAB Transmit Resin (TAAB, England). Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined with a Philips electron microscope.

2.8. 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 $p < 0.05$ were accepted as significant.

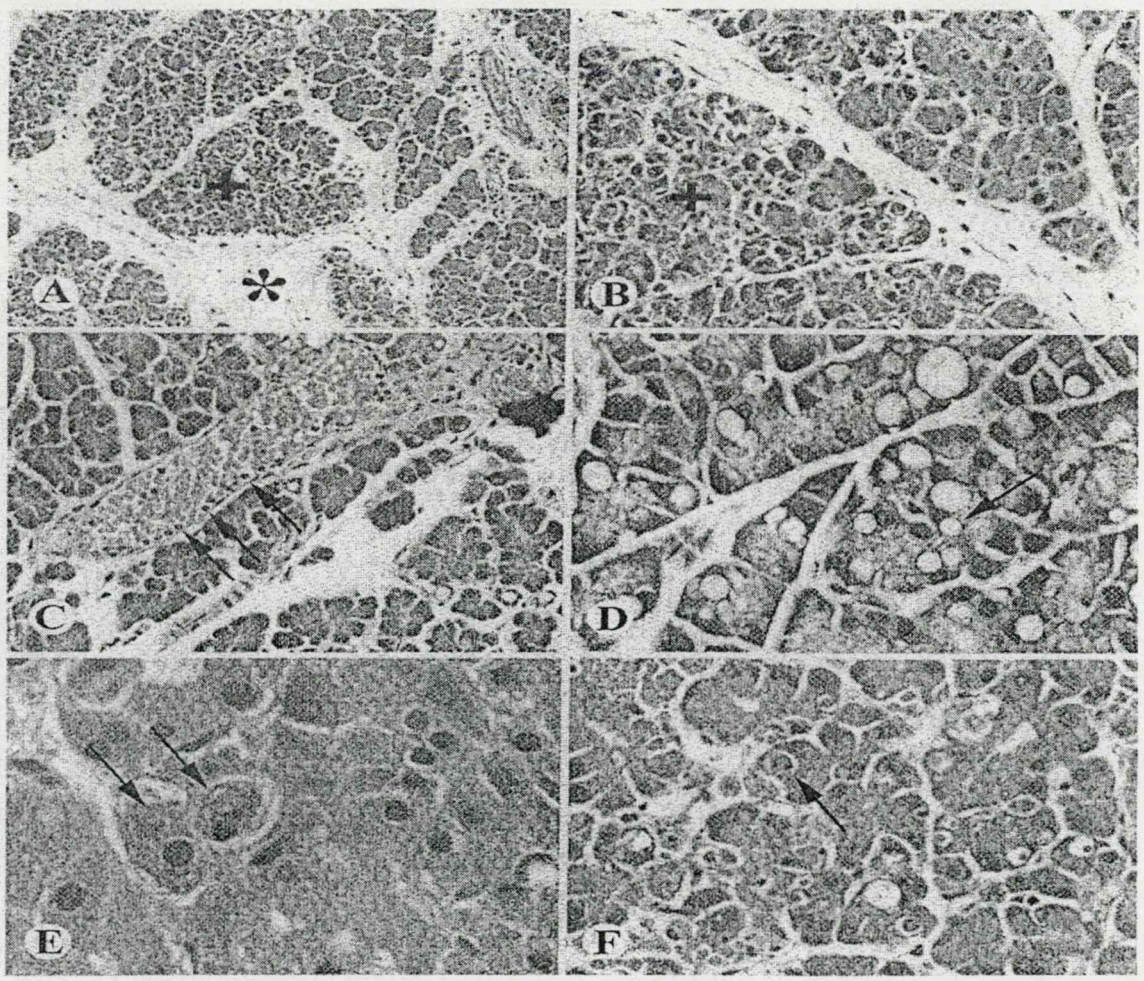

Fig. 5. Morphological features of experimental acute pancreatitis in rat. The features include **A., B.** interstitial oedema (asterisk), necrosis of pancreatic acinar cells (plus), **C.** adherence of leukocytes to the endothelium of venules (arrows), **D.** vacuolization of acinar cells (arrow), **E.** basophilic lamellation of the cytoplasm of acinar cells (arrows), and **F.** apoptosis of acinar cells (arrow) (hematoxilin and eosin, original magnifications were changed during image processing).

3. RESULTS

3.1. Specificity of the HSP60 antibody.

The specificity of the HSP60 antibody is shown in Fig. 6. Only the 60 kD band was stained.

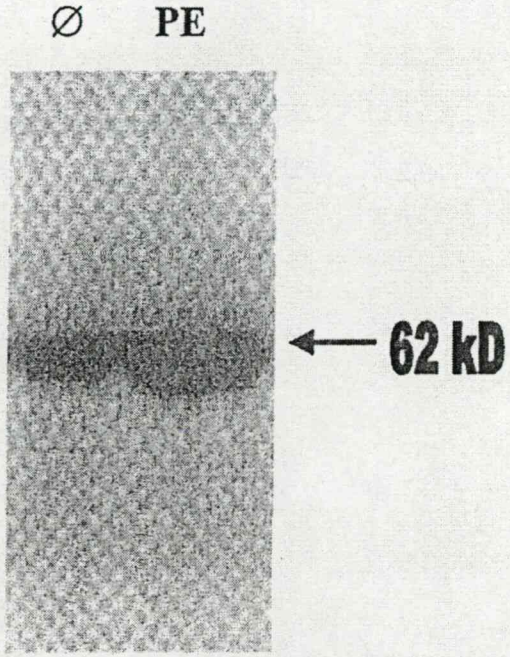

Fig. 6. Specificity of the home-made HSP60 antibody. The Figure shows a picture of a Western blot in which a 60 kD protein band is stained both in the pancreas homogenate of an untreated rat (∅) and in the protein extract (PE) of bacteria overexpressing rat HSP60. 20 micrograms of protein was loaded per lane. Samples were separated on an 8% sodium dodecylsulfate-polyacrylamide gel according to the method of Laemmli (44), 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, Denmark).

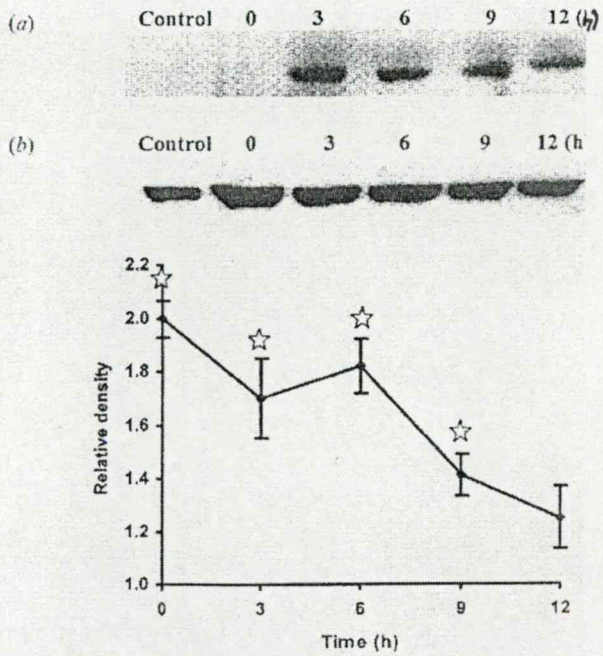

Fig. 7. Effect of water immersion stress on pancreatic HSP synthesis. Representative Western immunoblots of protein lysates (20 μg/lane) extracted from pancreata harvested over a time course after the water immersion treatments (0-12 h). The control did not receive any treatment. **A.** Expression of pancreatic HSP72 after HWI. HSP72 could not be detected in the unstressed control, but its expression was significantly increased at 3 h after 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 4 animals at each time point. ☆Significant difference ($p < 0.05$) vs the unstressed control group.

3.2. Induction of HSPs.

3.2.1. Cold- and hot water immersion 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 (Fig. 7/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 of the immersion, and the levels remained significantly elevated over the next 9 h (Fig. 7/B). The body temperature of rats during CWI and HWI stress is shown in Annex No. I.

3.2.2. Sodium arsenite injection.

The progressive doses of SA produced an increased expression of HSP72 in the pancreas and lungs (Fig. 8). The time-course of HSP72 expression after SA treatment was obtained by using the 10 mg/kg b.w. dose. HSP72 was already significantly increased at 3 h, peaked at 9-24 h after the SA injection and remained elevated until 48 h in both organs (Fig. 9). HSP60 is constitutively expressed in the pancreas and lungs. HWI and SA did not have a significant effect on its expression (results not shown). A 25% mortality rate was observed in the group of animals that received 14 mg/kg b.w. of SA. Moreover, most of the animals in this group appeared lethargic and anorectic. No mortality occurred at lower doses of SA.

