Acute pancreatitis is an inflammatory disease of the pancreas, which varies greatly in course and severity. Severe forms are associated with serious local and/or systemic complications, and eventually death. The pathobiology of acute pancreatitis is complex. Animal models have been developed to investigate pathobiological processes and identify factors determining disease course. We performed a time-course proteomic analysis using a rat model of severe necrotizing acute pancreatitis induced by taurocholate perfusion in the pancreatic ducts. Results showed that levels of proteins associated to a given biological process changed in a coordinated fashion after disease onset. It was possible to follow the response of a particular pathobiological process to pancreatitis induction and to compare the course of protein pathways. Proteins involved in acinar cell secretion were found to follow a different kinetics than other cellular processes. After an initial decrease, secretory pathway-associated proteins raised again at 18 h post-induction. This phenomenon coincided with a burst in the expression of pancreatitis-associated [...]
Time-course proteomic analysis of taurocholate-induced necrotizing acute pancreatitis☆☆☆

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\begin{abstract}
Acute pancreatitis is an inflammatory disease of the pancreas, which varies greatly in course and severity. Severe forms are associated with serious local and/or systemic complications, and eventually death. The pathobiology of acute pancreatitis is complex. Animal models have been developed to investigate pathobiological processes and identify factors determining disease course. We performed a time-course proteomic analysis using a rat model of severe necrotizing acute pancreatitis induced by taurocholate perfusion in the pancreatic ducts. Results showed that levels of proteins associated to a given biological process changed in a coordinated fashion after disease onset. It was possible to follow the response of a particular pathobiological process to pancreatitis induction and to compare the course of protein pathways. Proteins involved in acinar cell secretion were found to follow a different kinetics than other cellular processes. After an initial decrease, secretory pathway-associated proteins raised again at 18 h post-induction. This phenomenon coincided with a burst in the expression of pancreatitis-associated protein (REG3A), an acute phase protein produced by the exocrine pancreas, and with the decrease of classical markers of pancreatic injury, suggesting that the expression of proteins associated to the secretory pathway may be a modulating factor of pancreas injury.

Biological significance
Acute pancreatitis (AP) is a complex inflammatory disease, the pathobiology of which is not yet fully understood. Various animal models, relying on different mechanisms of disease induction, have been developed in order to investigate pathobiological processes of AP. In this study, we performed a time-course proteomic analysis to investigate changes of the pancreas proteome occurring in an experimental model of AP induced by perfusion of
\end{abstract}

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AP, acute pancreatitis; MS, mass spectrometry; MPO, myeloperoxidase; IHC, immunohistochemistry; LACB, bovine \textbeta;-lactoglobulin; CV, coefficient of variation; GO, gene ontology; A1I3, alpha-1-inhibitor 3; REG3A, pancreatitis-associated protein 1; GP2, pancreatic secretory granule membrane major glycoprotein; COPD, coatomer delta; COPB, coatomer beta.
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1. Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas that varies greatly in course and severity. Most cases correspond to mild forms of the disease, which resolve spontaneously without serious morbidity. In contrast, up to 20% of the patients develop a severe form of AP associated with local and/or systemic complications, such as extensive necrosis of the pancreas, secondary infection of the necrotic tissue, multiple organ dysfunctions (in particular pulmonary failure), or hypovolemic shock [1]. Systemic complications are related to the development of a systemic inflammatory response syndrome; the inflammatory process within the pancreas being out of control and extending to remote organs [2]. Severe forms of AP are associated with a high mortality rate (up to 15%) and patients developing such complications need to be rapidly identified for transfer to an intensive care unit.

The pathobiology of AP is still not fully understood. Schematically, AP results from the uncontrolled activation of digestive proteases within pancreatic acinar cells causing pancreatic tissue damage. This triggers inflammatory response with the recruitment of inflammatory cells and the secretion of various mediators of inflammation by activated acinar and inflammatory cells. However, the underlying mechanisms are complex and numerous aspects remain unclear regarding both disease induction and disease course. AP pathobiology involves indeed several, tightly interconnected, intracellular and extracellular pathways, including inflammatory response, edema, microcirculation dysfunction, cellular stress response, oxidative stress response, apoptosis, and necrosis [2]. The complexity of the interactions between these different pathways makes it difficult to understand which are the key factors in controlling disease course. In order to investigate pathobiological processes of AP, different experimental models of the disease have been developed in mammals [2-4]. These experimental models differ in the mechanisms of disease induction, the nature and extent of the pancreatic injury, the importance of the systemic response. They represent different features of disease and provide therefore complementary information on the disease.

In this context, we believe that proteomic approaches can bring new highlights on the disease by providing a global view on protein pathways involved in cellular and pathobiological processes. In previous studies, we used proteomic and peptidomic analyses to investigate the most widely used experimental model of AP in which disease is induced by parenteral injection of supramaximal doses of cerulein, a cholecystokinin analog [5-7]. This model is characterized by a relatively mild disease severity with pancreatic inflammation, apoptosis, mild necrosis and systemic inflammation [2]. Results from those studies showed that proteomic analysis, while being obviously not comprehensive, allowed obtaining a general picture of the main proteins pathways involved in AP pathobiology. Mass spectrometry (MS) analysis also provided for some proteins, such as protease inhibitor alpha-1-inhibitor 3, precise sequence information that could be correlated with specific pathobiological processes [5,6]. Finally, investigation of the protective effect of thermal stress suggested that proteins associated to the acinar cell secretory pathway play an important role in the modulation of AP severity [7].

