Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Secretome profiling of primary human skeletal muscle cells ☆

Sonja Hartwig [a,k], Silja Raschke [b,k], Birgit Knebel [a,k], Mika Scheler [c,k], Martin Irmler [c,k], Waltraud Passlack [a,k], Stefan Muller [d], Franz-Georg Hanisch [d], Thomas Franz [e], Xinping Li [e], Hans-Dieter Dicken [f], Kristin Eckardt [b,k], Johannes Beckers [c,g,k], Martin Hrabe de Angelis [c,g,k], Cora Weigert [h,i,k], Hans-Ulrich Häring [h,i,k], Hadi Al-Hasani [a,k], D. Margriet Ouwens [a,j,k], Jürgen Eckel [b,k], Jorg Kotzka [a,k], Stefan Lehr [a,k,*]

[a] Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Duesseldorf, Germany
[b] Paul-Langerhans-Group for Integrative Physiology, German Diabetes Center, Duesseldorf, Germany
[c] Institute of Experimental Genetics, Helmholtz Zentrum Muenchen, German Research Center for Environmental Health (GmbH), Neuherberg, Germany
[d] Center for Molecular Medicine Cologne, Cologne, Germany
[e] Max Planck Institute for Biology of Ageing, Cologne, Germany
[f] Multimedia Center, Heinrich-Heine-University Duesseldorf, Duesseldorf, Germany
[g] Experimental Genetics, Center of Life and Food Sciences Weihenstephan, Technische Universität München, Freising-Weihenstephan, Germany
[h] Division of Endocrinology, Diabetology, Angiology, Nephrology, Pathobiochemistry and Clinical Chemistry, Department of Internal Medicine, University Tuebingen, Germany
[i] Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Zentrum Muenchen at the University of Tuebingen, Tuebingen, Germany
[j] Department of Endocrinology, Ghent University Hospital, Ghent, Belgium
[k] German Center for Diabetes Research (DZD), Germany

ARTICLE INFO

Article history:
Received 11 April 2013
Received in revised form 31 July 2013
Accepted 11 August 2013
Available online xxxx

Keywords:
Myokine
Combined proteomic profiling
Two-dimensional gel electrophoresis
Mass spectrometry

ABSTRACT

The skeletal muscle is a metabolically active tissue that secretes various proteins. These so-called myokines have been proposed to affect muscle physiology and to exert systemic effects on other tissues and organs. Yet, changes in the secretory profile may participate in the pathophysiology of metabolic diseases. The present study aimed at characterizing the secretome of differentiated primary human skeletal muscle cells (hSkMC) derived from healthy, adult donors combining three different mass spectrometry based non-targeted approaches as well as one antibody based method. This led to the identification of 548 non-redundant proteins in conditioned media from hSkmc. For 501 proteins, significant mRNA expression could be demonstrated. Applying stringent consecutive filtering using SignalP, SecretomeP and ER_retention signal databases, 305 proteins were assigned as potential myokines of which 12 proteins containing a secretory signal peptide were not previously described. This comprehensive profiling study of the human skeletal muscle secretome expands our knowledge of the composition of the human myokinome and may contribute to our understanding of the role of myokines in multiple biological processes. This article is part of a Special Issue entitled: Biomarkers: A Proteomic Challenge.

1. Introduction

The search for an "exercise factor" that communicates the energy demand of the working muscle to other organs added the skeletal muscle to the list of secretory active tissues [1]. These muscle-derived secretory proteins, termed myokines, can exert auto-, para- or endocrine effects, founding a new paradigm for understanding how muscles communicate with other organs, such as adipose tissue, liver, or pancreas [2]. The discovery of the skeletal muscle as the origin of cytokines released during physical activity focuses the research on these myokines to understand the beneficial effects of exercise on metabolic disturbances like insulin resistance or type-2-diabetes. One prominent example is interleukin 6 (IL6) which is released into circulation from contracting muscles influencing glucose as well as lipid metabolism [3–5]. While exercise is still considered as one of the major contributors to the release of proteins from the muscle, other physiological and pathophysiological conditions that induce the release of myokines have been identified [6–9]. Recent studies of the past few years indicate that the skeletal muscle secretome comprises several peptides and proteins including many yet unidentified biological active factors, being involved in various biological processes [2]. Thus, the identification of the complex nature of the human myokine pattern may contribute to the understanding of various physiological crosstalks between the muscle and other organs as well as disease development and its prevention.

