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Complete List of Authors:	Fetaud-Lapierre, Vanessa; Geneva University, Department of Bioinformatics and Structural Biology Pastor, Catherine; Geneva University Hospitals, Laboratory of pathophysiology and molecular imaging Farina, Annarita; Geneva University, Department of Bioinformatics and Structural Biology Hochstrasser, Denis; Geneva University Hospitals, Department of Genetics and Laboratory Medicine; Geneva University, Department of Bioinformatics and Structural Biology Frossard, Jean-Louis; Geneva University Hospitals, Division of Gastroenterology and Hepatology Lescuyer, Pierre; Geneva University Hospitals, Department of Genetics and Laboratory Medicine; Geneva University, Department of Bioinformatics and Structural Biology

Proteomic analysis of heat shock-induced protection in acute pancreatitis

Vanessa Fétaud-Lapierre [1], Catherine M. Pastor [2], Annarita Farina [1], Denis F. Hochstrasser [1, 3], Jean-Louis Frossard [4], Pierre Lescuyer [1, 3]

[1] Biomedical Proteomics Research Group, Department of Bioinformatics and Structural Biology, Geneva Faculty of Medicine, Geneva, Switzerland.

[2] Laboratory of pathophysiology and molecular imaging, Geneva University Hospitals, Geneva, Switzerland.

[3] Clinical Proteomics Laboratory, Department of Genetics and Laboratory Medicine, Geneva University Hospitals, Geneva, Switzerland.

[4] Division of Gastroenterology and Hepatology, Geneva University Hospitals, Geneva, Switzerland.

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Corresponding author: Pierre Lescuyer

SML/DMGL

Hôpitaux Universitaires de Genève

Rue Gabrielle-Perret-Gentil 4

1211 Geneva 14

Switzerland

Tel: +41 (0) 22 372 73 61 Fax: +41 (0) 22 379 55 02

Email: pierre.lescuyer@hcuge.ch

Abbreviations: AP: Acute pancreatitis, HSP: heat shock protein, TEAB: triethylammonium hydrogen carbonate buffer, LACB: bovine ß-lactoglobulin, TFA: trifluoroacetic acid, FDR: false discovery rate, CV: coefficient of variation, CTL: control, CTL-HT: controls with hyperthermia, AP-HT: acute pancreatitis with hyperthermia, A1I3: Alpha-1-Inhibitor 3, REG3A: Regenerating Islet-Derived Protein 3 Alpha, GP3: Pancreatic Lipase-Related Protein 2, COPG: coatomer gamma.

Keywords: acute pancreatitis, heat shock protein, quantitative proteomics, inflammation, secretory pathway.

Abstract

Acute pancreatitis is an inflammatory disease of the pancreas, which can result in serious morbidity or death. Acute pancreatitis severity can be reduced in experimental models by preconditioning animals with a short hyperthermia prior disease induction. Heat shock proteins 27 and 70 are key effectors of this protective effect. In this study, we performed a comparative proteomic analysis using a combination of LC-MS/MS analysis and isobaric tagging to investigate changes in pancreatic proteins expression that were associated with thermal stress, both in healthy rats and in a model of caerulein-induced pancreatitis. In agreement with previous studies, we observed modulation of heat shock and inflammatory proteins expression in response to heat stress or pancreatitis induction. We also identified numerous other proteins, whose pancreatic level changed following pancreatitis induction, when acute pancreatitis severity was reduced by prior thermal stress, or in healthy rats in response to hyperthermia. Interestingly, we showed that the expression of various proteins associated to the secretory pathway was modified in the different experimental models, suggesting that modulation of this process is involved in the protective effect against pancreatic tissue damage.

1. Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas characterized by a great variability in course and severity. Most patients develop a mild form of the disease that resolves spontaneously without serious morbidity. Conversely, in up to 20% of the cases, local and/or systemic complications occur [1]. Local complications are pancreatic necrosis and, potentially, secondary infection of the necrotic tissue. Systemic complications are related to the extension of the inflammatory process to remote organs leading to a systemic inflammatory response syndrome (SIRS) and multiple organ dysfunctions, including pulmonary and renal failure, and hypovolemic shock [1, 2]. These severe forms of AP are associated with a high mortality rate (15%) and require a rapid transfer of the patient to an intensive care unit.

The use of experimental models of AP, mostly in rodents, allowed understanding the initial events and identifying the main pathobiological pathways involved in the disease [3, 4]. AP is caused by the uncontrolled activation of digestive proteases within pancreatic acinar cells leading to pancreatic tissue damage. This pancreatic tissue injury then triggers an inflammatory response with the recruitment of inflammatory cells and secretion of various mediators of inflammation by activated acinar and inflammatory cells. AP triggers a number of intracellular and extracellular pathobiological pathways: inflammatory response, edema, microcirculation dysfunction, cellular stress response, oxidative stress response, apoptosis, and necrosis [3]. Induction of AP in genetically modified mice showed that all these processes are playing an important role in modulating the extent of local and systemic damages [5]. However, these pathobiological processes are tightly interconnected and the complexity of the interactions makes difficult to understand which are the key factors in controlling disease severity. Nevertheless, numerous studies have pointed out the critical role of heat shock proteins (HSPs) in AP pathobiology. The expression of several inducible HSPs, such as HSP27 and HSP70, is strongly increased in the pancreas following induction of experimental AP [6-8]. Moreover, it was shown that preconditioning of animals with either

a thermal or a chemical stress, prior to the induction of experimental AP, stimulates HSPs expression and causes a reduction in disease severity [9-12]. HSP27 and HSP70 were shown to be direct effectors in mediating this protective effect against pancreatic tissue injury [13, 14].

In the present study, we investigated changes in pancreatic proteins expression associated with the protective effect against AP induced by heat shock. We performed a comparative proteomic analysis using a combination of LC-MS/MS and isobaric tagging. We first compared pancreatic protein extracts from rats with experimental AP and healthy controls. This comparison allowed us to monitor changes in pancreatic proteins expression induced by our experimental model of AP. These data were used as a basis for the interpretation of further comparisons. Two others set of experiments were then performed. Firstly, we compared pancreatic extracts from healthy rats that were exposed or not to thermal stress. Secondly, pancreatic protein extracts from rats with experimental AP were compared with pancreatic extracts from rats exposed to a short thermal stress prior to the induction of AP. These comparisons were done to identify changes in pancreas proteome induced by thermal stress in normal rats and those associated with the decrease in disease severity induced by thermal stress in AP. The goal was to determine whether these modifications might explain the protection against pancreatic injury.

2. Materials and methods

2.1 Experimental induction of acute pancreatitis and sample collection

AP was induced in Sprague-Dawley rats (male, 250 g; n = 3) by two intraperitoneal injections, at 1-hour interval, of a supramaximally stimulating dose (10 μg/kg) of caerulein. A second group of rats (n = 3) was exposed to a short thermal stress (42°C water bath for 20 minutes) twelve hours before the induction of AP [15]. Control rats with (n = 3) and without (n = 3) thermal stress conditioning received similar injections of saline solution. The animals were sacrificed with a pentobarbital sodium injection (50 mg/kg intraperitoneal) 5 hours after the last caerulein or saline injection. Pancreas and serum were collected and stored at -80°C. Samples were labeled as follows: control (CTL), control with hyperthermia (CTL-HT), acute pancreatitis (AP), acute pancreatitis with hyperthermia (AP-HT). The animal welfare committee of the University of Geneva and the veterinary office approved the protocol. The study conformed to the American Veterinary Medical Association guidelines on humane treatment of laboratory animals. Pancreatic tissue extracts were prepared by homogenization with a T18 Basic Ultra-Turrax disperser (IKA®-Werke, Staufen, Germany) in 1 ml of PBS containing a protease inhibitor cocktail (Complete Mini EDTA-free, Roche, Basel, Switzerland). Homogenates were centrifuged and supernatants were stored at -80°C until analysis. Protein concentration in pancreatic extracts was determined using the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA). Protein samples integrity was controlled by gel analysis. Twenty μg of protein extract were separated by SDS-PAGE on a homemade Tris-Glycine gel (12.5% T, 2.6% C). The gel was stained with Coomassie blue R250 and protein patterns were visually inspected to check for the absence of protein degradation. Amylase and lipase activities were measured in serum samples using Synchron® System reagents on Unicel® DxC 800 clinical chemistry analyzers (Beckman Coulter, Fullerton, CA, USA). Myeloperoxidase concentration was determined in pancreatic tissue extracts by ELISA (Hycult Biotechnology, Uden, The Netherlands).

2.2 Sample preparation and iTRAQ labeling

One pancreatic tissue extract from each group (CTL, CTL-HT, AP, and AP-HT) was selected for comparative proteomic analysis. The experiment, including iTRAQ labeling, peptide fractionation, and LC-MS/MS analysis, was performed in duplicate using the same pancreatic extracts. For each sample, 80 µg of proteins were mixed with 0.5M triethylammonium hydrogen carbonate (TEAB) buffer pH 8.0 to a final volume of 100 µl. An equal amount (1.6 µg) of bovine ß-lactoglobulin (LACB) was spiked in each sample to serve as an internal standard for experimental bias correction. Proteins were reduced by adding 1 µl of 1% SDS and 2 µl of 50 mM Tris (2-carboxyethyl) phosphine (TCEP) and heating at 60°C for 1 hour. Free thiol groups of cysteine residues were alkylated by adding 1 µl of 400 mM iodoacetamide and incubating 30 minutes at room temperature in the dark with agitation. Proteins were then digested at 37°C overnight with 8 µl of a trypsin solution at 0.2 µg/µl in TEAB (protein/trypsin ratio = 50/1). The resulting peptides were tagged with the 4plex iTRAQ™ reagents (AB Sciex, Foster City, CA, USA). Each sample was labeled with one of the isobaric tag reconstituted with 70 µl of ethanol by incubating for 1 hour at room temperature. Sample/isobaric tag combinations were changed between the two experimental replicates. The labeling reaction was stopped by adding 8 µl of 5% hydroxylamine and by incubating 15 minutes at room temperature. The four peptides mixtures were then pooled and dried under vacuum.

2.3 Peptide fractionation using OFFGEL electrophoresis

The mixture of iTRAQ labeled peptides was dissolved in 1.5 ml of 5% CH3CN, 0.1% trifluoroacetic acid (TFA) and loaded onto an OASIS® HLB 30mg extraction cartridge (Waters, Milford, MA, USA). Elution was performed with 2 times 1 ml of 50% CH3CN, 0.1% TFA using a Visiprep™ SPE Vacuum Manifold (Sigma-Supelco, Park City, Bellefonte State, PA). The sample was then dried under vacuum and dissolved in 720 µl of deionized water. A solution containing 6% glycerol and 0.15% IPG Buffer pH 3-10 (Agilent, Santa Clara, CA, USA) was added to a volume of 3.6 ml. Peptides were fractionated according to their p*I* on

an Agilent 3100 OFFGEL Fractionator using commercial 24 cm IPG pH 3-10 linear strips (GE Healthcare, Chalfont St. Giles, UK). The strip was rehydrated with 20 µl of rehydration solution (4.8% glycerol, 0.12% IPG Buffer pH 3-10) per well. After 30 minutes of incubation, 150 µl of the sample solution were loaded per well. The isoelectric focalization was carried out at 20°C until a total voltage of 50 kV/h with a maximum current of 50 µA and a maximum power of 200 mW. After the focalization, peptidic fractions were recovered in separate tubes and pH values were measured to check for the accuracy of the pH gradient. Fractions were then dried under vacuum, dissolved in 1 ml of 5% CH3CN, 0.1% TFA and loaded onto OASIS® HLB 10mg extraction cartridges (Waters). Elution was performed as previously described with 50% CH3CN, 0.1% TFA. Eluates were dried under vacuum and stored at -20°C until MS analysis.

