Differentially expressed kinase genes associated with trypsinogen activation in rat pancreatic acinar cells treated with taurolithocholic acid 3-sulfate

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Abstract. Trypsinogen activation is the initial factor involved in the development of all types of acute pancreatitis (AP) and has been suggested to be regulated by protein kinases. In the present study, AR42J rat pancreatic acinar cells were treated with taurolithocholic acid 3-sulfate (TLC-S), and trypsinogen activation was detected with bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride (BZiPAR) staining and flow cytometry. Differentially expressed protein kinase genes were screened by Gene Chip analysis, and the functions of these kinases were analyzed. A significantly increased activation of trypsinogen in AR42J cells following treatment with TLC-S was observed. A total of 22 differentially expressed protein kinase genes were found in the TLC-S group, among which 19 genes were upregulated and 3 were downregulated. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, kinase genes of the same KEGG pathways were connected to create a network through signaling pathways, and 10 nodes of kinases were identified, which were mitogen-activated protein kinase (Mapk)8, Mapk14, Map2k4, interleukin-1 receptor-associated kinase 3 (Irak3), ribosomal protein S6 kinase, 90 kDa, polypeptide 2 (Rps6ka2), protein kinase C, alpha (Prkca), v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (Lyn), protein tyrosine kinase 2 beta (Ptk2b), p21 protein (Cdc42/Rac)-activated kinase 4 (Pak4) and FYN oncogene related to SRC, FGR, YES (Fyn). The interactions between signaling pathways were further analyzed and a network was created. MAPK and calcium signaling pathways were found to be located at the center of the network. Thus, protein kinases constitute potential drug targets for AP treatment.

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Introduction

During the development of acute pancreatitis (AP), trypsinogen in pancreatic acinar cells is aberrantly activated by external stimuli; the activated trypsin then activates additional digestive zymogens, leading to the damage and self-digestion of pancreatic acinar cells. Subsequently, during inflammatory cell infiltration, a large number of inflammatory molecules enter the circulatory system, causing systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS) and even death.

During this process, the abnormality of calcium concentration and protein kinase, the reduction in adenosine triphosphate (ATP) levels, the oxidative stress, as well as other reactions in pancreatic acinar cells contribute to the development of AP. All of these mechanisms are based on protein-kinase-mediated intracellular signal transduction; thus, research on AP treatment involving the targeting protein kinases may prove useful for the treatment of multiple pathogeneses and may systematically inhibit the development of AP.

In acute biliary pancreatitis, pancreatic enzyme activation is caused by bile or some of its components, as well as other causes. Bile acid directly acts on pancreatic acinar cells by activating phosphatidylinositol 3-kinase (PI3K) to pathologically increase the calcium level and activate digestive zymogens, cell injury/death and inflammation pathways in cells (1). Bile acid also irritates inositol 1,4,5-trisphosphate (IP3) and ryanodine receptors, releasing the calcium stored in the endoplasmic reticulum and zymogen granules of acinar cells (2), thus leading to pathological calcium transients in cells. In 2010, Perides *et al* (3) demonstrated that taurolithocholic acid 3-sulfate (TLC-S) causes an alteration in the calcium concentration in cells by acting on the acinar cell surface (Gpbar 1), and they concluded that biliary AP may be a receptor-mediated disease.

The aim of this study was to provide evidence for the development of AP treatment strategies targeting protein kinases. Consequently, pancreatic acinar AR42J cells were treated with

TLC-S. Trypsinogen activation in pancreatic acinar cells was observed, the differentially expressed protein kinase genes were screened by gene chip analysis, and the functions of these kinases were analyzed.

Materials and methods

Cell culture and processing. The AR42J rat pancreatic acinar cell line was purchased from the China Center for Type Culture Collection (CCTCC; Wuhan, China) and cultured in Ham's F12 medium (F12K; Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA). The cells were cultivated in an incubator containing 5% CO_2 at 37˚C. The activation of trypsinogen and the change in the proteome were assessed 20 min following TLC-S (Sigma) treatment.

