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RESEARCH ARTICLE

Regulatory role of PGC-1α/PPAR signaling in skeletal muscle metabolic recruitment during cold acclimation

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SUMMARY

This study examined the molecular basis of energy-related regulatory mechanisms underlying metabolic recruitment of skeletal muscle during cold acclimation and possible involvement of the L-arginine/nitric oxide-producing pathway. Rats exposed to cold (4±1°C) for periods of 1, 3, 7, 12, 21 and 45 days were divided into three groups: untreated, L-arginine treated and N^{ω}-nitro-L-arginine methyl ester (L-NAME) treated. Compared with controls (22±1°C), there was an initial increase in the protein level of 5′-AMP-activated protein kinase α (day 1), followed by an increase in peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and peroxisome proliferator-activated receptors (PPARs): PPARα and PPARγ from day 1 and PPARδ from day 7 of cold acclimation. Activation of the PGC-1α/PPAR transcription program was accompanied by increased protein expression of the key metabolic enzymes in β-oxidation, the tricarboxylic acid cycle and oxidative phosphorylation, with the exceptions in complex I (no changes) and ATP synthase (decreased at day 1). Cold did not affect hexokinase and GAPDH protein levels, but increased lactate dehydrogenase activity compared with controls (1–45 days). L-arginine sustained, accelerated and/or intensified cold-induced molecular remodeling throughout cold acclimation. L-NAME exerted phase-dependent effects: similar to L-arginine in early cold acclimation and opposite after prolonged cold exposure (from day 21). It seems that upregulation of the PGC-1α/PPAR transcription program early during cold acclimation triggers the molecular recruitment of skeletal muscle underlying the shift to more oxidative metabolism during prolonged cold acclimation. Our results suggest that nitric oxide has a role in maintaining the skeletal muscle oxidative phenotype in late cold acclimation but question its role early in cold acclimation.

Key words: cold acclimation, skeletal muscle, transcription control, metabolism.

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INTRODUCTION

Skeletal muscle is recognized as an important player in the tissue triad, including brown and white adipose tissues, which plays an important role in maintaining energy homeostasis and body temperature in homeothermic animals. Shivering thermogenesis in skeletal muscle and non-shivering thermogenesis in brown adipose tissue are the major forms of thermogenesis in mammals (Heldmaier et al., 1989). White adipose tissue supplies skeletal muscle and brown adipose tissue with energy fuel and provides hormonal control of their thermogenic functions. Our previous studies showed that structural and metabolic remodeling of brown (Petrović et al., 2010; Vucetic et al., 2011) and white (Jankovic et al., 2013) adipose tissues are phase dependent, reflecting their functional recruitment during 45 days of cold acclimation. In contrast to the clear role of skeletal muscle shivering thermogenesis in thermoregulation early in cold acclimation, its role during prolonged cold exposure, when non-shivering is activated, is not clear. Recent data showed that sustained cold exposure in animals with brown adipose tissue causes metabolic and structural changes in skeletal muscle that indicate their shivering activity (Meyer et al., 2010; Mineo et al., 2012), similar to those observed following endurance exercise training (Schaeffer et al., 2003). It is known that shivering-related metabolic recruitment, in terms of fuel selection, can be achieved in three ways: (1) by recruitment of specific subpopulations of fibers within the same muscle; (2) by recruitment of muscles varying in fiber composition; and (3) by recruitment of different metabolic pathways within the same fibers.

Tight regulation of metabolic pathways involves the rapid modulation of the activity of specific proteins (enzymes, transporters), but also, on a long-term basis, changes in their quantity. This can be achieved by modulating their transcription rate through the action of specific transcription factors. The discovery of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors and their co-activator [peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α)] revealed the mechanism of the strong link between lipid/glucose availability and long-term metabolic adaptation. Three PPAR isoforms (PPARα, PPARγ and PPARδ) have been identified to date (Mukherjee et al., 1997). The most abundant isoform in skeletal muscle is PPARδ. It plays a role in the regulation of glucose and lipid uptake, handling and oxidation (Holst et al., 2003). PPARα and PPARγ are less abundant in skeletal muscle under basal conditions, and may be alternatively implicated in the regulation of the same target genes in various physiological conditions, changing the homeostasis of metabolic substrates (exercise, fasting, etc.) (Tunstall et al., 2002). However,

data concerning their role in skeletal muscle during shivering and especially during sustained cold exposure are unclear.

In addition to exercise and thermogenesis, metabolic recruitment of skeletal muscle can be induced by some nutriceutical agents, including L-arginine. In skeletal muscle, L-arginine regulates glucose uptake and oxidation, fatty acid oxidation (Jobgen et al., 2006), mitochondriogenesis (Puigserver et al., 1998), contractile function (Joneschild et al., 1999; Maréchal and Gailly, 1999) and blood flow (Brevetti et al., 2003). L-arginine is the precursor for nitric oxide (NO) synthesis. As such, supplementation of L-arginine is a widely used approach to examine the metabolic effects of NO. Such studies are usually complemented with the use of N^{ω}-nitro-L-arginine-methyl ester (L-NAME), an inhibitor of nitric oxide synthases (NOSs), in order to confirm the NO dependency of L-arginine effects.

In the present study, we extended our research with the aim of characterizing key bioenergetic pathways and their regulation in skeletal muscle during cold acclimation. To this end, the expression profile of PPAR isoforms and PGC-1α, along with the key enzymes in glucose and lipid metabolism and ATP production, were examined. To complete the picture regarding the role of the L-arginine–NO pathway in the regulation of oxidative metabolism during cold acclimation, rats were treated with the NO-manipulating agents L-arginine and L-NAME.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Ethical Committee for the treatment of experimental animals of the Institute for Biological Research, Belgrade, Serbia (certificate number: 05-09). The total number of 114 Mill Hill hybrid hooded, 4-month-old male rats [*Rattus norvegicus* (Berkenhout 1769)] 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 and (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 (Saha et al., 1996; Petrović et al., 2005; Petrović et al., 2008). 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. Body mass and food and fluid intake were recorded daily for each animal. The obtained data were reported previously (Petrović et al., 2008).