2.4 LC-MS/MS analysis

Each peptidic fraction was dissolved in 160 µL of 3% CH3CN, 0.1% TFA. Twenty-one µL of peptide solution was loaded on a 10 cm long homemade column with an internal diameter of 100 µm, packed with C18AQ Magic 5µm, 200Å stationary phase (Michrom Bioresources, Auburn, CA, USA). A gradient from 10 to 98% solvent B in solvent A (solvent A: 3% CH3CN, 0.1% TFA; solvent B: 95% CH3CN, 0.1% TFA) was developed over 55 min as follow: 10% solvent B for 2 minutes, 10 to 50% solvent B from 2 to 45 minutes, 50 to 98% tampon B from 50 to 55 minutes. A constant flow-rate of 400 nl/minute was used during the whole chromatographic cycle. Samples were eluted directly on a MALDI target using a homemade spotting robot. Matrix (5 mg/ml α-cyano-4-hydroxycinnamic in 50% CH3CN, 0.1% TFA, 10 mM $NH_4H_2PO_4$) was applied and dried. Peptides were analyzed in MS and MS/MS mode using a 4800 MALDI-TOF/TOF tandem mass spectrometer (AB Sciex). Argon was used as the collision gas. MS scan was conducted from 800 to 4000 m/z, and the 15 most abundant peaks with signal to noise ratio >10 were selected for MS/MS.

2.5 Database searching

Peak lists were generated as .mgf files using the 4000 Series Explorer™ software (AB Sciex). Peak lists corresponding to the 24 fractions were merged into a single file that was searched against International Protein Index-RAT database (release 3.42, April 2008) using Phenyx software (version 2.5, GeneBio, Geneva, Switzerland). MALDITOF/TOF was selected as the instrument type with TOF/TOF iTRAQ specified. The taxonomy selected was Rat. Trypsin was selected as the proteolytic enzyme with one missed cleavage allowed and the normal cleavage mode used. Oxidized methionine was selected as variable modification. Carbamidomethylation of cysteines and iTRAQ-labeled peptides on the amino terminus and lysine were set as fixed modifications. The parent ion tolerance was set to 1.1 Da. Turbo search mode was selected. The minimum peptide length allowed was six amino acids. Acceptance criteria were AC score 6.5, peptide Z-score 6.5, peptide p value 1×10^{-4}. Using these criteria, false discovery rates (FDR) of 1.6% and 2.7% were obtained in the first and second replicates, respectively. FDR was calculated by analyzing data sets against a reverse database. Only proteins identified with at least two unique peptides were considered. The same file was searched against Swiss-Prot database (release 56.0, July 2008) restricted to *Bos taurus* for identification of spiked LACB.

2.6 Relative protein quantification using isobaric tagging

Protein quantification was obtained from Phenyx data. The quantification values were extracted from the reporter peak intensities. An isotopic correction was applied to the intensities obtained for the different reporters (114, 115, 116, and 117) according to information provided on the iTRAQ reagents certificate of analysis. Quantification data were then normalized using values obtained for LACB spiked into pancreatic samples, in order to correct for experimental bias. After applying these two corrections, reporter intensities measured for a particular peptide were normalized by the sum of all reporter intensities obtained for this peptide. This allowed determining the relative abundance of each peptide in the compared experimental models. Then, for each protein, the mean, the standard deviation

and the coefficient of variation (CV) of relative peptide intensities were calculated in the four experimental conditions. Redundant peptides, whose sequence was shared by several proteins, were not included in this calculation. Only proteins with at least two non-redundant peptides were used for quantification. When the CV was above 20%, outliers were searched using one of the following statistical tests: IQR test for proteins quantified with more than five peptides and Dixon test for proteins quantified with 3-5 peptides. Outliers were not used for calculation of the mean relative peptide intensity. Mean relative peptide intensities represent relative protein abundances measured in the compared experimental setups. These values were used to calculate inter-experimental protein ratios. In this study, the following inter-experimental ratios were calculated: CTL versus AP, CTL versus CTL-HT, and AP versus AP-HT. Finally, each series of inter-experimental ratios was normalized by its median to correct for potential differences in the total amount of protein used for the different samples. Graphical representations of the distribution of normalized ratios log(2) values were made using Prism 4 software (GraphPad Software, San Diego, CA, USA).

2.7 Immunoblotting

Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T, 2.6% C). Proteins were then electroblotted onto a nitrocellulose or PVDF membrane essentially as described by Towbin *et al.* [16]. Membranes were stained in Ponceau red (nitrocellulose) or Amido-black (PVDF), destained in water and scanned to control homogeneity of sample loading. Immunodetection was performed as follows. Membranes were blocked in PBS, 0.05% Tween 20, 5% nonfat dry milk for 1 hour at room temperature and incubated with primary antibody overnight at 4°C. Primary antibodies were used at the following dilution in PBS, 0.05% Tween 20, 1% nonfat dry milk: 1:250 for rabbit polyclonal anti-actin (A2066, Sigma-Aldrich, Saint-Louis, MO, USA), 1:1000 for rabbit polyclonal anti-alpha 1 inhibitor 3 (ab61338, Abcam, Cambridge, UK), 1:5000 for mouse monoclonal anti-amylase (sc-46657, Santa-Cruz Biotechnology, Santa Cruz, CA, USA), 1:500 for rabbit polyclonal anti-annexin A4 (sc-1930-R, Santa-Cruz Biotechnology), 1:1000

for goat polyclonal anti-carbonic anhydrase 3 (sc-50715, Santa-Cruz Biotechnology), 1:500 for goat polyclonal anti-coatomer gamma (sc-14167, Santa-Cruz Biotechnology), 1:2000 for chicken polyclonal anti-GP3 (ab37599, Abcam), 1:5000 for mouse monoclonal anti-HSP72 (inducible HSP70) (MAB1663, R&D Systems, Minneapolis, MN, USA), 1:5000 for goat polyclonal anti-HSP27 (AF15801, R&D Systems), 1:500 for goat polyclonal anti-regenerating islet-derived protein 3 alpha (AF1745, R&D Systems), 1:1000 for mouse monoclonal anti-ribosomal protein L10a (sc-100827, Santa-Cruz Biotechnology), 1:200 for rabbit polyclonal anti-ribosomal protein L32 (sc-133977, Santa-Cruz Biotechnology), 1:10'000 for rabbit monoclonal anti-alpha-tubulin (ab52866, Abcam), 1:500 for mouse monoclonal anti-beta-tubulin (sc-53140, Santa-Cruz Biotechnology). Membranes were washed with PBS, 0.05% Tween 20, 1% nonfat milk and incubated with secondary antibody in PBS, 0.05% Tween 20, 1% nonfat milk for 1 hour at room temperature. Rabbit polyclonal anti-goat immunoglobulins (P0449, Dako, Glostrup, Denmark), goat polyclonal anti-rabbit immunoglobulins (P0448, Dako), and goat polyclonal anti-mouse immunoglobulins (P0447, Dako) were used diluted at 1:2000. Goat polyclonal anti-chicken IgY (ab6877, Abcam) was used diluted at 1:5000. Membranes were washed in PBS, 0.05% Tween 20, developed with BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland), and visualized on X-ray films.

2.8 Measurement of pancreatic hemoglobin concentration

Hemoglobin concentration in pancreatic tissue extracts was measured on a RX Daytona clinical chemistry analyzer (Randox Laboratories, Crumlin, Co. Antrim, UK) using DiscretePak™ Plasma Free Hemoglobin Reagent Kit from Catachem (Bridgeport, CT, USA). Hemoglobin measurements in pancreatic extracts were normalized using total protein concentration determined with the Bradford method (Bio-Rad Protein Assay).

2.9 Haptoglobin ELISA

Haptoglobin concentration in pancreatic tissue extracts was measured using Rat Haptoglobin ELISA kit from GenWay Biotech (San Diego, CA, USA). Samples were diluted 1/100 (CTL-

HT) or 1/500 (CTL, AP, AP-HT) and processed following manufacturer's instructions. A four-parameter logistics standard curve was used for data analysis. Haptoglobin measurements in pancreatic extracts were normalized using total protein concentration determined with the Bradford method (Bio-Rad Protein Assay).

2. Results

2.1 Animals models

Induction of experimental AP in rats was controlled by measuring amylase and lipase activities in serum and myeloperoxidase concentration in pancreatic extracts 5 hours after the second caerulein injection. Amylase and lipase are digestive enzymes released from damaged acinar cells and are therefore markers of pancreatic tissue injury. Myeloperoxidase is a marker of inflammatory cells at the site of injury. As shown in Figure 1, all three markers were strongly increased in AP and AP-HT rats compared to controls. Furthermore, the three markers were markedly decreased in AP-HT compared to AP samples, which confirmed the heat shock-induced decrease in disease severity. Expression of HSP70 and HSP27 in response to thermal stress was assessed by immunoblotting assays. Results confirmed that these two proteins were strongly increased in pancreatic tissue extracts from CTL-HT and AP-HT rats (Figure 2). The fact that a lower HSP27 level was observed in CTL-HT 1 and AP-HT 2 samples might be explained by the kinetics of HSP27 expression. Indeed, HSP27 expression in the pancreas was shown to be maximal 24 hours after the initial stress [7]. As pancreas was collected before this time limit, differences in the kinetics of HSP27 expression could explain the differences in tissue concentration between animals. In contrast, similar levels were measured for HSP70, which has a faster kinetics of expression [7]. The quality of pancreatic protein extracts was controlled by SDS-PAGE analysis. Results indicated the absence of protein degradation in both control and pancreatitis samples (Supplementary Figure 1).

2.2 LC-MS/MS analysis

A total of 573 proteins were identified by LC-MS/MS analysis with a minimum of two unique peptides from the two replicate experiments (Supplementary Table 1). In the first experiment, 504 proteins were identified, 452 were identified in the second, and 383 were common between the two replicates. MS data are presented in Supplementary Tables 2 and 3.

Relative abundance ratios were calculated for all identified proteins between the following pairs of samples: AP/CTL, CTL-HT/CTL, and AP/AP-HT (Supplementary Table 1). Details of ratio calculation are presented in Supplementary Tables 2 and 3. For proteins identified in both experiments, CVs of relative abundance ratios were calculated (Supplementary Table 1). Mean CVs obtained for AP/CTL, CTL-HT/CTL, and AP/AP-HT were 9.31%, 11.31%, and 10.87%, respectively. These results demonstrated the good reproducibility of relative quantification experiments. For further data analysis, proteins identified in both replicates with a relative abundance ratios CV < 25% and protein identified in a single experiment were included. Using these criteria, 562 proteins were quantified between AP and CTL, 584 between CTL-HT and CTL, and 545 between AP and AP-HT. For proteins identified in both experiments, mean ratios were used. Log(2) values of the relative abundance ratios were calculated and distributions obtained for the three sample comparisons are shown in Supplementary Figure 2. For the three sample comparisons, the distribution was centered on zero indicating that most proteins were not changing in abundance between the two experimental conditions compared.