3.2.3. Administration of BRX-220.

The expressions of pancreatic HSP60 and HSP72 were significantly decreased in the animals with pancreatitis (B, ØB) vs the untreated animals (Ø, not receiving BRX-220 and CCK) (Fig. 10). In group B, pancreatic HSP60 (1.8 x) and HSP72 (2.9 x) levels were significantly increased vs group ØB (Fig. 10).

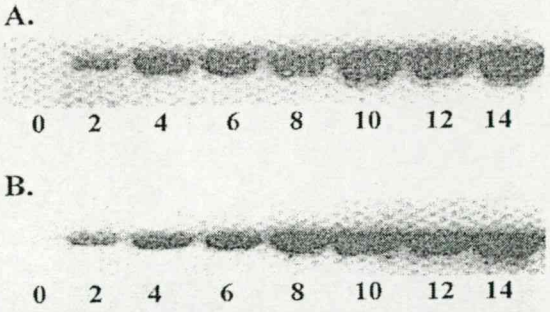

Fig. 8. Effects of progressive doses of SA injection on the synthesis of HSPs in the pancreas and lungs. The Figure depicts representative Western immunoblot analysis of protein lysates (50 μg/lane) from **A.** the pancreata and **B.** the lungs of rats, showing the expression of HSP72 after the injection of progressive doses of SA. Twenty-eight animals were injected i.p. with progressive doses of SA (2 to 14 mg/kg b.w.) [Merck, Darmstadt, Germany]; the control rats [0] (n=4) received a physiological saline injection i.p. The rats were sacrificed 12 h after the injection, and the pancreas and lungs were quickly isolated and frozen at –70 °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 b.w. of SA.

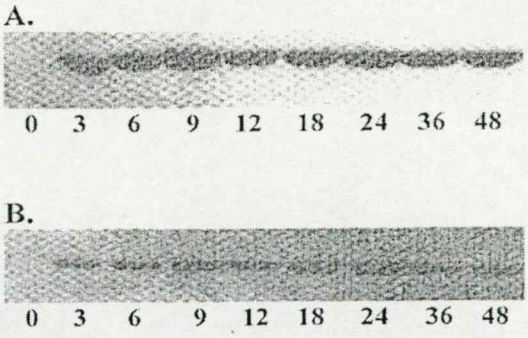

Fig. 9. Effects of SA injection on the synthesis of HSPs in the pancreas and lungs as a function of time. Representative Western immunoblot analysis of protein lysates (50 μg/lane) from **A.** the pancreata and **B.** the lungs of rats, showing the expression of HSP72 as a function of time after SA injection. A group of 32 animals were injected with 10 mg/kg b.w. of SA i.p. and were killed at different time-points after the injection (3-48 h). The control animals (0) (n=4) received a physiological saline injection i.p. and were killed after 12 h. HSP72 could not be detected in the control group, but its expression was already significantly increased at 3 h and peaked at 9-24 h after the SA injection, and remained elevated until 48 h 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).

3.3. Pancreatic reduced glutathione level, and the activities of glutathione peroxidase, superoxide dismutase and catalase after cold- and hot-water immersion stress

Both HWI and CWI significantly decreased the pancreatic GSH content, and the activities of GPx, Mn-SOD, Cu/Zn-SOD and catalase immediately after the end of the water immersion treatment (as compared to the unstressed control). Only the activity of Cu/Zn-SOD recovered soon after the CWI (3 h). The level of other antioxidants after water immersion remained significantly decreased throughout the examined time period (see Annex No. III).

3.4. The effects of cold- and hot-water immersion pretreatment on experimental acute pancreatitis models (I-III).

The administration of CCK, TC or Arg induced the typical laboratory and morphological changes of acute pancreatitis (3-6).

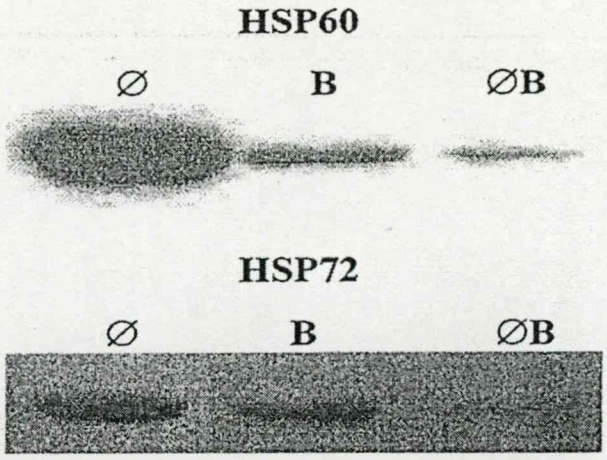

Fig. 10. Western blot analysis of pancreatic HSP60 and HSP72 expression. Representative Western blots of protein (20 μg/lane) extracted from the pancreata of untreated rats (∅), and rats treated (B) or not treated (∅B) with the HSP co-inducer BRX-220 (20 mg/kg) during CCK-induced acute pancreatitis are shown (see the detailed description in the 2.5.3 Experimental protocol). Supramaximal doses of CCK (B, ∅B) reduce the pancreatic levels of HSP60 and HSP72 vs animals not receiving any BRX-220 and 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.

Pw/bw, serum amylase activity (Fig. 11). In group CC, pw/bw (3.24 ± 0.13 mg/g) and the serum amylase activity (9,690 ± 1,114 U/l) were significantly decreased vs group ∅CC (3.69 ± 0.15 mg/g and 23,400 ± 4,625 U/l, respectively). In group HC, pw/bw (4.00 ± 0.15 mg/g) and the serum amylase activity (23,330 ± 1,412 U/l) were significantly decreased vs group ∅HC (4.65 ± 0.29 mg/g and 30,063 ± 1,676 U/l, respectively). In group CT, pw/bw (4.20 ± 0.28 mg/g) and the serum amylase activity (6,523 ± 536 U/l) were significantly decreased vs group ∅CT (5.84 ± 0.76 mg/g and 10,360 ± 720 U/l, respectively).

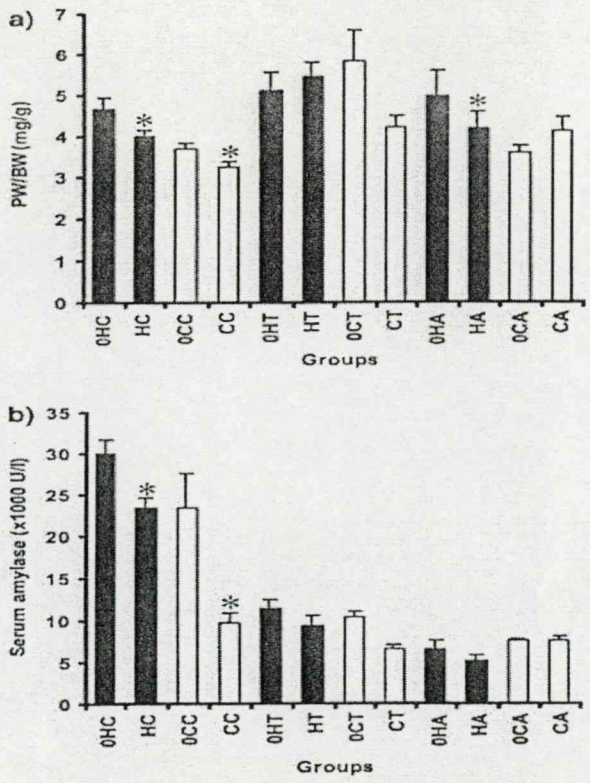

Fig. 11. A. Pancreatic weight/body weight ratio (pw/bw) and **B.** serum amylase levels in the rats with CCK-, TC- and Arg-induced acute pancreatitis. Groups were treated as indicated in the text (2.5.1.). Means ± SEM for 6 animals are shown. *, Significant difference ($p < 0.05$) vs the respective control group.

Serum cytokine levels (Fig. 12). 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). In group CC, the serum TNF-α (76.0 ± 8.0 U/ml) level was significantly elevated vs group ∅CC (not detected). In group CT, the serum level of IL-6 (91.7 ± 5.9 pg/ml) was significantly decreased vs 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 vs 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 TNF-α (15.0 ± 10.0 U/ml) and IL-1 (18.9 ± 0.7 pg/ml) levels were significantly decreased vs group ∅CA (76.7 ± 1.2 U/ml and 88.4 ± 33.2 pg/ml, respectively).