Complementary mass spectrometry based proteomic profiling technologies have contributed to the identification of hundreds of proteins found in conditioned media (CM) from multiple tissue derived cell

☆ This article is part of a Special Issue entitled: Biomarkers: A Proteomic Challenge.

* Corresponding author at: Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Auf'm Hennekamp 65, 40225 Duesseldorf, Germany. Tel.: +49 211 3382 531.
E-mail address: stefan.lehr@ddz.uni-duesseldorf.de (S. Lehr).

1570-9639/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbapap.2013.08.004

lines, including the rodent skeletal muscle cell lines L6 and C_2C_{12} [9–12]. Nevertheless, little is known about the secretome of adult human skeletal muscle [13–16]. Especially the knowledge about the secretome of mature, thus fully differentiated hSkMC obtained from healthy adults is still sparse.

In the current study we have utilized an integrated genomic and proteomic approach to define the secretome of differentiated hSkMC derived from adult donors. We applied a combination of three different mass spectrometry methods (*2-D PAGE MALDI-MS*, *SDS-PAGE LC–ESI-MS/MS and LC/MS^E*) as non-targeted approaches and multiplex immunoassay (MIA) as a targeted approach to enable a comprehensive analysis of the entire human muscle secretome and the expression of identified proteins was validated by transcriptomics. Our study expands our knowledge of the human skeletal muscle secretome by the identification of 12 potential myokines previously not described as muscle-derived factors.

2. Experimental procedures

2.1. Culture and preparation of conditioned media (CM) from primary human skeletal muscle cells

Cohort 1. Primary hSkMC from five healthy caucasian donors (three males and two females; 31 ± 6 yr) were used for the preparation of CM. The cells were supplied as proliferating myoblasts (PromoCell, Heidelberg, Germany) and cultured as described previously [17]. Briefly, myoblasts were seeded in six-well plates (1×10^5 cells/well), and were cultured in α-modified Eagle's (αMEM)/Ham's F-12 medium containing skeletal muscle cell growth medium supplement pack (PromoCell, Heidelberg, Germany) up to near-confluence. The cells were then differentiated to myotubes in αMEM containing 2% (v/v) horse serum (Gibco, Berlin, Germany) for 5 days. On day 6 of differentiation, cells were washed twice with PBS, serum-free medium was added and CM for proteomic profiling was collected after 24 h. In total more than 1 l of CM was centrifuged at 85,000 ×*g* and concentrated to mg/ml range using Amicon™ Ultra 15 centrifugal filter devices (Millipore, Billerica, USA) with a cut-off mass of 3 kDa. Protein concentrations were determined using Advanced Protein Assay (Tebu-bio, Offenbach, Germany) according to the manufacturer's instructions and aliquots of pooled CM were stored at −80 °C for non-targeted proteomic analysis.

Cohort 2. For targeted proteomic profiling (multiplex analysis (MIA)) and expression profiling, hSkMC obtained from percutaneous needle biopsies performed on the lateral portion of quadriceps femoris (vastus lateralis) muscle of twelve healthy subjects (three females, nine males; 25.6 ± 4.4 yr) were used. They gave informed written consent to the study and the protocol was approved by the Ethics Committee of the University of Tuebingen (Number: 179/97). Cell culture and collection of CM was performed as described above.

2.2. Non-targeted proteomic profiling

For non-targeted proteomic profiling three independent methods were used, i.e. 1-D PAGE/LC/ESI-MS/MS, data independent LC-MS/MS (LC/MS^E) and 2-D PAGE/MALDI-MS.