2.3 AP versus controls

Differential expression was defined by an inter-experimental ratio ≥ 1.50 or ≤ 0.66. Using these criteria, 83 proteins were found differentially expressed between CTL and AP samples, 46 being increased in AP and 37 being decreased. These proteins are listed in Table 1 and the biological or pathobiological process to which they are related is indicated. Most proteins found overexpressed in AP were associated to inflammatory and stress responses, which are major pathobiological pathways of AP. In contrast, proteins found decreased in AP compare to CTL were mainly components of the zymogen granules and proteins related to metabolic processes. Immunoblot experiments on pancreatic extracts were performed to verify MS data obtained for some of these proteins (Figure 3). Immunoblot of alpha-1-inhibitor 3 (A1I3) confirmed the strong increase of the pancreatic concentration of this acute phase inflammatory protein in response to AP induction. The protein was mainly detected as

fragments resulting from its anti-protease activity [17]. Immunoblot also confirmed the increased expression in AP samples of regenerating islet-derived protein 3 alpha (REG3A), a protein involved in cellular stress response. Two zymogen granules proteins, which expression was found decreased in AP samples by LC-MS/MS analysis, were analyzed by immunoblotting: amylase and pancreatic lipase-related protein 2 (GP3). For amylase, immunoblot did not show any evidence of a decreased protein level in AP samples but detected several bands of lower molecular weight probably corresponding to proteolytic fragments. Interestingly, the low molecular weight fragments were strongly elevated in AP samples suggesting an increased amylase degradation following AP induction. For GP3, immunoblot analysis did not show any significant difference in protein level between AP and CTL samples and therefore did not confirmed LC-MS/MS results. Actin, which was not found differentially expressed between AP and CTL samples (mean ratio = 1.13), was also measured by immunoblot. In that case, immunoblot results confirmed the absence of change in protein expression. Annexin A4 was also analyzed but results were not consistent between CTL samples (Supplementary Figure 3). It was therefore not possible to validate LC-MS/MS data, which suggested that the expression of this protein was increased in AP samples. Hemoglobin concentration was also measured in pancreatic tissue since LC-MS/MS results indicated a decreased pancreatic concentration in AP. The mean hemoglobin concentration was actually slightly reduced in AP samples but with an important overlap between AP and CTL groups (Supplementary Figure 3).

2.4 Controls with thermal stress versus controls

Between CTL and CTL-HT samples, 66 proteins were found differentially expressed by a factor ≥ 1.5. Among them, 14 were increased in CTL-HT compared to CTL and 52 were decreased (Table 2). As expected, several HSPs, including HSP70, were strongly overexpressed in CTL-HT. HSP27 (HSP beta 1) was not listed in Table 2 since the CV of relative abundance ratios was too high (43.2%). Nevertheless, this protein was found overexpressed in CTL-HT in the two LC-MS/MS experiments with a mean CTL-HT/CTL ratio

of 2.08 (Supplementary Table 1). The majority of down-regulated proteins in CTL-HT were zymogen granules components and proteins related to metabolic processes, as observed for AP samples. Cytoskeletal proteins, proteins involved in intracellular protein transport and proteins related to inflammatory response were also decreased in CTL-HT compared to CTL. Immunoblots were performed for several proteins, which pancreatic level was found decreased in CTL-HT by LC-MS/MS analysis: two zymogen granule proteins, amylase and GP3, a cytoskeletal protein, actin, and a protein involved in biosynthetic protein transport, coatomer gamma (COPG). Immunoblot results are shown in Figure 4. Amylase immunoblot did not show any difference in native protein concentration between CTL and CTL-HT samples. However, as observed with AP samples, strong bands corresponding to proteolytic fragments were detected in CTL-HT samples, suggesting protein degradation in response to thermal stress. For GP3 and actin, protein level was significantly reduced in two of the three CTL-HT samples tested. Results from COPG immunoblot also suggested increased protein degradation in CTL-HT samples. Therefore, despite some variability between CTL-HT samples, immunoblot data suggest that heat stress induced a decreased expression and/or increased degradation of the four proteins tested. In order to obtain additional information on pancreatic tissue response to thermal stress, microtubule-associated proteins were also analyzed by immunoblot. For tubulin alpha 1A, the mean CTL-HT/CTL ratio measured by LC-MS/MS analysis was 0.58 but the protein was not included in Table 2 since the relative abundance ratio CV was 32% (Supplementary Table 1). For tubulin alpha 4A, tubulin beta-2B and tubulin beta-2C, CTL-HT/CTL ratios of 0.80, 0.73, and 0.75 were obtained, respectively (Supplementary Table 1). Thus, LC-MS/MS data suggested that tubulin chains were decreased in the pancreas following heat shock. Immunoblots confirmed these results and showed a strong reduction of tubulin alpha and tubulin beta pancreatic level in CTL-HT samples (Figure 5). Additional immunoblot experiments were performed for two other proteins found differentially expressed by LC-MS/MS: annexin A4, which was found increased in CTL-HT samples, and carbonic anhydrase 3, which was found decreased in CTL-HT samples. However, results obtained for these two proteins did not allow to confirm

LC-MS/MS data since detected levels were not homogenous in both CTL and CTL-HT sample groups (Supplementary Figure 4). Finally, an increased level of hemoglobin alpha 2 chain was found in CTL-HT using LC-MS/MS analysis. Measurement of free hemoglobin concentration in pancreatic tissue showed that the mean hemoglobin concentration was increased in CTL-HT but with an important overlap with CTL group (Supplementary Figure 4).

2.5 Pancreatitis versus pancreatitis with thermal stress

A total of 102 proteins were found differentially expressed by a factor ≥ 1.5 between AP and AP-HT pancreatic extracts (Table 3). Among this list, 70 proteins were increased in AP compared to AP-HT samples and 32 were decreased. Surprisingly, 49 proteins found overexpressed in AP samples were ribosomal proteins. Immunoblot verifications were performed for ribosomal proteins L10a and L32. Results obtained suggested that the differences in ribosomal proteins concentration observed by LC-MS/MS were linked to inter-individual variations rather than a general effect of thermal stress on experimental AP (Supplementary Figure 5). Several proteins related to inflammatory response were found increased in AP versus AP-HT samples (Table 3). Furthermore, analysis of the distribution of relative abundance ratios of inflammatory proteins showed that the majority has a positive AP/AP-HT Log(2) value, indicating a higher pancreatic concentration in AP sample (Supplementary Figure 6). These findings were in agreement with the fact that heat pretreatment lowered pancreatic inflammation and AP severity. Another expected finding was the strong overexpression of several HSPs, notably HSP70 and HSP27, in AP-HT samples (Table 3). Among the other proteins found differentially expressed, tubulin alpha 4A was increased in AP-HT compared to AP samples (Table 3). Interestingly, LC-MS/MS results obtained for other tubulin chains also suggested a slightly higher concentration in AP-HT samples, with AP/AP-HT ratios of 0.84 for tubulin alpha 1A, 0.86 for tubulin beta 2B, and 0.87 for tubulin beta 2C. Immunoblot experiments were performed that confirmed the higher level of tubulin alpha and tubulin beta in AP-HT pancreatic extracts (Figure 6). In this study,

we observed in CTL-HT samples a concomitant decrease of tubulin chains and COPG. A similar phenomenon has also been described in caerulein-induced AP [18]. These data may be explained by the fact that tubulin and COPG are functionally associated for intracellular vesicle transport [19]. We therefore decided to measure COPG using immunoblot in AP and AP-HT samples despite the fact that LC-MS/MS did not detect a differential expression for this protein (ratio =0.95). Interestingly, immunoblot results clearly showed that the level of native COPG was higher in AP-HT compared to AP samples, as observed for tubulin chains (Figure 6). In contrast, the cytoskeletal protein actin was not found differentially expressed between AP and AP-HT samples (mean ratio = 1.04). This finding was confirmed by immunoblotting. Immunoassay-based verification experiments were also performed for haptoglobin since this protein exhibited a particular expression pattern in AP-HT samples. Like other inflammatory proteins, it was strongly increased in pancreatic tissue following AP induction (Table 1) and was decreased in response to heat stress (Table 2). Interestingly, LC-MS/MS analysis suggested, despite a high inter-experimental CV, that this protein was strongly decreased in AP-HT compared to AP samples, with AP/AP-HT ratios of 4.30 and 2.43, respectively. Quantitative ELISA confirmed these findings (Figure 7). As a matter of comparison, only slightly reduced levels were found in AP-HT samples for most proteins involved in inflammatory or stress responses, such as A1I3 or REG3A (Supplementary Figure 7). Additional immunoblot experiments were performed for carbonic anhydrase 3, a protein involved in the response to oxidative stress that was found overexpressed in AP compared to AP-HT samples. Immunoblot indeed detected higher levels of pancreatic carbonic anhydrase 3 in AP compared to AP-HT samples (Supplementary Figure 8) but these data should be considered with caution due to the high variability of pancreatic carbonic anhydrase 3 concentration in both CTL and CTL-HT samples (Supplementary Figure 4). Finally, LC-MS/MS analysis identified lower levels of several hemoglobin chains in AP versus AP-HT samples. Measurement of hemoglobin concentration in pancreatic tissue was performed. As for previous comparisons, mean hemoglobin concentration was in accordance with LC-MS/MS results but data were not consistent for individual samples

(Supplementary Figure 8).

Discussion

Comparative proteomic analysis was used to investigate modifications of the pancreas proteome induced by heat shock in healthy rats and in rats with caerulein-induced AP. Values of inter-experimental ratio ≥ 1.50 or ≤ 0.66 were chosen to define differential expression because they allowed selecting a reasonable number of candidates, but also based on knowledge on protein expression changes induced by experimental pancreatitis from previous studies of our group and others. In addition, these values were not considered as an absolute prerequisite for selecting a protein for verification experiments. Accordingly, immunoblot were performed for proteins, which had inter-experimental ratios below the defined cut-offs, but that appeared of potential interest due to their biological function. We first compared pancreatic extracts from control and AP rats, without prior thermal stress. These data allowed to validate our analytical workflow and served as a reference for interpretation of further comparisons involving animals exposed to thermal stress. The approach combining LC-MS/MS and isobaric tagging allowed visualizing numerous changes in protein abundance related to the induction of experimental AP. In particular, many proteins involved in inflammatory response were found strongly increased in AP samples, including acute phase proteins, such as A1I3 [20] or alpha-1-acid glycoprotein, leukocyte-derived proteins, such as annexin A1 [21] or protein S100A9 [22], complement factors, and kininogens. Several stress proteins that play a role in the protection against pancreatic tissue injury or in modulating inflammatory response were also found increased in AP: REG3A [23, 24], clusterin [25], and metallothionein-1 [26, 27]. In addition, MS analysis identified numerous zymogen granule proteins, which expression was found decreased in AP samples. LC-MS/MS data were in agreement with several studies that described a disturbed maturation and structure alterations of zymogen granules in caerulein-induced AP [28-31]. They were also in accordance with results from a recent proteomic study of the pancreatic rough endoplasmic reticulum that showed a decrease of digestive enzymes, including amylase, in both arginine-induced AP and caerulein-induced AP [32]. The decrease was

moderate in caerulein-induced AP, 6 hours after disease induction, but was severe in the arginine model, 24 hours after disease onset. However, immunoblot experiments performed for amylase and GP3, assumed to be representative of zymogen granules proteins, did not allow to confirm the decrease in concentration of these proteins in our AP samples. Yet, an increased amylase proteolysis was observed in AP samples. Thus, interpretation of data obtained for zymogen granules proteins appeared quite complex and would therefore require additional verification experiments. In this context, it is noteworthy that the presence of multiple protein fragments, as observed for amylase, might interfere with isobaric reagents-based quantification. LC-MS/MS analysis also detected a reduced level of tubulin beta-2B chain in AP. Proteolysis of tubulin alpha and tubulin beta has been previously described on the same experimental model of AP using peptidomic and immunoblot analyses [18]. These finding were in agreement with the fact that induction of caerulein-induced AP results in disorganization of the microtubule network [33, 34]. Additional proteins, which, to our knowledge, were described for the first time in AP, have been found differentially expressed between CTL and AP pancreatic extracts. Some of these proteins might be involved in the regulation of inflammatory response, such as annexin A6 [35], or in the regulation of cell death pathways, such as prothymosin alpha [36, 37]. However, further studies are warranted to assess their potential role in AP pathobiology. Indeed, increased expression of annexin A4, a protein described both as a regulator of NF-kB transcriptional activity [38] and as a component of pancreatic zymogen granules [39, 40], was detected in AP samples by LC-MS/MS analysis but immunoblot experiments did not confirm this finding.

