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Research paper

A ^{1}H NMR metabolic profiling to the assessment of protein tyrosine phosphatase 1B role in liver regeneration after partial hepatectomy

Sara Samino [a,b,1], Jesús Revuelta-Cervantes [b,c,1], Maria Vinaixa [a,b], Miguel Ángel Rodríguez [a,b,*], Ángela M. Valverde [b,c], Xavier Correig [a,b]

[a] Metabolomics Platform, IISPV, Universitat Rovira i Virgili, Avda. Països Catalans, 26, 43007 Tarragona, Spain
[b] Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Barcelona, Spain
[c] Instituto de Investigaciones Biomédicas Alberto Sols, CSIC-UAM, Madrid, C/Arturo Duperier, 4, 28029 Madrid, Spain

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ABSTRACT

Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of the tyrosine kinase growth factor signaling pathway, which is involved in major physiological mechanisms such as liver regeneration. We investigate early hepatic metabolic events produced by partial hepatectomy (PHx) for PTP1B deficient (PTP1B KO) and wild type (WT) mice using proton nuclear magnetic resonance spectroscopy. Metabolic response of the two genotypes produced 24 h upon PHx is compared using magic angle spinning high-resolution nuclear magnetic resonance (^{1}H-HR-MAS-NMR) on intact liver tissues. In addition, genotype-associated metabolic profile changes were monitored during the first 48 h after PHx using high-resolution nuclear magnetic resonance (^{1}H-HR-NMR) on liver extracts. A marked increase of lipid-related signals in regenerating livers was observed after 24 h PHx in either intact tissues or liver extracts studies. In spite of this common initial metabolic response, results obtained 48 h after PHx on liver extracts indicate a genotype-differential metabolic pattern. This metabolic pattern resulted in line with well known regenerative features such as more sustained cell proliferation, a better management of lipids as energy fuel and lessened liver injury for PTP1B KO mice as compared to WT. Taken together, these findings suggest the metabolic basis to the pivotal role of PTP1B in liver regeneration.

1. Introduction

The role of Protein Tyrosine Phosphatase 1B (PTP1B) as a regulator of signal transduction by dephosphorylation of Receptor Tyrosine Kinases (RTK) is well known [1–5] and it has been established as a regulator of many signaling pathways associated with human diseases, including diabetes [6,7] obesity [8,9] and cancer [10,11]. Different studies have explored regulatory links between PTP1B and the insulin receptor [12–14]. The development of PTP1B knockout mice [15,16] has allowed further study of the role of PTP1B in diabetes and obesity [17]. PTP1B KO mice have been shown to grow up normally without any significant difference in weight gain or fertility as compared to wild type (WT) mice [15]. Furthermore, PTP1B KO mice maintain lower glucose concentrations with significantly reduced amounts of insulin, displaying a tissue-specific increased sensitivity to insulin [15,16]. Currently, the inhibition of PTP1B is under study as a potential therapy target for diabetes [2,3,18–20]. In addition, PTP1B has been implicated in the control of cell adhesion, which regulates interactions with the extracellular matrix at focal adhesion complexes [21–23].

On the other hand, since liver regeneration involves tyrosine phosphorylation-mediated signaling we have recently investigated the role of PTP1B in hepatic regeneration by performing partial hepatectomy (PHx) in WT and PTP1B KO mice [24]. The essential role of PTP1B in the response to the cytokines and growth factors that initiate hepatic regeneration was assessed, as well as in the control of its termination. PTP1B KO mice triggered an earlier response to hepatic injury as compared to WT, i.e., a more rapid increase in intrahepatic lipid accumulation; an accelerated phosphorylation of JNK1/2 and STAT3; enhanced EGFR and HGFR mediated signaling and enhanced expression of proliferative cell markers such as the proliferative cell nuclear antigen (PCNA) and cyclins D1 and E. Based on this evidence, a metabolomic study was planned to investigate the metabolic rearrangements produced in

* Corresponding author. Metabolomics Platform (URV-CIBERDEM), Campus Sescelades, SRCiT Universitat Rovira i Virgili, Avda. Països Catalans, 26, 43007 Tarragona, Spain. Tel.: +34 977 256 587; fax: +34 977 55 8261.
E-mail address: miguelangel.rodriguez@urv.cat (M.Á. Rodríguez).
URL: http://www.metabolomicsplatform.com
[1] These authors contributed equally to this work.

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the liver upon PHx in PTP1B KO with the rationale that they would further shed light on the metabolic processes involved in such early triggered response. Thus, a time-course study along the initial stages of liver regeneration using an NMR-based untargeted metabolomic approach was assayed on partial hepatectomized PTP1B KO and WT mice. The first part of the study was performed on intact liver tissue using High Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy in which PTP1B KO was compared to WT mice at time points 0 and 24 h after PHx. The second part of the study consisted of the same comparison involving an extra time point (48 h after injury), performed on aqueous (acetonitrile/water) and lipidic (chloroform/methanol) liver extracts.

2. Materials and methods

2.1. Animals

The animals were 12-week-old male wild type (WT, *Ptpn1*$^{+/+}$) and PTP1B Knock Out (PTP1B KO, *Ptpn1*$^{-/-}$) mice of the mixed genetic background 129 sv x C57/BL6 as previously described [24]. Animal care followed the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) in terms of health monitoring. The use of animals in these experimental procedures was approved by the Consejo Superior de Investigaciones Científicas (CSIC) Animal Care and Use Committee. Mice were fed a standard chow diet.

