SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Characterization of mesotrypsinogen proteolysis and some natural pancreatic lipase mutations

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1. INTRODUCTION

The human pancreas contributes significantly to digestion by the expression and secretion of numerous hormones and digestive enzymes. Chronic pancreatitis is an irreversible inflammatory disease of the pancreas which can affect both exocrine and endocrine functions. Intensive alcohol consumption and smoking are the main risk factors of chronic pancreatitis but mutations in digestive enzymes also contribute to the development of the disease. Genetic risk factors can act through two pathological pathways, the trypsin-dependent and the trypsin-independent pathways.

The human pancreas secretes three trypsin precursor isoforms: cationic, anionic and mesotrypsinogen. In the trypsin-dependent pathological pathway, early activation of trypsin is observed within the pancreas which can lead to self-digestion of the organ. Insufficient regulation of trypsin activation may be responsible for the development of the disease. A minor chymotrypsin isoform (chymotrypsin C, CTRC) and a small inhibitory protein (SPINK1) produced by the exocrine pancreas play a major role in this regulation. CTRC can proteolytically cleave cationic and anionic trypsinogens within the pancreas. However, calcium, a major constituent of the pancreatic juice, protects trypsinigen from degradation. Besides that, SPINK1 can selectively inhibit cationic and anionic trypsins with high affinity thereby protecting the pancreas against autodigestion and pancreatitis.

Mesotrypsin is a minor trypsin isoform that has unique properties due to an evolutionary mutation in the active site. In contrast to the major trypsin isoforms, mesotrypsin cannot be autoactivated or effectively inhibited by trypsin inhibitors (SPINK1, SBTI). Instead, mesotrypsin degrades and inactivates these trypsin inhibitor proteins. The regulation of mesotrypsin by CTRC remained elusive so far, therefore our aim was to study the role of CTRC-mediated cleavages on mesotrypsin activity and function.

Trypsin-independent pathway is an alternative disease mechanism for chronic pancreatitis, in which certain missense mutations cause defective folding and intracellular aggregation of secretory proteins. The retention of misfolded proteins in the endoplasmic reticulum (ER) may induce ER stress if prolonged.

Recently, four rare heterozygous pancreatic lipase (PNLIP) mutations (A174P, G233E, C254R, V454F) characterized by secretion defect were identified in chronic pancreatitis patients and healthy controls. Our aim was to examine the role of these misfolded PNLIP variants in the development of ER stress and chronic pancreatitis.

2. AIMS

The role of CTRC in mesotrypsinogen inactivation

- Calcium protects cationic trypsinogen against proteolytic cleavage therefore our aim was to investigate the effect of calcium on mesotrypsinogen cleavage by CTRC.
- Our goal was to determine the catalytic properties of CTRC-cleaved mesotrypsin on both short peptide substrates and longer protein substrates.
- Our further aim was to examine the digestive properties of CTRC-cleaved mesotrypsin inhibitors.

Investigation of the role of pancreatic lipase mutations in chronic pancreatitis

- We aimed to generate wild-type and mutant PNLIP plasmids and adenovirus vectors.
- Our goal was to examine the secretion and intracellular retention of PNLIP variants in HEK 293T and AR42J rat pancreatic cells.
- Our further aim was to study the effect of mutant PNLIP intracellular accumulation on the levels of ER stress markers (XBP1 mRNA splicing, BiP expression) in HEK 293T and AR42J cell lines.

3. METHODS

In vitro mutagenesis

The mesotrypsinogen L81A and PNLIP mutations (A174P, G233E, C254R, V454F) were generated by overlap extension PCR mutagenesis and cloned into pTrapT7 or pcDNA3.1(-) vectors.

Generation of adenovirus vectors

Recombinant adenoviral vectors containing wild-type and mutant lipases were generated using AdenoONE Cloning and Expression kit (Sirion Biotech) then transfected HEK 293AD cells using jetPEI transfection reagent. Cells were lysated by repeated freeze-thaw cycles and adenoviruses were purified using AdenoOne Purification kit (Sirion Biotech). The infectious adenovirus concentration was determined by AdEasy Viral Titer kit (Agilent) and expressed in IFU/ml.

Protein expression

Human pancreatic trypsinogens were expressed in *E. coli* BL21 (DE3) cells then extracted and *in vitro* refolded. Other recombinant proteins were expressed in HEK 293T cells using polyethylenimine transfection reagent. Rat pancreatic cells (AR42J) were differentiated with dexamethasone and transduced with recombinant adenovirus vectors containing $5x10^7$ IFU/ml lipase.

Purification of recombinant proteins

The human trypsinogen isoforms were purified by ecotin affinity chromatography. The proteins carrying a C-terminal His-tag were purified by nickel affinity chromatography using the Äkta Prime FPLC system and dialyzed.

