Journal of Chromatography B, 879 (2011) 3393–3400

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Quantitative subproteomic analysis of age-related changes in mouse liver peroxisomes by iTRAQ LC–MS/MS

Hanna Amelina[a,*], Marcus O.D. Sjödin[b], Jonas Bergquist[b], Susana Cristobal[a,c,d]

[a] Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden
[b] Department of Physical and Analytical Chemistry, Uppsala University, SE-751 24 Uppsala, Sweden
[c] Department of Clinical and Experimental Medicine, Linköping University, SE-581 03 Linköping, Sweden
[d] IKERBASQUE, Basque Foundation for Science, Department of Physiology, University of the Basque Country, Leioa 48940, Spain

ARTICLE INFO

Article history:
Received 15 April 2011
Accepted 26 August 2011
Available online 10 September 2011

Keywords:
Liquid chromatography
Tandem mass spectrometry
Peroxisomes
Aging
Liver
iTRAQ

ABSTRACT

Aging is a complex multifactorial phenomenon, which is believed to result from the accumulation of cellular damage to biological macromolecules. Peroxisomes recently emerged as another important source of reactive oxygen species (ROS) production in addition to mitochondria. However, the role of these organelles in the process of aging is still not clear. The aim of this study was to characterize the changes in protein expression profiles of young (10 weeks old) versus old (18 months old) mouse liver peroxisome-enriched fractions. We have applied shotgun proteomic approach based on liquid chromatography and tandem mass spectrometry (LC–MS/MS) combined with iTRAQ (isobaric tags for relative and absolute quantitation) labeling that allows comparative quantitative multiplex analysis. Our analysis led to identification and quantification of 150 proteins, 8 out of which were differentially expressed between two age groups at a statistically significant level ($p < 0.05$), with folds ranging from 1.2 to 4.1. These proteins involved in peroxisomal β-oxidation, detoxification of xenobiotics and production of ROS. Noteworthy, differences in liver proteome have been observed between as well as within different age groups. In conclusion, our subproteomic quantitative study suggests that mouse liver proteome is sufficiently maintained until certain age.

1. Introduction

Aging is the most complex phenomenon currently known, as it becomes manifest in all organs and tissues, leads to the organism's physiological decline, affects functions at all levels and increases susceptibility to all major chronic diseases. It is a multifactorial process: protein turnover is impaired [1], telomeres shorten [2], somatic mutations increase [3] and chromatin modifications become more and more evident [4]. Nevertheless, aging is still poorly defined at the mechanistic level, even though in recent years much progress has been made by studying genetic mutations altering the life span.

The traditional scientific approach based upon reductionism has not been proved to be particularly efficient in deciphering very complex biological processes. Therefore there was a great promise that aging research would benefit from recently emerged large-scale technology platforms (genomics, transcriptomics, proteomics, metabolomics), which are designed to provide a global description of changes in the system and define inter-relationship between its elements.

Prior efforts to comprehensively identify and evaluate potential factors that contribute to the aging process have benefited from microarray analysis. Multiple studies have addressed the effect of aging on the transcriptional level in different tissues, including skeletal muscle in the leg (gastrocnemius) of mice [5], mouse brain [6], human and mouse kidney [7,8]. However, it has been found that the correlation between mRNA abundance and the quantity of the corresponding functional protein present within a cell is rather poor [9], therefore transcriptomic information alone is not sufficient to clarify the mechanism of aging.

Proteomics technology is an approach to examine and identify protein dynamics of biological pathways and multifactorial disease processes. Because these techniques can analyze the final end products of genes, they provide more functional insight. During the last decade a number of proteomic gel-based studies on aging tissues have been performed: 2-DE study of aging brain in mice [10], human normal colon epithelia [11], aging mouse kidney [8] among others. These studies reported on average up to 50 proteins with significant differential expression in old tissues compared to young. It has been observed that consequences of aging are tissue-specific

Abbreviations: ROS, reactive oxygen species; ESI, electrospray; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PCA, principle component analysis.

* Corresponding author. Tel.: +46 8 16 2378; fax: +46 8 15 3679.
E-mail address: hanna.amelina@dbb.su.se (H. Amelina).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.08.044

[12,13], which may be due to the organs' differing regenerating activity. For instance liver has a remarkable capacity to regenerate after injury, compared to other organs such as kidney and brain that undergo irreversible functional decline.

Recently a great progress in the field of high-resolution shotgun mass spectrometry-based proteomics has been made, allowing for accurate identification and quantification of complex protein samples. These techniques have already been applied to study age-related changes in biological systems, and one of such examples is a quantitative proteomic profiling of age-related changes of human cerebrospinal liquid using ICAT and mass spectrometry [14]. Another recent study from M. Mann laboratory [15], in which SILAC metabolic labeling of mice has been utilized to examine changes of the mouse tissue proteomes during aging, surprisingly reported only few proteins that differentially expressed with age.

In the present study, iTRAQ-based quantitative proteomic approach has been utilized to investigate the changes in the liver proteome during the age course. In order to reduce sample complexity and the masking effect of highly abundant proteins we have focused on peroxisome-enriched fractions.

