Biochemical and Biophysical Research Communications xxx (2017) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Quantitative proteomics reveals key proteins regulated by eicosapentaenoic acid in endothelial activation

Lu Zhang [a], Kunhong Xiao [b], Xuyang Zhao [c], Xiaoli Sun [a], Jianshu Zhang [a], Xian Wang [a], Yi Zhu [d], Xu Zhang [a, d, *]

[a] Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China
[b] Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261, USA
[c] Institute of Systems Biomedicine, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China
[d] Department of Physiology and Pathophysiology, Tianjin Medical University, Tianjin 300070, China

ARTICLE INFO

Article history:
Received 15 April 2017
Accepted 17 April 2017
Available online xxx

Keywords:
Proteomics
Eicosapentaenoic acid (EPA)
Arachidonic acid (AA)
Tumor necrosis factor-α (TNFα)
Endothelial activation
Atherosclerosis

ABSTRACT

Eicosapentaenoic acid (EPA), an n-3 polyunsaturated fatty acid (PUFA), has been shown to decrease the risk of atherosclerosis by attenuating endothelial activation. In this study, we used mass spectrometry-based label-free quantitative proteomics to study the protective mechanisms of EPA and to identify key proteins that regulated by EPA in endothelial activation. Arachidonic acid (AA) was used as a control. HUVECs were pretreated with each of the two PUFAs, and then stimulated with TNFα as a model of endothelial activation. A total of 3391 proteins were identified, and 1958 proteins were quantified. Pearson's correlation coefficients revealed the excellent biological reproducibility of the proteomic results. Gene Ontology and KEGG enrichment analysis of differentially expressed proteins was performed, thus leading to the identification of the glutathione metabolism, oxidation reduction, and DNA replication as the most significantly enriched pathways. Seven key proteins were identified: elongation factor Tu (mitochondrial, TUFM), integrin alpha 6 (ITGA6), catalase (CAT), annexin A6 (ANXA6), heat shock 70 kDa protein 1A (HSPA1A), glutamate-cysteine ligase regulatory subunit (GCLM), and heme oxygenase 1 (HMOX1). Further connections among these proteins were also revealed by protein-protein interaction analysis. The mRNA levels of CAT, GCLM, and HMOX1 were verified with real-time PCR. The protein level of CAT was verified using Western blotting. This study is an in-depth proteomics analysis of EPA-treated cells and may provide possible insights into the molecular mechanisms of EPA's cytoprotective and atheroprotective effects.

1. Introduction

Endothelial cells play a critical role in regulating functions of the cardiovascular system. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), mediate the expression of adhesion molecules on the endothelial cell surface, thus recruiting leukocytes from the blood, which then accumulate in the arterial intima [1]. This process, called endothelial activation, triggers the initiation of atherosclerosis. Activated endothelial cells and leukocytes continue to produce pro-inflammatory cytokines and growth factors, thereby aggravating the severity of plaques and potentially leading to acute cardiovascular events, such as stroke or myocardial infarction [2]. The suppression of endothelial activation may reverse atherogenesis and prevent future cardiovascular events [3].

In epidemiological studies and clinical trials, nutritional intake of fish oil or n-3 polyunsaturated fatty acids (PUFAs) has been proven to decrease the risk of atherosclerosis [4]. As one of the major n-3 PUFAs, eicosapentaenoic acid (EPA) exerts various cardiovascular protective effects that decrease plasma triglyceride levels, inhibit thrombus formation, and attenuate pro-inflammatory cytokine-induced endothelial activation [4,5]. The potential mechanisms mediating the cytoprotective effects of EPA have been reported to be associated with several receptors and signaling pathways, such as the membrane receptor GPR120 [6],

Abbreviations: EPA, eicosapentaenoic acid; AA, arachidonic acid; TNFα, tumor necrosis factor-α; PUFAs, polyunsaturated fatty acids; MS, mass spectrometry; HUVECs, human umbilical vein endothelial cells.

* Corresponding author. Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China.
E-mail address: xuzhang@tmu.edu.cn (X. Zhang).

http://dx.doi.org/10.1016/j.bbrc.2017.04.091
0006-291X/

the PPAR nuclear receptor family, and NF-κB signaling [5]. Although physicians recommend n-3 PUFAs as an alternative therapy for atherosclerosis [7], studies focused on the potential targets of n-3 PUFAs are lacking. Moreover, EPA capsules usually contain a small proportion of pro-inflammatory saturated and monounsaturated fatty acids that have opposite effects of EPA [8]; therefore, the identification of potential targets of EPA and development of relevant inducers and inhibitors may improve the efficacy of this atherosclerosis therapy.

In addition to the direct function of EPA itself, the balance between EPA and arachidonic acid (AA) is thought to play an indirect role in maintaining endothelial homeostasis. EPA and AA share the same set of metabolic enzymes [9]. AA metabolites are often thought to exert pro-inflammatory effects that may increase the risk of atherosclerosis [9]. EPA may displace AA from the cell membrane and partially antagonize the pro-inflammatory activity of AA metabolites [10]. According to recent clinical studies, a higher (EPA/AA) ratio in human blood is significantly associated with a lower risk of cardiovascular events [8]. Because EPA regulates endothelial functions in a comprehensive and complex manner, a systematic study is needed to improve understanding of the underlying mechanisms and would provide possible insights into the treatment of atherosclerosis.

