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PROTEOMIC EFFECTS OF THE COAGULATION PROTEINASE THROMBIN ON LX-2 HEPATIC STELLATE CELLS

PROTEOMIČKI UTICAJ KOAGULACIJSKE PROTEINAZE TROMBINA NA LX-2 STELATNE ĆELIJE JETRE

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Summary

Background: The aim of this study was to characterize the effects of the coagulation proteinase thrombin on proteomic level in human hepatic stellate LX-2 cells.
Methods: Proteomic analyses were performed using surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF-MS). The protein profiles obtained from LX-2 cell lysates using strong anion exchanger Q10 ProteinChip arrays were statistically analyzed.
Results: The peak intensities of 50 protein/peptide clusters were identified as being different between nonstimulated and LX-2 cells treated with thrombin for 6 h and 24 h, respectively. As the most significantly enhanced single signal in LX-2 cells stimulated with thrombin, a protein with a molecular mass of 13.560 kDa has been identified that corresponds exactly to calcium dependent phospholipase 2 (cPLA2). Thrombin-induced increase in the cPLA2 protein expression in LX-2 cells was confirmed by using the Western blotting technique.
Conclusions: Together with the finding that thrombin induced phosphorylating activation of cPLA2 in LX-2 cells, our data point to an important function of the thrombin-mediated modulation of cytosolic phospholipase A2 in hepatic stellate cells.

Keywords: thrombin, hepatic stellate cells, LX-2, proteomic profiling, mass spectrometry

Kratak sadržaj

Uvod: Cilj ove studije bio je da se odredi uticaj koagulacijske proteinaze trombina na proteomički nivo u humanim stelatnim LX-2 ćelijama jetre.
Metode: Proteomičke analize izvršene su pomoću tehnike SELDI-TOF-MS. Statistički su analizirani proteinski profili dobijeni iz lizata LX-2 ćelija pomoću jakog anjonskog izmenjivača i *Q10 ProteinChip arrays*.
Rezultati: Identifikovani su pik intenziteti 50 klastera proteina/peptida i utvrđeno je da se razlikuju između nestimulisanih i LX-2 ćelija tretiranih trombinom tokom 6, odnosno 24 h. Kao najznačajnije unapređen pojedinačni signal u LX-2 ćelijama stimulisanim trombinom, identifikovan je protein s molekulskom masom od 13,560 kDa koji tačno odgovara fosfolipazi 2 zavisnoj od kalcijuma (cPLA2). Porast proteinske ekspresije cPLA2 izazvan trombinom u LX-2 ćelijama potvrđen je pomoću tehnike vestern blotinga.
Zaključak: Pored nalaza da je trombin izazvao fosforilacijsku aktivaciju cPLA2 u LX-2 ćelijama, naši podaci ukazuju i na važnu funkciju modulacije citosolne fosfolipaze A2 u stelatnim ćelijama jetre posredstvom trombina.

Ključne reči: trombin, stelatne ćelije jetre, LX-2, proteomičko profilisanje, masena spektrometrija

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Introduction

Beside its critical role in blood coagulation, the serine proteinase thrombin (EC 3.4.21.5) is known to evoke biological responses from a variety of cells, e.g. platelets, fibroblasts, vascular smooth muscle cells and monocytes (1–3), and may influence a number of cellular responses that play a role in subsequent pro-inflammatory and profibrotic processes in different organs including the liver (4).

Recent data suggest a role for thrombin in the activation of hepatic stellate cells (HSCs) that has been recognized as a central event in the development of liver fibrosis and finally cirrhosis and malignancy. More specifically, thrombin has been shown to stimulate matrix synthesis and the regulation of MCP1-production of cultured hepatic stellate cells (6–8). Hepatic stellate cells also express the proteinase activated receptors 1 and 4 (8), members of a novel subfamily of G-protein coupled receptors mediating the cellular effects of thrombin in different cell types.

Surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS) is a proteomic analysis (10) that has been demonstrated to be a very suitable approach to evaluate complex variations on protein level in tissue and cells (11, 12).

In this study, we used SELDI-MS to evaluate the effect of thrombin on protein expression profiles in cells of the human stellate cell line LX-2 that has been characterized comprehensively as a suitable model for investigations on liver fibrosis (13). For that purpose, lysates from nonstimulated LX-2 cells and LX-2 cells stimulated with thrombin (1.0 NIH-U/mL) for 6 h and 24 h, respectively, were evaluated using strong anion exchanger Q10 ProteinChip arrays.

Materials and Methods

Reagents

Human alpha-thrombin (3085 NIH-Units/mg protein) was purchased from Haemochrom Diagnostica Supplies (Essen, Germany). All the other reagents were of the highest purity available.