In the present study, we used proteomic analysis to investigate an experimental model of AP induced by perfusion of taurocholate, a bile acid, into the pancreatic duct [8]. This mechanism of disease induction yields a significantly different phenotype than the cerulein model explored in previously published proteomic studies on AP. Taurocholate-induced AP is indeed characterized by a more severe disease with, in particular, severe pancreatic necrosis and systemic inflammation [2,5]. The objectives of this study were to highlight changes in the pancreas proteome occurring in the early steps of the experimental disease, to determine the kinetics of functionally related proteins, and to correlate these results with parameters classically used to assess disease severity and with proteomic data previously obtained from the cerulein model. Proteomic analysis was performed on pancreatic protein extracts collected before disease induction and at 2 h, 6 h and 18 h post-induction. The different time-points were compared using a robust quantitative proteomic approach involving isobaric tagging [9,10]. This provided information on pancreas proteome dynamic during the course of AP and allowed establishing kinetics of protein expression according to specific pathobiological pathways. Changes in abundance observed for specific proteins were verified using immunoblot and immunohistochemistry.

2. Material and methods

2.1. Induction of acute pancreatitis and sample collection

Male Sprague–Dawley rats (360 g ± 10 g, n = 12) were anesthetized by isoflurane inhalation and ventilated before AP induction. After laparotomy, taurocholic acid (4%, 0.5 ml sodium salts, Sigma) was perfused over 1 min through a PE10
catheter placed into the biliopancreatic duct. A clamp was placed close to the liver during the perfusion to direct taurocholic acid mainly to the pancreatic ducts. At the end of the perfusion, the clamp was removed and the end of the catheter introduced into the duodenum to prevent postoperative bile leakage into the peritoneum. Before abdomen closure, rats received saline solution (5 ml, intraperitoneal) to prevent severe hypovolemic shock. Postoperative analgesia was induced with buprenorphine (0.05 mg/kg, sc). In the T18h group, rats received another buprenorphine injection 12 h after the first one. Following surgery, rats were looked after in the laboratory until sample collection. Then, rats had a short anesthesia with isoflurane inhalation for sample collection. Control rats \((n = 4)\) had only the short anesthesia before sample collection. They were not ventilated nor received taurocholic acid perfusion. In order to verify that laparotomy and anesthesia had no adverse effect on the pancreas, sham-operated rats were included in the study. Anesthetized and ventilated sham rats \((n = 4)\) had laparotomy without taurocholic acid perfusion and postoperative analgesia. These rats had sample collection 2 h after abdomen closure. For AP rats, blood sample (from the inferior vena cava) and pancreas collection were performed at 2 h \((n = 4, T2h)\), 6 h \((n = 4, T6h)\), or 18 h \((n = 4, T18h)\) after taurocholic acid administration. This protocol was reviewed and approved by the Ethics Committee for Animal Research at the University Medical Center and by the Veterinary Office in Geneva, Switzerland. The study conformed to the American Veterinary Medical Association guidelines on humane treatment of laboratory animals.

Whole blood hemoglobin and bicarbonate concentrations, \(\text{pCO}_2\), \(\text{pH}\), and hematocrit were directly determined with an automatic analyzer. Pancreas and serum were stored at \(-80 \degree \text{C}\) until analysis. Pancreatic tissue extracts were prepared from half of the pancreas by homogenization with a T81 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 at 20,000 \(g\) for 5 min to remove whole cells, cellular debris, and nuclei. Using these centrifugation conditions, some cellular organelles (mitochondria, lysosomes) were probably lost in the pellet but, since we did not use any specific protocol for solubilizing membranes, it was necessary to obtain a clear lysate to avoid interferences in further analytical steps, such as iTRAQ labeling. Supernatants were stored at \(-80 \degree \text{C}\) until analysis. Protein concentration in pancreatic extracts was determined using the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA). Protein sample integrity was controlled by gel analysis. Twenty \(\mu\)g of protein extract was 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. Samples showing evidences of broad-range protein degradation (smearing) were excluded from the study Amylase and lipase activities were measured in serum samples usingSynchro® System reagents on Unicel® Dx800 clinical chemistry analyzers (Beckman Coulter, Fullerton, CA, USA). Myeloperoxidase (MPO) concentration was determined in pancreatic tissue extracts by ELISA (Hycult Biotechnology, Uden, The Netherlands).

The second half of the pancreas was used for histology and immunohistochemistry (IHC). Tissue sections were rapidly removed at the time of sacrifice, fixed, embedded in paraffin, and sectioned (4 \(\mu\)m). After staining with hematoxylin-eosin, the extent of cell necrosis and edema was quantified. Histological scores were expressed in percentage of total acinar tissue (\(\geq 20\) fields in each section).

