Biochimica et Biophysica Acta 1812 (2011) 836–841

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Proteomics for the discovery of nuclear bile acid receptor FXR targets ☆

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ARTICLE INFO

Article history:
Received 9 December 2010
Received in revised form 23 February 2011
Accepted 15 March 2011
Available online 23 March 2011

Keywords:
Nuclear receptor
FXR
Proteomic analysis
Expression profiling
Post-transcriptional modification
Mitochondrial

ABSTRACT

Nuclear receptors (NRs) are important pharmacological targets for a number of diseases, including cancer and metabolic disorders. To unmask the direct role of NR function it is fundamental to find the NR targets. During the last few years several NRs have been shown to affect microRNA expression, thereby modulating protein levels. The farnesoid X receptor (FXR), the main regulator of bile acid (BA) homeostasis, also regulates cholesterol, lipid and glucose metabolism. Here we used, for the first time, a proteomics approach on mice treated with a FXR ligand to find novel hepatic FXR targets. Nineteen spots with a more than two-fold difference in protein amounts were found by 2D-DIGE and 20 proteins were identified by MALDI-TOF MS as putative novel FXR targets. The most striking feature of the protein list was the great number of mitochondrial proteins, indicating a substantial impact of FXR activation on mitochondrial function in the liver. To examine if the differences found in the proteomics assay reflected differences at the mRNA level, a microarray assay was generated on hepatic samples from wild type and $FXR^{-/-}$ mice treated with a FXR ligand and compared to vehicle treatment. At least six proteins were shown to be regulated only at a post-transcriptional level. In conclusion, our study provides the impetus to include proteomic analysis for the identification of novel targets of transcription factors, such as NRs. This article is part of a Special Issue entitled: Translating nuclear receptors from health to disease.

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1. Introduction

Nuclear receptors (NRs) are important pharmacological targets for treatment of a number of diseases, including cancer and metabolic disorders [1]. Great efforts are made to find substances that are specific to a NR subtype or that affect only certain aspects of the NR function [2]. To understand all the effects and possible side effects of NR activation or repression it is fundamental to find the NR targets. Since NRs are transcription factors, it is natural to look for tissue specific expression patterns and their target genes by gene expression profiling [3,4]. A lot of information has been gained from such studies. However, bearing in mind the diverse ways of regulation of RNAs and proteins, including the effects of microRNAs on mRNA translation and degradation, maybe a proteomics approach should be applied as well. Indeed, during the last few years several NRs have been shown to affect microRNA expression, thereby modulating protein levels [5–10].

The farnesoid X receptor (FXR), the main regulator of bile acid (BA) homeostasis [11–13], also regulates cholesterol, lipid and glucose metabolism [14]. The importance of FXR for the metabolic homeostasis in the gut-liver axis has been revealed in whole body and tissue specific FXR loss-of-function ($FXR^{-/-}$) mouse models [15–17]. Furthermore, FXR has been shown to play a role in processes such as liver regeneration [18], carcinogenesis [19–21], inflammation and bacterial overgrowth in the intestine [22,23]. There are therapeutic potentials for selective FXR modulators in diseases such as the metabolic syndrome, diabetes, gallstone disease, hypertriglyceridemia, steato-hepatitis and colon cancer [14,21]. A recent study of genomic FXR binding in mouse liver and intestine suggests a greater number of FXR target genes than is known thus far [24]. Also a high degree of tissue-specific binding was revealed, where only 11% of the binding sites were shared between the tissues, indicating a high degree of tissue-specific effects of FXR activation. The hepatic FXR target genes found up till now encode proteins involved in BA, lipid and glucose metabolism as well as in the detoxification of xenobiotics [25]. In a recent study FXR has also been shown to inhibit the expression of a microRNA, miR-34a [8].