At the end of the cold exposure period, animals were killed by decapitation and the gastrocnemius was dissected and rinsed with physiological saline to wash out traces of blood. 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 mol l^{-1} sucrose, 0.1 mmol l^{-1} EDTA and 50 mmol l^{-1} Tris buffer, at pH 7.4, and sonicated.

SDS-PAGE and western blotting

Western blots were conducted as described previously (Petrović et al., 2010) using antibodies against: the *Ndufa9* subunit of complex I (2.5 μg ml^{-1}), cytochrome *c* (2 μg ml^{-1}), ATP synthase (0.8 μg ml^{-1}), hexokinase II (1:5000), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:500), medium chain fatty acids acyl-CoA dehydrogenase (ACADM; 1:10,000), succinyl-CoA synthetase (SCAS, 1:1000), β-actin (1:1000) (all purchased from Abcam, Cambridge, UK), phospho 5′-AMP-activated protein kinase α (AMPKα; 2 μg ml^{-1}) (Millipore International, Billerica, MA, USA) and glycogen phosphorylase (PYGB; 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Quantitative analysis of immunoreactive bands was conducted with ImageQuant software (Uppsala, Sweden). 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.

LDH activity

LDH activity was tested (Borgmann et al., 1974) 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.

Additional assays and statistical analysis

Protein content was estimated using bovine serum albumin as a reference (Lowry et al., 1951). ANOVA was used to test within-group comparisons. If the *F*-test indicated an overall difference, Tukey's test was applied to evaluate the significance of the differences. Statistical significance was set at $P<0.05$.

RESULTS

Expression patterns of PGC-1α and PPAR isoforms

As shown in Fig. 1, compared with the control, cold induced an increase in the protein level of PGC-1α from day 3 (Fig. 1A), PPARδ from day 7 (Fig. 1B) and both PPARα (Fig. 1C) and PPARγ (Fig. 1D) at all examined time points (from day 1 to day 45). L-arginine treatment accelerated and intensified the increase in PGC-1α protein levels, thus the protein level of this transcriptional coactivator was higher than the control throughout cold acclimation and higher than the untreated group at days 1, 3, 7 and 45. L-arginine also increased the protein level of PPARα during cold acclimation throughout the entire 45-day period. PPARγ was increased compared with the untreated groups at days 12, 21 and 45 during cold acclimation. In contrast to L-arginine, the effects of L-NAME were different in the early and late periods of cold acclimation. On day 1, the protein levels of PPARs were higher in the L-NAME-treated group than in the control and untreated groups. However, L-NAME subsequently induced a decrease in the expression of PGC-1α and PPARs compared with untreated groups: PGC-1α and PPARδ at days 21 and 45 and PPARγ at day 45. PPARα protein levels were lower than those observed in the control and untreated groups starting from day 12 to the end of cold acclimation (day 45).

Changes in the expression of the components of the electron transport chain and ATP synthesis

In contrast to complex I, which showed no changes in expression during cold acclimation, cytochrome *c* protein content was increased compared with the control throughout the period of cold acclimation, whereas after an slightly initial decrease in the protein level of ATP synthase at day 1, an increase from day 3 to day 21 was observed (Fig. 2). In the L-arginine-treated group, the protein expression of complex I and cytochrome *c* was significantly increased compared with the control and untreated animals throughout the entire cold acclimation period. The L-arginine treatment increased protein content of ATP synthase during the whole cold-acclimation period compared with the control, and also