2.2. Sample preparation and iTRAQ labeling

One pancreatic tissue extract from each of the following groups was selected for comparative proteomic analysis: Control \((T0)\), T2h, T6h, and T18h. The experiment, including iTRAQ labeling, peptide fractionation, and LC-MS/MS analysis, was performed in duplicate using a different sample from each group. For each sample, 70 \(\mu\)g of proteins was mixed with 0.1 M triethylammonium hydrogen carbonate (TEAB) buffer pH 8.0 to a final volume of 100 \(\mu\)l. An equal amount (1 \(\mu\)g) of bovine \(\beta\)-lactoglobulin (LACB) was spiked in each sample to serve as an internal standard for experimental bias correction. Proteins were reduced by adding 1 \(\mu\)l of 1% SDS and 2 \(\mu\)l of 50 mM Tris (2-carboxyethyl) phosphate (TCEP) and heating at 60 \(\degree \text{C}\) for 1 h. Free thiol groups of cysteine residues were alkylated by adding 1 \(\mu\)l of 400 mM iodoacetamide and incubating 30 min at room temperature in the dark with agitation. Proteins were then digested at 37 \(\degree \text{C}\) overnight with 7 \(\mu\)l of a trypsin solution at 0.2 \(\mu\)g/\(\mu\)l in TEAB 0.1 M (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 isotopic tags reconstituted with 70 \(\mu\)l of ethanol by incubating for 1 h at room temperature. Sample/isobaric tag combinations were changed between the two experimental replicates. The labeling reaction was stopped by adding 8 \(\mu\)l of 5% hydroxyamine and by incubating 15 min 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 400 \(\mu\)l of 5% CH\(_3\)CN, 0.1% formic acid (FA) and loaded onto a Macrospin column (Harvard). Elution was performed with 400 \(\mu\)l of 50% CH\(_3\)CN, 0.1% FA. The sample was then dried under vacuum and dissolved in 720 \(\mu\)l 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 pI 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 \(\mu\)l of rehydration solution (4.8% glycerol, 0.12% IPG buffer pH 3–10) per well. After 30 min of incubation, 150 \(\mu\)l of the sample solution was loaded per well. The isoelectric focialization was carried out at 20 \(\degree \text{C}\) until a total voltage of 50 kV/h with a maximum current of 50 \(\mu\)A 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 200 \(\mu\)l of 5% CH\(_3\)CN, 0.1% FA and loaded onto Microspin column (Harvard Apparatus). Elution was performed with 2 times 100 \(\mu\)l of 50% CH\(_3\)CN, 0.1% FA. Eluates were dried under vacuum and stored at –20 \(\degree \text{C}\) until MS analysis.
2.4 LC-MS/MS analysis

ESI LTQ-OT MS was performed on a LTQ Orbitrap XL and/or LTQ Orbitrap velos from Thermo Electron (San Jose, CA, USA) equipped with a NanoAcquity system from Waters. Each peptide fraction was dissolved in 22 μl of 5% CH3CN, 0.1% FA and 4 μl of peptide solution were loaded onto the LC system. Peptides were trapped on a home-made 5 μm 200 Å Magic C18 AQ (Michrom) 0.1 x 20 mm pre-column and separated on a home-made 5 μm 100 Å Magic C18 AQ (Michrom) 0.75 x 150 mm column with a gravity-pulled emitter. The analytical separation was run for 65 min using a gradient of H2O/FA 99.9%/0.1% (solvent A) and CH3CN/FA 99.9%/0.1% (solvent B). The gradient was run as follows: 0-1 min 5% B, then to 35% B at 55 min, and 80% B at 65 min at a flow rate of 220 nl/min. For MS survey scans, the OT resolution was set to 60,000 and the ion population was set to 5 x 10^5 with an m/z window from 400 to 2000. A maximum of 3 precursors was selected for both collision-induced dissociation (CID) in the LTQ and high-energy C-trap dissociation (HCD) with analysis in the OT. For MS/MS in the LTQ, the ion population was set to 1 x 10^4 [7 x 10^3 for velos] (isolation width of 2 m/z) while for MS/MS detection in the OT, it was set to 2 x 10^6 (isolation width of 4 m/z [or 2.5 for velos]), with resolution of 7500, first mass at m/z = 100, and maximum injection time of 750 ms. The normalized collision energies were set to 35% for CID and 60% for HCD.

2.5 Database searching

Peak lists were generated from raw data using the embedded software from the instrument vendor (Read.exe, version 4.2.1). After peak list generation, the CID and HCD spectra were merged for simultaneous identification and quantification [11] (http://www.expasy.org/tools/HCD_CID_merger.html). The monoisotopic masses of the selected precursor ions were corrected using an in-house written Perl script [12]. The corrected idj files, combined from the 24 analyzed off-gel fractions, were searched against UniProt/SwissProt database (release 2011_02 of 08-Feb-2011) using Phenyx (version 57.11, GeneBio, Geneva, Switzerland) via Easyprot interface. The taxonomy selected was Rattus norvegicus (7583 entries). Trypsin was selected as the proteolytic enzyme with one missed cleavage allowed. Oxidized methionine was selected as variable modification. Carbamidomethylation of cysteines and iTRAQ-labeled peptides on the amino terminus and lysine sequence was shared by several proteins, were not included in this calculation. Only proteins with at least two non-redundant peptides were therefore assigned to the corresponding protein group. In the report table, the protein with the highest score within the group is indicated. If two proteins shared the highest score, the one with the highest coverage is shown. The same file was searched against Swiss-Prot database (release 2011_02 of 08-Feb-2011)) restricted to Bos taurus (5818 entries) for identification of spiked LACB.

