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Short Communication

Effects of cold exposure on metabolites in brown adipose tissue of rats

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ABSTRACT

Brown adipose tissue (BAT) plays an important role in regulation of energy expenditure while adapting to a cold environment. BAT thermogenesis depends on uncoupling protein 1 (UCP1), which is expressed in the inner mitochondrial membranes of BAT. Gene expression profiles induced by cold exposure in BAT have been studied, but the metabolomic biological pathway that contributes to the activation of thermogenesis in BAT remains unclear. In this study, we comprehensively compared the relative levels of metabolites between the BAT of rats kept at room temperature (22 °C) and of those exposed to a cold temperature (4 °C) for 48 h using capillary electrophoresis (CE) time-of-flight mass spectrometry (TOFMS) and liquid chromatography (LC)-TOFMS. We identified 218 metabolites (137 cations and 81 anions) by CE-TOFMS and detected 81 metabolites (47 positive and 34 negative) by LC-TOFMS in BAT. We found that cold exposure highly influenced the BAT metabolome. We showed that the cold environment lead to lower levels of glycolysis and gluconeogenesis intermediates and higher levels of the tricarboxylic acid (TCA) cycle metabolites, fatty acids, and acyl-carnitine metabolites than control conditions in the BAT of rats. These results indicate that glycolysis and β-oxidation of fatty acids in BAT are positive biological pathways that contribute to the activation of thermogenesis by cold exposure, thereby facilitating the generation of heat by UCP1. These data provide useful information for understanding the basal metabolic functions of BAT thermogenesis in rats in response to cold exposure.

1. Introduction

Adipose tissue is a major metabolic organ and plays a key role in energy homeostasis. Based on cell morphology and tissue function, there are two types of adipose tissue, white and brown adipose tissue (WAT and BAT, respectively) in mammals. The physiological roles of these adipose tissues are different: WAT is highly adapted to store excess energy in the form of triglycerides, whereas BAT dissipates energy to produce heat by converting glucose and fatty acids to the resulting proton-motive force. BAT thermogenesis depends on uncoupling protein 1 (UCP1), which is specifically expressed in brown fat mitochondria and responsible for the unique metabolic function of BAT. UCP1 is known to dissipate the proton gradient across the inner mitochondrial membrane, thus uncoupling the electron transfer system from adenosine triphosphate (ATP) synthesis, which in turn causes the energy to be dissipated as heat [1–3]. Thus, BAT is a crucial tissue for regulation of energy expenditure for adaptation to a cold environment.

To understand the mechanisms of BAT thermogenesis, we previously reported the gene expression profiles involved in energy metabolism in BAT compared to WAT in rats [4–6]. The transcript levels of the proteins involved in the transport and catabolism of glucose and fatty acids in BAT were elevated in response to 48 h of exposure to 4 °C. Particularly, UCP1 was expressed only in BAT, and cold exposure elevated its transcript level [4]. In addition, we reported the transcript levels of various genes involved in the activation of thermogenesis in BAT through exposure to the cold using microarray analysis and revealed that cold exposure leads to transcriptional upregulation of thermogenic genes such as sarcomeric mitochondrial creatine kinase and myoglobin expressions in BAT [7]. Consistent with our reports, a previous study demonstrated that expression of UCP1 mRNA was increased in the BAT of mice in response to 24 h of exposure to the cold compared with room temperature controls [8]. Moreover, fatty acids are one of the major energy sources in the BAT of rats in response to cold exposure [1]. The intracellular fatty acid-binding proteins (FABPs)

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bind long-chain fatty acids and act as fatty acid transport proteins [9]. FABPs are known to exist in eight isoforms in mammals. We previously found that the transcript level of the heart-type fatty acid-binding protein 3 (FABP3) was elevated 100-fold in BAT of rats exposed to the cold compared to those kept at room temperature [4,10,11]. Although gene expression profiles are useful to understand the mechanisms underlying the activation of thermogenesis in BAT, the products of individual protein expression on several transduction pathways remains unclear.

Integration of omics data generated by the different high-throughput technologies including genomics, transcriptomics, proteomics, and metabolomics has widely expanded the understanding of the cellular mechanisms in the systems biology field. Metabolome analysis has been recognized as a useful approach to analyze chemical processes involving low molecular weight metabolites and helped to understand pathological conditions occurring in an organism [12]. Although gene expression profiles induced by exposure to the cold in BAT are well studied [4,7,11], metabolomic biological pathway contributions to the activation of thermogenesis in BAT has not been clearly elucidated. Recently, Lu et al. showed that acute cold exposure induced many significant metabolic changes in BAT and increased lipid metabolites such as diglyceride, monoglyceride, and fatty acid levels in the BAT of mice during the early phase of cold exposure (up to 6 h) [13]. They also demonstrated that glycolysis and pentose phosphate pathway metabolites had significant changes in gene expression in BAT after 4 h of cold exposure. Moreover, it was reported that loss of the mechanistic target of the rapamycin complex 1 (mTORC1) affected glucose, lipid, and oxidative metabolism in the BAT of mice following chronic cold exposure (2 weeks) using metabolomics [14]. However, little is known about the many metabolic statuses including those of glucose, fatty acid metabolism, and tricarboxylic acid (TCA) cycle involved in BAT thermogenesis of rats in response to cold exposure for 48 h compared with the gene expression profiles. Therefore, in this study, we utilized metabolomic approaches to focus on the comprehensive metabolic changes between the BAT of rats kept at room temperature (22 °C) and those exposed to a cold temperature (4 °C) for 48 h using capillary electrophoresis (CE) time-of-flight mass spectrometry (TOFMS) and liquid chromatography (LC)-TOFMS.