Using results from the AP/CTL comparison as a reference, the effect of heat shock preconditioning on the pancreas proteome of rats with AP was then analyzed. As evidenced by measurements of serum amylase, serum lipase and pancreatic MPO, heat pretreatment resulted in a reduction of AP severity. Comparison of AP-HT and AP samples was then a way to compare pancreas proteomes from two experimental models of AP with different severities. In AP-HT samples, LC-MS/MS analysis identified the increased expression of HSP27 and HSP70 resulting from thermal stress. In agreement with the reduced disease

severity, most proteins associated to inflammatory response were found decreased in AP-HT compared to AP samples, even if the relative abundance ratio was above the threshold set for differential expression for only a few of them. Previous works showed that the effect of hyperthermia preconditioning on inflammatory response in AP involves the modulation of NF-kB binding activity [15]. A very interesting finding from the comparison of AP-HT and AP pancreatic extracts was that tubulin alpha and beta chains were increased in AP-HT samples, suggesting that hyperthermia had a limiting effect on microtubule disturbances caused by AP. These data are of interest since it was shown that microtubule disorganization is an early and critical event in AP pathobiology [33, 34]. Moreover, a similar effect was observed for COPG, which expression was found increased in AP-HT compared to AP samples. This apparent correlation between COPG and tubulin chains expression could be explained by the fact that microtubules and COPI vesicles are functional partners in vesicular transport [19]. Furthermore, a connection can be made between our findings and results from a recent study showing, in a model of caerulein-induced pancreatitis, that a mild decrease of tubulin and other microtubule proteins expression was associated with an important disorganization of the Golgi apparatus [41]. Taken together, these data suggest that the protective effect of thermal stress against acinar cell injury involves the maintenance of microtubules and Golgi components, which are implicated in the early steps or the secretory pathway [42-44].

Finally, data from the comparison of healthy rats untreated or exposed to hyperthermia were examined to determine whether the differences highlighted between AP and AP-HT samples could be related to the pancreatic response to heat stress. Hyperthermia triggered the expression of several stress proteins in healthy pancreas: HSP27, HSP70, HSP90, HSP105, and metallothionein-1. Overexpression of HSPs in the pancreas following thermal stress is a well-known phenomenon [9-12] and both HSP27 and HSP 70 were shown to be direct mediators of the stress-induced protection in AP [13, 14, 45]. Metallothionein-1 was also described as a protective factor against pancreatic injury [26, 27]. Interestingly, results obtained indicated that hyperthermia, in addition to the induction of stress proteins

expression, had a direct effect on proteins associated to the secretory pathway in acinar cells. Indeed, decreased expression and/or increased degradation was observed for zymogen granules proteins, such as amylase and GP3 [46-48], for cytoskeleton proteins, such as actin [49], for microtubule proteins, such as tubulin alpha and beta chains [42], and for proteins participating in intracellular protein transport, such as COPG [43, 44]. Inter-individual differences in the response to hyperthermia were observed for some of these proteins, such as GP3 or actin, but the global picture emerging from the analysis of LC-MS/MS and immunoblot data was clearly a down-regulation of proteins from the secretory pathway. Surprisingly, changes observed for amylase, alpha-tubulin, beta-tubulin, and COPG in healthy pancreas after thermal stress were similar to those occurring after induction of experimental AP. However, in contrast to AP, a short hyperthermia did not induce inflammatory response or cell injury. The mechanisms underlying these effects on proteins associated to the secretory pathway remain to be clarified and it is not known whether the similar changes induced by hyperthermia and AP are mechanistically related. One hypothesis is that a short heat shock has a preconditioning effect setting acinar cell activity at rest and decreasing expression of proteins of the secretory pathway. The fact that acinar cell is in a long lasting resting state could allow a better response to cellular injury at the time of AP induction [50]. In particular, the decreased expression of proteins of the secretory pathway could be a way to prevent the colocalization of digestive and lysosomal enzymes and thereby to limit trypsinogen activation. Accordingly, Bhaghat and colleagues have proposed that the protective effect of HSP70 against pancreatitis involves blocking the changes of intracellular trafficking that ultimately lead to zymogen activation [13]. In this context, the higher levels of tubulin chains and COPG observed in AP-HT samples might reflect the early recovery of acinar cells that have been protected against damage caused by pancreatitis. In contrast, changes observed in caerulein-induced AP without prior thermal stress could result from both proteolytic degradation of cellular proteins and cellular response to stress and injury.

Results obtained in this study indicate that proteomic analysis of pancreatic tissue extracts using LC-MS/MS and isobaric tagging is an efficient approach for investigating AP pathobiology. Changes were observed for proteins involved in secretory pathway, inflammation or stress response and were discussed in the context of AP pathobiology and heat stress-induced protection. We also detected proteome modifications that we could not link to a particular biological mechanism. One example was haptoglobin, which expression level was strongly increased after AP induction but was almost similar to healthy controls in AP-HT samples. This was in contrast to most inflammatory proteins, whose pancreatic level was only moderately decreased in AP-HT compared to AP samples. It remains to determine whether this difference in haptoglobin level between AP and AP-HT pancreatic extracts could be related to AP severity. It is noteworthy that for some proteins, different results were obtained from LC-MS/MS quantification and immunoblots. These discrepancies were probably related to the fact that an intense proteolytic activity is present in pancreatic tissue during AP. As a consequence, the occurrence of multiple proteolytic fragments from a protein probably interferes with its quantification by LC-MS/MS. Despite these limitations, we showed that using comparative LC-MS/MS analysis as a screening method allowed identifying several changes in pancreatic protein expression that may be involved in the protection against pancreatic tissue damage in AP and/or play a role in modulating AP severity.

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Tables

Table 1. List of the proteins found differentially expressed between CTL and AP samples by LC-MS/MS analysis. Relative protein quantification was performed using iTRAQ™ reagents and LC-MS/MS. AP/CTL ratios were calculated as described in Material and Methods section. Differential expression was defined by a relative abundance ratio ≥ 1.50 or ≤ 0.66. The potential link of each protein with AP pathobiology was determined using data from the literature and from Swiss-Prot and Gene Ontology databases. CTL: control, AP: acute pancreatitis.

Accession number	Protein Name	AP/CTL ratio	Biological process
Proteins increased in AP versus CTL			
IPI00566099	118 KDA PROTEIN (homolog to Murinoglobulin 1)	2.61	Inflammatory response
IPI00780167	164 KDA PROTEIN (homolog to Murinoglobulin 1)	1.78	Inflammatory response
IPI00191715	ALPHA-1-ACID GLYCOPROTEIN	1.90	Inflammatory response
IPI00324019	ALPHA-1-ANTIPROTEINASE	1.83	Inflammatory response
IPI00201262	ALPHA-1-INHIBITOR 3	3.32	Inflammatory response
IPI00326140	ALPHA-1-MACROGLOBULIN	2.68	Inflammatory response
IPI00327469	ALPHA-2-HS-GLYCOPROTEIN	2.54	Inflammatory response
IPI00231615	ANNEXIN A1	1.67	Inflammatory response
IPI00197703	APOLIPOPROTEIN A-I	2.56	Inflammatory response
IPI00190701	APOLIPOPROTEIN E	2.08	Inflammatory response
IPI00213036	COMPLEMENT C4	2.16	Inflammatory response
IPI00325847	CERULOPLASMIN.	2.88	Inflammatory response
IPI00382202	HAPTOGLOBIN	3.14	Inflammatory response
IPI00195516	HEMOPEXIN	2.01	Inflammatory response
IPI00370486	IG LAMBDA-2 CHAIN C REGION	2.92	Inflammatory response
IPI00188541	INTER-ALPHA-INHIBITOR H4 HEAVY CHAIN	3.09	Inflammatory response
IPI00326984	INTER-ALPHA-TRYPSIN INHIBITOR HEAVY CHAIN H3	1.53	Inflammatory response
IPI00515829	KININOGEN 1	1.78	Inflammatory response
IPI00655254	LRRGT00161	2.71	Inflammatory response
IPI00207947	LEUKOTRIENE A4 HYDROLASE.	1.54	Inflammatory response
IPI00365935	PROSTAGLANDIN E SYNTHASE 3.	1.59	Inflammatory response
IPI00231262	PROTEIN S100-A9.	6.84	Inflammatory response
IPI00189981	PROTHROMBIN (FRAGMENT).	2.79	Inflammatory response
IPI00886485	T-KININOGEN.	2.27	Inflammatory response
IPI00200591	SERINE PROTEASE INHIBITOR A3L.	2.54	Inflammatory response
IPI00200593	SERINE PROTEASE INHIBITOR A3K	2.91	Inflammatory response
IPI00679202	SEROTRANSFERRIN	2.53	Inflammatory response
IPI00191737	SERUM ALBUMIN	2.97	Inflammatory response
IPI00194097	VITAMIN D-BINDING PROTEIN	2.25	Inflammatory response
IPI00198667	CLUSTERIN	1.87	Response to stress
IPI00192524	METALLOTHIONEIN-1.	11.85	Response to stress
IPI00196686	REGENERATING ISLET-DERIVED PROTEIN 3 ALPHA	2.32	Response to stress
IPI00200668	TRYPSIN-4	2.33	Zymogen granules
IPI00214905	TROPOMYOSIN ALPHA-4	1.60	Cytoskeleton