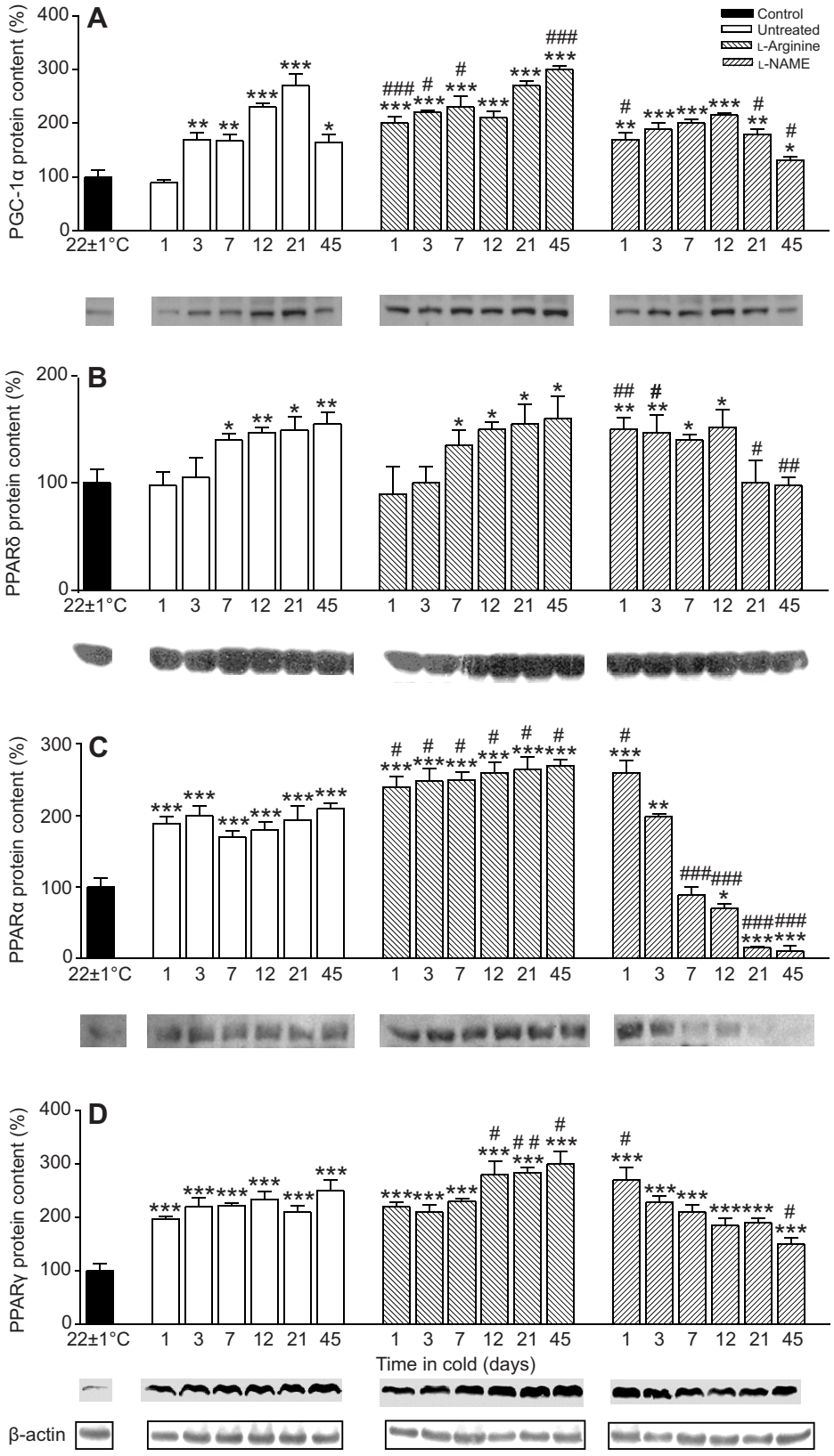

Fig. 1. Changes in the expression of PGC-1α (A), PPARδ (B), PPARα (C) and PPARγ (D) in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated rats during cold acclimation. Data showing protein levels, expressed relative to a control acclimated to room temperature (taken as 100%), represent the means ± s.e.m. of three independent experiments. *Compared with control: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{\#}$compared with untreated rats during the same period of cold acclimation: $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$.

at days 1 and 45 compared with untreated groups. In contrast to the similar trend in L-arginine action in all the examined components of the electron transport chain and ATP synthesis, the effects of L-NAME showed specificity related to the molecule. Complex I protein content was significantly decreased compared with the control and untreated groups during the acclimation period after L-NAME treatment. The effects of L-NAME on cytochrome *c* and ATP synthase showed phase specificity. Initially during cold acclimation, the effects of L-NAME were similar to those of L-arginine: cytochrome *c* protein level (from day 1 to day 21) was

