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Interscapular brown adipose tissue metabolic reprogramming during cold acclimation: Interplay of HIF-1α and AMPKα

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ABSTRACT

Background: Brown adipose tissue thermogenic program includes complex molecular and structural changes. However, energetic aspects of this process are poorly depicted.
Methods: We investigated time-dependent reprogramming of interscapular brown adipose tissue (IBAT) energy metabolism during cold-acclimation, as well as the effects of nitric oxide (•NO) on those changes. Rats were exposed to cold (4 ± 1 °C) for periods of 1, 3, 7, 12, 21, and 45 days, and divided into three groups: control, treated with L-arginine, and treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME).
Results: In the early phase of cold-acclimation (up to 7 days), the protein levels of all metabolic parameters and oxidative phosphorylation components were below the control. However, metabolic parameters and respiratory chain components entered a new homeostatic level in the late phase of cold-acclimation. These changes were accompanied with increased protein levels of phospho-AMP-dependent protein kinase-α (phospho-AMPKα) on the first day of cold-acclimation, and hypoxia-inducible factor-1α (HIF-1α) throughout early cold-acclimation. L-arginine positively affected protein expression of enzymes involved in glucose metabolism and β-oxidation of fatty acids in the early phase of cold-acclimation, and oxidative phosphorylation components throughout cold-acclimation. In contrast, L-NAME had the opposite effects.
Conclusion: Results suggest that IBAT structural remodeling is followed by energy metabolism reprogramming, which control might be orchestrated by the action of AMPKα and HIF-1α. Data also indicated the involvement of L-arginine-•NO in the regulation of IBAT metabolism.
General significance: Results obtained in this study might be of great importance for elucidating regulatory pathways governing energy metabolism in both physiological and pathophysiological states.

1. Introduction

Brown adipose tissue (BAT) is a highly specialized organ responsible for nonshivering thermogenesis, which enables endothermic animals to cope with low temperature conditions [1,2]. Recent studies showed that adult humans also possess active BAT [3–7]. This finding renewed global scientific interest in BAT, because of its capacity to regulate energy metabolism and potentially curb obesity or related disorders, such as metabolic syndrome and type 2 diabetes [8]. The release of norepinephrine during cold exposure triggers BAT activation and a thermogenic program consisting of complex tissue structure changes, *i.e.*, hyperplasia and hypertrophy of mature brown adipocytes, vascularization, mitochondriogenesis and remodeling of mitochondria, peroxisomal biogenesis, protein and DNA synthesis, and decreased rate of apoptosis [9–20]. All changes are directed to achieving the complete BAT thermogenic functionality by converting dormant tissue into an active thermogenic organ.

The high energetic cost of structural and functional remodeling means that these changes have to be followed by reprogramming at the level of BAT metabolism and energy production. As a consequence, the utilization and availability of the two major energy sources used by BAT, glucose and fatty acids, varies during cold-acclimation. Numerous studies have unambiguously shown the high activity of glycolytic and β-oxidation enzymes during the late acclimation phase, *i.e.*, prolonged cold-exposure [21–24], but the initial phase is poorly studied, as well

Abbreviations: BAT, brown adipose tissue; IBAT, interscapular BAT; L-NAME, N^{ω}-nitro-L-arginine methyl ester; phospho-AMPKα, phosphorylated AMP-activated protein kinase α; HIF-1α, hypoxia-inducible factor-1α; COX IV, subunit IV of cytochrome *c* oxidase; PDH, pyruvate dehydrogenase; SCAS, succinyl-CoA synthetase; ACADM, medium chain fatty acids acyl-CoA dehydrogenase; FAS, fatty acids synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PGC-1α, peroxisome proliferation-activated receptor-γ coactovator-1α; NOS, nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; PAS reagent, Period-Acid-Shiff reagent; TEM, transmission electron microscopy

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as a regulation of major metabolic parameters both in the early and late phase of cold-acclimation.

AMP-activated protein kinase (AMPK) and hypoxia-inducible factor-1 (HIF-1) are evolutionarily highly conserved sensors of cellular energy states, which function as the major drivers and regulators of orchestrated metabolism reprogramming. AMPK was viewed primarily as a sensor of acute intracellular energetic depletion, but its broader function in the long-term regulation of whole-body energy balance is now recognized [25–27]. In BAT, cold induces elevation of only α1 AMPK activity by a non-sympathetic mechanism [28–32]. A similar mechanism was reported in white adipose tissue, but not in other mitochondria-rich tissues such as the heart [29,30,33], which suggests a tissue-specific regulatory mechanism. Mulligan et al. [32] proposed a role for AMPK in coordinating the long-term control of thermogenic potential in BAT. HIF-1 was initially studied, because of its critical role in the tumor progression lifeline processes of angiogenesis and proliferation [34–36], and as a master switch from oxidative phosphorylation to glycolytic ATP production in hypoxic conditions. HIF-1 is also involved in regulation of metabolism in various tissues. For example, HIF-1 promotes free fatty acid uptake and triglyceride production in white adipose tissue and the liver [37]. Cold activates HIF-1 in BAT indicating its involvement in the cold-stimulus response [38], but its exact role in this tissue remains speculative. The unique ability of BAT to dissipate calories as heat via uncoupling, and its involvement in whole-body energy homeostasis, means that understanding the roles of AMPKα and HIF-1 in the regulation of BAT metabolism remodeling merits special attention.