IPI00193522	VESICLE-TRAFFICKING PROTEIN SEC22B	1.51	Intracellular protein transport
IPI00760126	14-3-3 PROTEIN BETA/ALPHA.	1.63	Unknown
IPI00231968	ANNEXIN A4	1.78	Unknown
IPI00421888	ANNEXIN A6	1.54	Unknown
IPI00366977	COPINE III	1.58	Unknown
IPI00870721	HISTONE CLUSTER 1, H2BN	2.27	Unknown
IPI00561052	HISTONE H4	2.38	Unknown
IPI00195148	LIVER CARBOXYLESTERASE 1	1.52	Unknown
IPI00393595	PROTHYMOSIN ALPHA	1.78	Unknown
IPI00765333	SIMILAR TO RIBOSOMAL PROTEIN L28	1.81	Unknown
IPI00767277	SIMILAR TO NONO/P54NRB HOMOLOG	1.62	Unknown
IPI00231196	TRANSGELIN	1.62	Unknown
Proteins decreased in AP versus CTL			
IPI00198466	ALPHA-AMYLASE.	0.45	Zymogen granules
IPI00212767	ANIONIC TRYPSIN-1	0.59	Zymogen granules
IPI00211548	BILE SALT-ACTIVATED LIPASE	0.36	Zymogen granules
IPI00193391	CARBOXYPEPTIDASE A2	0.43	Zymogen granules
IPI00211212	CATIONIC TRYPSIN-3	0.43	Zymogen granules
IPI00190598	CHYMOPASIN (Chymotrypsin A)	0.54	Zymogen granules
IPI00206309	CHYMOTRYPSINOGEN B	0.64	Zymogen granules
IPI00327729	ELASTASE-1	0.43	Zymogen granules
IPI00212792	ELASTASE-2A	0.37	Zymogen granules
IPI00211904	PANCREATIC ALPHA-AMYLASE	0.51	Zymogen granules
IPI00231487	PANCREATIC LIPASE-RELATED PROTEIN 2 (GP3)	0.63	Zymogen granules
IPI00192334	PHOSPHOLIPASE A2	0.58	Zymogen granules
IPI00211902	RIBONUCLEASE PANCREATIC BETA-TYPE	0.34	Zymogen granules
IPI00210065	SIMILAR TO SERPIN I2 (ZG-21p)	0.66	Zymogen granules
IPI00212367	SYNCOLLIN.	0.34	Zymogen granules
IPI00191680	TRYPSIN V-B	0.65	Zymogen granules
IPI00194721	ZYMOGEN GRANULE MEMBRANE PROTEIN 16	0.42	Zymogen granules
IPI00201413	3-KETOACYL-COA THIOLASE, MITOCHONDRIAL	0.55	Metabolic process
IPI00326436	5-OXOPROLINASE	0.63	Metabolic process
IPI00230901	ALANINE AMINOTRANSFERASE 1	0.59	Metabolic process
IPI00358059	ASPARTYL AMINOPEPTIDASE	0.66	Metabolic process
IPI00196107	ATP SYNTHASE SUBUNIT B, MITOCHONDRIAL	0.40	Metabolic process
IPI00205332	ELECTRON TRANSFER FLAVOPROTEIN SUBUNIT ALPHA, MITOCHONDRIAL	0.64	Metabolic process
IPI00411230	GLUTATHIONE S-TRANSFERASE MU 2	0.64	Metabolic process
IPI00200794	L-XYLULOSE REDUCTASE	0.63	Metabolic process
IPI00372191	METHYLCROTONOYL-COENZYME A CARBOXYLASE 1	0.56	Metabolic process
IPI00205018	METHYLMALONATE-SEMIALDEHYDE DEHYDROGENASE [ACYLATING], MITOCHONDRIAL	0.64	Metabolic process
IPI00326195	PEROXISOMAL TRANS-2-ENOYL-COA REDUCTASE	0.38	Metabolic process
IPI00211813	MYOSIN-10	0.43	Cytoskeleton
IPI00655259	TUBULIN BETA-2B CHAIN	0.64	Cytoskeleton
IPI00208026	SELENIUM-BINDING PROTEIN 1	0.66	Intracellular protein transport
IPI00287835	HEMOGLOBIN SUBUNIT ALPHA-1/2.	0.66	Hemoglobin complex
IPI00230897	HEMOGLOBIN SUBUNIT BETA-1.	0.65	Hemoglobin complex
IPI00369227	SIMILAR TO LA RELATED PROTEIN ISOFORM 2	0.56	Unknown
IPI00366436	EUKARYOTIC TRANSLATION INITIATION FACTOR 3, SUBUNIT 6 INTERACTING PROTEIN.	0.60	Unknown
IPI00373179	RIBOSOMAL PROTEIN L22 LIKE 1.	0.57	Unknown
IPI00231275	GALECTIN-1	0.40	Unknown

Table 2. List of the proteins found differentially expressed between CTL and CTL-HT samples by LC-MS/MS analysis. Relative protein quantification was performed using iTRAQ™ reagents and LC-MS/MS analysis. CTL-HT/CTL ratios were calculated as described in Material and Methods section. Differential expression was defined by a relative abundance ratio ≥ 1.50 or ≤ 0.66. The biological process was determined using data from the literature and from Swiss-Prot and Gene Ontology databases. CTL: control, CTL-HT: control with prior heat stress.

Accession number	Protein Name	CTL-HT/CTL ratio	Biological process
Proteins increased in CTL-HT versus CTL			
IPI00196751	HEAT SHOCK 70 KDA PROTEIN 1A/1B.	3.04	Response to stress
IPI00471834	HEAT SHOCK PROTEIN 105 KDA.	1.64	Response to stress
IPI00210566	HEAT SHOCK PROTEIN HSP 90-ALPHA.	1.72	Response to stress
IPI00192524	METALLOTHIONEIN-1.	2.04	Response to stress
IPI00207947	LEUKOTRIENE A4 HYDROLASE.	1.55	Inflammatory response
IPI00198916	PANCREATIC TRIACYLGLYCEROL LIPASE	1.55	Zymogen granules
IPI00205036	HEMOGLOBIN ALPHA 2 CHAIN	1.65	Hemoglobin complex
IPI00231968	ANNEXIN A4.	1.98	Unknown
IPI00421888	ANNEXIN A6.	1.55	Unknown
IPI00560977	CAPRIN-1	1.53	Unknown
IPI00366977	COPINE III	1.72	Unknown
IPI00373164	EUKARYOTIC TRANSLATION INITIATION FACTOR 2, SUBUNIT 2.	1.50	Unknown
IPI00768949	NUCLEASE-SENSITIVE ELEMENT-BINDING PROTEIN 1	2.13	Unknown
IPI00188079	SIMILAR TO RIBOSOME-BINDING PROTEIN 1	1.54	Unknown
Proteins decreased in CTL-HT versus CTL			
IPI00198466	ALPHA-AMYLASE.	0.39	Zymogen granules
IPI00212767	ANIONIC TRYPSIN-1	0.51	Zymogen granules
IPI00327713	CARBOXYPEPTIDASE A1	0.66	Zymogen granules
IPI00193391	CARBOXYPEPTIDASE A2	0.37	Zymogen granules
IPI00193393	CARBOXYPEPTIDASE B	0.63	Zymogen granules
IPI00211212	CATIONIC TRYPSIN-3	0.44	Zymogen granules
IPI00190598	CHYMOPASIN (Chymotrypsin A)	0.33	Zymogen granules
IPI00212799	COLIPASE	0.27	Zymogen granules
IPI00365289	ELASTASE 3B, PANCREATIC	0.64	Zymogen granules
IPI00327729	ELASTASE-1	0.41	Zymogen granules
IPI00212792	ELASTASE-2A	0.46	Zymogen granules
IPI00231193	NERVE GROWTH FACTOR, GAMMA (Kallikrein-1)	0.58	Zymogen granules
IPI00211904	PANCREATIC ALPHA-AMYLASE	0.38	Zymogen granules
IPI00231487	PANCREATIC LIPASE-RELATED PROTEIN 2 (GP3)	0.41	Zymogen granules
IPI00734632	PANCREATIC SECRETORY GRANULE MEMBRANE MAJOR GLYCOPROTEIN GP2	0.43	Zymogen granules
IPI00188485	PANCREATIC SECRETORY TRYPSIN INHIBITOR (Spink-1)	0.64	Zymogen granules
IPI00211902	RIBONUCLEASE PANCREATIC BETA-TYPE	0.32	Zymogen granules
IPI00324027	SERINE PROTEASE INHIBITOR KAZAL-TYPE 3	0.55	Zymogen granules

IPI00210065	SIMILAR TO SERPIN I2 (ZG-21p)	0.44	Zymogen granules
IPI00191680	TRYPSIN V-B	0.46	Zymogen granules
IPI00200668	TRYPSIN-4	0.63	Zymogen granules
IPI00189819	ACTIN, CYTOPLASMIC 1.	0.66	Cytoskeleton
IPI00480679	KERATIN, TYPE I CYTOSKELETAL 18.	0.55	Cytoskeleton
IPI00389571	KERATIN, TYPE II CYTOSKELETAL 8.	0.52	Cytoskeleton
IPI00211812	MYOSIN-10	0.43	Cytoskeleton
IPI00365944	MYOSIN LIGHT POLYPEPTIDE 6.	0.69	Cytoskeleton
IPI00780394	COATOMER SUBUNIT GAMMA.	0.61	Intracellular protein transport
IPI00766057	SIMILAR TO RAN BINDING PROTEIN 5	0.65	Intracellular protein transport
IPI00210116	PROTEIN ERGIC-53	0.65	Intracellular protein transport
IPI00382190	6-PHOSPHOGLUCONATE DEHYDROGENASE, DECARBOXYLATING.	0.63	Metabolic process
IPI00358058	ASPARTYL AMINOPEPTIDASE.	0.57	Metabolic process
IPI00396910	ATP SYNTHASE SUBUNIT ALPHA, MITOCHONDRIAL	0.66	Metabolic process
IPI00196107	ATP SYNTHASE SUBUNIT B, MITOCHONDRIAL	0.46	Metabolic process
IPI00195155	DIPEPTIDYL-PEPTIDASE 3.	0.62	Metabolic process
IPI00200661	FATTY ACID SYNTHASE.	0.64	Metabolic process
IPI00192043	METHYLCROTONOYL-COA CARBOXYLASE BETA CHAIN, MITOCHONDRIAL	0.65	Metabolic process
IPI00372191	METHYLCROTONOYL-COENZYME A CARBOXYLASE 1.		Metabolic process
IPI00326195	PEROXISOMAL TRANS-2-ENOYL-COA REDUCTASE	0.45	Metabolic process
IPI00215243	PROTEASOME SUBUNIT ALPHA TYPE-7.	0.65	Metabolic process
IPI00422011	B-FACTOR, PROPERDIN.	0.39	Inflammatory response
IPI00212708	FETUB PROTEIN.	0.66	Inflammatory response
IPI00382202	HAPTOGLOBIN	0.57	Inflammatory response
IPI00326984	INTER-ALPHA-TRYPSIN INHIBITOR HEAVY CHAIN H3	0.61	Inflammatory response
IPI00515828	KININOGEN 1	0.54	Inflammatory response
IPI00194097	VITAMIN D-BINDING PROTEIN	0.69	Inflammatory response
IPI00230788	CARBONIC ANHYDRASE 3.	0.42	Unknown
IPI00209264	GAMMA-GLUTAMYL HYDROLASE	0.65	Unknown
IPI00231275	GALECTIN-1.	0.55	Unknown
IPI00231692	40S RIBOSOMAL PROTEIN S15.	0.66	Unknown
IPI00199865	PHENYLALANYL-TRNA SYNTHETASE ALPHA CHAIN.	0.59	Unknown
IPI00869834	PROTEIN DISULFIDE ISOMERASE ASSOCIATED 2	0.56	Unknown
IPI00373179	RIBOSOMAL PROTEIN L22 LIKE 1.	0.55	Unknown

1

Table 3. List of the proteins found differentially expressed between AP and AP-HT samples by LC-MS/MS analysis. Relative protein quantification was performed using iTRAQ™ reagents and LC-MS/MS. AP/AP-HT ratios were calculated as described in Material and Methods section. Differential expression was defined by a relative abundance ratio ≥ 1.50 or ≤ 0.66. The potential link of each protein with AP pathobiology was determined using data from the literature and from Swiss-Prot and Gene Ontology databases. AP: acute pancreatitis, AP -HT: acute pancreatitis with prior heat stress.