Cell culture

LX-2 cells (gift from Prof. Scott Friedman, Mount Sinai School of Medicine, New York, USA) were routinely cultured in Dulbeccos modified Eagle's medium (DMEM) supplemented with 2% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed every 2–3 days. For subculturing, treatment with trypsin/EDTA was used.

For stimulation experiments, LX-2 cells were cultured in 6-well plates, serum starved for 16 h and subsequently stimulated with thrombin (Haemochrom Diagnostica Supplies; 1.0 NIH-Unit/mL).

ProteinChip array and analysis

Cells were transferred into tubes with lysis buffer (100 mmol/L Na_2HPO_4, 2 mmol/L $MgCl_2$, 5 mmol/L EDTA, 3 mmol/L β-mercaptoethanol, 0.1% CHAPS, 500 μmol/L leupeptin, 0.1 mmol/L PMSF). After 1h incubation on ice with mixing every 15 minutes, the samples were centrifuged with 14 000 rpm at 4 °C to remove cell debris.

Proteins were analyzed on strong anion exchange arrays (Q10; BioRad) as described elsewhere (14). In brief, array spots were preincubated by a washing/loading buffer containing 100 mmol/L Tris-buffer, pH 8.5 with 0.02% Triton X-100 for Q10 arrays and 100 mmol/L Tris-buffer, pH 4.5 with 0.02% Triton X-100 for CM10 arrays followed by application of 2 μL of sample extract on ProteinChip Arrays, which were incubated at room temperature for 90 min in a humidity chamber. After washing three times with the same buffers and two final washing steps with water, 2 × 0.5 μL sinapinic acid (saturated solution in 0.5% TFA/50% acetonitrile) were applied. Mass analysis was performed in a ProteinChip system (PCS 4000, Ciphergen Biosystems Inc, Fremont, CA) according to an automated data collection protocol. The instrument was externally calibrated with a ProteinChip OQ Kit (Biorad) for improved mass accuracy. Spectra were normalized with total ion current and cluster analyses of the detected signals and the determination of respective P-values were carried out with the CiphergenExpress Program (Version 3.0; Ciphergen Biosystems Inc, Fremont, CA). For P-value calculation, normalized spectra with signals in the range between 2.5 and 200 kDa exhibiting a signal-to-noise ratio (S/N) of at least 10 were selected and analyzed with the Mann–Whitney U test for nonparametric data.

Western blotting analysis

LX-2 cells were collected by centrifugation at 1000 × g for 5 min (4 °C), washed with PBS containing bacitracin (100 μg/mL), PMSF (0.1 mmol/L), pepstatin A (1.0 μg/mL) and leupeptin (2.0 μg/mL), pH 7.4, and centrifuged again. The pellet was treated with lysis buffer [(PBS, containing 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate and 0.1% (w/v) SDS] for 30 min at 4 °C, resuspended and centrifuged at 30000 × g for 15 min (4 °C).

Protein was determined using the DC Protein Assay System from BioRad Laboratories according to the manufacturer's instructions.

Proteins from cell lysates were separated on a 12% SDS/PAGE and transferred to nitrocellulose membranes (BioRad). After blocking in 1% BSA/1% skimmed milk for 1 h, the nitrocellulose strips were incubated overnight with the respective first antibody. For the estimation of phosphorylating activation of cPLA2 phospho (Ser 505) cPLA2-antibody (Cell Signaling Technology, No. 2831) and for estimation of the cPLA2 protein expression, an antibody to total cPLA2 (Cell Signaling Technology, No. 2832) was used. Strips were washed two times with 0.05% (v/v) Tween 20 washing buffer, incubated for 45 min with the secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase) and washed again two times as described above. The immunoblots were stripped and reprobed with an antibody to total cPLA2 (cPLA2 activation experiments) or with an antibody to beta-actin (Sigma-Aldrich, No. A 5441; cPLA2 protein expression experiments) to confirm equal protein loading. Secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase or anti-mouse IgG conjugated to horseradish peroxidase) was detected by using the chemiluminescence (ECL) Western blotting detection system (Amersham) and exposure to Kodak X-Omat films.

Immunoreactive bands for phosphorylated cPLA2, total cPLA2 and beta-actin were quantified using the image processing program Image J 1.43 (National Institutes of Health, Bethesda, Maryland, USA).

Results

Protein lysates from nonstimulated LX-2 cells and LX-2 cells stimulated with thrombin (1.0 NIH-Units/mL) for 6 h and 12 h, respectively, were applied to strong anion exchanger Q10 ProteinChip arrays and analysed individually by SELDI–MS on a PCS 4000 instrument to detect any differentially expressed proteins. In the low range of 2.5–20 kDa, up to 50 peaks were detected. After evaluation with CipherGenExpress Program, a mass with a highly significant P-value was identified. This signal of 13.560 kDa was upregulated in thrombin-stimulated LX-2 cells discriminated significantly between nonstimulated LX-2 cells and cells stimulated with thrombin (1.0 NIH-Units/mL) for both 6 h ($P=0.05$) and 24 h ($P=0.01$). The measured intensities for nonstimulated and thrombin-treated LX-2 cells are depicted in *Figure 1*. By enquiring the ExPasy protein database (http://www.expasy.org) with a specified Mw range of 13560 Da +/– 0.4 % and a pI range from 6 to 10, we found that the size of the protein with the mass of 13.560 kDa matches to the chain 21-138 of calcium-dependent phospholipase A2 (UniProt: PA2G5_HUMAN; P39877; Mw13591, pI 8.73).