Assessment of trypsinogen activation. Acinar cells were cultured for 20 min in HBS-EDTA (5 mM HEPES, 0.15 M NaCl, 2 mM EDTA; pH 7.35) with 5 mM rhodamine 110, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride (BZiPAR; Molecular Probes®; Invitrogen), with the cell concentration adjusted to 10^5 cells/ml. Trypsinogen activation was then assessed using a flow cytometer (FACSDiva, version 6.1; BD Biosciences, San Jose, NJ, USA) at an excitation wavelength of 485 nm following treatment with 200 μM/l TLC-S for an additional 20 min. The experiment was performed in triplicate.

Detection of gene expression using DNA microarrays. The cells were collected, and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Gene expression analyses were performed with the Rat 12x135K Gene Expression Array (NimbleGen Systems, Inc., Madison, WI, USA). Preparation of cDNA from 5 μg of total RNA, hybridizations, washes and detection were performed in accordance to the NimbleGen gene expression analysis protocol (NimbleGen Systems, Inc.); the slides were scanned using the Axon GenePix 4000B microarray scanner.

Data analysis. Scanned images (TIFF format) were then imported into NimbleScan software (version 2.5) for grid alignment and expression data analysis. Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. The Probe level (*_norm_RMA.pair) files and Gene level (*_RMA.calls) files were generated following normalization. All the gene level files were imported into GeneSpring GX software (version 11.5.1; Agilent Technologies, Inc., Santa Clara, CA, USA) for further analysis. Differentially expressed genes were identified through fold change filtering.

Based on the latest database of protein kinase (http://kinasource.co.uk/Database/substrates.html), differentially expressed kinase genes were selected. The differentially expressed kinases genes which were singled out were annotated in the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.jp) rat pathway. A visualized network was drawn with the software cytoscape (version 2.6.3) to exhibit the correlations between the kinases. Finally, each signaling pathway that annotated the differentially expressed kinase genes was imported into KEGG to search all related pathways, and the correlation was exhibited with a visualized network using software cytoscape (version 2.6.3).

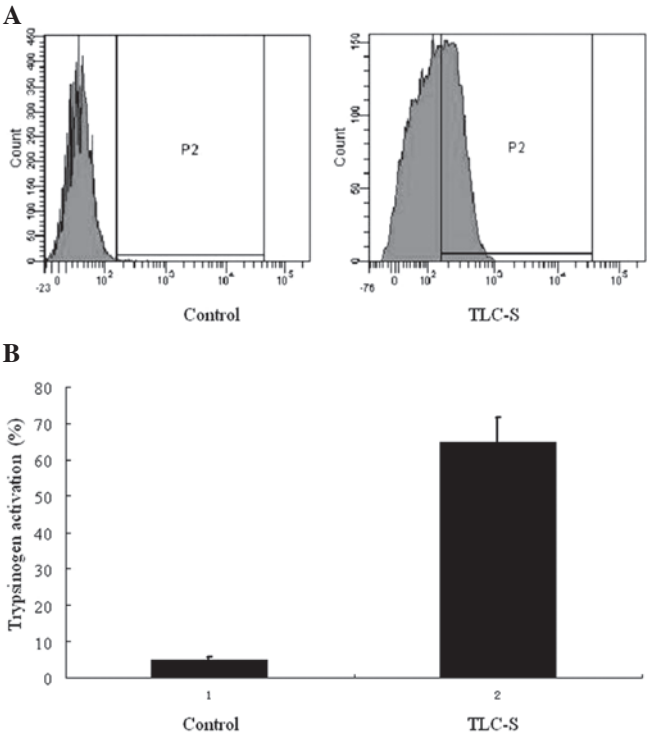

Figure 1. Trypsinogen activation in AR42J cells treated with taurolithocholic acid 3-sulfate (TLC-S). (A) Histogram of flow cytometry; (B) statistical chart. AR42J cells were stained with BZiPAR; BZiPAR was enzymatically lysed by activated trypsinogen (trypsin) and emited green fluorescence excited by an argon laser at 488 nm, which was detected using a flow cytometer. Increased activation of trypsinogen was observed following treatment with TLC-S. The results are expressed as the means ± SD (n=3). P<0.05.