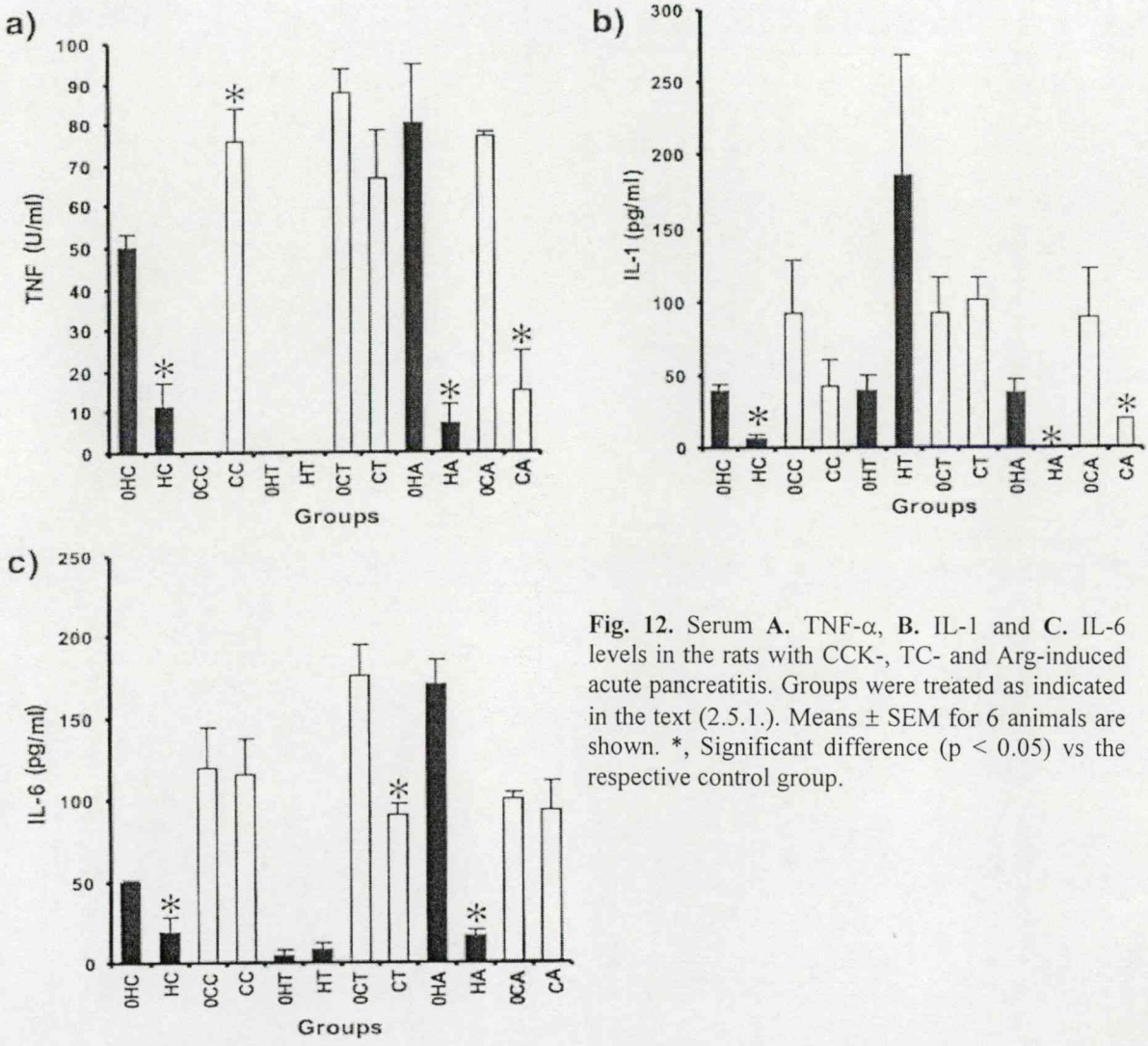

Fig. 12. Serum **A.** TNF-α, **B.** IL-1 and **C.** IL-6 levels in the rats with CCK-, TC- and Arg-induced acute pancreatitis. Groups were treated as indicated in the text (2.5.1.). Means ± SEM for 6 animals are shown. *, Significant difference ($p < 0.05$) vs the respective control group.

Pancreatic contents of protein, DNA, amylase, trypsinogen and lipase (Fig. 13). 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). The pancreatic contents of amylase, lipase and trypsinogen were significantly decreased in group CC (1,008 ± 216 IU/pancreas, 169.1 ± 8.4 IU/pancreas and 3.16 ± 0.60 IU/pancreas, respectively) vs group ∅CC (3,612 ± 1,007 IU/pancreas, 198.0

± 15.1 IU/pancreas 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, 6,464 ± 519 IU/pancreas, 209.6 ± 26.3 IU/pancreas and 5.09 ± 0.50 IU/pancreas, respectively) vs group ∅HC (124.9 ± 16.3 mg/pancreas, 10,244 ± 1,470 IU/pancreas, 343.3 ± 30.4 IU/pancreas and 7.08 ± 1.30 IU/pancreas, respectively). In group HA, the pancreatic contents of protein (76.5 ± 13.3 mg/pancreas) was significantly decreased vs group ∅HA (113.0 ± 10.8 mg/pancreas). The pancreatic contents of amylase and trypsinogen were significantly elevated in group HA (5,568 ± 1,719 IU/pancreas and 6.7 ± 1.0 IU/pancreas, respectively) vs group ∅HA (2,514 ± 421 IU/pancreas and 3.6 ± 1.2 IU/pancreas).

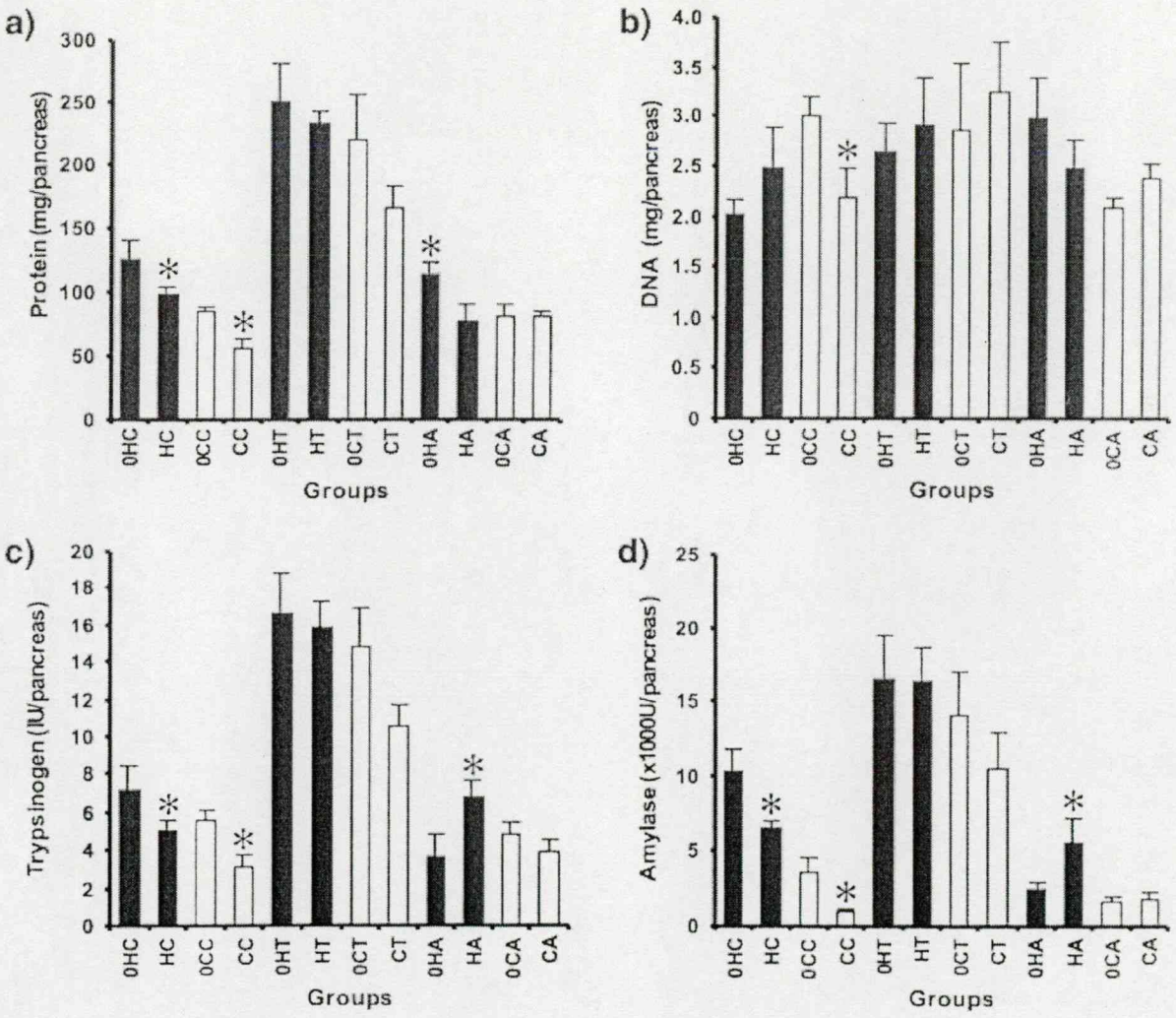

Fig. 13. Pancreatic contents of **A.** protein, **B.** DNA, **C.** trypsinogen, and **D.** amylase in the rats with CCK-, TC- and Arg-induced acute pancreatitis. Groups were treated as indicated in the text (2.5.1.). Data represent means ± SEM for 6 animals. *, Significant difference ($p < 0.05$) vs the respective control group.

