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The influence of AICAR - direct activator of AMP-activated protein kinase (AMPK) - on liver proteome in apoE-knockout mice

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ABSTRACT

There is a growing body of evidence that altered functioning of apoE may aggravate cellular energy homeostasis and stress response, leading to oxidative stress, mitochondrial dysfunction, endoplasmic reticulum (ER) stress and inflammation, leading to hypercholesterolemia, dyslipidemia, liver steatosis and neurodegeneration. One of the key cellular responses to mitochondria and ER-stress related processes and cellular energy imbalance is AMP-activated protein kinase (AMPK), considered as a cellular master energy sensor and critical regulator of mitochondrial homeostasis.

The aim of our study was to use differential proteomics and transcriptomics approach to elucidate the effect of direct AMPK activator AICAR on liver proteome in apoE$^{-/-}$ mice – experimental model of atherosclerosis and moderate nonalcoholic steatosis. We applied Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) labeling and two-dimensional chromatography coupled with mass spectrometry (2DLC-MS/MS) MudPIT strategy, as well as RT-PCR to investigate the changes in mitochondrial and cytosolic proteins and transcripts expression in 6-month old AICAR-treated apoE$^{-/-}$.

AICAR elicited induction of proteins related to mitochondrial β-oxidation, protein degradation and energy producing pathways (i.a. tricarboxylic acid cycle members and mitochondrial adenylate kinase 2). On the other hand, AICAR repressed inflammatory and pro-apoptotic markers in the apoE$^{-/-}$ mice liver, alongside reduction in several peroxisomal proteins, possibly suggesting induction of anti-oxidative pexophagy.

1. Introduction

Apolipoprotein E (apoE) is a pivotal constituent of lipoproteins, which modulates plasma lipoprotein and cholesterol concentrations, i.a. by maintaining lipoprotein integrity and facilitating their solubilization in the blood (Eichner et al., 2002). Majority of apoE is synthesized by parenchymal cells in the liver, however among other sources of significant amounts of apoE are the brain, kidneys, adipocytes and macrophages (Getz and Reardon, 2009; Williams et al., 1985), where apoE predominantly governs lipoprotein metabolism, including reverse cholesterol transport (Zanotti et al., 2011). However, apoE has been demonstrated to possess multiple additional cellular functions beyond its role in lipid metabolism, since apoE knockout mice (apoE$^{-/-}$) not only spontaneously develop dyslipidemia and arterial lesions (Eichner et al., 2002; Jawien, 2012), accompanied by low-grade non-alcoholic fatty liver disease (NAFLD) (Bonomini et al., 2010;

Abbreviations: 2DLC-MS/MS, two-dimensional chromatography coupled with mass spectrometry; ACN, acetonitryle; ADP, Adenosine monophosphate; AICAR, [(2*R*,3*S*,4*R*,5*R*)-5-(4-Carbamoyl-5-aminoimidazol-1-yl)-3,4-dihydroxyoxolan-2-yl]methyl dihydrogen phosphate; AK2, adenyate kinase 2; AMP, Adenosine diphosphate; AMPK, AMP-activated protein kinase; ATP, Adenosine triphosphate; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1- propanesulfonate; ChREBP, carbohydrate response element-binding protein; COX-IV, cytochrome *c* oxidase subunit IV; DTT, dithiothreitol; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ER, endoplasmic reticulum; ETC, electron transport chain; FA, formic acid; GS, glutamine synthetase; HCD, Higher energy Collisional Dissociation; HRP, horseradish peroxidase; IRE1, inositol-requiring enzyme 1; iTRAQ, Isobaric Tags for Relative and Absolute Quantitation; K_{ATP}, ATP-sensitive K^+ channels; LC-MS, liquid chromatography – mass spectrometry; MAM, mitochondria-associated ER membranes; MS/MS, tandem mass spectrometry; mTOR, Mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; MudPIT, Multidimensional Protein Identification Technology; NAFLD, nonalcoholic fatty liver disease; NF-κB, Nuclear factor NF-kappa-B; PBS, Phosphate-buffered saline; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PI3K, phosphoinositide 3-kinase; PMSF, phenylmethanesulfonyl fluoride; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SCX, strong cation exchange; SDS, sodium dodecyl sulfate; SREBP, Sterol regulatory element-binding protein; TCA, tricarboxylic acid cycle; TFA, trifluoroacetic acid; TPP, Trans-Proteomic Pipeline; TTBS, Tris-Buffered Saline-Tween 20 buffer; UPR, unfolded protein response; VSMC, vascular smooth muscle cells

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Stachowicz et al., 2012; Suski et al., 2011) but also exhibit neurodegenerative changes (Robertson et al., 2000), insulin resistance (Moghadasian et al., 2001) and immune defects (de Bont et al., 1999; Roselaar and Daugherty, 1998).