Results

Trypsinogen activation. BZiPAR coupled with fluorescein rhodamine 110, can not be excited to emit fluorescence prior to enzyme cleavage. However, following trypsin enzymatic lysis, rhodamine 110 emits green fluorescence excited by argon laser at 488 nm. As a result, the degree of trypsinogen activation can be determined by detecting the fluorescence intensity. In this experiment, there were only trace amounts of activated trypsinogen found in the control group, while a significantly higher level was detected by flow cytometry following 20 min of treatment with TLC-S in the AR42J cells (Fig. 1).

Identification of differentially expressed protein kinase genes. A total of 11,292 genes were detected to be expressed in the AR42J cells. In the TLC-S group, a total of 1,124 genes were upregulated and 498 were downregulated. Through bioinformatics analysis, 22 differentially expressed protein kinase genes were found in the 1,124 differentially expressed genes, among which 19 genes were upregulated and 3 were downregulated (Table I).

Table I. Differently expressed protein kinase genes in AR42J cells treated with taurolithocholic acid 3-sulfate (TLC-S).

Gene name	Gene ID	Description	Mode of regulation	Fold change
Sgk196	306549	Sugen kinase 196	Upregulated	2.0006287
Map2k4	287398	Mitogen-activated protein kinase kinase 4	Upregulated	2.0351782
Ptk2b	50646	Protein tyrosine kinase 2 beta	Upregulated	2.3132236
Cdk1	54237	Cyclin-dependent kinase 1	Upregulated	4.0139747
Lyn	81515	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	Upregulated	3.2749004
Mapk14	81649	Mitogen-activated protein kinase 14	Upregulated	2.2141974
Cdc42bpb	113960	CDC42 binding protein kinase beta (DMPK-like)	Upregulated	2.1441314
Stk17b	170904	Serine/threonine kinase 17b	Upregulated	2.80085
Tesk2	170908	Testis-specific kinase 2	Upregulated	2.557841
Rps6ka2	117269	Ribosomal protein S6 kinase, 90 kDa, polypeptide 2	Upregulated	3.3565648
Sgk3	171498	Serum/glucocorticoid regulated kinase 3	Upregulated	2.0300796
Ttk	315852	Tramtrack	Upregulated	2.8380172
Melk	362510	Maternal embryonic leucine zipper kinase	Upregulated	4.2185326
Mast2	313819	Microtubule-associated serine/threonine kinase 2	Upregulated	2.1736362
Pak4	292756	p21 protein (Cdc42/Rac)-activated kinase 4	Upregulated	2.865645
Bub1	296137	Budding uninhibited by benzimidazoles 1 homolog	Upregulated	6.3756404
Irak3	314870	Interleukin-1 receptor-associated kinase 3	Upregulated	2.1035454
Prkca	24680	Protein kinase C, alpha	Upregulated	2.1087615
Mapk8	116554	Mitogen-activated protein kinase 8	Upregulated	2.7886798
Fyn	25150	FYN oncogene related to SRC, FGR, YES	Downregulated	-2.3253782
Mst1	24566	Macrophage stimulating 1	Downregulated	-3.9783108
Ptk7	301242	Protein tyrosine kinase 7	Downregulated	-3.8359256