Light microscopy. In group CC, the total pancreatic damage (3.71 ± 0.53 points) was significantly decreased vs group ØCC (5.07 ± 0.45 points). In group CT, the total pancreatic damage (8.01 ± 0.29 points) was significantly elevated vs group ØCT (6.02 ± 0.83 points). The point values for each of the scored parameters are shown in Table 2.

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

	Group ØHC	Group HC	Group ØCC	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 ØHT	**Group HT**	**Group ØCT**	**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 ØHA	**Group HA**	**Group ØCA**	**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.

Electron microscopy. An electron microscopic study was performed to evaluate the cause of the basophilic lamellation of the cytoplasm of the acinar cells. Figure 14 shows that this was due to tightly packed rough endoplasmic reticulum.

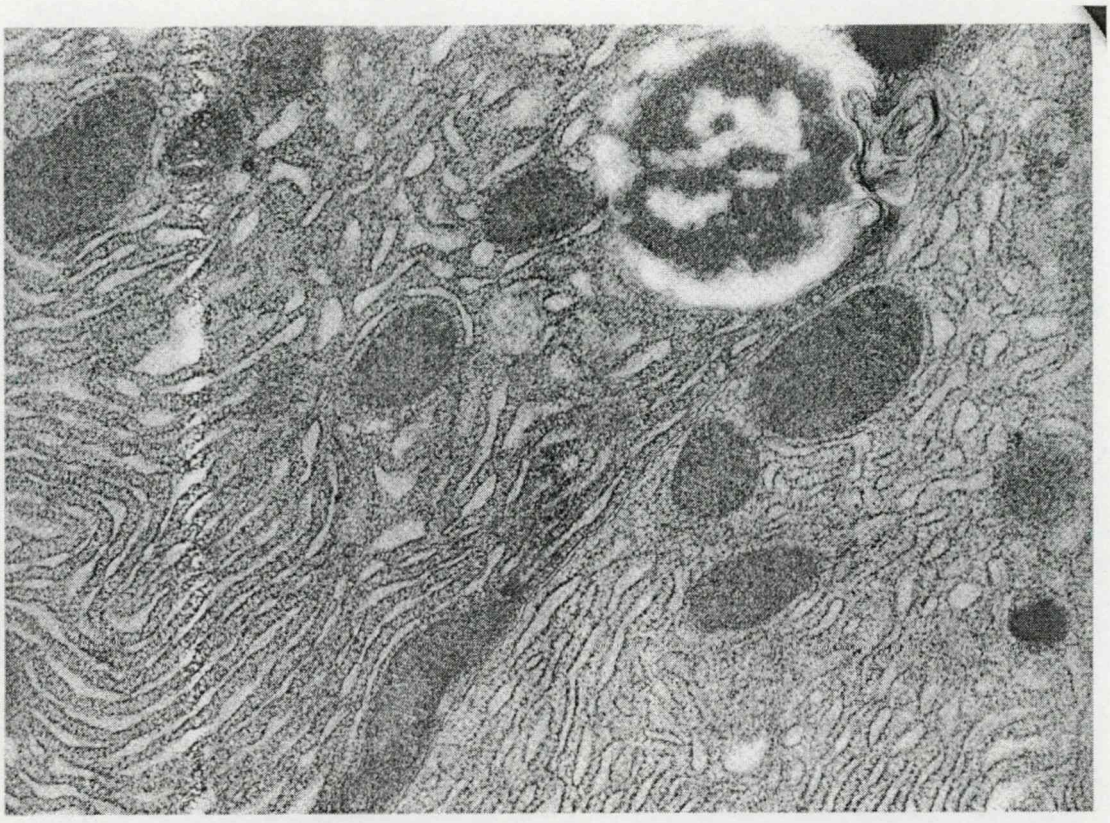

Fig. 14. Transmission electron micrograph of the pancreas from a rat 2 hours after the last of three s.c. injections of 75 μg/kg body weight of CCK (Group HC). Acinar cells exhibit tightly packed rough endoplasmic reticulum corresponding to the areas of basophilic lamellation of the cytoplasm of the cells (original magnification 4600x).

3.5. The effect of sodium arsenite pretreatment on CCK-induced acute pancreatitis (IV).

The administration of 3 x 75 μg/kg b.w. CCK induced the typical laboratory and morphological changes of experimental acute pancreatitis (3).

The pw/bw ratio and serum amylase activity (Fig. 15). In group HC, the pw/bw (4.05 ± 0.27 mg/g) and the serum amylase activity (12,428 ± 1,134 U/l) were significantly decreased vs group ∅HC (4.89 ± 0.28 mg/g and 18,251 ± 1993 U/l, respectively). In group AC-2, the serum amylase activity (10,515 ± 1,539 U/l) was significantly decreased vs group SC-2 (16,150 ± 1,633 U/l). Otherwise, SA pretreatment had no effect on pw/bw and serum amylase activity.

Serum cytokine levels (Fig. 16). 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 vs the corresponding values in group ∅HC (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 vs the respective controls.

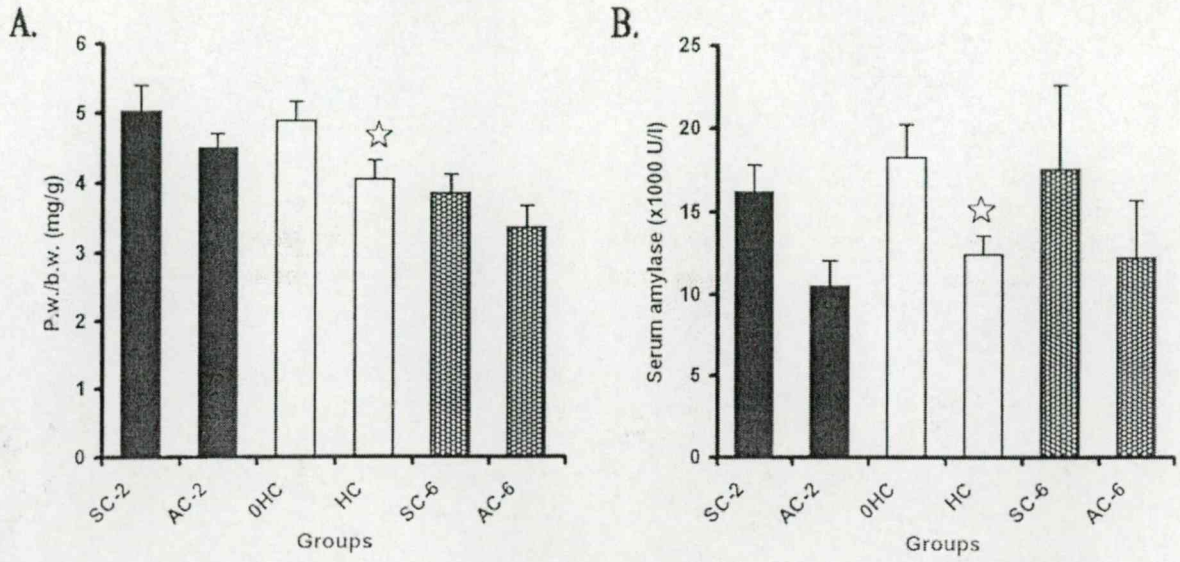

Fig. 15. A. The pancreatic weight/body weight ratio (pw/bw) and **B.** serum amylase levels in groups ∅HC, HC, SC-2, AC-2, SC-6 and AC-6. The groups were treated as indicated in Figs. 1/B and 4. Means ± SEM for 6 animals are shown.☆ Significant difference ($p < 0.05$) vs the respective control group.

