Human platelets are a source of collagen I

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Research Design and Methods

Reagents

Thrombin was from Hemochrom Diagnostica (Essen, Germany). The anti-collagen I antibodies were from Cell Signaling (New England Biolabs, Frankfurt, Germany). All other compounds were from Merck (Darmstadt, Germany).

Study subjects

A total of 32 PCOS patients diagnosed according the Rotterdam criteria, attending the Endokrinologikum Frankfurt for fertility problems or for routine control were included in the present study. Thirty-five age-matched female subjects without PCOS, diabetes or insulin resistance served as the control group. None of the participants took any medication known to interfere with platelet aggregation for at least 10 days before blood sampling. The study protocol was approved by the ethics committee of the Goethe University Hospital (No. E 61/09 Geschäfts Nr 86/09) and the Landesärztekammer Hessen. All of the participants gave written informed consent.

Platelet isolation

Human platelets were isolated as described [1] and samples were either directly used for further experiments or snap frozen and stored at -80°C until use.

Quantification of free amino acids

Platelets were lysed in Triton X-100 lysis buffer and lysates containing 100 µg protein were used for amino acid analysis. Sample preparation was performed using the EZ:faast Liquid chromatography (LC) /mass spectrometry (MS) free amino acid analysis kit (Phenomenex, Aschaffenburg, Germany) according to the manufacturer's instructions, with minor modifications. Internal standards (10 µL) were applied to all samples and to the standard curve. Analysis of metabolites was performed by LC-MS/MS using the EZ:faast AAA-MS HPLC column on an Agilent 1290 Infinity LC system (Agilent, Waldbronn, Germany) coupled to a QTrap 5500 mass spectrometer (Sciex, Darmstadt, Germany). Electro spray ionization in positive mode was employed. The intensity of the measured metabolite was normalized to internal standards. Analyst 1.6.2 and MultiQuant 3.0 (Sciex, Darmstadt, Germany), were used for data acquisition and analysis, respectively.

Mass spectrometry

Platelet lysates were subjected to SDS-PAGE and the gel was stained with coomassie blue. Each lane was cut into 16 slices equal fractions and collected in 96 filter well plates (30-40 µm PP/PE, Pall Corporation). The gel pieces were destained in 60% Methanol, 50 mmol/L ammoniumbicarbonate (ABC). Proteins were reduced in 10 mmol/L DTT, 50 mmol/L ABC for

one hour at 56°C and alkylated for 45 minutes in 30 mM iodoacetamide. Samples were digested for 16 hours with trypsin (sequencing grade, Promega) at 37°C in 50 mmol/L ABC, 0.01% Protease Max (Promega) and 1 mmol/L $CaCl_2$. Peptides were eluted in 30% ACN and 3% formic acid, centrifuged into a fresh 96 well plate, dried in speed vacuum and resolved in 1% acetonitrile and 0.5% formic acid.

LC-MS/MS was performed using a Thermo Scientific Q Exactive Plus equipped with an ultra-high performance LC unit (Thermo Scientific Dionex Ultimate 3000) and a Nanospray Flex Ion-Source (Thermo Scientific). Peptides were loaded on a C18 reversed-phase precolumn (Thermo Scientific) followed by separation on a 2.4 µm Reprosil C18 resin (Dr. Maisch GmbH) in-house packed picotip emitter tip (diameter 100 µm, 15 cm long from New Objectives), using a gradient from 4% ACN, 0.1% formic acid to 50 % eluent B (99% acetonitrile, 0.1% formic acid) for 30 minutes with a flow rate 300 nL/min and washout with 99% B for 5 minutes.

MS data were recorded by data-dependent acquisition. The full MS scan range was 300 to 2000 m/z with a resolution of 70000, and an automatic gain control (AGC) value of 3E6 total ion counts with a maximal ion injection time of 160 ms. Only higher charged ions (2+) were selected for MS/MS scans with a resolution of 17500, an isolation window of 2 m/z and an automatic gain control value set to E5 ions with a maximal ion injection time of 150 ms. MS1 Data were acquired in profile mode.

Mass spectrometry data were analysed by MaxQuant v1.6.1.0 [2], Proteins were identified using human reference proteome database UniProtKB with 71785 entries, released in 2/2018. Acetylation (+42.01) at N-terminus, oxidation of methionine (+15.99) and hydroxyproline (+15.99) were selected as variable modifications and carbamidomethylation (+57.02) as a fixed modification on cysteines. The enzyme specificity was set to Trypsin. False discovery rate (FDR) for the identification of protein and peptides was 1%.

Measurement of collagen content

Total collagen was determined using a commercially available kit (K218-100-BV, Biovision, Biocat, Heidelberg, Germany), according to manufacturer's instructions.

Western blotting

Platelets were lysed in triton-X 100 lysis buffer and lysate was subjected to SDS-PAGE followed by immunoblotting as described [3].

In vitro treatment of pro-collagen with thrombin

Recombinant human pro-collagen 1 (1 µg; R&D system, Bio-Techne, Wiesbaden, Germany) was incubated with thrombin (1 U/ml) in the presence or not of hirudin (1U/ml) for 30 minutes at 37°C in phosphate buffered saline. Reactions were stopped by adding reducing SDS-PAGE

buffer and heating for 5 minutes at 95°C. Samples were subjected to SDS-PAGE followed by protein silver-staining.