2. Materials and methods

2.1. Animals and tissue sampling

Specific pathogen-free male Wistar rats (5 weeks) from Japan SLC Inc. (Hamamatsu, Japan) were used according to ethical guidelines for Animal Experiments of Tokushima University with approval of the Institutional Animal Care and Use Committee of Tokushima University (Approval number: T28–29). In this experiment, six rats were randomly assigned to two subgroups ($n = 3$ in each group) as follows: (1) rats kept at room temperature (22 °C) for 48 h; (2) rats kept at 4 °C for 48 h. Prior to the experiment, rats were housed in groups at room temperature for one week under a 12-h light/dark cycle and provided a normal diet and water ad libitum. All rats were euthanized by cervical dislocation. An interscapular BAT was isolated from three rats in each group, and then the samples were combined to avoid differences between individuals. BAT samples were stored at −80 °C until metabolome analysis.

2.2. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from BAT using an RNeasy Lipid Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized from 500 ng total RNA using PrimeScript RT Master Mix (Perfect Real Time, TaKaRa Bio, Otsu, Japan). Quantitative real-time PCR was performed using StepOne Plus (Applied Biosystems, Foster City, CA, USA). Template cDNA (5 ng/μl) was mixed with Fast SYBR® Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), distilled water, and primers (final concentration 500 nM). The reaction was performed at 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. The following primer sets were used: UCP1 forward, 5′-GTACCCAGCTGTGC AATGAC-3′, and UCP1 reverse, 5′-GATGACGTTCCAGGATCCGA-3′; FABP3 forward, 5′-ACCAAGCCGACCACAATCAT-3′, and FABP3 reverse, 5′-TCCCACTTCTGCACATGGAC-3′; RPLP0 forward, 5′-ATTGGC TACCCGACTGTTGC-3′, and RPLP0 reverse, 5′-CCGCAAATGCAGATGG ATCG-3′. The relative mRNA levels of the various genes were normalized to that of ribosomal protein lateral stalk subunit P0 (RPLP0) mRNA as an internal control.

2.3. Sample preparation

Metabolite extraction and metabolome analysis were conducted at Human Metabolome Technologies Inc. (HMT, Tsuruoka, Yamagata, Japan) as follows. For CE-TOFMS analysis, approximately 200 mg of each frozen BAT sample was plunged into 6 ml of 50% acetonitrile/Milli-Q water containing 10 μM of an internal standard solution (H3304-1002, HMT) at 0 °C. The tissue was homogenized using a homogenizer (Micro Smash MS100R, Tomy Digital Biology Co., Ltd., Tokyo, Japan) at 1500 rpm for 120 s 5 times on ice and then the homogenate was centrifuged at 2300 ×*g* at 4 °C for 5 min. After that, 800 μl of the upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter (UltrafreeMC-PLHCC, HMT) at 9100 ×*g* at 4 °C for 120 min to remove macromolecules. The filtrate was centrifugally concentrated and re-suspended in 50 μl Milli-Q water for CE-TOFMS analysis at HMT.

For LC-TOFMS analysis, approximately 50 mg of each frozen BAT sample was plunged into 500 μl of 1% formic acid/acetonitrile containing 20 μM of an internal standard solution (H3304–1002, HMT) at 0 °C. The tissue was homogenized using a homogenizer (Micro Smash MS100R) at 1500 rpm for 120 s 3 times on ice. The mixture was homogenized again after adding 167 μl Milli-Q water, and then the homogenate was centrifuged at 2300 ×*g* at 4 °C for 5 min. The supernatant was then mixed with 500 μl of 1% formic acid/acetonitrile and 167 μl Milli-Q water, and the solution was centrifugally filtered through 3-kDa cutoff filter (NANOCEP 3 K OMEGA, PALL Corporation, Michigan, USA) at 9100 ×*g* at 4 °C for 120 min to remove macromolecules, and then phospholipids were removed using a Hybrid SPE phospholipid cartridge (55261-U, Supelco, Bellefonte, PA, USA). The filtrate was desiccated and then re-suspended in 100 μl of 50% isopropanol/Milli-Q water for LC-TOFMS analysis at HMT.

2.4. CE-TOFMS measurements

Metabolome analysis was conducted using a *Dual Scan* package at HMT using CE-TOFMS and LC-TOFMS for ionic and non-ionic metabolites, respectively, based on previously described methods [15,16]. The CE-TOFMS analysis were carried out using an Agilent CE system (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent 6210 TOFMS (Agilent Technologies) at a service facility at HMT. Cationic metabolites were analyzed with a fused silica capillary (50 μm internal diameter × 80 cm total length) with cationic electrophoresis buffer (H3301-1001, HMT) as the electrolyte. The sample solution was injected at a pressure of 50 mbar for 10 s. The applied voltage was set at 27 kV. Electrospray ionization-mass spectrometry (ESI-MS) was conducted in the positive-ion mode and the capillary voltages were set at 4000 V. The spectrometer was scanned from m/z 50 to 1000. Anionic metabolites were analyzed with a fused silica capillary (50 μm × 80 cm) with anionic electrophoresis buffer (H3302-1021, HMT) as the electrolyte. The sample solution was injected at a pressure of 50 mbar for 25 s. The applied voltage was set at 30 kV. ESI-MS was conducted in the negative-ion mode and the capillary voltages were set at 3500 V. The spectrometer was scanned from m/z 50 to 1000.