To find hepatic FXR targets we used, for the first time, a proteomics approach with mice treated with the potent FXR ligand 6-ethyl chenodeoxycholic acid (6-ECDCA or INT-747) [26]. In addition, a

Abbreviations: NR, nuclear receptor; FXR, farnesoid X receptor; BA, bile acid; $FXR^{-/-}$, FXR loss-of-function

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doi:10.1016/j.bbadis.2011.03.009

microarray assay was carried out to examine if the differences found in the proteomics assay reflected differences at the mRNA level.

2. Materials and methods

2.1. Animals and treatments

Ten weeks old wild type C57BL/6J male mice and FXR$^{-/-}$ C57BL/6J male mice were treated with 10 mg/kg/day INT-747 (Intercept Pharmaceuticals) or only the vehicle, 1% methylcellulose, by gavages for at least three days. The animals were fasted overnight and given their last gavages 3 h before the sacrifice. The liver samples were snap-frozen in liquid nitrogen and kept at −80 °C until used. The Ethical Committee of the Consorzio Mario Negri Sud approved this experimental set-up, which was also certified by the Italian Ministry of Health according with internationally accepted guidelines for the animal care.

2.2. Proteomic analysis

2.2.1. Liver protein extraction for proteomic analysis

Individual mouse liver samples were ground into powder under liquid nitrogen, dissolved in a buffer containing 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) Zwittergent 3-10 detergent (Calbiochem, Merck Biosciences, Darmstadt, Germany) 50 mM DTT (Sigma Aldrich, Milano, Italy) and protease inhibitor cocktail set III (Calbiochem). After a centrifugation at 100,000*g* for 30 min at 12 °C the pellets were discarded and the supernatants taken as the cytosol fraction. The protein content was determined by ETTAN™ procedure, using a protein assay kit from GE Healthcare (Chalfont St. Giles, Bucks, UK).

2.2.2. Two-dimensional difference gel electrophoresis (2D-DIGE) and quantitative gel image analysis

Five vehicle and five INT-747 samples (each 50 μg of protein) were labeled separately with either 200 pmol Cy3 or Cy5, and the internal standard (25 μg of each of the ten samples) was labeled with Cy2. One vehicle, INT-747 and standard sample forming a set of Cy2, Cy3 and Cy5 labeled samples were combined for each of five gels and were diluted in the rehydration solution, composed of 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) Zwittergent, 40 mM DTT and 0.5% IPG buffer for pH 3–10 linear gradient (GE Healthcare). Isoelectric focusing (IEF) was carried out on immobilized IPG strips with a broad pH 3–10 linear gradient, by using an IPGphor Isoelectric Focusing System (GE Healthcare). After a rehydration step at 30 V for 16 h, focusing started at 200 V. The voltage was increased step by step to 1000 V, then gradually up to 8000 V and kept constant for further 5 h for a total 46,000 Vh. Following IEF, individual protein strips were reduced by rocking for 15 min in a solution containing 6 M urea, 50 mM Tris–HCl, pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS, 1% DTT. Proteins were subsequently alkylated by replacing DTT with 100 mM iodoacetamide for 15 min. The strips were placed on the top of 12.5% SDS-PAGE (160 × 160 × 1 mm) and run at 10 mA, for molecular size electrophoresis. Protein size was determined by running standard protein markers (Rainbow, GE Healthcare), in the range of 14.3–220.0 kDa.

Images were visualized using the pharos-FX imager from Bio-Rad. The gels were scanned using a 488 nm laser and an emission filter of 530 nm BP (band Pass) 40, a 532 nm laser and an emission filter of 695 nm DF (discriminating filter) 50, a 635 nm laser and 695 nm DF 55 emission filter to acquire the Cy2, Cy3, and Cy5 image respectively. All gels were scanned at 200 μm resolution. Images were then processed using the PD-Quest software (Bio-Rad) protocol. Protein spots were matched and gels were normalized using the internal standard present in all gels.

An overall total of around 1500 protein spots were visualized in the present study and a p-value < 0.05 (Student's t-test) was considered statistically significant. Only the spots showing at least a two-fold difference were further analyzed.