Activation and titration of digestive enzymes

Trypsinogen and CTRC were activated by the addition of human enteropeptidase or cationic trypsin, respectively. Concentrations of the active enzymes were determined by active site titration. The activity of trypsin and CTRC was measured with a plate reader at 405 nm.

Gel electrophoresis and densitometry

Reduced proteins were separated by 12 or 15 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized by Coomassie Brilliant Blue staining and quantified by semi-quantitative densitometry analysis.

Mesotrypsinogen cleavage and activation

L81A mesotrypsinogen was incubated with CTRC at 37 °C in the autolysis loop cleavage assay. At the indicated time points aliquots were withdrawn, precipitated with TCA and analyzed with 15 % reducing SDS-PAGE. To produce cleaved mesotrypsin, L81A mesotrypsinogen was incubated with CTRC at 37 °C and activated with human enteropeptidase.

Mesotripsin activity measurement

Mesotrypsin activity was measured using 0.3 mM chromogenic peptide trypsin substrate with a plate reader at 405 nm.

Examination of casein degradation

Bovine β-casein was incubated with non-sulfated and sulfated CTRC-cleaved and intact L81A mesotrypsin at 37 °C. Aliquots were precipitated with TCA at the indicated time points. Casein degradation was examined by 15 % reducing SDS-PAGE.

SBTI digestion study

Soybean trypsin inhibitor (SBTI) was incubated with L81A mesotrypsin (non-sulfated, sulfated and CTRC cleaved non-sulfated and sulfated forms) at 37 °C. At indicated time points, aliquots were taken from the reaction mixture and precipitated with TCA. SBTI cleavage was determined by 15 % reducing SDS-PAGE.

SPINK1 degradation assay

SPINK1 was incubated with L81A mesotrypsin (non-sulfated, sulphated, CTRC cleaved sulfated and non-sulfated forms) at 37 °C. SPINK1 degradation was determined with cationic trypsin inhibition assays. The activity of cationic trypsin was determined with a chromogenic peptide substrate.

Enzyme kinetic measurements

Michaelis-Menten kinetic parameters of mesotrypsin were determined by using chromogenic peptide substrates. K_m and k_{cat} values were calculated using hyperbolic fits on reaction rates versus substrate concentration plots. The Michaelis-Menten parameters of intact and CTRC-cleaved sulfated L81A mesotrypsins were measured at increasing SBTI concentrations, from which inhibitory constant (K_i) values for SBTI were determined.

Lipase activity measurement

PNLIP activity in the conditioned media of transfected HEK 293T cells was measured with p-nitrophenyl palmitate substrate with a plate reader at 405 nm.

Preparation of cell lysates

For the preparation of protein cell lysates HEK 293T and AR42J cells were lysed and the total protein concentration of the samples was determined with BCA Protein Assay kit.

Ultracentrifugation

Transfected HEK 293T cell lysates were ultracentrifugated 48 hours after transfection. The distribution of target protein in the supernatant and pellet fraction was examined by SDS-PAGE and western blot analysis.

Protein immunoblot

Target protein in the media of transfected HEK 293T/transduced AR42J cells and in cell lysates was detected by SDS-PAGE and immunoblotting. To detect PNLIP, HRP-conjugated penta-His antibody was used. BiP protein was detected with rabbit anti-GRP78 antibody and anti-rabbit HRP-conjugated secondary antibody. GAPDH protein was detected with anti-GAPDH primary and HRP-conjugated anti-rabbit IgG secondary antibodies. Protein bands were visualized by using WesternBright ECL HRP substrate.

RNA isolation and reverse transcription

Total RNA was isolated from transfected HEK 293T and transduced AR42J cells and reverse transcribed.

XBP1 splicing assay

Semiquantitative PCR was used to detect X-box binding protein-1 (XBP1) mRNA processing. PCR products were separated by 2.5 % agarose gel to determine spiced and unspliced XBP1 mRNA. The ratio of immature and mature XBP1 cDNA bands was determined by densitometry.

BiP expression assay

Immunoglobulin binding protein (BiP) mRNA expression was determined by qPCR using TaqMan primers and TaqMan Universal PCR Mastermix. Comparative CT (ΔΔCT) value was used to quantify gene expression. Data were calculated using the $2^{-\Delta\Delta CT}$ formula.