Our data obtained with the use of proteinchip technology singled out a role for cPLA2 in thrombin's signalling activity in LX-2 hepatic stellate cells. To confirm these data, we investigated the effect of thrombin on cPLA2 protein expression by Western blotting. We found that stimulation of LX-2 cells with thrombin (1.0 NIH-U/mL) resulted in a significant increase of cPLA2 protein expression (*Figure 2A*). Since thrombin is known to activate cPLA2 in different cell types (15–17), we wished furthermore to evaluate thrombin-mediated activation of the enzyme in LX-2 cells, using a Western blot approach that monitors increases in phospho(P)-cPLA2 following the exposure of cells to thrombin. As demonstrated in *Figure 2B*, stimulation of LX-2 cells for 20 min caused an approximately 2-fold increase in P-cPLA2 immunoreactivity, relative to the signal observed in nonstimulated LX-2 cells.

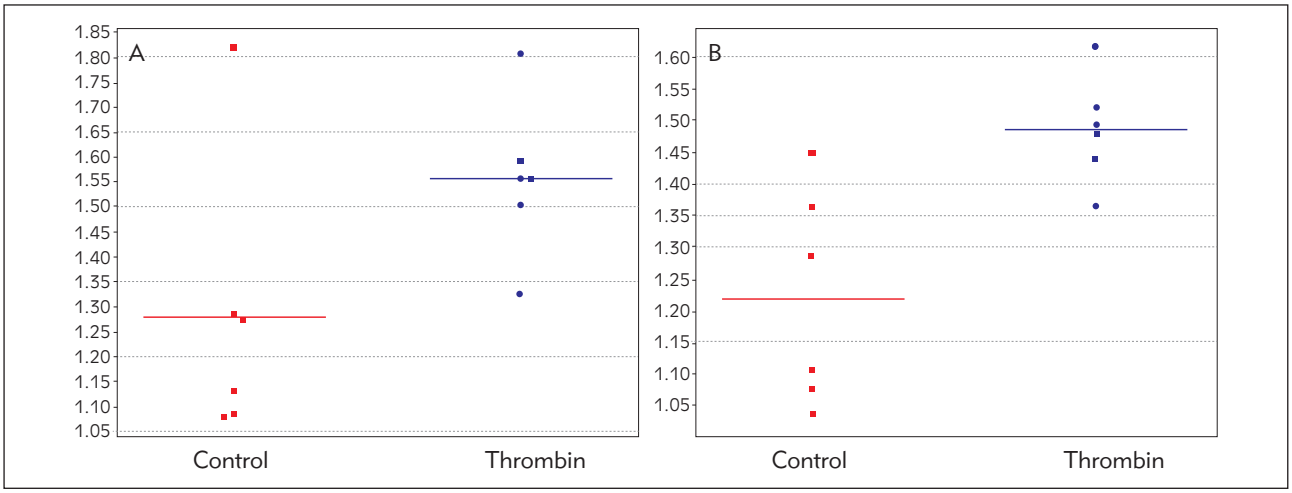

Figure 1 Distribution of the intensities of the peak at 13.560 kDa expressed significantly differently in nonstimulated (control) and LX-2 cells stimulated with thrombin (1.0 NIH-Unit/mL) for 6 h (A) and 24 h (B). X-axis indicates the sample groups, Y-axis the intensity (μA).