2.2.3. Protein identification by MALDI-TOF MS analysis

An additional gel was made using 300 μg of total protein pooled from each of the five vehicle and INT-747 samples analyzed run under conditions identical to the analytical gels except that the proteins were unlabeled (non DIGE). Selected protein spots were *in situ* digested and analyzed by MALDI-TOF MS. Briefly, protein bands were excised from SDS-PAGE and after washing, cysteins were reduced with DTT and alkylated with iodoacetamide. Gels were digested *in situ* by incubation with sequencing-grade trypsin (Promega, Madison, WI, USA) in 40 mM ammonium bicarbonate under slight shaking on a thermomixer at 37 °C overnight [27]. The reaction was stopped with H_2O/TFA 0.1% at 30 °C, for 15 min. Tryptic peptides were extracted, desalted with ZipTip C_{18} columns (Millipore Corp, Bedford, MA, USA), eluted and crystallized in 50% (v/v) ACN/H_2O saturated solution of alfa-cyano-4-hydroxy-cinnamic acid. Peptide mass spectra were obtained by a time-of-flight mass spectrometer (Reflex IV®, Bruker Daltonics, Bremen, Germany), equipped with a nitrogen laser with an emission wavelength of 337 nm. Mass spectra were acquired in positive ion Reflectron-mode with delayed extraction and an accelerating voltage of 20 kV. An external calibration was performed for each measurement, using a mixture of seven standard peptides (average mass accuracy better than 20 ppm). All mass spectra were acquired using a minimum number of 250 laser shots. Spectra were internally calibrated with trypsin autolysis products. Peptide matching and protein searches were performed submitting peptide mass lists to database search on NCBInr and/or SWISS PROT, using the MASCOT and ProFound search engines. The main search parameters were as follows: no restriction on molecular weight and isoelectric point (MW and pI); taxonomy, mause; one missed cleavage allowed; carboxymethylation of cystein; oxidation of methionine; 50–100 ppm peptide mass tolerance. Proteins listed as significant matches in MASCOT were considered when a threshold score allowing a $p < 0.05$ was achieved.

2.2.4. Western blot analysis

Western blot analysis was performed on pooled mouse liver samples, five in each group, processed as described above. Total proteins were quantified by ETTAN™ procedure, using a protein assay kit from GE Healthcare (Chalfont St. Giles, Bucks, UK).

For one-dimensional (1-DE) gel electrophoresis, samples of cytosol fractions were dissolved in SDS sample buffer, composed of 12.5 mM Tris–HCl, pH 6.8, 1% SDS, 10% glycerol, 0.025% bromophenol blue, boiled for 10 min, and applied to 12.5% (w/v) SDS-PAGE. Following 1-DE separation, proteins were transferred to nitrocellulose and after transfer, the nitrocellulose blots were checked by Ponceau red staining to ensure an homogeneous transfer efficiency. The blots were then probed with a primary antibody anti-GSTM1 (kindly provided by Dr. B. Favaloro, Ce.S.I., University G. D'Annunzio, Chieti, Italy), ATP5A (C-15):sc-49162 antibody , Laminin-R antibody (G-7):sc-74531,(Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), Annexin V antibody (ab 14196) Abcam plc,330 Cambridge science park, Cambridge CB4 OFL, UK. Blots were visualized by ECL chemiluminescence detection system (GE Healthcare) according to the manufacturer. Protein abundance was quantified by densitometric analysis, with Quantity ONE software (Bio-Rad) and the results normalized against β-Actin.

2.3. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using QIAzol (Qiagen) and the integrity of the RNA was assessed on a formaldehyde gel. cDNA was synthesized from total RNA by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) after DNase I treatment using the DNA-free Kit from Ambion. Relative amounts of *Shp* and cyclophilin mRNA were obtained on a 7500 Fast Real-Time PCR System machine (Applied

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