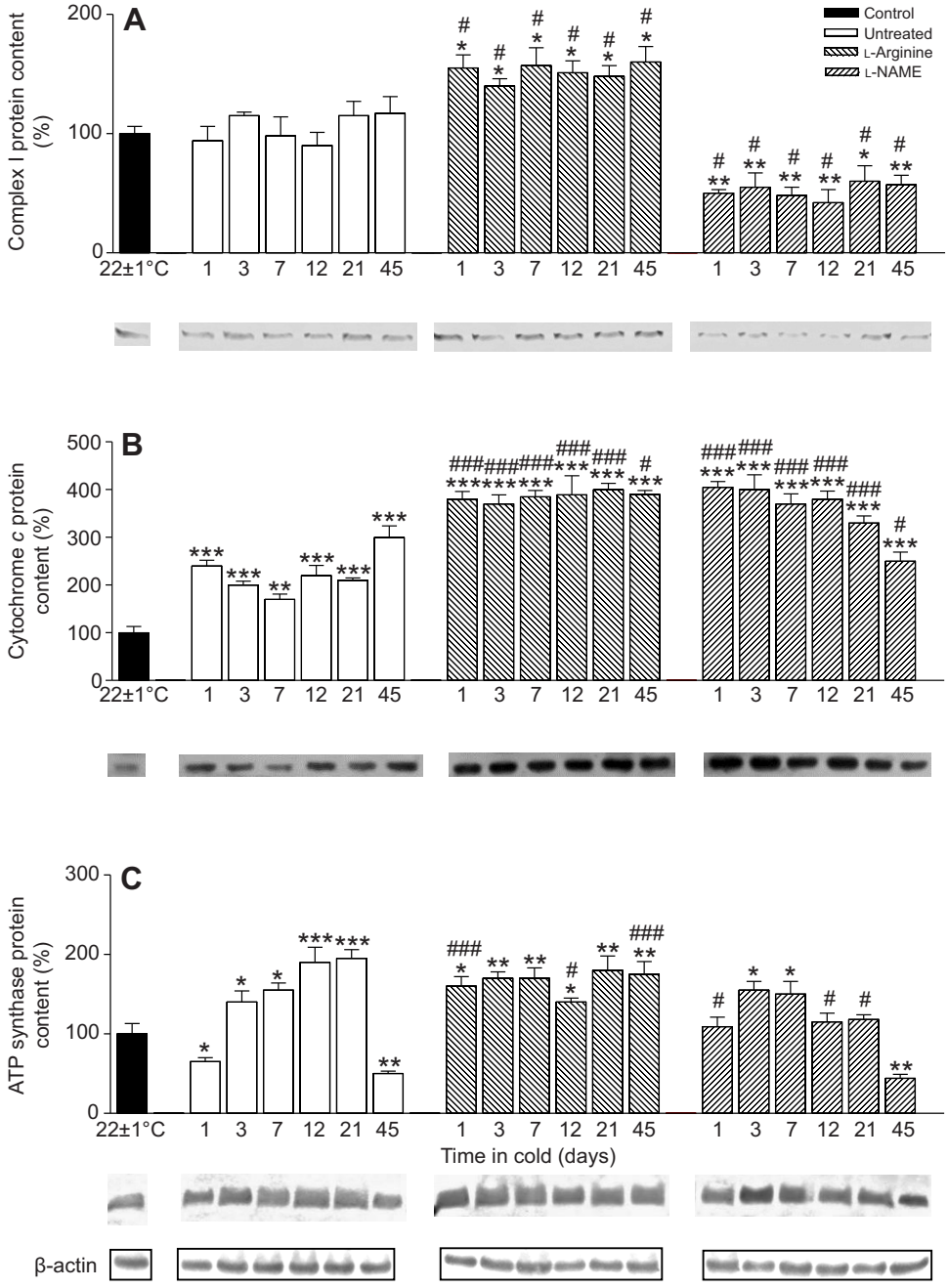

Fig. 2. Time course of changes in the expression of complex I (A), cytochrome *c* (B) and ATP synthase (C) in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated rats during cold acclimation. Protein content is expressed relative to a control acclimated to room temperature, which was standardized as 100%. The results of a representative example from three observations are shown. Values represent means ± s.e.m. *Compared with control: **P*<0.05; ***P*<0.01; ****P*<0.001; #compared with untreated rats during the same period of cold acclimation: #*P*<0.05; ###*P*<0.001.

higher than that observed in the control and untreated groups, and ATP synthase protein expression was restored to the control level at day 1. However, the protein level of cytochrome *c* was subsequently (day 45) lower in L-NAME-treated animals compared with untreated animals, while the expression of ATP synthase decreased compared with both untreated animals (days 12 and 21) and the control (day 45).

Changes in enzymes involved in glucose metabolism

Fig. 3 summarizes the results of the protein expression patterns and the activity of the enzymes involved in glucose metabolism. It can be seen that GAPDH protein level (Fig. 3A) was not affected by either cold, L-arginine or L-NAME treatment. Similarly, hexokinase II protein level (Fig. 3B) was unchanged during cold acclimation, while PYGB protein content (Fig. 3C) was decreased compared to the control during early cold acclimation (days 1 and 3). L-arginine treatment restored the cold-induced decrease in protein level of PYGB during early cold acclimation and its expression was significantly higher than that in the control and untreated animals throughout the entire 45-day cold acclimation period. In contrast, the protein level of hexokinase II was upregulated after only 1 day of L-arginine treatment during cold acclimation. Acting in a manner similar to that of L-arginine, L-NAME initially induced an increase in PYGB protein content compared with untreated animals from day 1 to day 12 of cold acclimation. In contrast, hexokinase II protein levels were lower in L-NAME-treated rats compared with the control and untreated animals during early cold acclimation (days 1 and 3).

Cold acclimation induced an increase in LDH activity compared with the control (except at the 12-day time point)