Peroxisomes are important cell organelles involved in oxidative metabolism in nearly all eukaryotes. They harbor enzymes involved into a variety of essential metabolic processes, such as β-oxidation of long-chain fatty acids and the synthesis of ether lipids in men [16]. Peroxisomes contain hydrogen peroxide producing oxidases and hence, contribute to the cellular production of reactive oxygen species (ROS) that accumulate with age and can damage important macromolecules such as DNA, proteins and lipids.

Until recently mitochondria were considered a key player in ROS generation, and their role in aging has been addressed in a number of proteomic studies [17–19]. However, soon it became evident that in specific cells or tissues (e.g. liver) peroxisomes represent major ROS producers. Moreover, peroxisomal dysfunction has been shown to be associated with cellular aging and molecular pathologies that often lead to age-related degenerative diseases [20,21]. These facts altogether make peroxisomes another appealing organelle for studying the mechanisms of aging.

In our study we applied a mass spectrometry-based quantitative proteomic method to explore how aging affects the peroxisomes. Here, we show that the peroxisomal proteome of mouse liver undergoes only slight changes during aging, and that inter-individual variation must be considered in aging studies.

2. Materials and methods

2.1. Chemicals

Optiprep was purchased from Axis-Shield, Norway. Complete Protease Inhibitor Cocktail was from Roche Diagnostics, Mannheim, Germany. Anti-catalase antibody was purchased from Rockland Immunochemicals, Inc.; anti-prohibitin (H-80) and anti-epoxide hydrolase (A-5) antibodies were from Santa Cruz Biotechnology, Inc. iTRAQ reagents and trypsin were from Applied Biosystems AB (Foster City, CA). 3-(N-morpholino) propanesulfonic acid (MOPS), acetonitrile (ACN), formic acid (FA) and acetic acid (HAc) were obtained from Merck (Darmstadt, Germany). Sucrose, EDTA and urea were from Sigma–Aldrich (St. Louis, MO, USA). All water used was Milli-Q ultra-pure (Millipore, Bedford, MA, USA).

2.2. Animals

Frozen livers from male C57BL6/J mice of 10-week-old and 18-month-old were purchased from Janvier laboratories (Le Genest-St-Isle, France). Livers from six biological replicates per age group were used in this study. Organs were dissected according to standard protocols, snap-frozen in liquid nitrogen and air-transported with dry ice on the same day.

Table 1
Experimental design.

Experimental run order	Labels			
	114	115	116	117
1	pool	young 1	young 2	old 1
2	pool	old 2	old 3	young 3
3	pool	young 4	young 5	old 4
4	pool	old 5	old 6	young 6

2.3. Cell fractionation and isolation of peroxisome-enriched fraction

Frozen tissues were thawed on ice, minced and homogenized in the ice-cold homogenization buffer (250 mM sucrose, 5 mM MOPS, 0.5 M EDTA, 0.1% ethanol) in the presence of Complete Protease Inhibitor Cocktail (1 tablet dissolved in 50 mL buffer). Four individual livers that included samples from both age groups were processed simultaneously, enabling us to reduce possible "batch effect". The subcellular fractionation by differential and density centrifugation was performed as described previously [22] with a few modifications outlined below. The main subcellular fractions were termed according to the nomenclature used by Volkl and Fahimi [23]. Thus, the crude homogenate was termed A, the heavy mitochondrial fraction was termed B, postmitochondrial faction was termed C, the light mitochondrial or peroxisome-enriched fraction was termed D, the cytosolic fraction was termed E, and the microsomal was termed F. Two milliliters of the resuspended D fraction were carefully layered on the top of 10 mL of 28% iodixanol (v/v), 5 mM MOPS, 0.1% ethanol, 1 mM EDTA solution and 1 mL of 50% iodixanol cushion and centrifuged at 131,000 g_{av} for 2 h. The peroxisome-enriched fractions were collected from the interface between 28 and 50% iodixanol. The efficiency of purification procedure was assessed using protein immunoblot against peroxisomal catalase and mitochondrial prohibitin (Fig. 1). Protein concentration in obtained fractions was determined according to Bradford [24]. The fractions corresponding to 90 μg of proteins were then freeze-dried and then processed according to the iTRAQ reagents protocol (Applied Biosystems AB). As none of the substances present in the homogenization buffer were listed as interfering with the iTRAQ reagents protocol, protein precipitation step prior digestion was omitted in order to minimize protein losses.

2.4. Experimental design

The samples were divided into four 4-plex experiments (Table 1), and a pooled reference sample prepared by combining aliquots from 6 young and 6 old samples, was incorporated into each experiment. This experimental setup enabled us to simultaneously analyze the data across the four 4-plex experiments, and to correct for experimental variability across multiple experiments. For every 4-plex experiment equivalent amount of protein from each biological replicate and pooled reference sample was reduced, alkylated, digested and labeled with one of the isobaric tags. The reference standard was allocated into each experiment using the same labeling tag, and the rest of samples were allocated to the remaining tags.

2.5. Protein reduction, alkylation and trypsin digestion

Protein denaturation, reduction, cysteine blocking and digestion were performed according to the protocol provided by the