2.6 Relative protein quantification using isobaric tagging

Reporter peak intensities (114, 115, 116, and 117) extracted from Phenyx data were used for protein quantification. In the first experiment, control sample was labeled with the tag 115, sample T2h with the tag 116, sample T6h with the tag 117, and sample T18h with the tag 114. In the second experiment, control sample was labeled with the tag 116, sample T2h with the tag 117, sample T6h with the tag 114, and sample T18h with the tag 115. Isotopic correction was applied to raw intensities obtained for the different reporters according to information provided on the iTRAQ reagents certificate of analysis (tab “Protein Details”: i114, i115, i116 and i117). Quantification data were then normalized using values obtained for LACB spiked into pancreatic extracts in order to correct for experimental bias (tab “Protein Details”: n114, n115, n116 and n117). The following ratios were then calculated: T2h/T0, T6h/T0, and T18h/T0. The median of each series of inter-experimental ratios was calculated and used as a normalization factor to correct for potential differences in the total amount of protein loaded for the different samples (tab “Quant Resume All Peptides” and tab “Protein Details”: N114, N115, N116 and N117). After applying these corrections, relative reporter intensities were calculated for the three ratios. Each individual reporter value was normalized by the sum of the two reporter intensities used for calculating the ratio (tab “Protein Details”: IR.N114, IR.N115, IR.N116 and IR.N117). This allowed determining the relative abundance of each peptide in the compared experimental models. For each protein, the mean, the median, the standard deviation, and the coefficient of variation (CV) of relative peptide intensities were then calculated in the four experimental conditions. Inter-experimental protein ratios T2h/T0, T6h/T0, and T18h/T0 were calculated using the median since it was assumed that distribution were not Gaussian (tab “Libra”). 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.

2.7 Immunoblotting

Ten μg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris–Glycine gels (10% or 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: 1:5000 for rabbit polyclonal anti-alpha 1 inhibitor 3 (ab61338, Abcam, Cambridge, UK), 1:500 for goat polyclonal anti-regenerating islet-derived protein 3 alpha (AF1745, R&D Systems), 1:350 for rabbit monoclonal anti-cytochrome c (ab76237, Abcam), 1:5000 for mouse monoclonal anti-amylose (sc-46657, Santa-Cruz Biotechnology, Santa Cruz, CA, USA), 1:400 for rabbit anti-GP2 (HPA015739, Sigma), 1:500 for rabbit polyclonal anti-coatamer beta (ab24359, Abcam), 1:250 for rabbit polyclonal anti-coatamer delta (ab72314, Abcam), 1:200 for rabbit polyclonal anti-alpha-tubulin (ab15246, Abcam), 120,000 for rabbit monoclonal anti-beta-tubulin (ab52623, Abcam), and 1:250 for rabbit polyclonal anti-actin (A2066, Sigma-Aldrich, Saint-Louis, MO, USA). 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 h 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. Membranes were washed in PBS, 0.05% Tween 20, developed with BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland), and visualized on X-ray films. Immunoblot band volumes were measured using TotalLab Quant v12.2 Software (TotalLab Ltd, Newcastle upon Tyne, UK). Statistical analysis of immunoblot data was performed using Mann and Witney test and Kruskal Wallis trend test (Statistica™ software, StatSoft, Tulsa, OK, USA). Graphics with box and whiskers were prepared using Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA).

2.8. Immunohistochemistry

Rat pancreas sections (4 μm thick) were analyzed by immunohistochemistry (IHC) using anti-COPB (Abcam, ab24359), anti-COPG (Santa Cruz, sc-14167), anti-alpha tubulin (Abcam, ab52866), and anti-actin (Sigma, A2066) antibodies with the Ventana Discovery automated staining system (Ventana Medical Systems, Tucson, AZ, USA). Ventana reagents for the entire procedure were used. Antigens were retrieved by heating slides with CCI cell conditioning solution for 36 min (Tris-based buffer pH 8.4) using a standard protocol. Primary antibodies were used at the dilutions 1:200, 1:100, 1:1200, and 1:150 in Dako diluent (S2022) for COPB, COPG, alpha-tubulin, and actin respectively and incubated 30 min at 37 °C. Detection of primary antibodies was carried out using the anti-rabbit OmniMap-DAB kit for (Ventana Medical Systems), based on conversion of dianimobenzidine to a dye with multimeric horseradish peroxidase (HRP). For COPG staining, detection was made by incubating the secondary antibodies rabbit biotynylated anti-goat 30 min at 37 °C and by using the DABMap kit from Ventana.

3. Results

3.1. Experimental acute pancreatitis

Different experiments were performed to check for induction of experimental AP and to follow disease course. First, amylase and lipase activities were measured in serum. Amylase and lipase are digestive enzymes synthesized in pancreatic acinar cells. Increase in serum activities of these two enzymes is a marker of pancreatic tissue injury. Second, MPO concentration was measured in pancreatic extracts. MPO is a marker of inflammatory cells at the site of injury and was used to quantify pancreatic tissue inflammation. Third, histological scores of pancreatic edema and necrosis were determined. As shown in Fig. 1, all these markers of pancreatic insult were strongly increased in AP rats compared to controls. In sham-operated rats, enzyme activities, MPO concentration, and histological scores were similar to those in control rats, indicating that pancreatic injury was related to taurocholate administration and not to laparotomy or anesthesia. In AP rats, serum amylase and lipase activities reached a maximum at 6 h, remaining elevated at 18 h. A similar kinetics was observed for pancreatic edema and necrosis scores, suggesting that the severity of pancreatic insult was decreasing at the later time point. On Hematoxylin Eosin stained tissue sections, the morphology of the pancreatic tissue was indicative of AP with large pericinar and perilobular spaces and vacuolization of the acinar cells (Supplemental Information 1). The lobular architecture was however roughly preserved. Infiltration of leucocytes was also visible. In contrast, pancreatic MPO showed a different kinetics. MPO concentration was indeed strongly increased at 18 h compared to the 2 h and 6 h time-points. In addition to these markers of pancreatic injury, several parameters were measured in whole blood as indicators of systemic disease activity (Supplemental Information 2). Hemoglobin and hematocrit were used as markers of hemococoncentration while pCO2, pH, and bicarbonate concentrations were used as markers of acidosis. As previously seen with markers of pancreatic damage, acidosis and hemococoncentration were more severe at the early time-points (2 h and 6 h) and partial recovery was observed at 18 h after disease induction.