Mass spectrometry (MS)-based proteomics is a powerful tool that has been successfully applied for the identification and quantification of proteins associated with diseases and treatments. In this study, we used a label-free quantitative proteomics approach to study the protein profiles of EPA-treated human umbilical vein endothelial cells (HUVECs) to examine EPA's cytoprotective effect, and we used AA as a control in our experiments. TNFα was applied to cells as a model of endothelial activation. A total of 3391 proteins were identified through MS-based proteomics, and subsequent bioinformatics analysis was performed to explore the differentially expressed proteins and pathways. This study aimed to provide a deeper understanding of the cytoprotective mechanisms of EPA and identify differentially regulated proteins that may play important roles in regulating endothelial functions.

2. Materials and methods

Cell culture. HUVECs were cultured for at most five passages with previously described methods [11]. One day before the experiments, HUVECs were plated in 10 cm dishes at 50% confluency to minimize cell overgrowth during the next three days of experiments. On the day of experiments, EPA (Cayman, Ann Arbor, MI) was mixed with 10% fatty-acid-free BSA (Sigma-Aldrich, St. Louis, MO) buffer at a molar ratio of 4:1 before being added to the dishes. Cells were cultured with EPA at 37 °C for 42 h, and then stimulated with 1 ng/ml TNFα (Sigma-Aldrich, St. Louis, MO) for 6 h. AA (Cayman, Ann Arbor, MI) was used in the same manner. The proteins were then extracted from HUVECs and stored at −80 °C until use.

Western blotting. Proteins were quantified with BCA protein assays (Thermo Scientific, Waltham, MA). The same amount of proteins from each sample was separated on 10% SDS-PAGE gels and transferred to a PVDF membrane. Rabbit anti-VCAM-1, mouse anti-CAT and mouse anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 1: 1000 dilutions.

Sample preparation. Proteins were quantified with BCA protein assays. Thirty micrograms of each lysate sample was loaded and separated on 10% SDS-PAGE gels, and the gels were subjected to Coomassie blue staining with Brilliant Blue G (Sigma-Aldrich, St. Louis, MO). Each gel lane was cut into 9 slices. A total of 162 slices (3 biological replicates x 6 treatments x 9 slices) were in-gel digested [12] with sequencing-grade modified trypsin (Promega, Madison, WI). Peptides were extracted from the gel slices and desalted with Stage Tips (Thermo Scientific, Waltham, MA).

LC MS/MS data acquisition. Desalted peptides were analyzed with an EASY-nLC system (Thermo Fisher Scientific) coupled online to an LTQ-Orbitrap Elite (Thermo Scientific) at the PKU Institute of Systems Biomedicine (see the Supplemental Methods for details).

Raw data analysis. Raw mass spectrometry data were analyzed with MaxQuant software [13] version 1.5.3.30 and searched against the human UniProt database UP000005640 (http://www.uniprot.org) [14]. The false discovery rates (FDRs) for both proteins and peptides were 1%. The “match between runs” and “label-free quantification (LFQ) algorithm” features were also activated in the MaxQuant software to optimize the protein identification and quantification procedures (see the Supplemental Methods for details).

Bioinformatics analysis. The downstream bioinformatics analysis was performed with Perseus software version 1.5.2.6 [13], including Pearson's correlation coefficient, Welch's *t*-test, volcano plots, and Z-scored heat map. The LFQ intensities from the MaxQuant output were Log_2 transformed. At least twelve valid values from eighteen LFQ intensities (six treatments in triplicates) and at least one valid value from each treatment were used as criteria for quantified proteins. Welch's t-tests were performed with a permutation-based FDR of 0.05 for the quantified proteins. Welch's difference was defined as the difference between the medians among triplicates.

Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov) version 6.7 was used for the functional annotation and enrichment analysis of Gene Ontology Biological Processes (GOBP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [15]. P values < 0.05 were the cutoff criteria for enriched categories.

The protein-protein interaction (PPI) network was assessed online using String database (http://string-db.org) version 10.0 [16], and only interactions with a confidence score > 0.4 were selected as significant. Other statistical details are reported in each figure legend.

Real-time PCR. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). The cDNAs were prepared with AMV Reverse Transcription System (Promega, Madison, WI). The real-time PCR was performed with Brilliant SYBR Green QPCR system.

The primer sequences were:

HMOX1-L-(5′-CAGGGCCATGAACTTTGTCC-3′), HMOX1-R-(5′−CCCCTCTGAAGTTTAGGCCA-3′), GCLM-L-(5′-CAGTTGACATGGCCTGTTCAG-3′), GCLM-R-(5′-AGCAAATGCAGTCAAATCTGGT-3′), CAT-L-(5′-AGAAAGCGGTCAAGAACTTCAC-3′), CAT-R-(5′-TTTGCACATCTAGCACAGGAG-3′), β-Actin-L-(5′-ATCTGGCACCACACCTTC-3′), and β-Actin-R-(5′-AGCCAGGTCCAGACGCA-3′).

3. Results

3.1. Label-free quantitative proteomics analysis of the cytoprotective effects of EPA

We established an endothelial activation model using HUVECs. TNFα significantly increased the expression of vascular cell adhesion molecule 1 (VCAM1) (Fig. 1), thus indicating that the endothelial cells were successfully activated [1]. Then, the distinct functions of EPA and AA were verified using Western blotting, and the optimal concentration was determined to be 100 μM (Fig. 1). Pretreatment with EPA, but not AA, significantly inhibited TNFα-induced VCAM1 expression in a dose-dependent manner. Using AA as the control may exclude some false-positive findings in our results.

Please cite this article in press as: L. Zhang, et al., Quantitative proteomics reveals key proteins regulated by eicosapentaenoic acid in endothelial activation, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.04.091