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Trypsin activity in secretagogue-induced murine pancreatitis is solely elicited by cathepsin B and does not mediate key pathologic responses

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The premature activation of trypsinogen to trypsin in the pancreas has been regarded as a fundamental pathogenic event in the development of pancreatitis. The mechanism of intrapancreatic trypsin activation, however, has remained controversial. Trypsinogen can be activated by the lysosomal protease cathepsin B (CTSB), but it also undergoes autoactivation, a self-amplifying reaction in which trypsin activates trypsinogen. Biochemical studies of trypsinogen mutations associated with human hereditary pancreatitis indicated that autoactivation rather than CTSB-mediated trypsinogen activation is the disease-relevant mechanism [1]. This notion was further supported by the severe pancreatitis phenotype of recently developed mouse models carrying rapidly autoactivating cationic trypsinogen mutants [2, 3]. However, the occurrence of CTSB-mediated intrapancreatic trypsinogen activation and its potential role in disease initiation is also supported by a body of published work. Thus, supramaximal stimulation of the rodent pancreas with the secretagogue cerulein resulted in elevated trypsin activity and redistribution of lysosomal cathepsins to a zymogen granule-enriched subcellular compartment. Furthermore, pharmacologic and genetic inhibition of CTSB abolished cerulein-induced intrapancreatic trypsin activity and ameliorated pancreatitis severity, suggesting that CTSB-generated trypsin mediates pathologic responses in this model [4, 5].

Here, we investigated to what extent CTSB and autoactivation contribute to cerulein-induced intrapancreatic trypsin activity in mice, and examined the role of intrapancreatic trypsin in pancreatitis onset and severity. We generated novel mouse models carrying trypsinogen mutations that inhibit autoactivation but either preserve or increase sensitivity to CTSB-mediated activation. Activation of trypsinogen to trypsin requires proteolytic cleavage after a conserved Lys residue in the activation peptide (Figure 1A). To render trypsinogen resistant to autoactivation and preserve activation by CTSB, we mutated the activation-site Lys to Gly in combination with Ala mutations of the preceding Asp residues in mouse cationic

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trypsinogen (isoform T7), thereby generating strains *T7D23A,K24G* and *T7D22A,K24G* (Figures 1A, 1B, and S1A). Biochemical analysis confirmed that both mutants were completely defective in autoactivation but were readily activated by CTSB [6]. When compared with wild-type T7, mutant D22A,K24G had significantly increased activation with CTSB, whereas mutant D23A,K24G was activated at the same rate as wild type. The mutant trypsinogens were expressed normally in the pancreas of *T7D23A,K24G* and *T7D22A,K24G* mice, as judged by reverse-transcription quantitative PCR and western blot analysis (Figures S1B and S1C). We also generated a novel global *Ctsb-KO* strain on a pure C57BL/6N genetic background (Figure S1D). Successful elimination of the mouse *Ctsb* gene was confirmed by reverse-transcription PCR of pancreatic RNA and western blotting of pancreas homogenates from *Ctsb-KO* mice (Figures S1E and S1F).

To investigate intrapancreatic trypsin activation, we administered single intraperitoneal injections of cerulein or saline to *T7D23A,K24G*, *T7D22A,K24G*, *Ctsb-KO*, and wild-type C57BL/6N mice, and analyzed the pancreas after 30 min. As expected, trypsin activity was significantly increased in the pancreas of the cerulein-treated *T7D23A,K24G, T7D22A,K24G*, and C57BL/6N strains compared with their saline-treated controls (Figure 1C). *T7D22A,K24G* mice exhibited a much more pronounced elevation in trypsin activity than *T7D23A,K24G* or C57BL/6N mice, which showed comparable trypsin levels. In contrast, intrapancreatic trypsin activity remained at basal levels in the pancreas of cerulein-injected *Ctsb-KO* mice (Figure 1C). These observations indicate that during murine pancreatitis induced by cerulein, intrapancreatic trypsin activity is generated exclusively by CTSB, and trypsinogen autoactivation plays no role in this process.