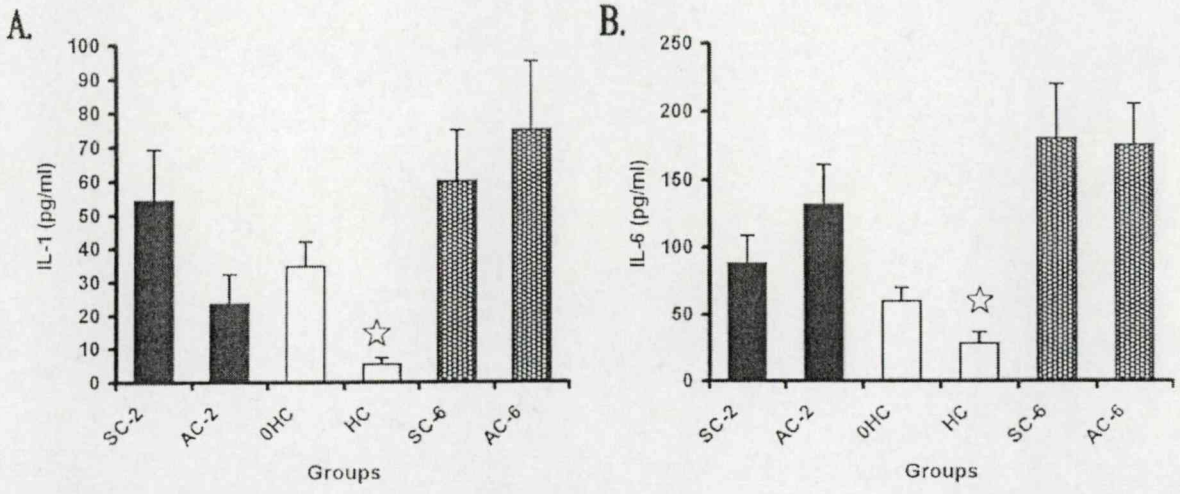

Fig. 16. Serum **A.** IL-1 and **B.** IL-6 levels in groups ∅HC, HC, SC-2, AC-2, SC-6 and AC-6. Groups were treated as indicated in Figs. 1/B and 4. Means ± SEM for 6 animals are shown.☆ Significant difference ($p < 0.05$) vs the respective control group.

Pancreatic contents of DNA, protein, amylase, trypsinogen and lipase (Fig. 17). The pancreatic contents of protein were significantly decreased in group HC (85 ± 6 mg/pancreas) vs group ∅HC (115 ± 11 mg/pancreas). The pancreatic contents of amylase, trypsinogen and lipase in group HC (6,280 ± 690 IU/pancreas, 7.98 ± 0.91 IU/pancreas, 310 ± 29 IU/pancreas, respectively) were significantly elevated vs group ∅HC (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 (4,692 ± 1,008 IU/pancreas) and trypsinogen (7.6 ± 1.2 IU/pancreas) were significantly decreased vs group SC-6 (126 ± 7 mg/pancreas, 8,369 ± 2,020 IU/pancreas and 11.1 ± 0.9 IU/pancreas, respectively).

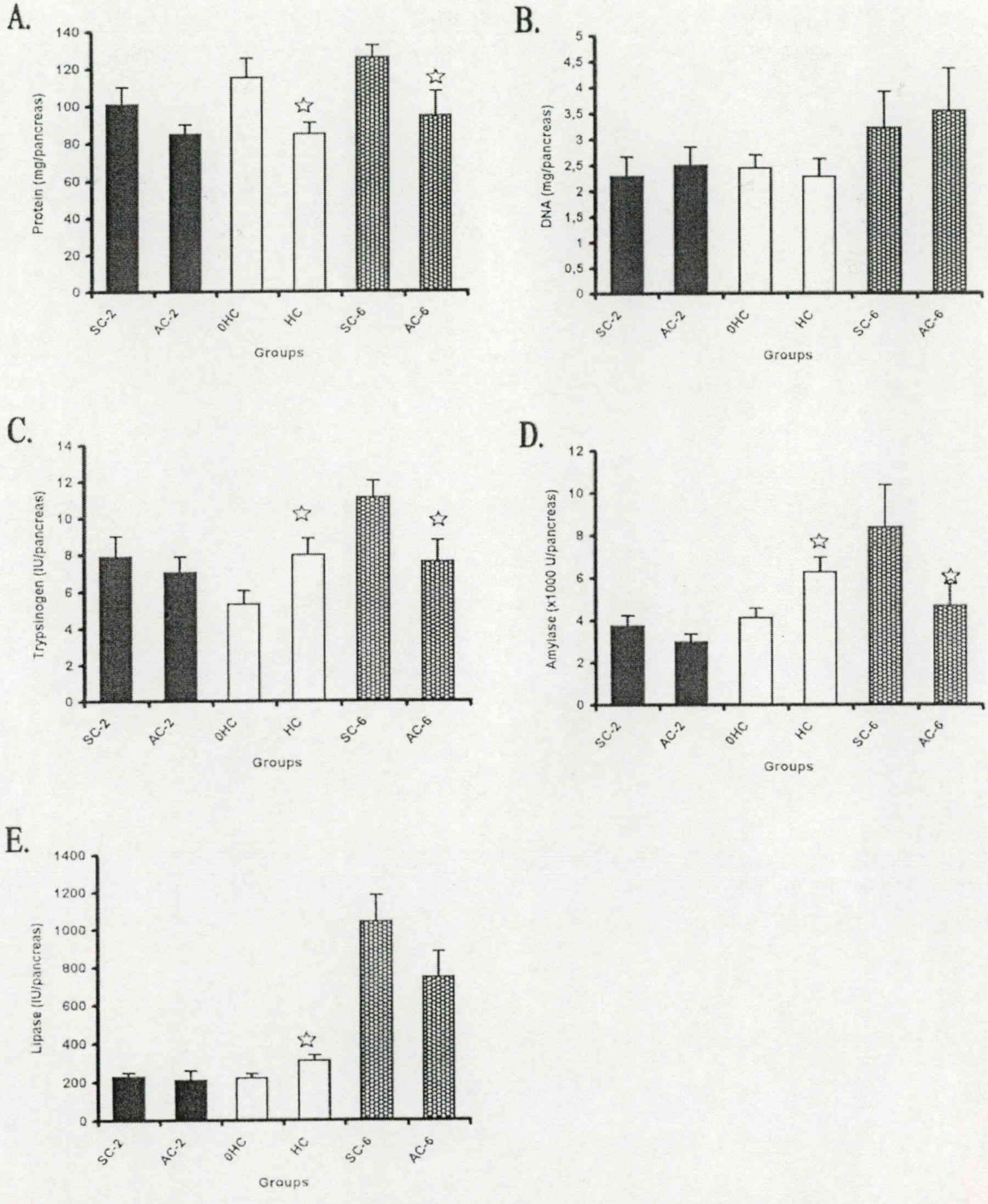

Fig. 17. Pancreatic contents of **A.** protein, **B.** DNA, **C.** trypsinogen, **D.** amylase, and **E.** lipase in groups ØHC, HC, SC-2, AC-2, SC-6 and AC-6. Groups were treated as indicated in Figs. 1/B and 4. Data are means ± SEM for 6 animals. ☆ Significant difference ($p < 0.05$) vs the respective control group.

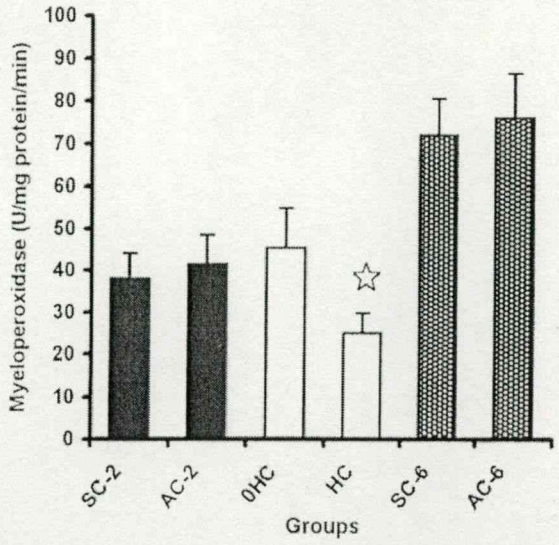

Fig. 18. Lung myeloperoxidase activities in groups ∅HC, HC, SC-2, AC-2, SC-6 and AC-6. Groups were treated as indicated in Figs. 1/B and 4. Data are means ± SEM for 6 animals. ☆ Significant difference ($p < 0.05$) vs the respective control group.

Lung myeloperoxidase activity (Fig. 18). In group HC, the lung myeloperoxidase activity (25.2 ± 4.7 U/mg protein/min) was significantly decreased vs group ∅HC (45.1 ± 9.6 U/mg protein/min). No significant change in lung myeloperoxidase activity was detected between the SA-treated and the control groups.

Light microscopy. No significant alteration was observed between the groups as concerns the total pancreatic damage. The point values for each of the scored parameters are shown in Table 3.

Table 3. Effects of sodium arsenite and hot-water immersion pretreatment on the histologic parameters in cholecystokinin-octapeptide-induced acute pancreatitis. Groups were treated as indicated in Figs. 1/B and 4. Data are means ± SEM for 6 animals. *, significant difference ($p < 0.05$) vs the respective control group.

	Group SC-2	*Group AC-2*	*Group ∅HC*	*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

3.6. The effect of BRX-220 on CCK-induced acute pancreatitis (V).

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 1317 ± 142 IU/l, respectively) (Fig. 19/A,B). In group B, the plasma TAP concentration (20 ± 5 nM/ml) was significantly decreased vs group ∅B (39 ± 6 nM/ml) (Fig. 19/C).

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. 20). No significant changes were detected in 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).