2.2. Partial hepatectomy surgery procedure

A subset of four to seven animals for each condition was subjected to a standard 70% PHx under isoflurane anesthesia, as previously described [25]. Analgesia was provided prior to surgery by subcutaneous injection of buprenorphine 50 μg/kg (Buprex, Schering-Plough). Briefly, the liver lobes were extruded, the median and left lateral lobes were ligated and removed as described above, and the wounds were sutured. The left lateral lobe was immediately frozen at −80 °C and stored until assayed. Animals were provided with water and food *ad libitum* and kept in a warm environment to avoid hypothermia. The remaining lobes were collected 24 or 48 h after PHx. Samples of tissue consisting of the distal portion of the anterior right lobe were weighed, chopped into smaller pieces of the desired size and immediately frozen and stored at −80 °C.

2.3. Oil red O staining

Portions of regenerating liver were fixed, embedded in O.C.T. (Tissue Tek, Sakura Finetek Europe) and flash frozen in liquid nitrogen. 5 μm tissue slices were stained with oil red O and counterstained with hematoxylin to reveal the presence of triglycerides and lipids in the liver.

2.4. Sample preparation and HR-MAS (ex-vivo) intact liver tissue measurements

Individual frozen tissues samples (9.8–19.5 mg) were placed in an insert for a 4 mm outer diameter ZrO_2 rotor, limiting the rotor inner volume to 20 μl. Then, the insert was filled with cooled D_2O, sealed and subsequently inserted into the ZrO_2 rotor. HR-MAS spectra were recorded on a Bruker Avance III 500 spectrometer operating at a proton frequency of 500.13 MHz. The instrument was equipped with a 4 mm triple resonance (^1H, ^{13}C, ^{31}P) gradient HR-MAS probe. A Bruker Cooling Unit (BCU-Xtreme) was used to keep the sample temperature at 4 °C. Samples properly prepared with D_2O and shimmed were spun at 4 kHz to keep the rotation sidebands out of the spectral region of interest [26]. One-dimensional (1D) ^1H spectra were acquired using a noesy presat sequence for water suppression. A total of 128 scans with a 90° flip angle were accumulated in 23 min, with a 7000 Hz (14 ppm) of spectral width and 8.0 s of relaxation delay to ensure full relaxation of magnetization between scans.

2.5. Liver tissue extracts preparation and high-resolution liquid NMR (in-vitro) measurement

Around 100 mg of liver (left lateral or anterior right lobes for baseline or experimental time-point, respectively) were homogenized in 2 ml of a cold mixture (CH_3CN:H_2O 1:1 v/v $T = 0$ °C, 5 min) by using a T10 basic Ultra-Turrax Disperser (IKA, Staufen, Germany). The resulting suspension was centrifuged (5000 × *g*, 15 min, $T = 4$ °C) and the supernatant was carefully transferred to a new eppendorf tube. This step was repeated three times and the combined aqueous phases were frozen, lyophilized and stored at −80 °C until further analysis. The resulting pellet was dried and extracted with 1 ml of a chloroform:methanol mixture at room temperature for 20 min (CH_3Cl:MeOH 3:1 v/v). Then, it was centrifuged for 5 min at 6000 × *g* at 4 °C and the supernatant (lipidic phase) was collected in a new eppendorf tube. The lipidic phase was dried under a nitrogen stream and stored at −80 °C. For NMR measurements, the hydrophilic extracts were reconstituted in 600 μl of D_2O containing 0.58 mM trisilylpropionic acid (TSP). The lipophilic extracts were subsequently extracted in 700 μl of a solution $CDCl_3$/CD_3OD (2:1) containing 0.73 mM tetramethylsilane (TMS) and samples were then vortexed, homogenized for 20 min, centrifuged for 15 min at 6000 × *g* at room temperature and transferred into 5 mm NMR tubes.

One- and two-dimensional ^1H NMR spectra were measured at a 600.20 MHz frequency using an Avance III-600 Bruker spectrometer equipped with an inverse TCI 5 mm cryoprobe®. For the 1D aqueous extract spectra, one-dimensional (1D) Nuclear Overhauser Effect Spectroscopy with a spoil gradient (noesygppr1d) was used. Solvent presaturation with low irradiation power (10 Hz) was applied during recycling delay and mixing time (tm = 100 ms) to suppress residual water. A total of 256 transients were collected across 12 kHz spectral width at 300 K into 64 k data points, and exponential line broadening of 0.3 Hz was applied before Fourier transformation. A recycling delay time of 8 s was applied between scans to ensure correct quantification. In the case of lipophilic extracts, a 90° pulse with a presaturation sequence (zgpr) was used. We performed measurements at 287 K, shifting the residual water signal to 4.65 ppm to allow for the quantification of the characteristic glycerol-backbone signals. In addition, residual water was pre-saturated during recycling delay (RD = 8 s) using a low irradiation power (10 Hz). A total of 256 FIDs of 12 kHz of spectral width were collected into 64 k data points, and exponential line broadening of 0.3 Hz was applied before Fourier transformation. The frequency spectra were phased, baseline corrected and then calibrated (TMS or TSP, 0.0 ppm) using TopSpin software (version 2.1, Bruker®, Germany).

2.6. Metabolite identification and quantification

Resonance assignments were done on the basis of literature values and different database search engines (Bioref AMIX 3.8 database from Bruker®; Chenomx NMRSuite 7.5 from Chenomx Inc. and Human Metabolome Database, HMDB http://www.hmdb.ca). Structural confirmations were based on two-dimensional (2D) ^1H–^1H COrrelation SpectroscopY (COSY) and 2D ^1H, ^{13}C Heteronuclear Single Quantum Correlation (HSQC). We selected 1D NMR regions based on our previously published 1D ^1H NMR lipidic and aqueous