2.2.1. Validation of CM collection procedure

To validate the collection of CM, murine C_2C_{12} skeletal muscle cell model system was used. Proliferating C_2C_{12} [18] myoblasts were seeded in six-well culture dishes at a density of 1×10^5 cells/well and cultured to near-confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) and 100 μg/ml streptomycin (Invitrogen, Paisley, UK). The cells were then differentiated in DMEM containing 2% (v/v) horse serum for 5 days. On day 6 cells were washed twice with PBS and three times with fresh serum-free DMEM, serum-free medium was added and CM for proteomic profiling was collected after 24 h. CM was centrifuged at 85,000 ×*g* and concentrated to mg/ml range using Amicon™ Ultra 15 centrifugal filter devices (Millipore, Billerica, USA) with a cut-off mass of 3 kDa. Aliquots of concentrated CM were subjected to two-dimensional difference gel electrophoresis 2-D DIGE. 2-D DIGE was performed as described previously [19]. Briefly, labeled samples (50 μg each) were separated in the first dimension by isoelectric focusing (IEF) on a MultiPhor II electrophoresis unit (Amersham Biosciences) using IPG strips (24 cm, pH 4–7 linear), followed by SDS-PAGE on 12.5% polyacrylamide gels (24 cm × 18 cm) using an EttanDalt 12 system (Amersham Biosciences). Subsequently, images of protein pattern were acquired using a Typhoon 9400 (Amersham Biosciences) laser scanner according to the manufacturer's recommendations (resolution of 100 μm, photomultiplier tube of 550 V).

2.2.2. 1-D PAGE and protein identification by liquid chromatography (LC)-MS/MS

For LC-MS analysis aliquots of concentrated CM (20 μg) were first separated by one-dimensional SDS-PAGE (5% to 15% gradient gel). Extracted peptides derived from 24 equally sized gel slices were subsequently analyzed as described previously [20]. Liquid chromatography (LC)-MS data were acquired on a HCT ETD II ion trap mass spectrometer (BrukerDaltoniks, Bremen, Germany). Raw data were processed using Data Analysis 4.0 (BrukerDaltoniks, Bremen, Germany) and xml formatted peak lists were transferred to Proteinscape 3.0 (BrukerDaltoniks, Bremen, Germany). MASCOT 2.2 (Matrix Science Ltd, London, UK) was used to search a composite decoy database which was built from SwissProt_2011 (532,146 sequences; 188,719,038 residues; and 20,249 human sequences). The composite database was generated with the Perl script *makeDecoyDB* (BrukerDaltoniks, Bremen, Germany) which added a randomized sequence and a tagged accession number for each entry. The tagged accessions were used for the calculation of false positive rate in Proteinscape 3.0. Searches were submitted via Proteinscape3.0 and the following parameter settings: enzyme "trypsin", species "human", fixed modifications "carbamidomethyl", optional modifications "Methionine oxidation" and missed cleavages "2". The mass tolerance was set to 0.4 Da for peptide and fragment spectra. Protein lists were compiled in Proteinscape3.0. Peptide hits were accepted when the ion score exceeded a value of 20. Protein hits required at least one peptide hit exceeding a peptide score of 40. In addition, the hits to decoy entries were used to calculate a minimal protein score which is required to keep the false positive rate below 2% on the protein level.

2.2.3. In-solution digestion of total protein sample and protein identification by data independent LC-MS/MS

20 μg of each sample was dissolved in 20 μl of 25 mM ammonium hydrogen carbonate. 2 μl of 1% RapiGest (Waters Corporation, Milford, USA) was added for denaturing the proteins. The protein solution was incubated at 80 °C for 10 min on a Thermo-mixer. After the addition of 1 μl aliquot of 50 mM DTT the solution was heated at 60 °C for 15 min. The protein solution was then cooled down to room temperature and centrifuged. After the addition of 1 μl aliquot of 150 mM iodoacetamide in 25 mM ammonium, the solution was stored in the dark at room temperature for 30 min. The tryptic digestion was performed by adding Trypsin Gold mass spectrometry grade (Promega, Madison, MI, USA) at a 1:50 (w/w) ratio and incubating at 37 °C overnight. 1 μl of 37% HCl was added to adjust the pH below 2. After being vortexed and centrifuged at 13,000 ×*g* for 30 min, the supernatant was collected and transferred to a clean microcentrifuge tube. The tryptic digest was desalted with PepClean™ C-18 Spin Column (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instruction, and eluted with 40 μl of 50% acetonitrile (ACN)/0.1% formic acid. After drying in a SpeedVac, the digest was re-suspended in 100 μl of 0.1% formic acid.

Protein identification was performed with a Xevo Q-Tof (Waters Corporation, Milford, USA) coupled with a nanoACQUITY UPLC™ (Waters Corporation, Milford USA). 3–4 μl of tryptic digest was directly loaded into an analytical column of 75 μm × 150 mm C18 BEH 1.7 μm

Please cite this article as: S. Hartwig, et al., Secretome profiling of primary human skeletal muscle cells, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbapap.2013.08.004