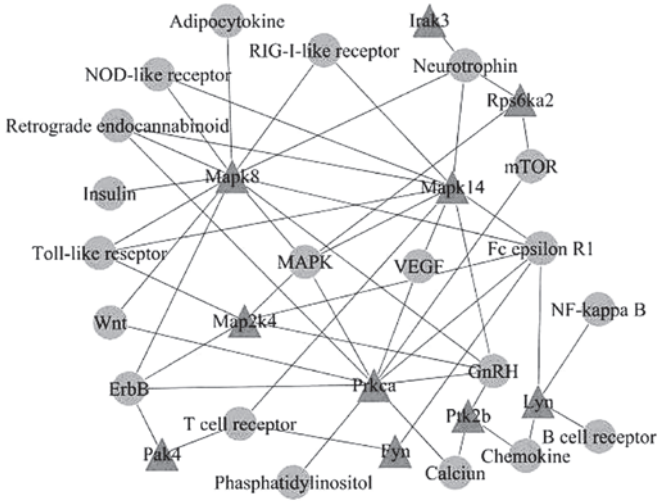

Figure 2. Network of interactions of the differentially expressed kinase genes. This network is composed of differentially expressed kinase genes and some related KEGG signaling pathways. Ellipse shape nodes refer to genes involved in signaling pathways (KEGG); triangle shape nodes refer to protein kinase genes.

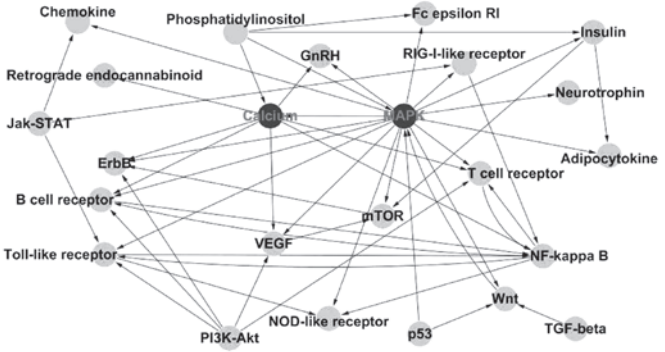

Figure 3. Network of interactions of signaling pathways. Each signaling pathway that showed differentially expressed kinase genes was imported into KEGG database, all the related pathways were searched, and the interactions were exhibited as a network. Each ellipse shape node refers to a signaling pathway.

Based on the KEGG database, kinase genes of the same KEGG pathways were connected to create a visualized network through signaling pathways, and 10 nodes of kinases were identified, which were mitogen-activated protein kinase (Mapk)8, Mapk14, Map2k4, interleukin-1 receptor-associated kinase 3 (Irak3), ribosomal protein S6 kinase, 90 kDa, polypeptide 2 (Rps6ka2), protein kinase C, alpha (Prkca), v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (Lyn), protein tyrosine kinase 2 beta (Ptk2b), p21 protein (Cdc42/Rac)-activated kinase 4 (Pak4) and FYN oncogene related to SRC, FGR, YES (Fyn) (Fig. 2).

Subsequently, the interactions between the signaling pathways were further analyzed and a network was created. MAPK and calcium signaling pathways were found to be located at the center of the network (Fig. 3).

Table II. Regulation of trypsinogen activation based on literature mining.

Regulators of trypsinogen activation	Journal	Year of publication	Authors/(Refs.)
Cathepsins			
Cathepsin B	J Clin Invest	2010	Reiser *et al* (6)
Cathepsin B	Pancreatology	2006	Lindkvist *et al* (7)
Cathepsin L	Gastroenterology	2010	Wartmann *et al* (8)
Calcium signaling pathway			
Calcineurin	Am J Physiol Gastrointest Liver Physiol	2009	Shah *et al* (9)
Calcium release	Proc Natl Acad Sci USA	2009	Gerasimenko *et al* (10)
Calpain	Int J Exp Pathol	2009	Weber *et al* (11)
Ryanodine receptor regulation calcium	Proc Natl Acad Sci USA	2005	Husain *et al* (12)
Protein kinase signaling pathway			
PKD/PKD1	Am J Physiol Gastrointest Liver Physiol	2011	Throwe *et al* (13)
Rho-kinase	Br J Pharmacol	2011	Awla *et al* (14)
MAPK	Am J Physiol Gastrointest Liver Physiol	2008	Namkung *et al* (15)
PKC	J Gastroenterol Hepatol	2008	Gorelick *et al* (16)
Pgk1, Pgk2	Pancreatology	2012	Li *et al* (17)
Endothelins			
Endothelins	Exp Toxicol Pathol	2011	Andrzejewska and Dlugosz (18)
Endothelin-1 receptor antagonists	World J Gastroenterol	2005	Andrzejewska *et al* (19)
Enterokinases			
Enteropeptidase	Front Biosci	2009	Zheng *et al* (20)
Enterokinase	Surgery	2007	Hartwig *et al* (21)