3.2. Proteomic analysis of pancreatic extracts

A total of 828 proteins were identified and quantified by LC-MS/MS analysis, with a minimum of two unique peptides, from the two biological replicates: 638 proteins in the first and 770 in the second (Supplemental information 3 and 4). The number of proteins identified in common between the two experiments was 580. Relative abundance ratios were calculated for all proteins. Control sample (T0) was taken as the reference for further analysis. To assess the reproducibility of relative protein quantification, the CV of abundance ratios between the two biological replicates was calculated for all proteins at all time-points. The mean CV was 24.9 % demonstrating the good reproducibility of relative quantification experiments.
3.3. Time-course analysis of pancreas proteome

In order to assess the dynamic of protein expression in the pancreas during the course of acute necrotizing pancreatitis, graphics presenting ratios at different time-points for specific biological and pathobiological pathways were prepared. Interestingly, proteins associated with a given process generally exhibited a homogeneous pattern. For example, proteins involved in the inflammatory response showed a 2 to 5-times increase at 2 h post-induction and remained at a similar level at 6 h (Fig. 2A and B). At 18 h post-induction, some remained stable but the pancreatic level of the majority of them was further increased. As shown in Fig. 2B, a few inflammatory proteins were very strongly elevated (>10 times) at the 18 h time-point. Interestingly, the pattern of expression of inflammatory proteins measured by proteomic analysis matched the one observed for MPO using ELISA measurement. One protein, PIGR, showed a particular pattern with a 35-fold increase at 6 h. Plasma proteins not directly involved in inflammatory pathways were also increased in

Fig. 1 – Markers of pancreas injury. AP was induced in rats by perfusion with taurocholic acid into the pancreatic duct. Animals were sacrificed at 2 h (n = 4), 6 h (n = 4), or 18 h (n = 4) after disease induction. Control rats (T0) (n = 4) did not receive taurocholic acid perfusion. Sham-operated rats (SO) (n = 4) underwent laparotomy but were not perfused with taurocholic acid. 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). Cell necrosis and edema were quantified on hematoxylin–eosin stained pancreatic tissue sections. Histological scores were expressed in percentage of total acinar tissue (≥20 fields in each section). Graphs show mean values and bars indicate standard deviations.
pancreatic extracts at all time-points compared to untreated controls (Fig. 2C). This phenomenon is most probably explained by protein extravasation resulting from increased permeability of pancreatic vessels during the course of AP. Red blood cells proteins were also found elevated in pancreatic extracts but with a different kinetics. A regular elevation was observed until the 6 h time-point but pancreatic level decreased at 18 h (Fig. 2D). Three proteins involved in stress response have also been quantified (Fig. 2E). All three have a similar kinetics with a relatively modest elevation of their pancreatic concentration at early time points and a strongly increased expression 18 h after AP induction.

In contrast, proteins involved in metabolic pathways showed decreased expression in pancreatic extracts during the course of AP. Proteins associated to lipids, carbohydrates, aminoacids and other metabolic processes exhibited overall a similar kinetics: ratios dropped significantly at 2 h post-induction and then decreased progressively until 18 h post-induction (Fig. 3A–D). A similar pattern was observed for proteins involved in protein synthesis and for the chaperone/protein folding group (Fig. 3E–F). For most of these proteins, the lower ratio was obtained at 18 h after AP induction. However, a few started to recover at this time point. The only exceptions were two proteins associated to lipid metabolism, fatty acid synthase (FAS) and mitochondrial
Fig. 3 – Time-course analysis of proteins involved in cellular and metabolic processes. Proteins were quantified by LC-MS/MS analysis from the two replicate experiments as described in Material and methods section. Relative abundance ratios were calculated using control samples (T0) as references. T2h/T0, T6h/T0, and T18h/T0 correspond to the mean value from the two biological replicates. Protein classification was done according to GO terms and bibliographic data. The corresponding proteins are listed in Supplemental Information 5. Proteins presented in Graphics are labeled using Uniprot Entry Name.
2,4-dienoyl-CoA reductase (DECR), which increased at 2 h before dropping at later time points (Fig. 3G). The response of ribosomal proteins to AP induction was delayed in comparison to previous groups. Their level was indeed globally stable at 2 h post-induction and showed a significant decrease at 6 h only (Fig. 3H). Proteins associated to redox metabolism, respiratory chain, RNA processing, and proteasome pathway were also quantified. These proteins had various kinetics but they all decreased following AP induction (Supplemental Information 6).