Accession number	Protein Name	AP/AP-HT ratio	Biological process
Proteins increased in AP versus AP-HT			
IPI00190701	APOLIPOPROTEIN E	1.91	Inflammatory response
IPI00325847	CERULOPLASMIN.	1.52	Inflammatory response
IPI00213036	COMPLEMENT C4	1.77	Inflammatory response
IPI00890355	IG GAMMA-2A CHAIN C REGION	1.65	Inflammatory response
IPI00655254	LRRGT00161.	1.71	Inflammatory response
IPI00231262	PROTEIN S100-A9.	6.21	Inflammatory response
IPI00886485	T-KININOGEN.	1.66	Inflammatory response
IPI00192524	METALLOTHIONEIN-1.	2.30	Response to stress
IPI00387709	12 KDA PROTEIN	1.93	Ribosomal protein
IPI00393567	13 KDA PROTEIN	2.29	Ribosomal protein
IPI00567087	18 KDA PROTEIN	2.00	Ribosomal protein
IPI00565717	27 KDA PROTEIN	1.57	Ribosomal protein
IPI00191142	40S RIBOSOMAL PROTEIN S10.	1.51	Ribosomal protein
IPI00366014	40S RIBOSOMAL PROTEIN S13.	1.56	Ribosomal protein
IPI00201500	40S RIBOSOMAL PROTEIN S14.	1.67	Ribosomal protein
IPI00231692	40S RIBOSOMAL PROTEIN S15.	1.65	Ribosomal protein
IPI00231474	40S RIBOSOMAL PROTEIN S15A.	1.67	Ribosomal protein
IPI00559098	40S RIBOSOMAL PROTEIN S19.	1.55	Ribosomal protein
IPI00475776	40S RIBOSOMAL PROTEIN S20.	1.54	Ribosomal protein
IPI00212776	40S RIBOSOMAL PROTEIN S3.	1.59	Ribosomal protein
IPI00475474	40S RIBOSOMAL PROTEIN S4, X ISOFORM.	1.77	Ribosomal protein
IPI00886474	40S RIBOSOMAL PROTEIN S5	1.51	Ribosomal protein
IPI00231202	40S RIBOSOMAL PROTEIN S8.	1.84	Ribosomal protein
IPI00421626	40S RIBOSOMAL PROTEIN S9.	1.94	Ribosomal protein
IPI00188804	60S ACIDIC RIBOSOMAL PROTEIN P2.	1.63	Ribosomal protein
IPI00230915	60S RIBOSOMAL PROTEIN L10.	1.99	Ribosomal protein
IPI00339012	60S RIBOSOMAL PROTEIN L10A.	2.01	Ribosomal protein
IPI00475561	60S RIBOSOMAL PROTEIN L12.	1.53	Ribosomal protein
IPI00475722	60S RIBOSOMAL PROTEIN L14.	1.50	Ribosomal protein
IPI00231445	60S RIBOSOMAL PROTEIN L15.	1.95	Ribosomal protein
IPI00210946	60S RIBOSOMAL PROTEIN L17.	2.09	Ribosomal protein
IPI00230917	60S RIBOSOMAL PROTEIN L18.	1.99	Ribosomal protein
IPI00192257	60S RIBOSOMAL PROTEIN L18A.	2.00	Ribosomal protein
IPI00202214	60S RIBOSOMAL PROTEIN L19.	2.04	Ribosomal protein
IPI00207980	60S RIBOSOMAL PROTEIN L23.	1.59	Ribosomal protein

IPI00203523	60S RIBOSOMAL PROTEIN L23A.	1.65	Ribosomal protein
IPI00230939	60S RIBOSOMAL PROTEIN L24.	2.14	Ribosomal protein
IPI00200552	60S RIBOSOMAL PROTEIN L26.	1.76	Ribosomal protein
IPI00371209	60S RIBOSOMAL PROTEIN L27A.	1.80	Ribosomal protein
IPI00555189	60S RIBOSOMAL PROTEIN L28.	2.64	Ribosomal protein
IPI00395285	60S RIBOSOMAL PROTEIN L3.	1.89	Ribosomal protein
IPI00231346	60S RIBOSOMAL PROTEIN L30.	1.88	Ribosomal protein
IPI00231042	60S RIBOSOMAL PROTEIN L31.	1.68	Ribosomal protein
IPI00197720	60S RIBOSOMAL PROTEIN L35A.	1.75	Ribosomal protein
IPI00230919	60S RIBOSOMAL PROTEIN L36A.	1.69	Ribosomal protein
IPI00569309	60S RIBOSOMAL PROTEIN L37A	1.73	Ribosomal protein
IPI00390823	60S RIBOSOMAL PROTEIN L38.	1.70	Ribosomal protein
IPI00202512	60S RIBOSOMAL PROTEIN L4.	1.86	Ribosomal protein
IPI00390343	60S RIBOSOMAL PROTEIN L6.	2.61	Ribosomal protein
IPI00889339	60S RIBOSOMAL PROTEIN L7	1.87	Ribosomal protein
IPI00215208	60S RIBOSOMAL PROTEIN L8.	1.69	Ribosomal protein
IPI00515778	60S RIBOSOMAL PROTEIN L9.	1.91	Ribosomal protein
IPI00780235	RIBOSOMAL PROTEIN L13A.	2.63	Ribosomal protein
IPI00764351	SIMILAR TO RIBOSOMAL PROTEIN L21	2.03	Ribosomal protein
IPI00765333	SIMILAR TO RIBOSOMAL PROTEIN L28	4.43	Ribosomal protein
IPI00765403	SIMILAR TO 60S RIBOSOMAL PROTEIN L17	2.25	Ribosomal protein
IPI00231609	SIMILAR TO 60S RIBOSOMAL PROTEIN L6	2.23	Ribosomal protein
IPI00358198	SIMILAR TO TRYPSIN 4	1.59	Zymogen granules
IPI00231148	GLYCEROL-3-PHOSPHATE DEHYDROGENASE [NAD+], CYTOPLASMIC.	1.74	Metabolic process
IPI00421931	METHIONINE AMINOPEPTIDASE	1.72	Metabolic process
IPI00204365	RIBOPHORIN I.	1.91	Metabolic process
IPI00230788	CARBONIC ANHYDRASE 3.	2.58	Unknown
IPI00870721	HISTONE CLUSTER 1, H2BN	1.93	Unknown
IPI00231650	HISTONE H1.2	1.61	Unknown
IPI00561052	HISTONE H4.	1.96	Unknown
IPI00409539	FILAMIN-A.	1.56	Unknown
IPI00561555	SIGNAL SEQUENCE RECEPTOR, ALPHA.	2.01	Unknown
IPI00766717	SIMILAR TO ALDO-KETO REDUCTASE FAMILY 1 MEMBER C13	1.51	Unknown
IPI00422076	THROMBOSPONDIN 1.	1.65	Unknown
IPI00231196	TRANSGELIN.	1.95	Unknown
Proteins decreased in AP versus AP-HT			
IPI00196751	HEAT SHOCK 70 KDA PROTEIN 1A/1B.	0.32	Response to stress
IPI00201586	HEAT SHOCK PROTEIN BETA-1 (HSP 27)	0.33	Response to stress
IPI00210566	HEAT SHOCK PROTEIN HSP 90-ALPHA.	0.63	Response to stress
IPI00339148	60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL	0.55	Response to stress
IPI00230901	ALANINE AMINOTRANSFERASE 1.	0.61	Metabolic process
IPI00326195	PEROXISOMAL TRANS-2-ENOYL-COA REDUCTASE	0.33	Metabolic process
IPI00188112	PHOSPHOSERINE PHOSPHATASE.	0.62	Metabolic process
IPI00200794	L-XYLULOSE REDUCTASE.	0.59	Metabolic process
IPI00464791	AMINOACYLASE-1A.	0.66	Metabolic process
IPI00211548	BILE SALT-ACTIVATED LIPASE	0.53	Zymogen granules
IPI00198916	PANCREATIC TRIACYLGLYCEROL LIPASE	0.59	Zymogen granules
IPI00191680	TRYPSIN V-B	0.57	Zymogen granules
IPI00194721	ZYMOGEN GRANULE MEMBRANE PROTEIN 16	0.39	Zymogen granules
IPI00362927	TUBULIN ALPHA-4A CHAIN.	0.65	Cytoskeleton
IPI00213611	ALPHA GLOBIN.	0.57	Hemoglobin complex

IPI00205036	HEMOGLOBIN ALPHA 2 CHAIN (homolog 81%, HBA_RAT, P01946)	0.53	Hemoglobin complex
IPI00287835	HEMOGLOBIN SUBUNIT ALPHA-1/2.	0.55	Hemoglobin complex
IPI00230897	HEMOGLOBIN SUBUNIT BETA-1.	0.44	Hemoglobin complex
IPI00231192	HEMOGLOBIN SUBUNIT BETA-2.	0.60	Hemoglobin complex
IPI00845876	FGA PROTEIN.	0.50	Blood coagulation
IPI00382134	FIBRINOGEN BETA CHAIN	0.45	Blood coagulation
IPI00230944	FIBRINOGEN GAMMA CHAIN	0.32	Blood coagulation
IPI00366397	102 KDA PROTEIN	0.64	Unknown
IPI00560977	CAPRIN-1	0.52	Unknown
IPI00230787	CARBONIC ANHYDRASE 2.	0.65	Unknown
IPI00195372	ELONGATION FACTOR 1-ALPHA 1.	0.66	Unknown
IPI00373164	EUKARYOTIC TRANSLATION INITIATION FACTOR 2, SUBUNIT 2.	0.59	Unknown
IPI00366436	EUKARYOTIC TRANSLATION INITIATION FACTOR 3, SUBUNIT 6 INTERACTING PROTEIN.	0.65	Unknown
IPI00231275	GALECTIN-1.	0.64	Unknown
IPI00779473	LEUKOCYTE ELASTASE INHIBITOR A.	0.57	Unknown
IPI00324986	RAB GDP DISSOCIATION INHIBITOR ALPHA.	0.48	Unknown
IPI00369227	SIMILAR TO LA RELATED PROTEIN ISOFORM 2	0.65	Unknown

Legends

Figure 1. Serum amylase and lipase activities and pancreatic myeloperoxidase concentration. AP was induced in rats by two intraperitoneal injections of caerulein, at 1-hour interval. Control rats received similar injections of saline solution. AP-HT and CTL-HT rats were exposed to a short thermal stress (42°C water bath for 20 minutes) twelve hours before the first injection of caerulein or saline solution. The animals were killed 5 hours after the last injection. Serum were collected and stored at -80°C until analysis. Enzymatic assays were performed on a clinical chemistry analyzer Unicel® DxC 800 using Synchron® System reagents (Beckman Coulter). Myeloperoxidase (MPO) concentration was determined in pancreatic tissue extracts by ELISA (Hycult Biotechnology). Graphs show mean values and bars indicate standard deviations. CTL: control, AP: acute pancreatitis, CTL-HT: control with prior heat stress, AP-HT: acute pancreatitis with prior heat stress.

Figure 2. Immunoblot of HSP70 and HSP27. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane. Immunodetection was performed as described in Material and Methods section using antibodies against HSP70 and HSP27. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, CTL: control, AP: acute pancreatitis, CTL-HT: control with prior heat stress, AP-HT: acute pancreatitis with prior heat stress.

Figure 3. Immunoblot verification of proteins found differentially expressed between AP and CTL samples. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose or PVDF membrane. Immunodetection was performed as described in Material and Methods section using antibodies against A1I3, REG3A, amylase, GP3 and

actin. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, CTL: control, AP: acute pancreatitis.

Figure 4. Immunoblot verification of proteins found differentially expressed between CTL-HT and CTL samples. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane. Immunodetection was performed as described in Material and Methods section using antibodies against amylase, GP3, actin, and COPG. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, CTL: control, CTL-HT: control with prior heat stress.

Figure 5. Immunoblot analysis of alpha and beta tubulins. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane. Immunodetection was performed as described in Material and Methods section using antibodies against alpha-tubulin and beta tubulin. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, CTL: control, CTL-HT: control with prior heat stress.

Figure 6. Immunoblot verification of proteins found differentially expressed between AP and AP-HT samples. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane. Immunodetection was performed as described in Material and Methods section using antibodies against alpha-tubulin, beta tubulin, COPG, and actin. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, AP: acute pancreatitis, AP-HT: acute pancreatitis with prior heat stress.

Figure 7. Measurement of haptoglobin pancreatic concentration. Haptoglobin concentration in pancreatic tissue extracts was measured by ELISA (GenWay Biotech) following manufacturer's instructions. Haptoglobin measurements in pancreatic extracts were normalized using total protein concentration determined with the Bradford method (Bio-Rad Protein Assay). CTL: control, CTL-HT: control with prior heat stress, AP: acute pancreatitis, AP-HT: acute pancreatitis with prior heat stress.