It has been shown that impairment of apoE function may aggravate cellular stress responses, such as endoplasmic reticulum (ER) stress and mitochondrial dysfunction (Dose et al., 2016). Interestingly, both pathways have been reported to play important role in apoE$^{-/-}$ pathologies (Wang et al., 2013; Shinozaki et al., 2013; Wang et al., 2014; Tang et al., 2014; Xiong et al., 2016; Yu et al., 2013; Harrison et al., 2011).

One of the key cellular compensatory responses to mitochondrial dysfunction and ER-stress is activation of AMP-activated protein kinase (AMPK), considered as a cellular master energy sensor and critical regulator of mitochondrial homeostasis (Hardie et al., 2012). In general, regulation of cellular energy metabolism upon AMPK activation is based on the induction of pathways that generate ATP, and inhibition of the biosynthetic pathways that consume ATP. The former include induction of glucose uptake and glycolysis, fatty acid uptake and oxidation, as well as mitochondrial biogenesis and autophagy, while the latter comprise of inhibition of synthesis of proteins, fatty acids, triglycerides, glycogen, cholesterol as well as arresting the transcription of lipogenic and gluconeogenic enzymes (Hardie et al., 2012). Such activities predispose AMPK to be the potential core target in the treatment/prevention of NAFLD. Indeed, it was demonstrated that NAFLD induced by high-fat-diet is associated with the lower activity of AMPK in the liver (Park et al., 2008; Sinha-Hikim et al., 2011) and that metformin, widely known, indirect activator of AMPK has been shown to attenuate liver steatosis in animal models and patients with NAFLD (Stachowicz et al., 2012; Garinis et al., 2010), however it is still unclear, whether such action of metformin depend exclusively on AMPK activation. In this regard, use of selective, direct AMPK activator - [(2*R*,3*S*,4*R*,5*R*)-5-(4-Carbamoyl-5-aminoimidazol-1-yl)-3,4-dihydroxyoxolan-2-yl]methyl dihydrogen phosphate (AICAR) may provide more precise information about the role AMPK in liver steatosis.

The aim of our study was to elucidate the effect of AICAR on liver proteome of apoE$^{-/-}$ mice by use of the methods of differential proteomics and transcriptomics. We applied Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) labeling and two-dimensional chromatography coupled with mass spectrometry (2DLC-MS/MS) MudPIT strategy, as well as RT-PCR to investigate the changes in mitochondrial and cytosolic protein and transcript expression in 6-month old AICAR-treated apoE$^{-/-}$ mice as compared to non-treated transgenic animals.

2. Materials and methods

2.1. Animals

The scheme of the study design is depicted on Fig. 1. All animal procedures were approved by the Jagiellonian University Ethical Committee on Animal Experiments. Female apoE-knockout mice on the C57BL/6J background were obtained from Taconic (Ejby, Denmark). Mice were maintained on 12-h dark/12-h light cycles in air-conditioned rooms (22.5 ± 0.5 °C, 50 ± 5% humidity) and access to diet and water ad libitum in Animal House of Chair of Immunology of JUMC. At the age of 8 weeks mice were put on chow diet made by Ssniff (Soest, Germany). Two groups of animals were studied: control group (apoE$^{-/-}$ mice w/o treatment, on chow diet as above, n = 12) and AICAR-treated mice (n = 8). In this group, 4 mg of AICAR dissolved in saline was injected i.p. to mice three times a week. At the age of 6 months mice from both groups were killed under anesthesia 5 min after injection of fraxiparine (1000 UI, Sanofi-Synthelabo, France) into the peritoneum. Finally, the liver was dissected and subjected to subcellular fractionation. Part of the liver (approximately 100 mg of wet tissue) was preserved for triglycerides assessment.