Discussion

Trypsinogen activation in pancreatic acinar cells is the initial factor involved in the development of all types of AP and causes the self-digestion of cells. Shortly after the induction of AP, immature secretory vesicles have been observed in the cytoplasm, and the activity of trypsin has been detected in this subcellular domain (4). However, the underlying mechanisms of trypsinogen activation remain to be elucidated. Several studies have shown that cathepsin and the increased calcium concentration in cells are essential for the activation of digestive enzymes (5). Over the past decade, the roles of protein kinases and the signaling pathways associated with trypsinogen activation have received increased attention (Table II).

Protein kinase, also known as protein phosphakinase, is a key regulator of numerous cellular functions. It changes the molecular structure by adding a phosphate group to substrate proteins in order to regulate protein activity, and it coordinates cellular processes by acting on different signaling pathways. Protein kinase is associated with almost every cellular function. Protein kinase, which is important in regulating cell behavior, is considered to be the target in the treatment for various diseases including tumors, diabetes, osteoporosis, phlogosis and oculopathy (22-24).

In the present study, the AR42J rat pancreatic acinar cell line was used to establish a model of trypsinogen activation in pancreatic cells following treatment with 200 μM TLC-S (17). Total RNA from the AR42J cells was then extracted to perform whole-genome expression profile analysis, and 22 differentially expressed protein kinase genes were identified. Ten nodes of protein kinase genes were listed in a correlation diagram through analyzing KEGG pathways. MAPK and calcium signaling pathways were found to play an important role in trypsinogen activation.

The present study suggests that calcium signaling pathways play an important role in trypsinogen activation, and that Prkca and Ptk2b are associated with this signaling pathway. The association between AP and $[Ca^{2+}]i$ in pancreatic acinar cells has been previously investigated (9). In 1995, Ward *et al* (25) hypothesized that calcium overload in acinar cells constitutes the main cause of AP development. Mooren *et al* (26) identified an increase in $[Ca^{2+}]i$ in acinar cells and an alteration of the normal calcium signals. The early use of BAPTA-AM, a calcium chelator, inhibits the above reaction. The abnormal release of calcium in cells and the aberrant activation of zymogen in acinar cells that cause self-digestion are known to be early events in the progression of AP (27). According to previous studies, the aberrant increase in $[Ca^{2+}]i$ in acinar cells has been shown to directly trigger trypsinogen activation and liquid cavity formation, since the treatment of pancreatic acinar cells with large doses of cholecystokinin (CCK, 10 nmol/l) triggers trypsin activation of $[Ca^{2+}]i$-dependent top granules in acinar cells (28), and large doses of CCK cause changes in the calcium dependence of acinar cells in cellular morphology and the extensive replacement of zymogen granules by liquid cavity. Raraty *et al* (29) showed

that the stably increased $[Ca^{2+}]i$ induces trypsinogen activation. Shah *et al* (9) observed that under the effect of FK506, a calcium/calmodulin-protein-dependent phosphatase inhibitor, the level of trypsinogen activation significantly decreased; a lower level of blood amylase and the inflammatory factor, interleukin (IL)-6, was also observed. The effect of FK506 was also investigated by Ozawa (30). Calcium/calmodulin calcineurin (PP2B) is controlled by many factors; however, the main mechanisms are the aberrant increase of $[Ca^{2+}]i$ in acinar cells and the alteration of calmodulin concentration (31). PP2B has also been shown to be the main target for the aberrant increase of $[Ca^{2+}]i$ in pancreatic acinar cells. The aberrant increase in $[Ca^{2+}]i$ in pancreatic acinar cells has been shown to activate PP2B, which is considered to be an important step in pathologic trypsinogen activation in pancreatic acinar cells.