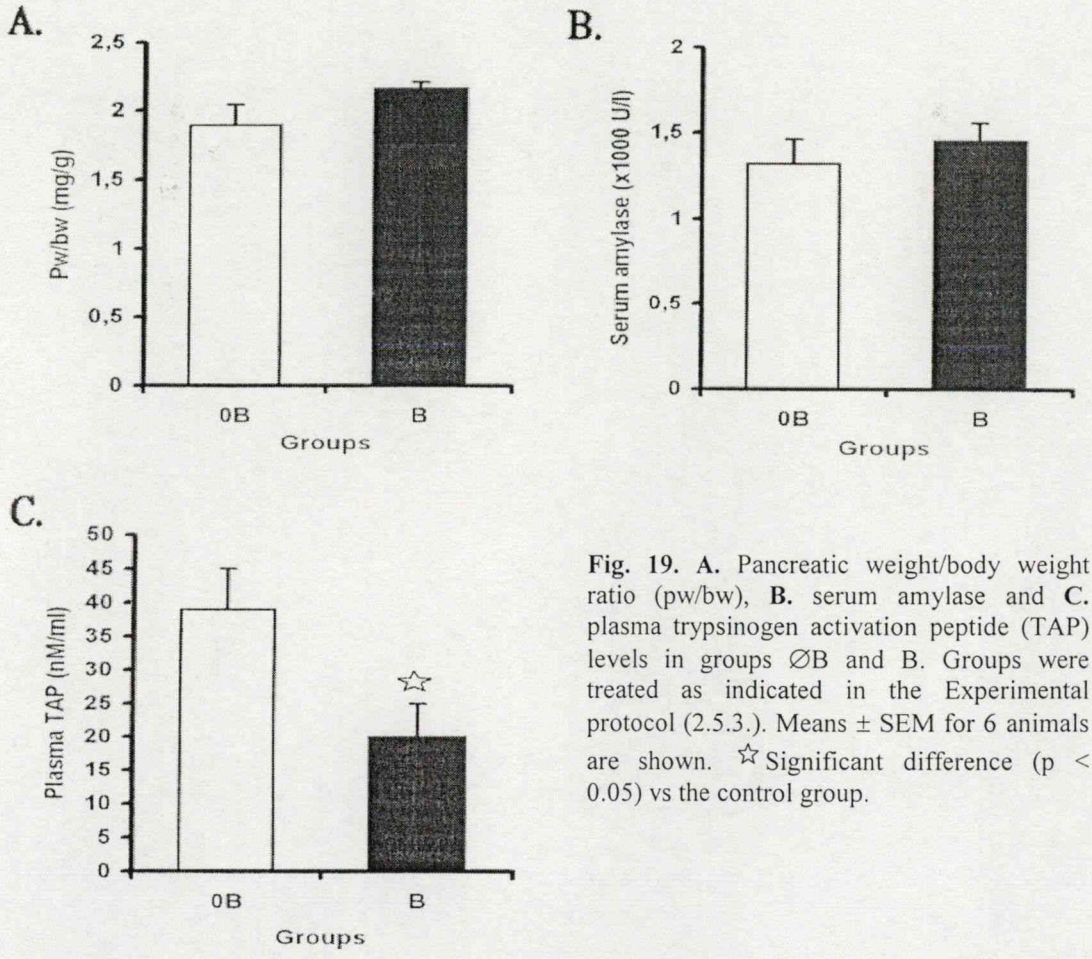

Fig. 19. A. Pancreatic weight/body weight ratio (pw/bw), **B.** serum amylase and **C.** plasma trypsinogen activation peptide (TAP) levels in groups ∅B and B. Groups were treated as indicated in the Experimental protocol (2.5.3.). Means ± SEM for 6 animals are shown. ☆ Significant difference ($p < 0.05$) vs the control group.

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. 21/A, B). The activity of pancreatic GPx was significantly increased in group B (2.07 ± 0.17 U/mg protein x 10^{-3}) vs group ∅B (not detected) (Fig.

21/C). 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 21/D). No significant alterations were observed in the pancreatic GSH level (1.00 ± 0.13 μM/mg protein x 10^{-2}), or the activities of Mn-SOD (not detected) and catalase (0.41 ± 0.06 U/mg protein x 10^{-3}) in group B vs group ∅B (0.86 ± 0.06 μM/mg protein x 10^{-2}, not detected, 0.54 ± 0.07 U/mg protein x 10^{-3}, respectively).

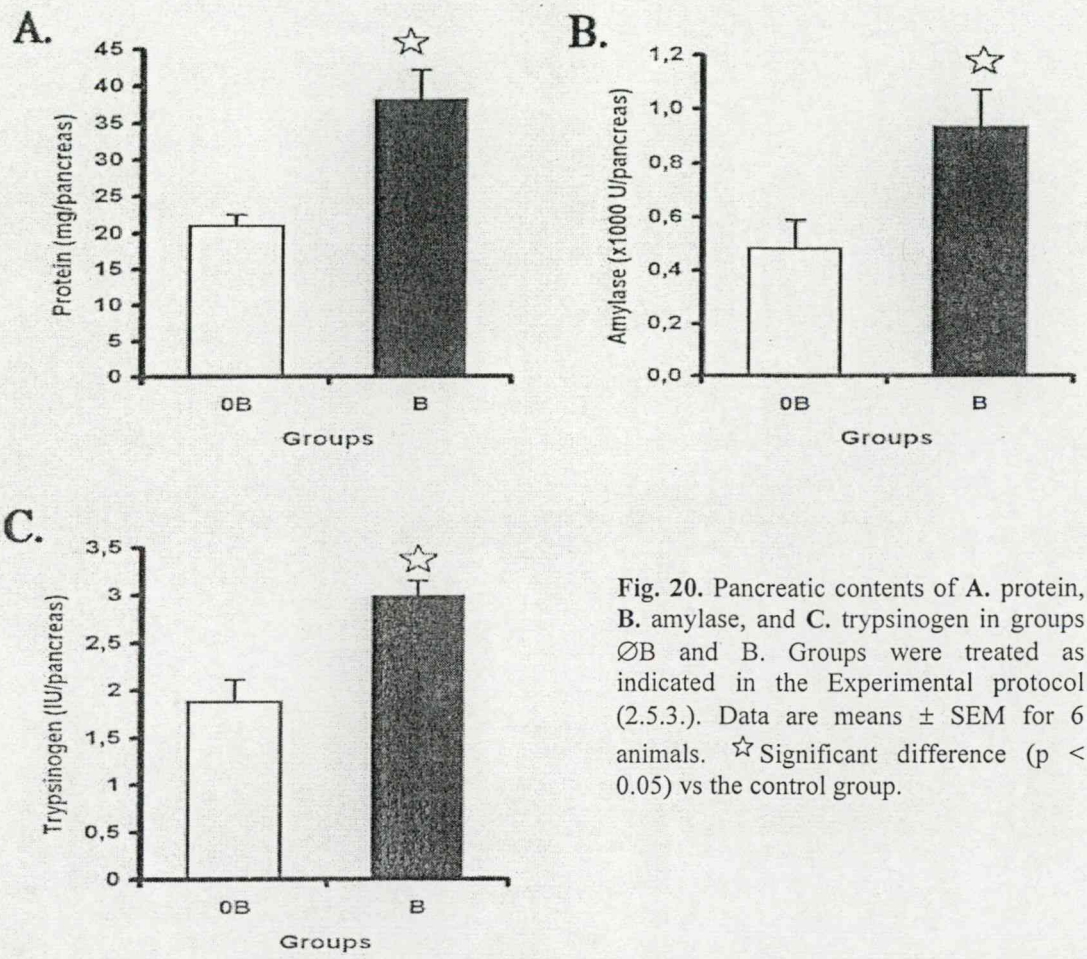

Fig. 20. Pancreatic contents of **A.** protein, **B.** amylase, and **C.** trypsinogen in groups ∅B and B. Groups were treated as indicated in the Experimental protocol (2.5.3.). Data are means ± SEM for 6 animals. ☆ Significant difference ($p < 0.05$) vs the control group.

Light microscopy. 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. 22). The point values for each of the scored parameters are shown in Table 4. BRX-220 treatment significantly ameliorated the pancreatic leukocyte infiltration and adherence and the vacuolization, necrosis and apoptosis of the acinar cells.