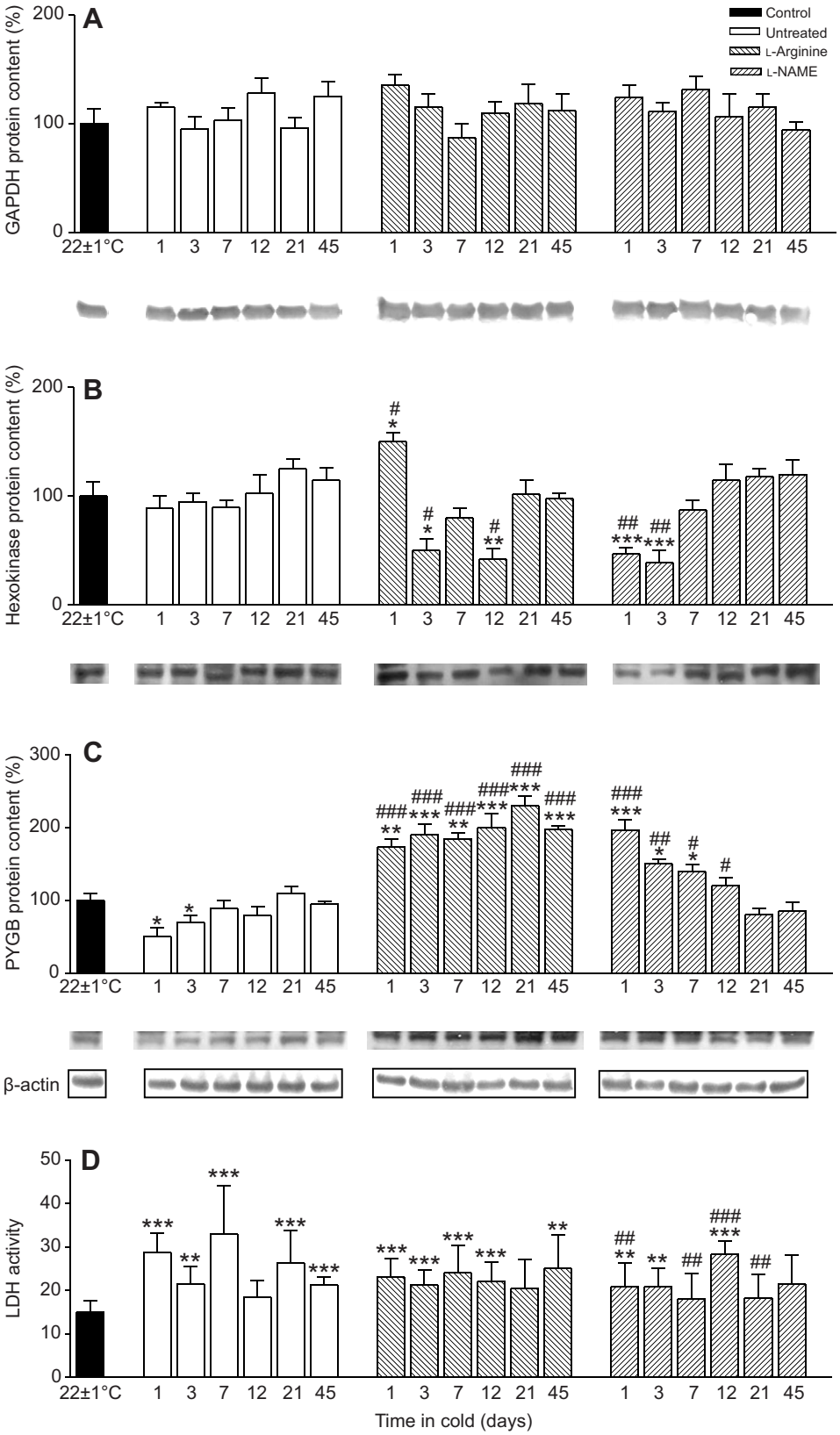

Fig. 3. Time-dependent changes in the protein expression of GAPDH (A), hexokinase II (B) and PYGB (C) and activity of LDH (D) in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated rats during cold acclimation. The results of the representative experiment and densitometric analysis are shown. The obtained data for protein content are expressed as a percentage of the control (taken as 100%) and represent the means ± s.e.m. of three independent experiments. *Compared with control: *P<0.05; **P<0.01; ***P<0.001; #compared with untreated rats during the same period of cold acclimation: #P<0.05; ##P<0.01; ###P<0.001.

(Fig. 3D). L-arginine treatment did not affect cold-induced changes in LDH activity; however, L-NAME treatment diminished the effects of cold acclimation on days 1, 7 and 21, i.e. at these time points LDH activity was lower than that in untreated cold-acclimated animals.

Expression patterns of enzymes involved in lipid metabolism and the Krebs cycle

It can be seen from Fig. 4 that ACADM protein content was increased at day 3, 7, 21 and 45 of cold acclimation (Fig. 4A), while the protein level of SCAS was higher than that in the control

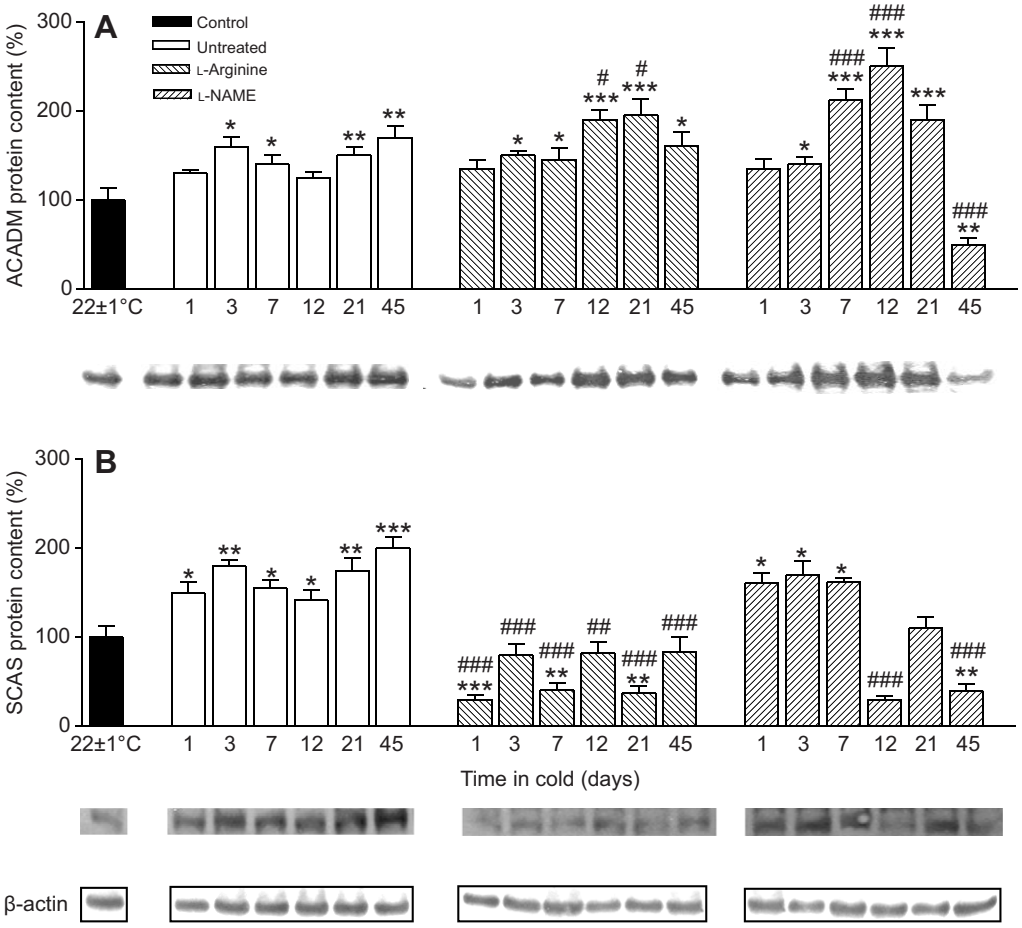

Fig. 4. Expression profiles of ACADM (A) and SCAS (B) in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated rats during cold acclimation. Data showing protein levels, expressed relative to a control acclimated to room temperature (taken as 100%), represent the means ± s.e.m. of three independent experiments. *Compared with control: *P<0.05; **P<0.01; ***P<0.001; #compared with untreated rats during the same period of cold acclimation: #P<0.05; ###P<0.001.

throughout the entire cold acclimation period (Fig. 4B). L-arginine treatment sustained the cold-induced increase in ACADM protein level starting from day 3 of cold acclimation and intensified the cold effects at days 12 and 21. However, L-arginine reduced the effect of cold on SCAS protein expression, decreasing the level to below that of the control. The expression pattern of ACADM in the L-NAME-treated groups was similar to that observed in the L-arginine-treated groups up to day 21 of cold acclimation; however, 45 days of L-NAME treatment induced a significant decrease in ACADM protein levels compared with the control and untreated groups. L-NAME sustained the cold-induced increase in the protein level of SCAS from day 1 to day 7 of cold acclimation, but induced a significant decrease in the SCAS protein level during late cold acclimation (12 and 45 days).