To study whether cerulein-induced pancreatitis severity would mirror intrapancreatic trypsin levels in *T7D23A,K24G*, *T7D22A,K24G*, *Ctsb-KO*, and C57BL/6N mice, we challenged the animals with 10 hourly cerulein injections, and analyzed disease parameters 1 h after the last injection (Figure S1G). We found that repeated cerulein injections induced similar pathology in all four strains investigated, as judged by plasma amylase activity (Figure 1D), pancreas edema (Figures 1E, S1H, and S1I), histopathological changes (Figure 1F), and scoring of pancreas sections for edema, inflammatory cells, and necrosis (Figures 1G, 1H, and 1I). When pancreas sections (n=5–6) were stained with immunohistochemistry for the neutrophil granulocyte marker myeloperoxidase (MPO) and the macrophage marker F4/80, we observed, on average, about 3-fold higher positivity for F4/80 than MPO. However, with the sole exception of slightly elevated MPO-positive cells in *Ctsb-KO* mice, no significant differences were observed in immune cell distribution among the 4 strains studied (reviewed but not shown). Taken together, the observations clearly indicate that there is no correlation between cerulein-induced pancreatitis severity and intrapancreatic trypsin levels. The high trypsin activity in the pancreas of *T7D22A,K24G* mice did not cause more severe disease than the lower trypsin activity in C57BL/6N controls or in *T7D23A,K24G* mice. Similarly, in *Ctsb-KO* mice, the absence of intrapancreatic trypsin activity did not mitigate pancreatitis. This latter observation is at odds with the results of Halangk et al. (2000) who found that *Ctsb-KO* mice exhibited partly reduced pancreas damage during cerulein-induced pancreatitis, as judged by serum amylase and lipase activity, pancreas edema, and acinar cell necrosis [5]. The discrepancy may be due to the mixed genetic background of the *Ctsb-KO*

mice used in the prior study. In agreement with our results, Halangk et al. also noted that *Ctsb* deletion did not affect inflammatory parameters of experimental pancreatitis.

Previously, the Steer laboratory (2002) demonstrated that the CTSB inhibitor CA-074me abolished intrapancreatic trypsin activation and decreased the severity of experimental pancreatitis induced by cerulein or bile acid in mice [4]. Subsequently, however, the authors found that while CA-074me prevented trypsin activation and organellar fragility, it had no impact on pro-inflammatory and cytoskeletal changes in the acinar cells during cerulein-induced pancreatitis [7]. The somewhat contradictory findings of these pharmacologic studies are difficult to interpret. The possible off-target effects of CA-074me may be a confounding factor.

In accord with our results, several studies using genetically modified mice documented a disconnect between intrapancreatic trypsin activity and pancreatitis severity. Thus, cerulein injections resulted in significantly increased intrapancreatic trypsin activity without affecting pancreatitis severity in mice lacking one of the mannose 6-phosphate receptors, which caused redistribution of CTSB to the secretory compartment [8]. Markedly increased intrapancreatic trypsin activity was seen in mice deficient in the trypsinogen degrading protease cathepsin L (CTSL), while pancreatitis severity was reduced in response to cerulein injections [9]. Furthermore, intrapancreatic trypsin activity was diminished in cerulein-treated T7 trypsinogen knock-out mice, but inflammatory parameters of acute pancreatitis were unchanged even though acinar cell necrosis was partially alleviated [10]. Finally, while this paper was under review, Chen et al. (2022) reported the effect of pancreas-specific conditional deletion of mouse CTSB and CTSL on cerulein-induced trypsin activity and pancreatitis [11]. Relative to C57BL/6N wild-type controls, single-knockout (*Ctsb*$^{\Delta pan}$ and *Ctsl*$^{\Delta pan}$) and double-knockout (*Ctsb*$^{\Delta pan}$,*Ctsl*$^{\Delta pan}$) mice exhibited altered intrapancreatic trypsin activity without significant changes in disease severity, as judged by serum amylase activity, pancreas histology, pancreatic cytokine mRNA expression, acinar cell apoptosis, and autophagy.

We note that CTSB may contribute to disease development through mechanisms unrelated to trypsin activity, e.g. by activating cell death pathways [12], although the phenotype of the *Ctsb-KO* mice argue against this possibility, at least in the mild, edematous model used here. It is conceivable that in severe experimental models, CTSB may play a more significant role. In this regard, a recent study showed that infiltrating macrophages may ingest trypsinogen during severe pancreatitis, and CTSB-mediated activation may stimulate pro-inflammatory macrophage function [13].

We conclude that in cerulein-induced murine pancreatitis, intrapancreatic trypsin activity is generated exclusively by CTSB, and trypsinogen autoactivation plays no role in this process. Furthermore, we find that CTSB-elicited trypsin activity is not required for pancreatitis onset, nor does it determine severity in this experimental model. This stands in contrast to the direct pathogenic role of trypsin generated by increased trypsinogen autoactivation in mouse models with hereditary-pancreatitis associated mutations [2, 3]. To explain why the activation mechanism of trypsin determines pathological outcomes, we speculate that autoactivation occurs in the interstitial space and may initiate pancreatitis

through cell-surface expressed proteinase activated receptors, which would be inaccessible to CTSB-elicited intracellular trypsin activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials:

All materials are available for research purposes upon request. All data are included in the manuscript.