2.2. Subcellular fractionation

Isolation of mitochondria was performed at 4 °C from freshly-harvested mouse liver. Homogenization was carried out in 250 mM sucrose, 1 mM EGTA, pH 7.8 with addition of PMSF (1 mM) and a mix of protease inhibitors (approximately 100 µl for 3 g of tissue) (Sigma, USA). Nuclei and unbroken cells were pulled down by centrifugation at 1000*g* for 10 min. Then, the mitochondrial fraction was obtained by centrifugation of the supernatant at 12,000*g* for 10 min. The mitochondrial pellet was then purified by 3 cycles of resuspension, homogenization and centrifugation (at 12,000*g* for 15, 20, 15 min). The cytosolic fraction was obtained by further centrifugation of the supernatant (90 min at 125,000*g*, 4 °C). Samples were collected and stored at − 80 °C until assayed.

2.3. Sample preparation

Protein lysis buffer contained 7 M urea, 2 M tiourea, 4% CHAPS, 1% DTT with and addition of protease inhibitors (Roche). Protein concentration of cytosolic and mitochondrial fractions was determined by Coomassie Plus™ Bradford protein assay (Thermo Scientific). 100 µg of protein content of each sample was precipitated overnight with ice-cold acetone (Sigma-Aldrich) (1:6 v:v). Samples were centrifuged at 6000 × *g* for 10 min at 4 °C. Acetone was carefully removed and precipitates were dissolved in 20 µl dissolution buffer with the addition of 1 µl denaturant solution, reduced with reducing agent for 1 h at 60 °C and finally alkylated with cysteine blocking reagent as recommended by iTRAQ protocol (ABSciex). Proteins were digested with Trypsin (Promega, Germany) overnight, with 1:50 (w:w) ratio, at 37 °C. Samples were labeled with iTRAQ reagents as follows: control apoE$^{-/-}$ mice - 113, 114, 115 AICAR-treated apoE$^{-/-}$ mice - 116, 117, 118. The same labeling procedure was applied to both cytosolic and mitochondrial fractions. Labeled samples within each cellular fraction were combined and dried in vacuum concentrator (Eppendorf, Germany). Next, peptides were dissolved in 5% acetonitrile (ACN), 0,1% formic acid (FA) and purified with C18 MacroSpin Columns (Harvard Apparatus). Eluate was dried in vacuum concentrator, reconstituted in 5% ACN, 0.1% FA, and subjected to 2DLC-MS analysis.

2.4. 2DLC-MS/MS

Each sample was injected on a Poros 10S SCX column (300 µm i.d. × 25 cm, Thermo Scientific Dionex) and peptide fractions were then eluted by consecutive 11 salt plugs injections (1 mM, 2 mM, 5 mM, 10 mM, 20 mM, 50 mM, 100 mM, 200 mM, 500 mM, 1000 mM and 2000 mM NaCl solutions, respectively) and concentrated on a trap column (Acclaim PepMap100 RP C18 75 µm i.d. × 2 cm column, Thermo Scientific Dionex). Each fraction was then injected on-line on PepMap100 RP C18 75 µm i.d. × 15 cm column (Thermo Scientific Dionex) and the peptides were separated in 55 min 7–55% B phase linear gradient (A phase – 2% ACN and 0.1% formic acid; B phase – 80% ACN and 0.1% formic acid) with a flow rate of 300 nl/min by UltiMate 3000 HPLC system (Thermo Scientific Dionex) and applied on-line to a Velos Pro (Thermo Scientific, USA) dual-pressure ion-trap mass spectrometer. The main working nanoelectrospray ion source (Nanospray Flex, Thermo Scientific) parameters were as follows: ion spray voltage 1.9 kV and capillary temperature 250 °C. Spectra were collected in full scan mode (400–1500 Da), followed by five Higher energy Collisional Dissociation (HCD) MS/MS scans of five most intense ions form the preceding survey full scan under dynamic exclusion criteria. Collected data were analyzed by the X!Tandem search algorithm (The GPM Organization) and statistically validated with PeptideProphet under the Trans-Proteomic Pipeline (TPP) suite of software (Institute for Systems Biology) (Deutsch et al., 2010). Search