Expression profile of phospho-AMPKα

The protein content of phospho-AMPKα showed phase-dependent changes during cold acclimation (Fig. 5). Compared with the control, during early cold acclimation (1 and 3 days) phospho-AMPKα protein expression was significantly increased, while during late cold acclimation (12 and 45 days) the level was below that of the control. L-arginine induced an additional increase in phospho-AMPKα protein levels throughout the entire cold acclimation period. The effect of L-NAME was similar to the effect of L-arginine during early cold acclimation (1–12 days), but had the opposite effect during late cold acclimation (days 21 and 45), where the protein content of phospho-AMPKα was significantly lower than that in the control and untreated groups.

DISCUSSION

The present study provides data on the molecular basis of metabolic remodeling of skeletal muscle during the course of cold acclimation. Our results suggest that during early cold acclimation there was transcriptional activation that drives skeletal muscle metabolism to be more oxidative and energy effective, as increases in the protein levels of PGC-1α and the three PPAR isoforms were coincident with increases in the key enzymes in lipid metabolism, the tricarboxylic acid cycle and oxidative phosphorylation (OXPHOS) from days 1 and 3 of cold acclimation. It seems that such metabolic perturbations are triggered by activation of AMPKα, given that the protein level of the phosphorylated form was increased at day 1 of cold acclimation. The observed molecular metabolic recruitment of skeletal muscle was maintained until the end of the examined period (45 days), suggesting a role for skeletal muscle in the regulation of body temperature and/or of whole-body metabolic homeostasis throughout cold acclimation. In addition, our results suggest that the L-arginine/NO-producing pathway is important in supporting skeletal muscle metabolic remodeling in late cold acclimation.

During the course of early cold exposure, shivering thermogenesis is activated. To overcome exposure to cold, nerve stimulation induces permanent skeletal muscle contractions and relaxations. Therefore, this period may be seen as muscle training, which is known to be an ATP-consuming process. Accordingly, we found an increased protein level of phospho-AMPKα (at days 1 and 3), a well-known energy sensor that when activated favors ATP producing pathways. Because LDH activity was markedly increased on day 1, it is likely that the energy demands of skeletal muscle at that time were mainly

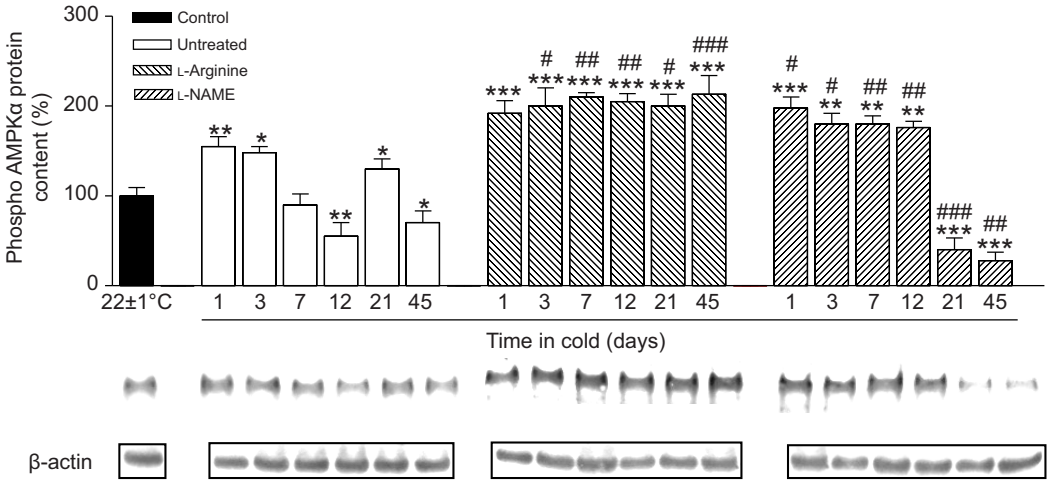

Fig. 5. Changes in the expression of AMPKα protein in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated rats during cold acclimation. Data obtained after quantification of AMPKα bands and expressed as a percentage of the control (taken as 100%) represent the means ± s.e.m. of three independent experiments. *Compared with control: *P<0.05; **P<0.01; ***P<0.001; #compared with untreated rats during the same period of cold acclimation: #P<0.05; ##P<0.01; ###P<0.001.

accomplished through partial metabolism of glucose to lactate, providing rapid ATP production. Our results also suggest that steady-state protein levels of hexokinase and GAPDH satisfied the constant flux of glucose through glycolytic pathways. However, the fact that protein levels of ATP synthase were only slightly decreased at day 1 of cold acclimation suggests that OXPHOS also takes part in ATP production initially during cold acclimation.

