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Original article

Early proteome analysis of rat pancreatic acinar AR42J cells treated with taurolithocholic acid 3-sulfate

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ABSTRACT

Background: Bile acids are the initiating factors of biliary acute pancreatitis. Bile acids can induce the activation of intracellular zymogen, thus leading injury in pancreatic acinar cells. Pathological zymogen activation in pancreatic acinar cells is a common feature of all types of acute pancreatitis. The proteins expressed in pancreatic acinar cells during the activation of zymogen may determine the severity of acute pancreatitis. The present study aims to determine the differentially expressed proteins in taurolithocholic acid 3-sulfate-stimulated pancreatic acinar cells as an *in vitro* model for acute pancreatitis.
Methods: Rat pancreatic acinar AR42J cells were treated with taurolithocholic acid 3-sulfate for 20 min. Laser confocal scanning microscopy and flow cytometry were used to detect activated trypsinogen in pancreatic acinar AR42J cells. After the determination of trypsinogen activation, proteome analysis was performed to identify the proteins differentially expressed in taurolithocholic acid 3-sulfate-treated cells and non-treated cells.
Results: After treatment with taurolithocholic acid 3-sulfate for 20 min, the activation of trypsinogen in AR42J cells was concurrent with changes in the protein expression profile. Thirty-nine differentially expressed proteins were detected; among these, 23 proteins were up-regulated and 16 proteins were down-regulated. KEGG analysis indicated that these proteins are involved in cellular metabolic pathways, cellular defensive mechanisms, intracellular calcium regulation and cytoskeletal changes.
Conclusion: The expression of proteins in the pancreatic acinar cell changes at the early stage of biliary acute pancreatitis. These differentially expressed proteins will provide valuable information to understand the pathophysiologic mechanism biliary acute pancreatitis and may be useful for prognostic indices of acute pancreatitis.

1. Introduction

Biliary acute pancreatitis (BAP) is one of the most common types of acute pancreatitis (AP). Eugene Opie proposed his "common channel" theory in 1901 to explain the pathogenesis of BAP [1]. This theory proposed that bile acids or some other ingredients in bile were initiating factors of AP. Bile acids can directly activate phosphoinositide-3-kinase-mediated inhibition of sarco(endoplasmic) Ca-ATPase in pancreatic acinar cells, leading to a pathological increase in the intracellular calcium concentration, activation of intracellular digestive zymogen, cell injury/death and activation of inflammatory pathways [2–4]. Bile acids in submicellar concentrations can also stimulate both the IP3 and ryanodine receptors; this leads to the release of calcium from both the endocytoplasmic reticulum and zymogen granules in pancreatic acinar cells [5]. This calcium release can result in pathological intracellular calcium transients.

Perides et al. [6] found an additional and novel mechanism for the effects of bile acid (taurolithocholic acid 3-sulfate, or TLC-S) on pancreatic acinar cells in 2010. They proposed that TLC-S affects G-protein-coupled bile acid receptor-1 (Gpbar1) at the luminal cell surface and induces changes in the intracellular calcium concentration; thus, it was deduced that BAP might be a surface-receptor-mediated disease. The investigation by Perides et al. revealed that TLC-S-induced cell injuries were dependent on the presence of

Abbreviations: TLC-S, taurolithocholic acid 3-sulfate; Gpbar1, G-protein-coupled bile acid receptor-1; BZiPAR, (CBZ-Ile-Pro-Arg) 2-rhodamine 110; SBTI, soybean trypsin inhibitor.

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Gpbar1, pathological calcium signals and undisturbed pH regulation, but the effects of TLC-S on amylase secretion seemed to be independent of Gpbar1. Furthermore, increase in TLC-S concentration was independent of the changes in the calcium concentration. These discrepancies indicate that the effects of bile acids on pancreatic acinar cells involve a complex process that can affect different cellular surfaces and organelles via both receptor-related and receptor-independent mechanisms; however, the precise mechanisms remain to be elucidated. The present study used proteomic methods to analyze the early effects of TLC-S on pancreatic acinar cells in order to identify pathological and physiological changes at early stages of the pathogenesis of BAP.

2. Materials and methods

2.1. Cell culture and treatment

The rat pancreatic acinar AR42J cells were obtained from the China Center for Type Culture Collection (Wuhan, China) and cultured in F12K medium (Sigma–Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) in a 5% CO_2 environment. The cells were treated with TLC-S (Sigma–Aldrich, St. Louis, USA) at a concentration of 200 μM and cultured for 20 min. The dose of TLC-S used and the duration of the exposure to the cells were adapted from the studies of Gerasimenko and Voronina [5,7]. Intracellular trypsinogen activation was detected using confocal microscopy and flow cytometry. Proteomic analysis was then performed to determine differential expression of proteins in response to TLC-S. In addition, five up-regulated proteins (Hyou1, Hspa5, Cfl1, Gstp1, Gapdh) and four down-regulated proteins (Eno1, Anxa7, Pgk1, Pa2g4) were analyzed by Western blotting.