Interestingly, a group of proteins associated to the acinar cell secretory pathway exhibited a distinct profile. Indeed, pancreatic level of these proteins decreased significantly from AP induction to 6 h but most of them raised again at 18 h (Fig. 4A). Several zymogen granules proteins, the end products of the acinar cell secretory pathway, have been quantified. Some of them, such as the pancreatic secretory granule membrane major glycoprotein (GP2), trypsin or amylase, were found to increase in pancreatic extracts during the course of AP while others, such as pancreatic triacylglycerol lipase, were decreased at 6 h after induction (Fig. 4B). Nevertheless, none of the identified zymogen granules proteins showed reduced expression at 18 h post-induction. Finally, two types of kinetics were observed for cytoskeletal and cytoskeleton-associated proteins. Some were decreased at all time points after AP induction (Fig. 4C). In this first group, several proteins, such as the transforming protein RhoA and alpha-centractin, had a pattern similar to that of the secretory pathway proteins while others, such as beta-tubulin or dynein light chain 2, reached their lower level at 18 h. In contrast, the second group of cytoskeleton-associated proteins showed increased ratios in response to AP induction (Fig. 4D). This group includes beta-actin and several actin-binding proteins: vinculin, thymosin beta-4, and tropomyosin chains.

### 3.4. Verification of proteomic data using immunoassays

Immunoblot and immunohistochemistry (IHC) experiments were performed on selected proteins to verify quantitative data obtained using proteomic analysis. Quantitative proteomic results obtained for the proteins analyzed by immunoassays are summarized in Table 1. Immunoblot statistics are summarized in Supplemental Information 7. Fig. 5A presents immunoblot data obtained for alpha-1-inhibitor 3 (A1I3), an acute phase protein, for pancreatitis-associated protein 1 (REG3A), a protein involved in acinar cell response to stress, and for cytochrome c. A1I3 was found increased in pancreatic extracts at all time-points compared to controls (Fig. 5B). The highest levels were observed at 6 and 18 h after AP induction. In addition to native A1I3, strong bands corresponding to A1I3 fragments were detected. These fragments resulted from A1I3 anti-protease activity [5,7]. REG3A showed a different kinetics: no change was detected at 2 h post-induction, a moderate increase in protein expression was observed at 6 h, and a strong overexpression was visible at 18 h. In contrast, the concentration of cytochrome c in pancreatic extracts was shown to decrease progressively from T0 to T18h. Immunoblot data obtained for these three proteins.
matched therefore accurately with the expression patterns
determined by proteomic analysis for proteins involved in
inflammation, cellular stress response, and metabolic processes.

Immunoassay verifications were also performed for proteins
associated with two different steps of the acinar cell secretory
pathway. First, two proteins from the zymogen granules were
analyzed by immunoblot: amylase and GP2 (Fig. 6). Proteomic
data suggested that amylase was slightly increased after AP
induction. Immunoblot rather showed a slightly decreased but
overall stable amylase level at all time points after AP onset. A
few bands of lower molecular weight were visible but this
degradation pattern was similar in control and AP samples. For
GP2, immunoblot demonstrated clearly an increased expression
in pancreatic extracts following induction in AP and confirmed
therefore quantitative proteomic data. Second, coatomer sub-
units, which are involved in the early steps of the secretory
pathway, were investigated. Coatomer subunits associate with
Golgi non-clathrin-coated vesicles and participate to the endo-
plasmic reticulum-Golgi transport during secretory protein
biosynthesis. Quantitative proteomic analysis suggested that
expression of these proteins decreased in early steps of
taurocholate-induced AP before going back up at 18 h post-
induction. Immunoblot performed on coatomer delta (COPD)
showed reduced expression at 2 h but levels returned to
baseline at 6 h and 18 h (Fig. 7). Analysis of coatomer beta
(COPB) yielded a more complex picture. In the four controls
samples, a band corresponding to native COPB was detected
along with faint bands of lower molecular weight corresponding
to degradation products. At 2 h post-induction, native COPB
was present at a similar or only slightly decreased level but
degradation products were clearly visible in all samples. At 6 h,
native COPB was almost undetectable in 3 out of 4 samples and
only fragments were observed. In the fourth pancreatic extract,
native COPB was detected along with degradation products. At
18 h, strong bands corresponding to degradation products were
observed in the four samples but native COPB was present again
in 3 out of 4 samples. In addition, IHC was performed for
coatomer subunits beta and gamma (Supplemental Information
8). Both subunits showed a decreased staining in acinar cells at 2
and 6 h after AP induction then levels rose again at 18 h. It is
noteworthy that COPG immunoblot and COPD IHC were
performed but did not yield reliable and interpretable results.

Finally, immunoassays were performed on actin and tubulin
chains. Accurate quantification of these structural proteins by
proteomic analysis is difficult since they belong to protein
families that include numerous members sharing high degrees

<table>
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<tr>
<th>UniProt ID</th>
<th>Description</th>
<th>Ratio T2/T0</th>
<th>Ratio exp. 1 T2/T0</th>
<th>Ratio exp. 2 T2/T0</th>
<th>Mean T2/T0</th>
<th>Mean WB band ratio T2/T0</th>
<th>Ratio T6/T0</th>
<th>Ratio exp. 1 T6/T0</th>
<th>Ratio exp. 2 T6/T0</th>
<th>Mean T6/T0</th>
<th>Mean WB band ratio T6/T0</th>
<th>Ratio T18/T0</th>
<th>Ratio exp. 1 T18/T0</th>
<th>Ratio exp. 2 T18/T0</th>
<th>Mean T18/T0</th>
<th>Mean WB band ratio T18/T0</th>
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<td>P35231 REG3A Regenerating islet-derived protein 3-alpha</td>
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<td>1.24</td>
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<td>0.63</td>
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<tr>
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<td>1.61</td>
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<td>P23514 COPB Coatamer subunit beta</td>
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<td>0.81</td>
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<tr>
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<tr>
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<tr>
<td>P60711 ACTB Actin, cytoplasmic 1, N-terminally processed</td>
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<td>0.86</td>
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of sequence homology. Accordingly, identification of a specific protein within the family using LC–MS/MS analysis is hampered by peptide redundancy between family members. Moreover, quantitative values obtained in this study by proteomic analysis were based on only a limited number of peptides since the redundant ones were excluded from ratio calculation. Quantitative proteomic data obtained for actin and tubulin chains should therefore be interpreted with caution (Supplemental)