In addition, it seems likely that AMPKα, through monitoring of perturbations in energy status in muscle during early cold acclimation (1 and 3 days), initiated molecular remodeling aimed at effectively responding to the energetic challenge in the long term. This includes reprogramming of the skeletal muscle toward high-capacity fatty acid burning during cold acclimation, driven by the well-known transcriptional coactivators and factors PGC-1α and PPARs. The PGC-1α protein level was increased throughout cold acclimation (45 days) starting from day 3 of cold exposure. This is in accordance with previous data that showed that both short-term (4 days) and long-term (4–5 weeks) cold exposure induced PGC-1α expression in skeletal muscle (Oliveira et al., 2004; Bruton et al., 2010). PGC-1α regulates several key processes of adaptive thermogenesis in skeletal muscle, including fuel utilization and switching, insulin sensitivity, glucose transport, gluconeogenesis and lipid oxidation, mitochondrial content and function, ATP synthesis and muscle fiber differentiation (Handschin et al., 2007; Jäger et al., 2007). PGC-1α acts by increasing the expression and activation of various transcriptional factors including PPARs (Wu et al., 1999). Accordingly, we observed that upregulation of the protein level of PGC-1α was coincident with an increase in the expression of PPARα and PPARγ from day 3 and of PPARδ from day 7 of cold acclimation. It was previously shown that cold exposure for 30 days increased the protein level of PPARδ (Seebacher and Glanville, 2010). To our knowledge, ours are the first data to show the effects of cold exposure on PPARα and PPARγ expression in skeletal muscle, suggesting their role in skeletal muscle metabolic remodeling during cold acclimation. The activation of different PPAR isoforms seems to have overlapping effects on fatty acid metabolism with similar molecular targets (including key regulatory enzymes in glucose and lipid metabolism, the tricarboxylic acid cycle and the electron transport chain) (Ferré, 2004). Accordingly, we found that in parallel with upregulation of PPARs during cold acclimation, increases in the protein levels of key metabolic enzymes in β-oxidation (ACADM), the tricarboxylic acid cycle (SCAS) and OXPHOS pathways (cytochrome *c* and ATP synthase) were observed.

Thus, during early cold acclimation there is transcriptional and translational recruitment of skeletal muscle to support lipid-based thermogenesis and shivering. This suggests underlying mechanisms for increased lipid oxidation during early cold acclimation, as reported by Vaillancourt et al. (Vaillancourt et al., 2009). Lipids are the preferred metabolic fuel during periods of sustained submaximal exercise (McClelland, 2004) and shivering thermogenesis (Vaillancourt et al., 2009) because of their high contribution to total energy reserves in mammals (80%) and an energy density one order of magnitude greater than that of carbohydrates (Weber, 2011). In addition, our results suggest that an established molecular basis for increased skeletal muscle lipid-based oxidative metabolism was maintained until the end of the examined time period, when shivering decreased and non-shivering thermogenesis took place. These results are in accordance with our previous data, which showed that from day 3 of cold acclimation there was a conspicuous increase in skeletal muscle catalase and glutathione peroxidase activities, enzymes that remove H_2O_2 when levels increase because of increased β-oxidation (Petrović et al., 2008). Such a shift toward lipid metabolism in skeletal muscle during prolonged cold exposure highlighted the physiological significance of our recently obtained data concerning white adipose tissue structural (Janković et al., 2009) and endocrine (Jankovic et al., 2013) remodeling during cold acclimation. In line with the role of fat tissue mobilization in response to skeletal muscle metabolic demand during cold acclimation, recruitment of lipid metabolism in skeletal muscle is coordinated with a significant reduction in white adipose tissue mass (Janković et al., 2009). Tight cooperation of the two tissues in terms of overall metabolic changes during cold acclimation also involves an endocrine component, as adiponectin expression in white adipose tissue was upregulated at the same time point (after 3 days of cold acclimation). It has been well documented that the regulatory role of adiponectin on lipid and glucose metabolism in skeletal muscle involves the abovementioned signaling cascade, including AMPKα, PPARs and their metabolic targets (Yoon et al., 2006; Amin et al., 2010).

Furthermore, our results suggest that along with increased lipid metabolism, PGC-1α/PPAR signaling regulated the contribution of the glycolytic pathway in response to the energy demands of skeletal muscle during cold acclimation. The established molecular basis for glycolysis early in cold acclimation (upregulated LDH activity and unchanged hexokinase II and GAPDH protein levels on day 1) was maintained throughout the cold acclimation period in parallel with upregulation of the PGC-1α/PPAR transcription program.

Constant flux through the glycolytic pathway may be sustained by increased glucose uptake, observed previously in skeletal muscle of cold-exposed and cold-acclimated rats (Vallerand et al., 1990).

The present data, along with the results of other studies (Meyer et al., 2010; Mineo et al., 2012), demonstrate increased oxidative metabolism in skeletal muscle after prolonged cold exposure; however, the physiological significance and the contribution of shivering to total thermogenesis, when non-shivering is activated, remain to be elucidated. In addition, our results show a signaling cascade leading to the training phenotype of skeletal muscle, suggesting the potential benefit of prolonged cold exposure and potential molecular targets in the management of metabolic disorders including obesity and type II diabetes.

L-arginine treatment sustained the observed increase in LDH activity and accelerated and intensified cold-induced molecular metabolic remodeling of skeletal muscle to a more oxidative phenotype. The role of L-arginine in the regulation of skeletal muscle fuel metabolism at rest and exercise is well documented (Puigserver et al., 1998; Maréchal and Gailly, 1999; Brevetti et al., 2003; Jobgen et al., 2006; Lee-Young et al., 2010). We also recently reported that L-arginine supplementation improves skeletal muscle antioxidative defense (Petrović et al., 2008). The present study extends the data on the role of L-arginine in the regulation of the skeletal muscle response to metabolic demand during cold acclimation. L-arginine induced additional upregulation of OXPHOS complexes, i.e. increased protein levels of complex I, cytochrome *c* and ATP synthase during cold acclimation starting on day 1. Such effects on mitochondrial oxidative metabolism by L-arginine were supported by increased protein expression of ACADM from day 12 of cold acclimation. In addition, L-arginine induced an increase in the protein level of hexokinase II on day 1, suggesting its involvement in the regulation of the skeletal muscle response to shivering-related energy demand (mainly based on glucose) early in cold acclimation. It is likely that L-arginine improves the recruitment of stored glucose to support glucose utilization throughout cold acclimation, as glycogen breakdown regulating protein (glycogen phosphorylase B) was upregulated after L-arginine treatment at all examined time points during cold acclimation.