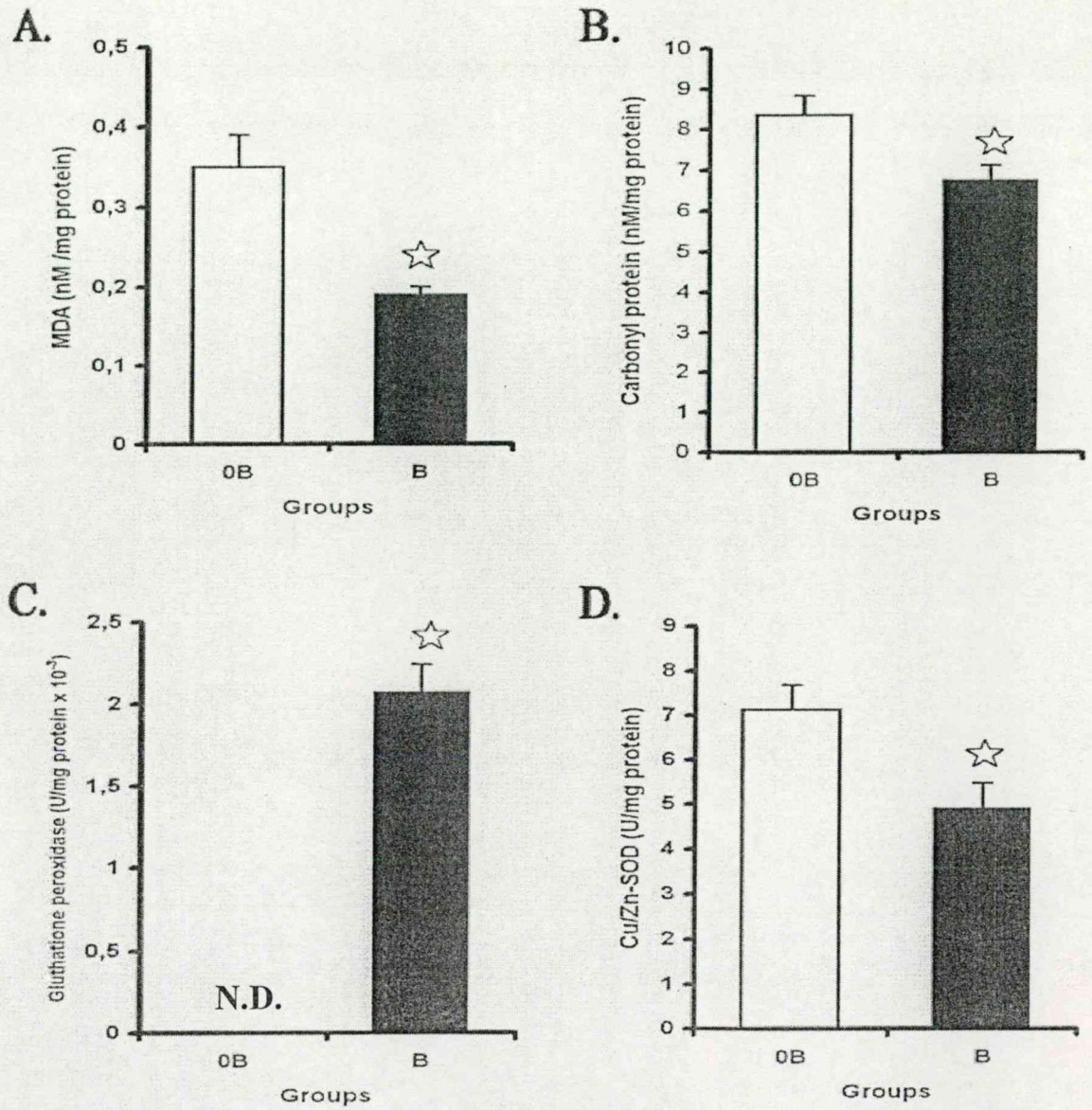

Fig. 21. Pancreatic **A.** malondialdehyde levels, **B.** protein carbonyl content and **C.** glutathione peroxidase, and **D.** Cu/Zn-superoxide dismutase activities in groups ØB and B. Groups were treated as indicated in the Experimental protocol (2.5.3.). Means ± SEM for 6 animals are shown. ☆ Significant difference ($p < 0.05$) vs the control group. N.D.: not detected.

	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*

Table 4. Effects of BRX-220 on the histologic parameters in cholecystokinin-octapeptide-induced acute pancreatitis. Groups were treated as indicated in the Experimental protocol (2.5.3.). Data are means ± SEM for 6 animals. *, Significant difference ($p < 0.05$) vs the control group.

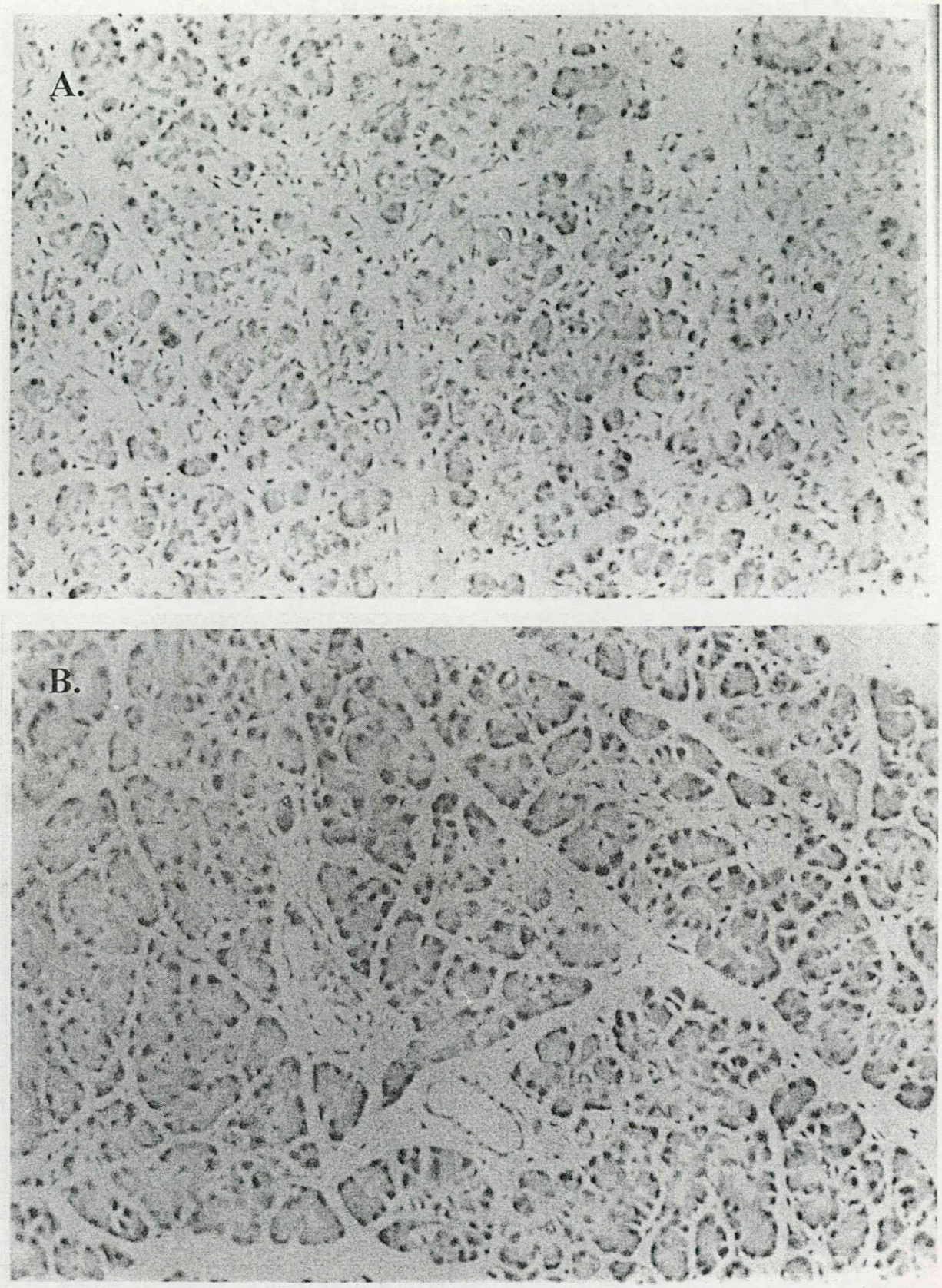

Fig. 22. Heat shock protein co-inducer BRX-220 protects against CCK-induced acute pancreatitis. The Figure shows the histological pictures of pancreata from rats either **A.** not treated (Group ∅B) or **B.** treated with BRX-220 (Group B) 12 hours after the last injection of 75 μg/kg body weight of 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 200x).

4. DISCUSSION

The members of the major cytoprotective HSPs are constitutively expressed or can be induced in the pancreas (14). Cells subjected to a mild, sublethal stress event sufficient to increase the levels of HSPs are able to survive a subsequent more serious stress event (9). HSP preinduction is known to protect the pancreas against cerulein- and dibutylin dichloride-induced pancreatitis in rats or against choline-deficient ethionine-supplemented diet model pancreatitis in mice (15-23). Our first set of experiments were designed to compare the effects of CWI and HWI pretreatment and the potential effects of HSP preinduction (HSP60 and HSP72) on three different acute pancreatitis models (I-III). 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.* (20), our results demonstrate that CWI specifically induces HSP60, while 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.* (19). The quantity of HSP72 was significantly increased at 3 h after HWI, and remained elevated until 12 h. Otaka *et al.* (20) reported the HSP72 expression peak at 6 h, which could be due to the differences in the experimental protocols.