2.2. Measurement of trypsinogen activation

Trypsin serine protease activity in intact living acinar cells was quantitated as previously reported in detail [8]. In brief, after an equilibration of 30 min, 200 μM TLC-S was added for up to 20 min at 37 °C. Acinar cells were then washed and resuspended in NaHEPES lacking TLC-S and supplemented with 10 μM of the cell-permeant synthetic trypsin substrate (CBZ-Ile-Pro-Arg) 2-rhodamine 110 (BZiPAR; Molecular Probes). BZiPAR is a specific substrate for trypsin that becomes fluorescent after cleavage of the two oligopeptide side chains. Activation may be observed by fluorescence of rhodamine 110 by using an excitation wavelength of 485 nm [9]. To ensure that the observed tryptic activity was solely from intracellular enzymes and not from trypsin released into the extracellular fluid, cells for these experiments were prepared in NaHepes containing 5 mM soybean trypsin inhibitor (SBTI; Sigma–Aldrich, St. Louis, USA); all the solutions used also contained SBTI. At this concentration, SBTI can inhibit 1000 units of trypsin per milliliter [10]. The trypsin activity was investigated by flow cytometry (FACSDiva Version 6.1) and confocal microscopy (LSM 510 Meta).

2.3. 2-DE separation and MALDI–TOF–MS/MS identification

After intracellular trypsinogen activation was detected, two-dimensional electrophoresis was utilized to separate the differentially expressed proteins.

2.4. Protein extraction

Cells at a density of 1.0 × 107/ml were treated with TLC-S, washed with PBS three times and then incubated in 500 μl of lysis buffer for 10 min. A cell scraper was then used to collect the lysate, which was then subjected to ultrasonic treatment (80 W, 10 s once for five times on ice with an interval of 15 s). Particulates were removed by centrifugation (12,000 × g, 45 min), and the supernatant was collected. The protein content was then quantitated by the Bradford method (Biomiga, USA) and distributed among small vials in 100 μg aliquots.

2.5. Two-dimensional electrophoresis

One hundred micrograms of protein were adsorbed onto a non-linear IPG strip (pH 3–10; Amersham) and electrophoresed by isoelectric focusing (IEF) at 30 V for 12 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 8 h and 500 V for 4 h. The Ettan IPGphor Isoelectric Focusing System (GE Amersham) was used. Following IEF, the strips were subjected to SDS-PAGE (15 mA/gel for 30 min, 30 mA/gel until the bromophenol blue was 0.5 cm away from the lower edge of the gel). Molecular weight separation was achieved using Hofer SE 600 (GE Amersham). Silver staining was then performed, and the stained gels were scanned using UMax Powerlook 2110XL (GE Amersham).

2.6. Image analysis

The stained gels were analyzed with Image Master Software (Amersham Pharmacia). Three separate gels visualized using either standard or MS-compatible silver staining were analyzed in order to minimize the contribution of experimental variations, and spots displaying the same pattern were selected for further analysis. The normalized relative intensities of spots were used for comparisons between non-treated cells and TLC-S-treated cells (the three gels used the same protein sample), and only those spots with significantly increased or decreased intensity in gels of TLC-S-treated cells (Student's t-test, $P < 0.05$, all with a greater than 1.5-fold increase or decrease) were selected for analysis by MS.

2.7. In-gel digestion

The differential protein spots were excised manually from the silver-stained gel, cut into small pieces, and transferred into 0.5 ml Eppendorf tubes. The gel pieces were destained by adding 50 μl of 30 mM $K_3Fe(CN)_6$/100 mM $Na_2S_2O_3$ (1:1), freeze-dried, and incubated in 5 μl of sequencing-grade trypsin (Promega) solution at 4 °C for 20 h. The solution was then transferred into new Eppendorf tubes. The old Eppendorf tubes were filled with 100 μl of 60% acetonitrile/0.1% trifluoroacetic acid and sonicated for 15 min, after which their contents were combined with the solution of the new Eppendorf tubes.

2.8. MALDI–TOF–MS/MS identification and database search

Each sample was resuspended with 5 mg/ml HCCA matrix. The analysis was performed on a 4800 Plus MALDI-TOF/TOFTM Analyzer (Applied Biosystems, USA). The proteins were identified by PMF and MS/MS using the program MASCOT (Matrix Science, London, UK) against an NCBInr database with GPS explorer software (Applied Biosystems). The search was performed in *Rattus* species. The type of search performed was peptide mass fingerprinting (MS/MS Ion Search). A maximum of one missed cleavage per peptide was allowed, and a mass tolerance of ±100 ppm fragment and MS/MS tolerance of 0.4 Da were used. Proteins with a protein score greater than 55 and a protein score C.I.% greater than 95% were considered significant.