RT-qPCR

Total RNA was isolated using peqGOLD TriFast reagent (VWR, Darmstadt, Germany) and mRNA was reversely transcribed using SuperScript III reverse transcriptase (Life Technologies, Darmstadt, Germany). Real-time qPCR from equal amounts of cDNA was performed using a Magnetic Induction Cycler (Biozym, Hessisch Oldendorf, Germany) and a SYBR Green master mix (Thermo Fisher Scientific, Dreieich, Germany). The relative expression levels of collagen Iα1 and collagen I β2 were calculated using the ΔΔCt method and normalized to 18S ribosomal RNA in each sample. The following primers were used collagen I α1: forward 5'-CCAAATCTGTCTCCCCAGAA-3', reverse 5'-TCAAAAACGAAGGGGAGATG-3'; collagen I β2 forward 5'-CTGCAAGAACAGCATTGCAT-3',reverse 5'-GGCGTGATGGCTTATTTGTT-3'; 18S forward 5'-CTTTGGTCGCTCGCTCCTC-3', reverse 5'-CTGACCGGGTTGGTTTTGAT-3' – all from Biospring (Frankfurt, Germany).

Platelet immunostaining

Resting platelets or platelets stimulated with thrombin were fixed with paraformaldehyde and added on poly-L-lysine-coated 8-well chamber microslides (Ibidi, Gräfelfing, Germany). After blocking with BSA (3%) in PBS for 30 minutes at room temperature (RT), platelets were stained overnight at 4°C with anti-pro-collagen I or anti-collagen I antibodies. After washing and staining with fluorescent secondary anti-rabbit or anti-mouse antibodies (Thermo Fischer Scientific, Darmstadt, Germany), and anti-CD42b antibody (BD transduction laboratories, Heidelberg, Germany) platelets were washed and mounted with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were obtained using a Zeiss LSM-510 Meta laser confocal microscope and analyzed using the imaging software AxioVision 4.8 (Zeiss, Jena, Germany). Data was expressed as the mean ratio of collagen-positive platelets to total platelets counted in 3-4 images that were randomly taken from each sample.

Statistical analysis

Data are expressed as mean ± SEM and statistical evaluation was performed using either Student's *t* test, or two-way ANOVA followed by Tukey's post-test where appropriate using Prism software (GraphPad 7). Values of $P<0.05$ were considered statistically significant.

Supplementary Table 1. Excel data reporting the concentrations of amino acids (nmol/µg protein) in platelets from healthy donors and from PCOS patients.

References

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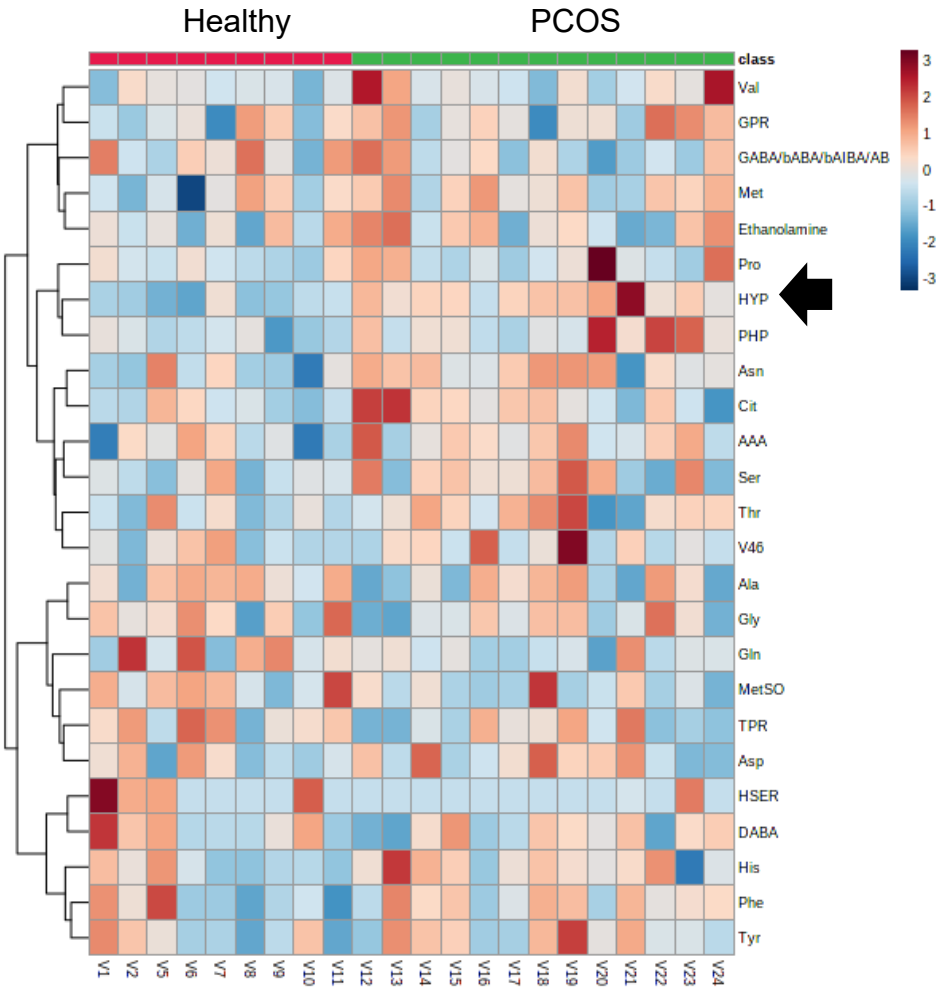

Supplementary Figure 1: Heatmap showing the 25 most significantly altered amino acids in platelet lysates from healthy donors and from PCOS patients. The color code describes high levels (red) and low levels (blue) of each amino acid; n=9-13.