All the aforementioned cold-induced BAT structural changes are regulated by nitric oxide (•NO) [17–20], but except for very few studies that have examined the influence of •NO on lipid and glucose metabolism [39], and •NO-dependent inhibition of some components of the electron transport chain [40], it is so far unknown whether •NO modulates energy metabolism in BAT during cold-acclimation.

We performed a comprehensive time-course study to elucidate phase-dependent changes of interscapular BAT (IBAT) energy metabolism during cold-acclimation, by investigating the protein expression profiles of key enzymes involved in glucose metabolism, metabolism of lipids, Krebs cycle, and components of the electron transport chain, ATP synthase, and transcriptional factors that may govern regulation of energy metabolism in BAT (AMPKα and HIF-1α). A particular emphasis of this study was to investigate the effects of the modulation of •NO production on BAT metabolism reprogramming, during the early and late phase of low temperature acclimation. To do this, cold-acclimated rats were chronically treated with L-arginine, as a substrate for •NO synthases (NOSs), and with N^{ω}-nitro-L-arginine methyl ester (L-NAME), as an inhibitor of its synthesis.

2. Material and methods

2.1. Animals

The experimental protocol was approved by the ethical committee for the treatment of experimental animals of the Institute for Biological Research, Belgrade, Serbia. Mill Hill hybrid hooded, 4-month-old male rats were divided into two groups: a control group kept at room temperature (22 ± 1 °C) for the duration of the experiment, and a group maintained in the cold (4 ± 1 °C). The cold-acclimated group was divided into three subgroups: 1, untreated; 2, L-arginine-treated; 3, L-NAME-treated. Drugs were administered in drinking water, as 2.25% L-arginine·HCl or 0.01% L-NAME·HCl, as described previously [41,17,18]. Rats were housed in individual plastic cages with drinking water and food *ad libitum*. The duration of cold-exposure ranged from 1 to 45 days (1, 3, 7, 12, 21, or 45 days), with six animals per experimental group. At the end of the cold exposure period, animals were sacrificed by decapitation and the IBAT was rapidly harvested on ice and halved. Small portions of IBAT were sampled from three animals per group and used for electron and light microscope examination. Tissue samples were homogenized, using a Janke and Kunkel Ka/Werke Ultra/Turrax homogenizer, for lactate dehydrogenase (LDH) activity determination at 0–4 °C in 0.25 M sucrose, 0.1 mM EDTA and 50 mM Tris buffer, at pH 7.4 and sonicated.

2.2. SDS-PAGE and western blotting

Western blots were conducted as described previously [17,20], using antibodies against: *Ndufa9* subunit of complex I (ab55521; 2.5 μg ml^{-1}), cytochrome *c* (ab18738; 2 μg ml^{-1}), complex III (ab14745; 0.5 μg ml^{-1}), subunit IV of cytochrome *c* oxidase (COX IV; ab14744; 0.1 μg ml^{-1}), ATP synthase (ab14730; 0.8 μg ml^{-1}), hexokinase II (ab78259; 1:5000), E1 beta subunit of pyruvate dehydrogenase (PDH; ab84588; 1 μg ml^{-1}), succinyl-CoA synthetase (SCAS; ab96172; 1:1000), medium chain fatty acids acyl-CoA dehydrogenase (ACADM; ab92461; 1:10,000), hypoxia-inducible factor-1α (HIF-1α; ab51608; 1:2000), beta actin (ab8226; 1:1000) (all purchased from ABCAM); phosphorylated AMP-activated protein kinase α (phospho-AMPKα; Millipore - Upstate, Chemicon International; # 07–681; 2 μg ml^{-1}), fatty acids synthase (FAS; Cell Signaling Technology #3180; 1:1000), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology sc-20357; 1:1000). Quantitative analysis of immunoreactive bands was conducted with ImageQuant software. Volume was the sum of all the pixel intensities within a band, *i.e.*, 1 pixel = 0.007744 mm^2. We averaged the ratio of dots per band for the target protein and actin in corresponding time-periods, from three similar independent experiments, and expressed them relative to the room temperature acclimated control, which was standardized as 100%. Data were then statistically analyzed.

2.3. LDH activity

LDH activity was tested [42] and enzymatic activity was expressed as U mg^{-1} protein. The reaction velocity was determined by the decrease in absorbance at 340 nm, which resulted from the oxidation of NADH. One unit caused the oxidation of 1 μmol of NADH per minute at 25 °C at pH 7.4, under the specified conditions.

2.4. Electron microscopy

IBAT samples were cut into small pieces immediately after removal and fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2), then postfixed in 2% osmium tetroxide using the same buffer, and dehydrated with serial alcohol solutions of increasing concentration before embedding in Araldite. Blocks were trimmed and cut with diamond knife (Diatome) on a UC6 ultramicrotome (Leica). Thin sections were contrasted using uranyl acetate and lead citrate (Leica EM AC20), and visualized with a Philips CM12 electron microscope (Philips/FEI) equipped with a Megaview III digital camera (Soft Imaging System).

2.5. PAS staining

Paraffin sections (4–6 μm thick) of IBAT were dewaxed, rehydrated, and stained with Period-Acid-Shiff (PAS) reagent to detect glycogen deposits, and counter-stained with hematoxylin. Duplicate sections served as negative controls, which were pretreated with diastase (Sigma Chemical Co.) before PAS staining to digest glycogen to glucose. Digital images were captured with a DMBL Leica light microscope (Leica Microsystem).