Fig. 5 – Immunoblot verification of proteins involved in inflammation, cellular stress response or metabolic process. A) Ten μg of pancreatic protein extracts was separated by SDS-PAGE on homemade Tris–Glycine gels (12.5% T or 10% for A1I3). 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, and cytochrome c. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers; CTL: control; T2h, T6h, and T18h: AP rats at 2 h, 6 h and 18 h after disease induction, respectively. B) Immunoblot band volumes were measured using TotalLab Quant v12.2 Software (TotalLab Ltd, Newcastle upon Tyne, UK). Graphics with box and whiskers were prepared using Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA).
Immunoblot verifications were performed for alpha-tubulin, beta-tubulin, and actin (Fig. 8). For beta-tubulin, a signal was detected in the four control samples but with various intensities. In AP samples, heterogeneous results were also obtained at all time-points: some samples gave strong signal, some only a faint band and others were negative. Conversely, results for alpha-tubulin were easily interpretable, showing a progressive decrease in pancreatic extracts from 2 h to 18 h after disease induction. Similar results were obtained by IHC confirming the down-regulation of alpha-tubulin in pancreatic acinar in the course of AP (Supplemental information 8). For actin, immunoblots were performed using a polyclonal antibody recognizing the different actin chains. Results showed a slightly reduced actin expression in pancreatic extracts at the 6 and 18 h time-points. These data were not in accordance with quantitative proteomics analysis where ACTB was found to increase during the course of AP. IHC was performed with the same polyclonal anti-actin antibody. Results showed also a slightly decreased actin expression in pancreatic acinar cells, particularly at 18 h post-induction, confirming therefore immunoblot data.

### 4. Discussion

The perfusion of taurocholate into the pancreatic duct induces a severe disease with interstitial edema, extensive necrosis of the pancreas, and hemorrhages [14–16]. This model is supposed to mimic quite accurately the natural course of severe acute AP in humans. This assumption is based on the hypothesis that the reflux of bile into the pancreatic duct system and the concomitant pressure increase in the pancreatic ducts are triggering events in the development of pancreatic lesions [2]. Animal models have shown that bile salts, such as taurocholate, have a direct toxic effect on the pancreatic tissue causing destruction of the pancreatic duct walls and of the adjacent lobules [14,17]. In a second phase, lesions extend to surrounding parenchyma not directly injured by bile salts, creating large areas of necrosis. Some region of the pancreas remain, however, free from necrosis.

Proteomic analysis was focused on the initial steps of the disease, from 2 h to 18 h after onset, in order to investigate pancreas proteome changes occurring during to the development of pancreatic injuries. One limitation of such an approach is that proteomic experiments were performed on whole pancreas extracts. Information about protein expression in specific cell types was then lost. However, as pancreatic acini represent the large majority of the pancreas mass, we assumed that the observed patterns were representative of changes occurring at the acinar cell level. Since we did not perform subcellular fractionation, we also do not have information on the subcellular localization of the identified proteins. Finally, information on whether proteins were intact or degraded prior LC–MS/MS analysis was lost because of protein digestion. Despite these limitations, quantitative proteomic analysis highlighted...
a number of proteins whose pancreatic level changed significantly during the time-course study. Interestingly, proteins belonging to a same biological or pathobiological process were shown to follow similar kinetics, suggesting that they were modulated in similar fashion. Not surprisingly, the concentration of acute phase and plasma proteins increased in pancreatic extracts in response to AP induction. These changes were obviously related to inflammatory processes, increased permeability of pancreatic vessels, and edema. Most inflammatory proteins, including MPO measured by ELISA, reached a maximum level at 18 h post-induction. In parallel, a general decrease of proteins involved in protein synthesis and metabolic processes was observed. This phenomenon could not be explained by a simple dilution effect resulting from pancreatic edema since the level of structural proteins, such as actin, was only slightly reduced. These findings rather suggested that the whole acinar cell metabolism was set at rest in response to injury. As observed for inflammatory proteins, the maximum effect was reached for most of these proteins at 18 h post-induction. In parallel, a general decrease of proteins involved in protein synthesis and metabolic processes was observed. This phenomenon could not be explained by a simple dilution effect resulting from pancreatic edema since the level of structural proteins, such as actin, was only slightly reduced. These findings rather suggested that the whole acinar cell metabolism was set at rest in response to injury. As observed for inflammatory proteins, the maximum effect was reached for most of these proteins at 18 h post-induction.