Although HSPs have been implicated as mediators of pancreatic protection, these proteins were mostly induced by thermal methods (16-23). Water immersion stress results in a number of stress-induced responses, like metabolic alterations, the inhibition of *nuclear factor kappa B* (NF-κB)-binding activity (17) and the synthesis of a variety of proteins besides HSP60 and HSP72, which may have protective roles in the prevention of a subsequent stress. For example, the antioxidant enzymes catalase and manganese SOD can also take part in the protective effect (24,25). Therefore, (besides HSPs) we also examined the levels of pancreatic antioxidants after the water immersion stress. However, we could not detect elevated levels of pancreatic antioxidants.

Previously reported data concerning the protective role of different HSPs (particularly HSP60 and HSP72) against cerulein-induced pancreatitis are conflicting. Wagner *et al.* (21) and Frossard *et al.* (17,18) demonstrated that the expression of HSP70, induced by hyperthermia, correlated best with the time course and degree of protection against cerulein-induced pancreatitis. Convincing evidence of the protective effects of pancreatic HSP72 was

provided by Bhagat *et al.* just recently (16). They have shown that hyperthermia induced pancreatic HSP70 expression, prevented intrapancreatic trypsinogen activation, and protected against pancreatitis. The administration of HSP70 antisense oligonucleotides reduced the thermal stress-induced HSP70 expression, restored the ability of supramaximal cerulein stimulation to cause intrapancreatic trypsinogen activation, and abolished the protective effect of prior thermal stress. Moreover, in non-thermally stressed animals, pretreatment with HSP70 antisense oligonucleotides before the induction of pancreatitis exacerbated all the parameters associated with pancreatitis. In contrast with these data, Otaka *et al.* (20) found that the specific preinduction of HSP72 by hot-water immersion (42 °C, 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.* (19). In fact, our studies confirm that HSP60 might indeed play a role in the protection. In the experiments of Otaka *et al.* (20) and Wagner *et al.* (21), 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. We considered that the lack of protection against CCK-induced pancreatitis in the case of HWI pretreatment by Otaka *et al.* (20) 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 against acute pancreatitis. In the present study, the duration of the HWI pretreatment was longer than that applied by Otaka *et al.* (20), because we hypothesised 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. However, we must note that HWI pretreatment and possibly even the higher level of HSP72 were not sufficient to significantly 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. *In vitro* studies have shown that culture stress-induced HSP72 can prevent the intra-acinar cell activation of trypsinogen caused by cerulein in freshly prepared rat pancreas segments (15). Frossard *et al.* (18) have found that both thermal and non-thermal (β-adrenergic stimulation by isoproterenol) protected against cerulein-induced pancreatitis. The upregulated HSP72 expression was localised to the cytoplasmic acinar cell vacuoles which are believed to be the site of trypsinogen activation. However, 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 (47). 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 (17) or hormonal release (48,49).

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 (50), which is supported by the fact that the animals in group CT exhibited a greater vascular involvement (hyperemia and hemorrhage) than that 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 the CWI (results not demonstrated 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 to the control. HWI pretreatment did not have any effect on the measured parameters of this severe acute necrohemorrhagic pancreatitis.

It is well known that the proinflammatory cytokine levels increase during experimental acute pancreatitis (3,51,52). 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 effect the serum IL-6 level in this acute pancreatitis model, which closely reflects the severity of acute pancreatitis (53).

The differential protective effects of HSPs were called to our attention by several investigators. Cumming *et al.* (54) reported that the overexpression of HSP70 protected the 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.

To bypass the confounding effects of the changes in the body temperature, rats were administered SA (IV) and BRX-220 (V) before the induction of edematous pancreatitis. SA itself does not influence the body temperature of the animals (55). Intravenous (i.v.) administration of SA is known to increase the level of HSP72 in the kidney, heart and liver of rabbits (56). Our results are in accordance with those of Ribeiro *et al.* (55), 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 i.v. to induce HSP72 (55). 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. Wijeweera *et al.* also showed that SA treatment does not influence HSP60 expression (whereas the level of HSP72 increases) in precision-cut rat lung slices (57).

Similarly to our earlier experiments, acute pancreatitis was induced near the peak of pancreatic HSP expression (9 h after the SA injection) by administering high doses of CCK s.c. While HWI pretreatment ameliorated most of the examined laboratory and some morphological parameters of CCK-induced pancreatitis, we did not find such an effect after SA pretreatment despite the similar levels of pancreatic HSP72 synthesis achieved after either thermal stress or SA treatment. Morover, the pancreatic enzyme contents were more depleted in one of the SA-pretreated groups (AC-6), which indicates a more severe pancreatitis. The extent of lung injury was assessed via the myeloperoxidase activity. HWI pretreatment decreased lung injury, whereas we did not see such an effect after SA treatment, despite the induction of HSP72.

Unfortunately, the study with SA does not confirm that increased pancreatic HSP60 synthesis can play a role in the protection against secretagogue-induced pancreatitis, since it was not influenced by HWI or SA injection. Although a simple upregulation of HSP72 does not seem to be 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 (58). It is also possible that SA produced some toxic side-effect on the animals which prevented the beneficial properties of HSP72.

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.

Pancreatic HSP preinduction by thermal or non-thermal methods before the onset of acute pancreatitis does not mimic the clinical reality. Study No. V was designed to investigate the potential effects of a non-toxic HSP co-inducer (induction of 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 (59-61). BRX-220 exerts its beneficial effects over a longer time period, and we therefore chose to administer the drug and CCK for 5 days.

Repeated supramaximal doses of CCK stimulation for 5 days 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 (62,63). 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-day CCK injections ($p = 0.08$). We believe that these cytoprotective effects are due to the beneficial effects of HSPs.

In addition to causing pancreatitis, supramaximally stimulating doses of cerulein act as stressors and have been shown to induce expression of HSPs in the pancreas (16,26). In accordance with Strowski *et al.* (29), we have shown that supramaximal doses of CCK paradoxically reduce the levels of HSP60 and HSP72. However, this decrease was ameliorated by the administration of the HSP co-inducer BRX-220. This non-toxic hydroxylamine derivative upregulated the expression of pancreatic HSP60 and HSP72 about two- to three-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 above. 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. Absolutely decisive proof of the protective effects of HSPs against secretagogue pancreatitis would require studies which either more specifically induce HSPs (for example transgenic animals) or interfere with the expression (for example, HSP knockout animals) or function of these proteins.

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 (14), by increasing the resistance of the cells to apoptosis (12,64) or necrosis (10), by decreasing pro-inflammatory cytokine levels (65), by their antioxidant effects (66), by preventing the pathologic rise in intracellular calcium concentration that follows supramaximal cerulein stimulation (15) and/or by preventing intracellular trypsinogen activation (15). Our findings support many of these possibilities.

CWI stress is known to increase the level of HSP60 (I-III,19,20). 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) (67). 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 (21). Furukawa *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 (68). Therefore, the above-mentioned data are not in contradiction with ours, since CWI was administered as a pretreatment and BRX-220 has no such side-effects. Moreover, this calls attention to the fact that the co-induction of HSPs should be achieved only by a method that does not have harmful effects on the disease to be treated. Non-toxic HSP-inducer drugs could be one convenient way to approach this problem.

In conclusion, we have shown that HWI and SA specifically induces the synthesis of pancreatic HSP72, while CWI specifically elevates the level of pancreatic HSP60. We demonstrated the differential protective effects of water immersion pretreatment on three acute pancreatitis models. Water immersion pretreatment (and possibly HSP60 and HSP72) exerts a definite protective effect in mild pancreatitis, whereas this was not seen in more severe acute pancreatitis models. On the other hand, the protective effect of hyperthermia seems to be independent of the increased HSP72 synthesis since the non-thermal induction of HSP72 by SA failed to reduce the severity of CCK-induced acute pancreatitis and

pancreatitis-associated lung injury. We have found that repeated injections of supramaximal doses of CCK for 5 days paradoxically decrease the levels of pancreatic HSP60 and HSP72. This reduction was ameliorated by the administration of the non-toxic HSP co-inducer, BRX-220. We were the first to demonstrate that a non-toxic HSP-inducer compound administered during the course of the disease can protect against CCK-induced pancreatitis. These cytoprotective effects of the drug are most probably due to the upregulated synthesis of HSPs. Our findings suggest the possible roles of HSP60 and HSP72, in the protection against interstitial acute pancreatitis and the potential therapeutic applications of HSP-inducer drugs in the treatment of acute pancreatitis. Further studies are needed to investigate the promising use of these compounds in humans.

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