MAPK, one of the serine/threonine protein kinases, presents in the majority of cells. It is one of the important signaling systems where eukaryotic cells transduce extracellular signals into cells to cause cellular reactions. Prior to activation, MAPK is located in the cytoplasm, and it enters the cell nucleus to activate the target gene following activation by phosphorylation upon stimulation. MAPK affects the gene transcription and regulation to influence the biological behavior of cells, such as cell proliferation, differentiation, transformation and apoptosis. The MAPK signaling pathway may be induced by pro-inflammatory molecules and stress, and is involved in most of the reactions of immunization and apoptosis caused by pro-inflammatory molecules (32). The present study identified the upregulated genes, Mapk14, Map2k4, Mapk8, Rps6ka2 and Prkca, that are associated with the MAPK signaling pathway, indicating that the MAPK signaling pathway plays an important role in trypsinogen activation.

There are 3 subgroups in the MAPK family, p38, ERK 1/2 and JNK. p38 has been proven to be specifically associated with the severity of AP and lung injury caused by severe AP. Thus, injuries in the lungs and part of the pancreas are reduced by inhibiting the activation of p38 (33). Certain studies have shown that during the inflammatory processes, oxidative stress and pro-inflammatory molecules are involved in the inflammatory cascade process by enabling the signaling transduction pathway, and that the activation of MAPK is of high importance during this process. Pereda *et al* (34) also demonstrated that the concurrent treatment of AP animal models with oxypurinol, a p38 inhibitor, and pentoxifyllin, an ERK 1/2 and JNK inhibitor, relieves inflammatory responses and reduces mortality. Additionally, the inhibition of the p38, ERK1/2 and JNK signaling pathways has been shown to have a less significant effect. The p38 signaling pathway specifically correlates with oxidative stress and the ERK 1/2 and JNK pathways are clearly associated with the expression of pro-inflammatory molecules. Since p38, ERK 1/2 and JNK have an independent effect on the process of AP development, concurrently inhibiting all 3 signaling pathways has a specific effect on relieving AP symptoms. It has been reported that oxidative stress causes MAPK activation and induces tumor necrosis factor (TNF)-α (35). TNF-α has also been suggested to be an initiator of cytokine cascade reaction during AP development. Williard *et al* (36) showed that p38 is involved in the activation of the pro-inflammatory nuclear transcription factor, nuclear factor (NF)-κB, to upregulate the expression of pro-inflammatory factors and aggregate AP. Samuel *et al* (37) demonstrated that the use of ERK 1/2 (PD98059), p38 (SB203580) and JNK (SP600125) inhibitors to suppress their activation in a biliary AP model, significantly reduced the expression of the pro-inflammatory cytokine in pancreatic tissue. Liu *et al* (38) supported this finding by comparing the serum and cytokine levels in the ascites of rats with severe and mild AP.

In conclusion, protein kinases are important in AP development, since they are associated with approximately every mechanism of AP development. As a result, protein kinases constitute potential drug targets for AP treatment. Gene therapeutic methods, such as gene transfection and RNA interference have proven to be successful in the treatment of AP in laboratory experiments; however, their use in clinical practice is still highly restricted. Targeting protein kinases as a form of therapy should be investigated in clinical trials, since a number of kinase inhibitors have been developed which may be used as a selective target. Therefore, further studies on the role of protein kinases in AP development are warranted, in order to develop a specific kinase inhibitor for the target therapy of AP.

Acknowledgements

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