Quantitative proteomic analysis allowed investigating additional changes in cellular protein pathways. The main function of pancreatic acinar cells is the secretion of digestive enzymes.
enzymes and the kinetics of expression of proteins associated to the secretory pathway is therefore of particular interest for understanding pancreatic response to injury. Proteomic analysis showed that, among proteins highlighted by comparative proteomic analysis, the ones related to the secretory pathway had a different course. All secretory pathway-associated proteins were indeed decreased in pancreatic extracts following induction of AP, reaching their lower level at 2 or at 6 h, but most of them were found to raise again at 18 h. This kinetics contrasted sharply with proteins involved in protein synthesis and metabolic processes, which continued decreasing at 18 h. There were differences in individual protein kinetics and, as shown by COPB immunoblot, inter-animal variations were observed for some proteins, but overall, corroborating data were obtained from proteomic analysis, immunoblot and IHC. Interestingly, comparative proteomics analysis identified several cytoskeleton-associated proteins that behaved similarly than proteins of the secretory pathway.

Fig. 8 – Immunoblot verification of cytoskeleton proteins. Ten μg of pancreatic protein extracts was 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 beta-tubulin, alpha-tubulin, and actin. Membranes were developed with BM Chemiluminescence Blotting Substrate (Roche). MW: molecular weight markers; CTL: control; T2h, T6h, and T18h: AP rats at 2 h, 6 h and 18 h after disease induction, respectively. Immunoblot band volumes were measured using TotalLab Quant v12.2 Software (TotalLab Ltd, Newcastle upon Tyne, UK). Graphics with box and whiskers were prepared using Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA).
group. These proteins have been described, albeit with different levels of evidence, as participating in protein trafficking in the secretory pathway: RhoA plays a role in actin cytoskeleton reorganization during pancreatic exocrine secretion [25], destrin is involved in secretory cargo sorting [26,27], alpha-centractin and myosin are associated to intracellular vesicular motility [28]. Taken together, these data suggest a relatively early recovery of the acinar cell secretory pathway in pancreas regions preserved from necrosis. Nevertheless, some key components of the secretory pathway, such as tubulin chains, remained expressed at low levels in pancreatic extracts at 18 h post-induction, suggesting that, if recovery actually occurred, it was probably not yet fully complete at this step of disease course. The assumption that the secretory pathway activity was restored early in the course of AP is to parallel with the fact that acinar cells were shown to produce pancreatitis-associated protein 1 (REG3A) in response to AP induction [29]. REG3A is indeed an acute phase protein with an exocrine nature, whose synthesis and processing follows the acinar cell secretory pathway, from the endoplasmic reticulum to the zymogen granules [29,30]. REG3A was shown to act as a protective factor against pancreas damage [19,31,32]. It is therefore remarkable that the rise in the concentration of secretory pathway-associated proteins observed by proteomic analysis and immunohistochemistry coincided with the burst of REG3A expression. Our findings indicate that recovery of secretory pathway proteins expression in acinar cells could be an important factor for modulating pancreas injury during AP by permitting synthesis of REG3A. This hypothesis of a functional link between the acinar cell secretory pathway, stress response and AP course is however only based on that stage on data from proteomic analysis and must be confirmed by additional experiments.

Finally, changes of the pancreas proteome detected in taurocholate-induced AP were analyzed in the light of previous observations made in a cerulein-induced model AP. Many features were similar between the two models (increase of inflammatory, plasma and cell stress proteins, decrease of tubulin chains, secretory pathway proteins, and metabolic enzymes) but one major difference concerned zymogen granules proteins. In cerulein-induced pancreatitis, almost all the zymogen granules proteins identified were found strongly decreased in pancreatic extracts [6]. Moreover, immunoblot of amylase indicated an important degradation [6]. Conversely, in the present study, most of the digestive enzymes and zymogen granules proteins identified had a rather stable or increased expression level along disease course. Only a few were decreased at 2 h and/or 6 h but they all returned to baseline or were elevated at 18 h. These results were confirmed by immunoblot for amylase and GP2. For amylase, no degradation product was detected at any time point, in accordance to previously published results [30,33,34]. Indeed, studies of pancreas tissue structure and ultra-structure in taurocholate-induced AP using light and electron microscopy indicated that, compared to other cellular organelles, zymogen granules were well preserved, even in severely damaged acinar cells [30,33]. Flow cytometry data also showed an increased enzyme load and an increased trypsin/amylase ratio in acinar cells after induction of AP by bile duct obstruction [34]. These differences regarding the fate of zymogen granules proteins are probably related to the different mechanisms of disease induction: altered acinar cell secretion and proteolysis of zymogen granules in the cerulein model [35] versus direct toxic effect of taurocholate on the pancreatic ducts and the adjacent lobules [14,17].

5. Conclusions

The present study provides for the first time an overview of protein expression in the pancreas during the course of taurocholate-induced necrotizing AP. Data obtained by proteomic analysis, immunoblot and IHC allowed determining the global kinetics of protein pathways that play key roles in AP pathobiology during the early steps of disease course. Correlation of these results with data obtained using proteomic or biochemical approaches in various experimental models of AP allowed building hypotheses on the biological processes underlying the observed changes and their potential implications for AP severity. Additional experiments are obviously requested to complement our findings and to confirm our assumptions but such proteomic screening studies allows highlighting new features, generating hypotheses and constitute therefore a strong and reliable basis for further targeted investigations. Proteomic studies targeting specific subcellular fractions instead of total tissue extracts, such as the work by Chen and colleagues on the rough endoplasmic reticulum [36], or studies investigating specific processes, such as protein degradation or protein translocation during the course of AP, would also be a way to better understand this disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.04.022.

REFERENCES