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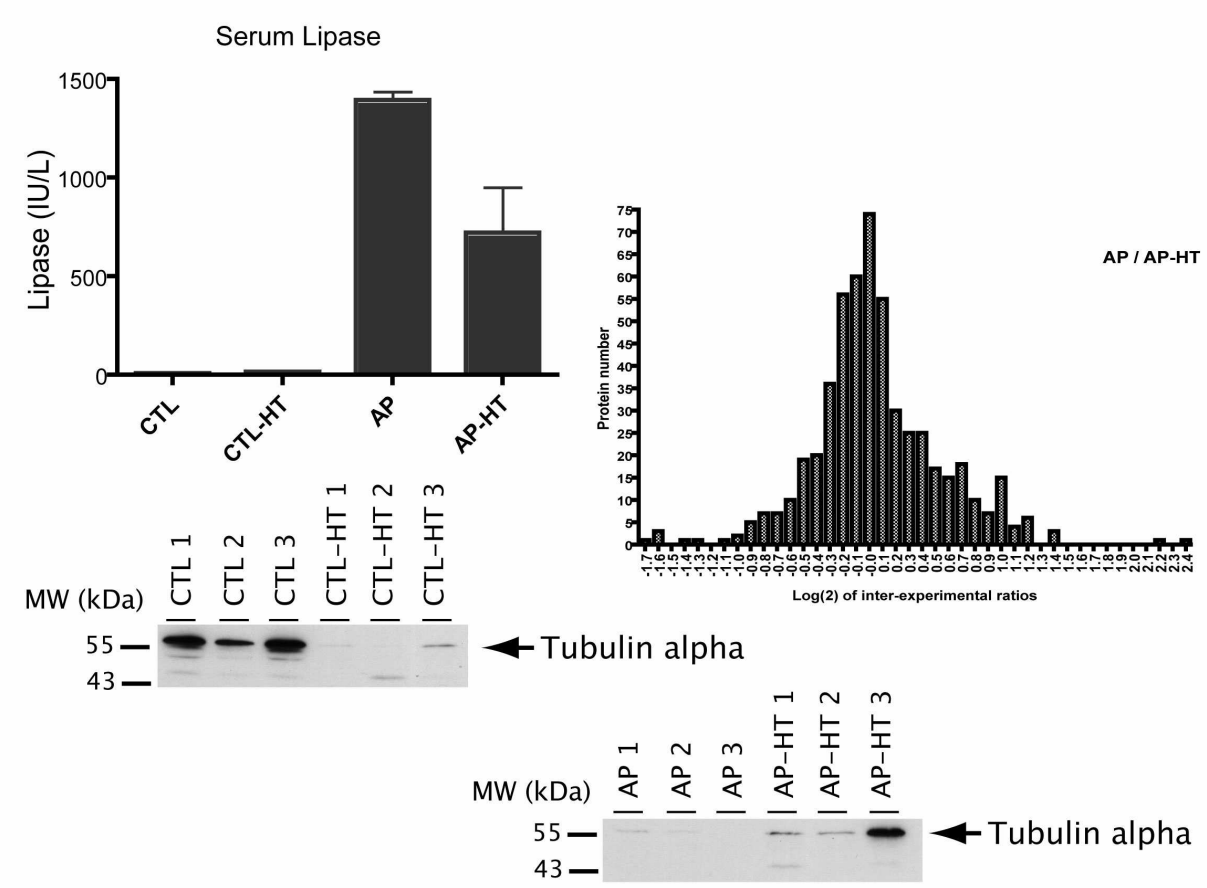
Synopsis:

A comparative proteomic analysis was performed to investigate changes in pancreatic proteins expression induced by heat shock in healthy rats and in rats with experimental acute pancreatitis. We identified numerous proteins, which pancreatic expression changed either in response to hyperthermia or when pancreatitis severity was reduced by thermal stress. Our data suggests that modulation of proteins of the secretory pathway is involved in the protective effect against pancreatic tissue damage.



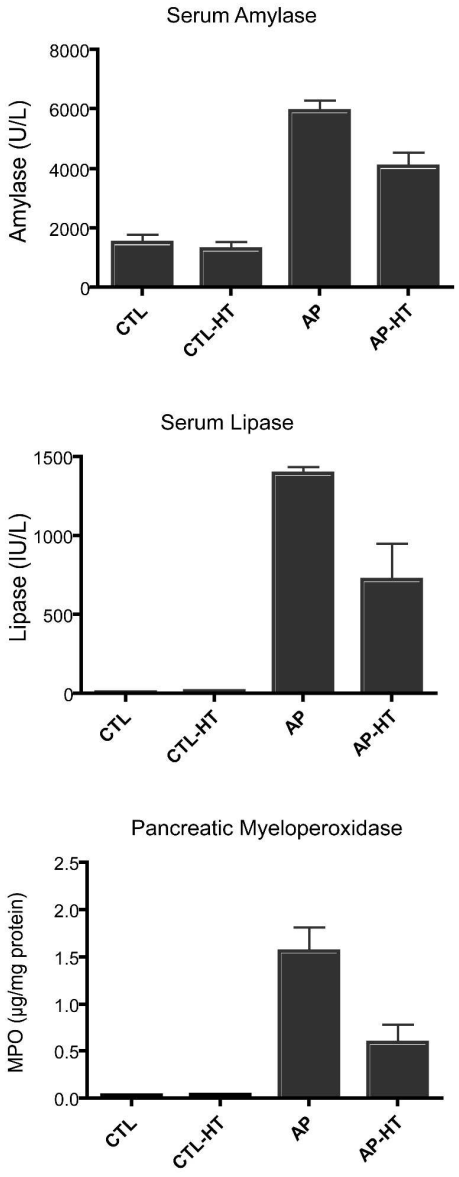


Figure 1. Serum amylase and lipase activities and pancreatic myeloperoxidase concentration. AP was induced in rats by two intraperitoneal injections of caerulein, at 1-hour interval. Control rats received similar injections of saline solution. AP-HT and CTL-HT rats were exposed to a short thermal stress (42°C water bath for 20 minutes) twelve hours before the first injection of caerulein or saline solution. The animals were killed 5 hours after the last injection. Serum were collected and stored at -80°C until analysis. Enzymatic assays were performed on a clinical chemistry analyzer Unicel® DxC 800 using Synchron® System reagents (Beckman Coulter). Myeloperoxidase (MPO) concentration was determined in pancreatic tissue extracts by ELISA (Hycult Biotechnology). Graphs show mean values and bars indicate standard deviations. CTL: control, AP: acute pancreatitis, CTL-HT: control with prior heat stress, AP-HT: acute pancreatitis with prior heat stress.
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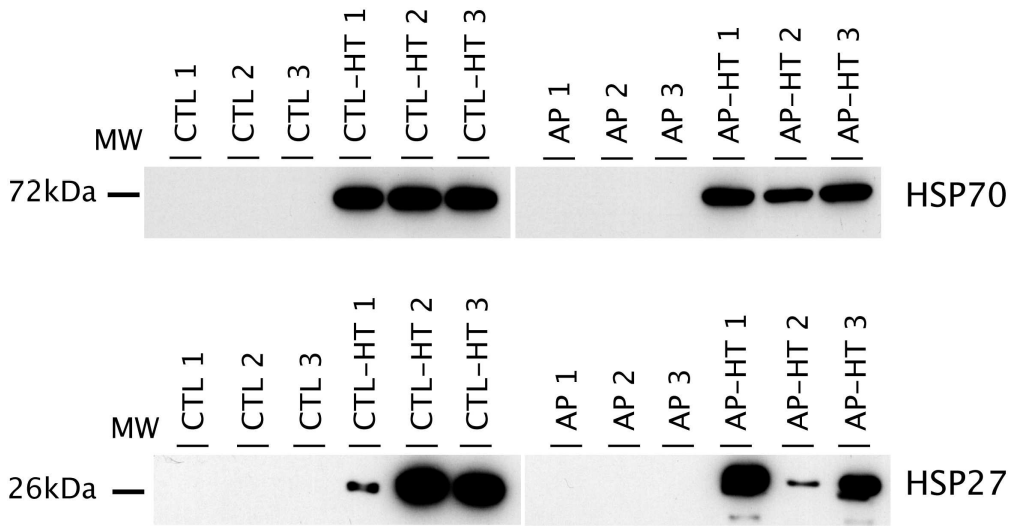

Figure 2. Immunoblot of HSP70 and HSP27. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane. Immunodetection was performed as described in Material and Methods section using antibodies against HSP70 and HSP27. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, CTL: control, AP: acute pancreatitis, CTL-HT: control with prior heat stress, AP-HT: acute pancreatitis with prior heat stress.
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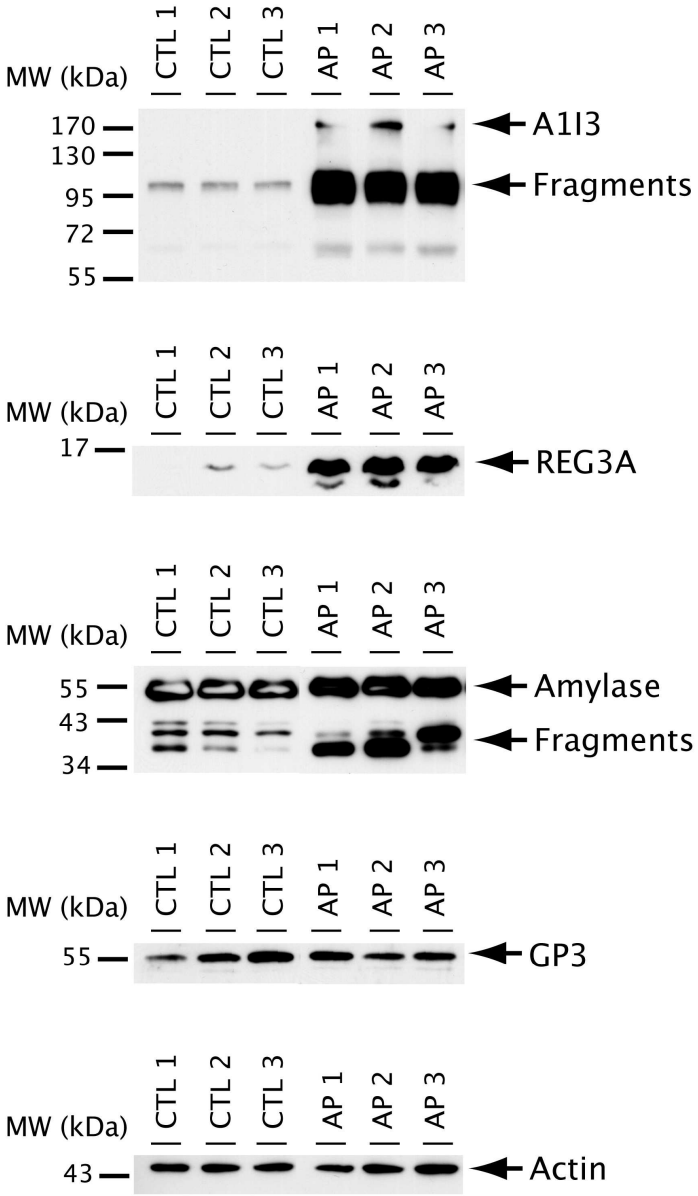