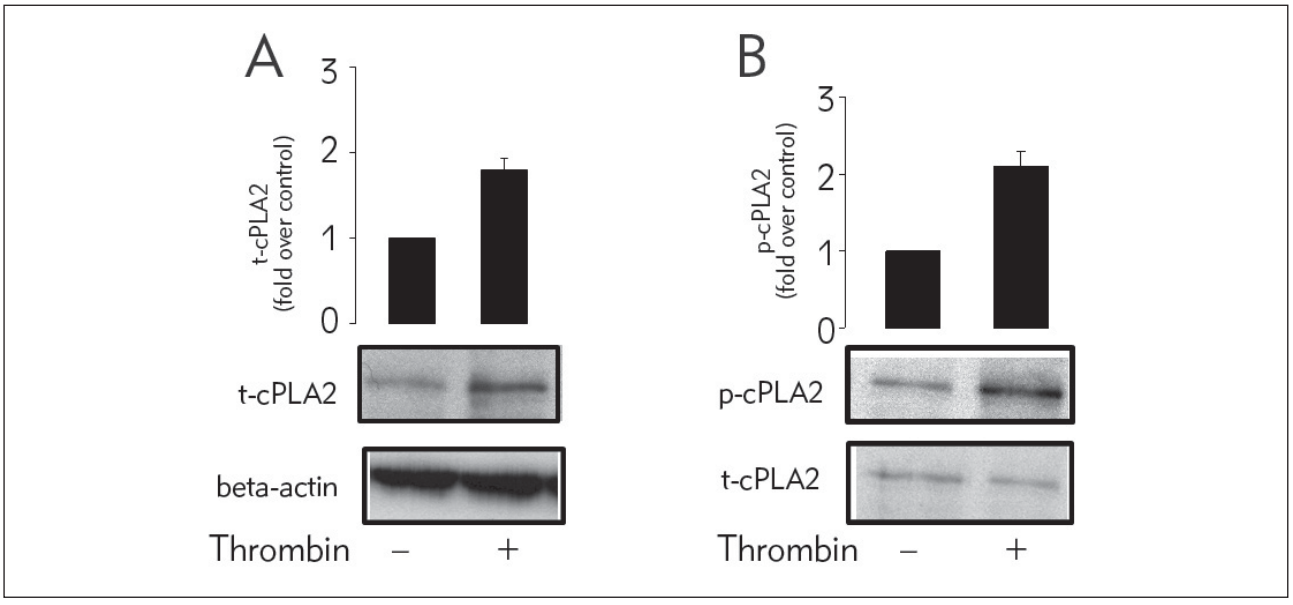

Figure 2 Thrombin induces an increase in A) cPLA2 protein expression and B) cPLA2 phosphorylation in LX-2 cells. LX-2 cells were stimulated with thrombin (1.0 NIH-U/mL) for A) 24 h or B) 20 min. Cell lysates were subjected to SDS-PAGE and Western blotting with A) an anti-cPLA2 antibody and reprobed with an anti-beta-actin antibody, B) an anti-phospho-cPLA2 antibody and reprobed with an anti-cPLA2 antibody. Immunoblot analyses from representative experiments are shown with A) beta-actin and B) total cPLA2 as control for constant protein loading in the lanes. In the histograms above the blots, normalized data are shown as the fold increase over untreated control (mean ± SD) from three independent experiments. P = phosphorylated, t = total.

Discussion

Liver fibrosis is the major complication of most chronic liver diseases, leading eventually to cirrhosis and finally to carcinoma. It is characterized by a massive deposition of extracellular matrix (ECM) components in the liver parenchyma. Synthesis of ECM components is performed by fibrogenic cells that derive from the activation of quiescent precursors such as hepatic stellate cells. Therefore, these cells play a key role in liver fibrosis and are also discussed as crucial cellular elements for the malignant process in the liver (5). In this study, we demonstrate an effect of the serine proteinase thrombin on the proteomic level in cells from the human hepatic stellate cell line LX-2 using SELDI ProteinChip technology. LX-2 cells have been extensively characterized and retain key features of cytokine signalling, neuronal gene expression, retinoid metabolism, and fibrogenesis, making them highly suitable for culture based studies of human hepatic fibrosis (13).

Proteomic analysis revealed that treatment of LX-2 cells with thrombin upregulated a single protein with a molecular mass corresponding to the chain 21-138 of calcium-dependent phospholipase A2. The effect of thrombin on the PLA2 protein expression was confirmed by Western blotting. Moreover, thrombin could be shown to induce phosphorylating activation of cPLA2 in LX-2 cells. Phospholipase A2 enzymes catalyze the hydrolysis of the sn-2 position of glycerophospholipids to release free arachidonic acid, which in turn is metabolized to prostaglandins by the cyclooxygenase pathway and to leukotrienes by the 5-lipoxygenase pathway (for review see e.g. 18). PLA2 is known to be involved in hepatic stellate cell activation (19) and is critically implicated in thrombin-induced signalling in different cell types including platelets (20, 21) and endothelial cells (22–24). In addition, from recently published data, a role for thrombin in liver fibrosis and hepatocellular carcinoma progression has been suggested (25, 26).

Our study provides evidence for a link between thrombin and PLA2 in hepatic stellate cells. Thrombin's role in HSC cell activation during liver fibrosis and carcinogenesis has to be explored in further studies, including experiments on primary cultures of hepatic stellate cells and animal models.

Taken together, our data demonstrate that proteomic analysis is a suitable approach to evaluate effects of a single stimulus on protein level in hepatic stellate cells. With our results we support the concept of a function for the coagulation proteinase thrombin in hepatic stellate cell activation, and more generally, for coagulation enzymes in liver fibrosis.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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