4. RESULTS

4.1. THE ROLE OF CTRC IN MESOTRYPSINOGEN INACTIVATION

Cleavage of mesotrypsinogen by CTRC

First we determined the effect of CTRC on mesotrypsin activity. We found that increasing concentrations of CTRC stimulated mesotrypsinogen activation but reduced its final activity. To study the molecular background of this phenomenon, we monitored mesotrypsinogen cleavage by SDS-PAGE and identified the cleavage sites by Edman degradation. Our results showed that mesotrypsinogen contains four CTRC cleavage sites: one in the activation peptide (Phe18), one in the calcium-binding loop (Leu81) and two in the autolysis loop (Leu148, Phe150). Based on our results, the increased mesotrypsin activation was due to the processing of the activation peptide, whereas the decreased final mesotrypsin activity was due to the cleavages in the calcium-binding and autolysis loops. Mesotrypsinogen was predominantly cleaved in the autolysis loop. To study mesotrypsinogen degradation in more details, we generated the L81A mesotrypsinogen variant and selectively monitored the effect of CTRC only on the autolysis loop. Human trypsin isoforms are post-translationally sulfated at the substrate binding site. We were curious about the effect of sulfation on mesotrypsin function. Our results showed that CTRC cleaved the sulfated L81A mesotrypsinogen more rapidly than its non-sulfated counterpart. In addition, our results showed that calcium prevents CTRC cleavage.

Catalytic properties of CTRC-cleaved mesotrypsin

We studied the effect of CTRC cleavage on the activity of mesotrypsinogen. Cleavages by CTRC significantly reduced the activity of mesotrypsin. Therefore, the catalytic properties of mesotrypsin forms were determined using short chromogenic peptide substrates. Mesotrypsin cleavage by CTRC increased its K_m value by an order of magnitude, while the k_{cat} value remained unchanged. Mesotrypsin activity was also determined on a large protein substrate, casein. Sulfated mesotrypsin cleaved casein faster than the non-sulfated protease. CTRC-cleaved mesotrypsin could not cleave the substrate efficiently. Taken together, cleavage by CTRC significantly affects the catalytic properties of the enzyme.

Inhibitor binding of CTRC-cleaved mesotrypsin

The binding of sulfated L81A mesotrypsin and SBTI was studied to test the effect of CTRC-mediated cleavage on inhibitor binding of mesotrypsin. We determined the K_i value of SBTI for both CTRC cleaved and uncleaved mesotrypsin. The value of the cleaved mesotrypsin is significantly higher than the uncleaved form. Our observations showed that mesotrypsin cleavage by CTRC reduced inhibitor binding by an order of magnitude.

Inhibitor digestion by CTRC-cleaved mesotrypsin

Our previous results demonstrated that the binding of inhibitors to CTRC-cleaved mesotrypsin is reduced. Thus, our next experiments aimed to determine whether weaker binding is associated with reduced trypsin inhibitor digestion. Sulfated L81A-mesotrypsin cleaved SBTI more than twice as fast as non-sulfated L81A-mesotrypsin. Digestion rate of SBTI was significantly reduced by CTRC-cleaved mesotrypsin. Both non-sulfated and sulfated L81A CTRC-cleaved mesotrypsins became almost completely inactive.

Finally, intact and CTRC cleaved L81A-mesotrypsin forms (non-sulfated and sulfated) were incubated with human SPINK1 inhibitor. We found that intact sulfated and non-sulfated L81A mesotrypsin efficiently degraded SPINK1 inhibitor. The sulfated mesotrypsin was found to be more active than the non-sulfated form. In contrast, CTRC cleaved L81A mesotrypsin was unable to digest SPINK1 so the inhibitory function of SPINK1 was reduced only to a negligible extent (sulfated form) or not at all (non-sulfated form).

4.2. ROLE OF PANCREATIC LIPASE MUTATIONS IN CHRONIC PANCREATITIS

Secretion of PNLIP variants in HEK 293T cells

The secretion of lipase variants was detected by Coomassie staining followed by SDS-PAGE and western blot analysis. Compared to the wild-type lipase, which was normally secreted, A174P, G233E, C254R and V454F PNLIP variants were not detectable in the media of transfected cells. Then we examined what causes the reduced secretion of lipase variants. We detected PNLIP protein in lysates of the transfected cells by western blotting. Our results showed that all the variants were expressed at approximately the same level as wild-type lipase. After demonstrating that the reduced secretion was not due to defective protein production, we examined whether the misfolded proteins form insoluble aggregates within the cell. Protein aggregates can be easily separated from the soluble proteins by ultracentrifugation. Therefore, the total cell lysates were ultra-centrifuged and PNLIP protein was detected both in the pellet and supernatant by immunoblotting. As expected, wild-type lipase was mostly found in the soluble fraction, whereas PNLIP mutants were mainly present in the insoluble precipitate.