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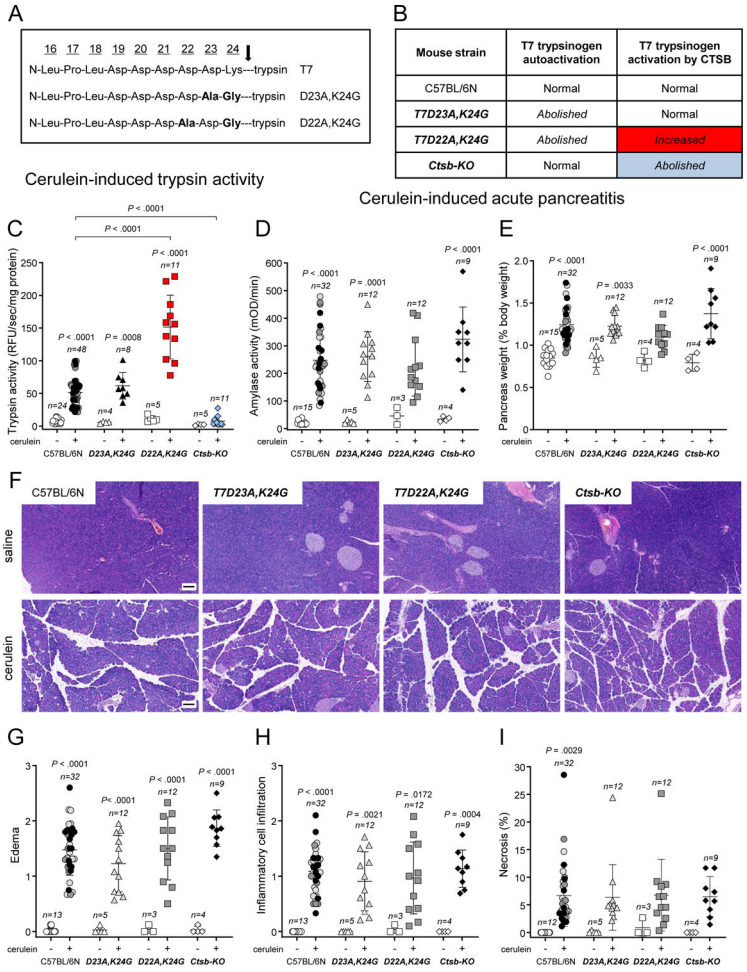

Figure 1.

Effect of trypsinogen mutations D23A,K24G and D22A,K24G, and cathepsin B deficiency on cerulein-induced intrapancreatic trypsin activity and acute pancreatitis in mice. The three mutant strains were analyzed in separate experiments with their own C57BL/6N controls. For clarity, all results were graphed together and the C57BL/6N data were pooled, with light gray (*T7D23A,K24G*), dark gray (*T7D22A,K24G*), and black (*Ctsb-KO*) symbols indicating the different experiments. **A**, Primary structure of the trypsinogen activation peptide in mouse cationic trypsinogen (T7). The position of mutations D23A,K24G and D22A,K24G are indicated. The arrow points to the activation cleavage site. **B**, Biochemical pathways of intrapancreatic trypsinogen activation in mouse models with mutated T7 trypsinogen and *Ctsb* deletion *(Ctsb-KO)* alleles. **C**, Cerulein-induced intrapancreatic trypsin activity in *T7D23A,K24G*, *T7D22A,K24G*, *Ctsb-KO*, and C57BL/6N mice. Red and blue symbols highlight the increased and diminished trypsin activities in the *T7D22A,K24G* and *Ctsb-KO* mice, respectively. Trypsin activity was determined 30 min after a single injection of saline or cerulein. **D**, **E**, **F**, **G**, **H**, **I**, Acute pancreatitis in *T7D23A,K24G, T7D22A,K24G, Ctsb-KO*, and C57BL/6N mice given 10 hourly injections of normal saline or cerulein. **D**, Plasma amylase activity. **E**, Pancreas weight. **F**, Representative hematoxylin-eosin stained pancreas sections. Scale bars correspond to 100 μm. **G, H, I**, Histology scoring of pancreas sections

for edema (G), inflammatory cells (H), and necrosis (I). Individual values with mean ± SD are shown. The difference of means between groups was analyzed by one-way ANOVA and Tukey-Kramer post-hoc test. See Methods in the Supplementary Material for experimental details.