Figure 3. Immunoblot verification of proteins found differentially expressed between AP and CTL samples. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose or PVDF membrane. Immunodetection was performed as described in Material and Methods section using antibodies against A1I3, REG3A, amylase, GP3 and actin. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, CTL: control, AP: acute pancreatitis.
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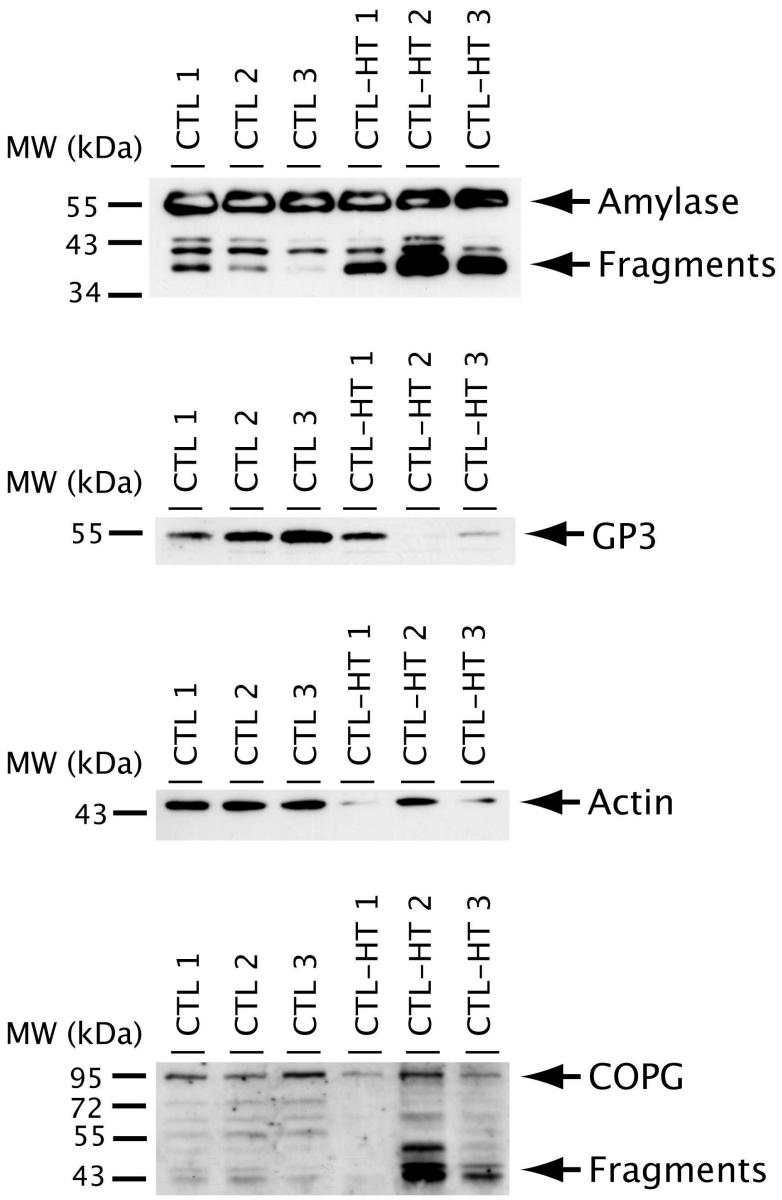

Figure 4. Immunoblot verification of proteins found differentially expressed between CTL-HT and CTL samples. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane. Immunodetection was performed as described in Material and Methods section using antibodies against amylase, GP3, actin, and COPG. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, CTL: control, CTL-HT: control with prior heat stress.
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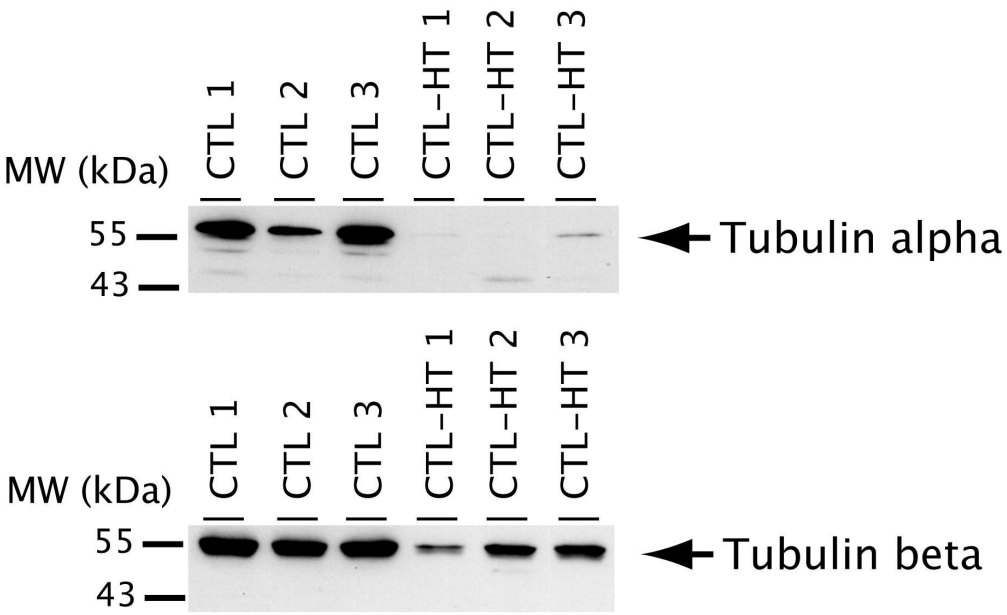

Figure 5. Immunoblot analysis of alpha and beta tubulins. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane. Immunodetection was performed as described in Material and Methods section using antibodies against alpha-tubulin and beta tubulin. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, CTL: control, CTL-HT: control with prior heat stress.
116x72mm (400 x 400 DPI)

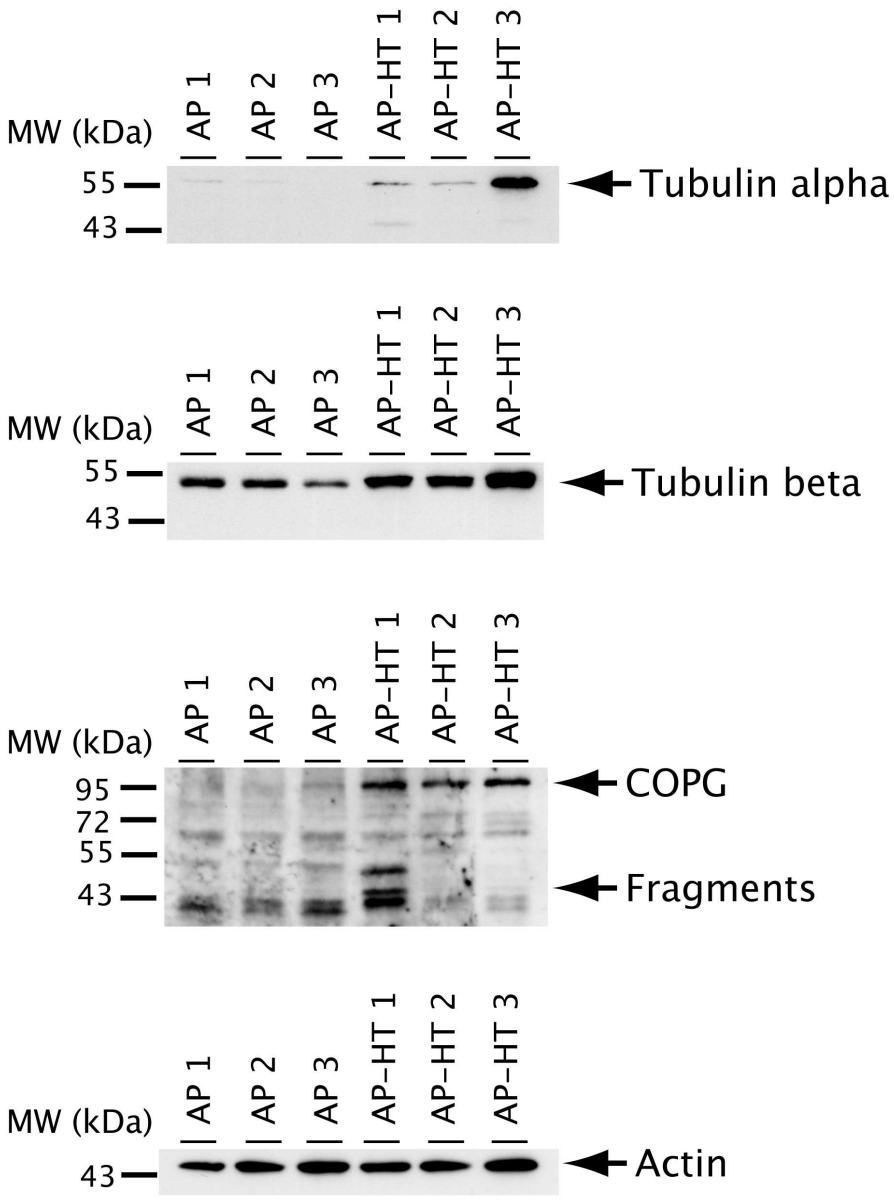

Figure 6. Immunoblot verification of proteins found differentially expressed between AP and AP-HT samples. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane. Immunodetection was performed as described in Material and Methods section using antibodies against alpha-tubulin, beta tubulin, COPG, and actin. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers, AP: acute pancreatitis, AP-HT: acute pancreatitis with prior heat stress.
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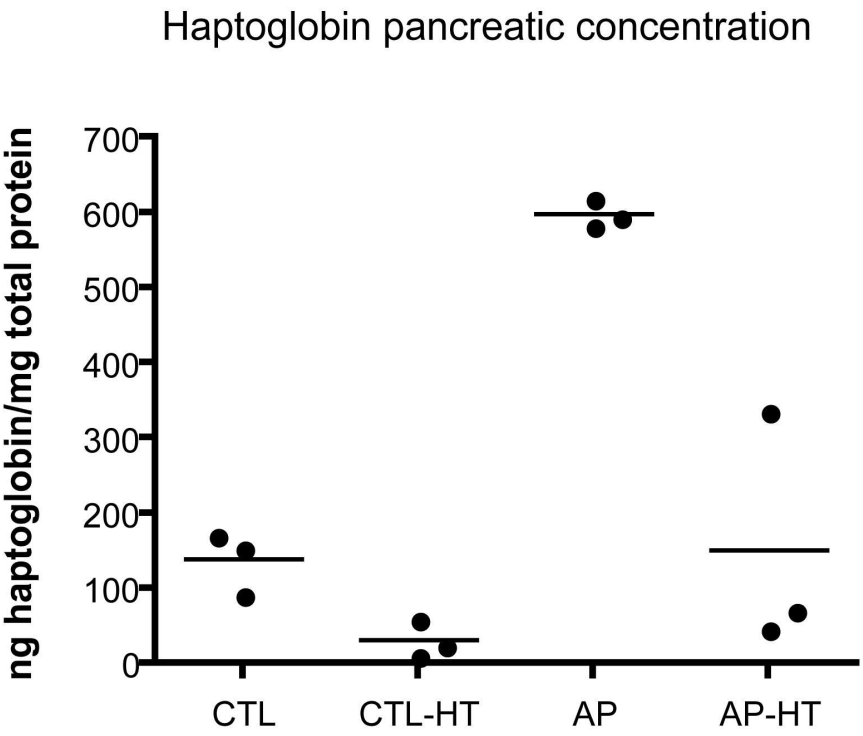

Figure 7. Measurement of haptoglobin pancreatic concentration. Haptoglobin concentration in pancreatic tissue extracts was measured by ELISA (GenWay Biotech) following manufacturer's instructions. Haptoglobin measurements in pancreatic extracts were normalized using total protein concentration determined with the Bradford method (Bio-Rad Protein Assay). CTL: control, CTL-HT: control with prior heat stress, AP: acute pancreatitis, AP-HT: acute pancreatitis with prior heat stress.
114x90mm (400 x 400 DPI)