Endoplasmic reticulum (ER) stress response in HEK 293T cells

Based on the literature, PNLIP secretion defect induces ER stress in mammalian cells. To test this notion, total RNA was isolated from transfected HEK 293T cells and reverse

transcribed. We found that the levels of ER stress markers (XBP1 mRNS splicing and BiP chaperone expression) were significantly increased in cells producing lipase mutants.

Secretion of PNLIP variants in AR42J cells

AR42J rat pancreatic cells are more suitable for studying the secretion and cellular effects of pancreatic protein variants. Adenoviral vectors carrying wild-type and mutant PNLIP coding DNAs were used to transduce AR42J cells. The secretion of PNLIP was monitored by examining the media of transduced cells. Our results demonstrated that PNLIP variants remained inside the cells in contrast to the wild-type lipase which was secreted. The protein levels of the intracellular G233E, C254R and V454F PNLIP variants were approximately the same as the wild-type lipase. In contrast, the amount of A174P variant was significantly reduced probably due to intracellular degradation.

Analysis of ER stress markers in AR42J cells

The secretion defect of PNLIP variants suggests that ER stress may be also developed in rat pancreatic cells. Therefore similar to experiments performed on the HEK 293T cell line, we examined the levels of two ER stress markers: XBP1 mRNA splicing and BiP mRNA expression. XBP1 mRNA splicing increased nearly two-fold in rat pancreatic cells transduced with poorly secreted PNLIP variants. In addition BiP mRNA expression levels were also increased in cells transduced with PNLIP variants. The largest effect was observed in the V454F PNLIP variant expressing cells. To confirm that mutant lipases cause ER stress not only at the mRNA level but also at the protein expression level we determined the amount of BiP protein in AR42J cells by western blot analysis. Surprisingly, the amount of BiP protein was nearly identical to wild-type lipase and to cells transduced with A174P, G233E and C254R variants. However, we found a two-fold change in protein amount only in case of V454F.

5. SUMMARY

Our research has focused on the proteolytic regulation of mesotrypsinogen by CTRC. In addition to the previously known Phe18 and Leu81 site in the mesotrypsinogen, we identified two additional cleavage sites in the autolysis loop of mesotrypsinogen at Leu148 and Phe150. We found that the cleavage of the autolysis loop was faster than that of Leu81. Our results demonstrated that the catalytic activity of mesotrypsin was significantly reduced as a result of autolysis loop digestion, detected on short peptide and protein substrates. Our experiments revealed that cleavage of the autolysis loop with CTRC significantly increased the K_m value of the protease while the k_{cat} value remained unchanged. In addition, CTRC cleaved mesotrypsin bound the trypsin inhibitor SBTI weaker and degraded the trypsin inhibitors SBTI and SPINK1 less efficiently than uncleaved mesotrypsin. In conclusion, CTRC prevents the pancreatitis-protective SPINK1 trypsin inhibitor from degradation by inactivating mesotrypsin through proteolytic cleavages.

Recently, four rare heterozygous pancreatic lipase (PNLIP) mutations (A174P, C254R, G233E, V454F) were identified with reduced PNLIP secretion in two European pancreatitis cohorts, in both young patients and controls. Our aim was to determine whether these mutations are pathogenic or harmless. We examined protein secretion, intracellular aggregation and levels of ER stress markers in HEK 293T and AR42J cell lines. Our results showed that mutations block PNLIP protein secretion due to defective folding. We confirmed that PNLIP variants accumulate inside the cells as insoluble aggregates. Expression of PNLIP mutants induced ER stress as indicated by increased XBP1 mRNA splicing and elevated levels of the ER chaperone BiP in HEK 293T and AR42J cells. Based on the available genetic data and our results, we conclude that the studied PNLIP mutations in heterozygous form do not cause chronic pancreatitis, but may increase the risk of the disease in the presence of other risk factors.

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List of publications related to the dissertation

1. **Toldi, V.**, Kassay, N., Szabó, A.: Missense PNLIP mutations impeding pancreatic lipase secretion cause protein misfolding and endoplasmic reticulum stress.
   Pancreatology. [Epub ahead of print], 2021.
   DOI: http://dx.doi.org/10.1016/j.pan.2021.07.008
   IF: 3.996 (2020)
2. **Toldi, V.**, Szabó, A., Sahin-Tóth, M.: Inactivation of mesotrypsin by chymotrypsin C prevents trypsin inhibitor degradation.
   J. Biol. Chem. 295 (11), 3447-3455, 2020.
   DOI: http://dx.doi.org/10.1074/jbc.RA120.012526
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List of other publications

3. Szabó, A., **Toldi, V.**, Gazda, L., Demcsák, A., Tőzsér, J., Sahin-Tóth, M.: Defective binding of SPINK1 variants is an uncommon mechanism for impaired trypsin inhibition in chronic pancreatitis.
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