It seems that the effects of L-arginine are mediated by AMPKα as phospho-AMPKα protein levels were increased throughout cold acclimation in L-arginine-treated rats. The role of AMPKα signaling in mediating the effects of L-arginine in lipid oxidation and overall energy metabolism in skeletal muscle has been previously reported *in vitro* (de Castro Barbosa et al., 2013) and during exercise *in vivo* (Lee-Young et al., 2010). Our results suggest that, besides AMPKα, the L-arginine-induced signaling cascade driving skeletal muscle metabolism to more oxidative metabolism during cold acclimation included upregulation of co-transcriptional/transcriptional machinery based on PGC-1α and PPARs, as L-arginine accelerated and intensified the increase in PGC-1α protein levels and additionally increased PPARα and PPARγ protein levels during cold acclimation. Therefore, the action of L-arginine on molecules regulating fuel metabolism is coordinated to meet the energy requirements of skeletal muscle related to its role during cold acclimation.

At this stage, we are unable to define the contribution of NO to the observed effects of L-arginine. It is well known that all three isoforms of NOS, endothelial (e), neuronal (n) and inducible (i), are expressed in skeletal muscle. Furthermore, Peralta et al. (Peralta et al., 2003) reported that mitochondrial NOS (mtNOS) is the major isoform in skeletal muscle that participates in the response to cold acclimation. Recently, de Castro Barbosa et al. (de Castro Barbosa et al., 2012) provided direct evidence of the stimulatory effect of L-arginine on glucose and lipid metabolism in skeletal muscle, which occurs *via* the NO/cGMP cascade. In the present study, the effects of L-arginine and L-NAME on the examined regulatory proteins and metabolism-related enzymes were not consistent during early cold acclimation, and showed opposite or similar trends. However, during late cold acclimation (21 and 45 days) the two 'antagonists' exerted clear opposite effects. This suggests that the observed effects of L-arginine on prolonged cold acclimation are NO dependent. Thus, it seems likely that the L-arginine/NO-producing pathway takes part in maintaining the skeletal muscle oxidative phenotype, established during early cold acclimation, after the period of intensive shivering. This hypothesis is supported by our previous results (Petrović et al., 2008), which showed a clear opposite effect of L-arginine and L-NAME on catalase and glutathione peroxidase activities on days 21 and 45 of cold exposure.

However, partial parallelism of the two physiological 'antagonists', observed during acute L-NAME treatment, requires further investigation. Similar parallel effects of L-arginine and L-NAME were reported previously under various (patho)physiological conditions (Henningsson et al., 2000; Vasilijević et al., 2007a). These analogous effects were attributed to the action of NO originating from non-enzymatic (Moroz et al., 1998) and iNOS-mediated (Henningsson et al., 2000) production in the presence of NOS inhibitors, or they may be related to other NO-independent metabolic effects of an L-arginine analog (Krippeit-Drews et al., 1996). Moreover, following *in vivo* administration of NOS inhibitors, the response may result from their systemic effects, and not only from local, peripheral effects (Bult et al., 1990; Jun et al., 1995). We noted during our previous study on the effects of L-NAME on brown adipose tissue (Petrović et al., 2009; Vucetic et al., 2011), pancreas (Vasilijević et al., 2007a; Vasilijević et al., 2007b) and white adipose tissue (Janković et al., 2009) that the response of specific physiological processes and their molecular basis were tissue specific.

The present study highlighted the molecular mechanism underlying the shift in skeletal muscle metabolism to more oxidative mode, preferring lipids as an energy substrate during prolonged cold acclimation. This included upregulation of the PGC-1α/PPARs transcriptional program in the early stage of cold acclimation, which triggered the molecular recruitment of β-oxidation, the tricarboxylic acid cycle and OXPHOS. The established molecular basis of metabolic remodeling early in cold acclimation was maintained until the end of cold acclimation, suggesting that the role of skeletal muscle in adaptation to cold is more than just shivering and includes regulation of whole-body energy homeostasis. Importantly, the L-arginine/NO-producing pathway takes part in the establishment of the oxidative skeletal muscle phenotype during cold acclimation. Taken together with earlier studies highlighting the role of L-arginine in oxidative metabolism in skeletal muscle, our findings suggest that this amino acid holds promise as an effective nutrient that may improve the metabolic profile of skeletal muscle in patients with obesity and type II diabetes.

LIST OF ABBREVIATIONS

ACADM	medium chain fatty acids acyl-CoA dehydrogenase
AMPKα	5′-AMP-activated protein kinase α
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LDH	lactate dehydrogenase
L-NAME	N^{ω}-nitro-L-arginine methyl ester
NO	nitric oxide
NOS	nitric oxide synthase
OXPHOS	oxidative phosphorylation
PGC-1α	peroxisome proliferator-activated receptor-γ coactivator-1α

PPAR	peroxisome proliferator-activated receptor
PYGB	glycogen phosphorylase
SCAS	succinyl-CoA synthetase

AUTHOR CONTRIBUTIONS

A.S. performed experiments, interpreted the data and drafted the paper. A.K. interpreted the data. B.B. revised the manuscript. V.O., A.J. and M.V. critically revised the manuscript and performed experiments. K.V., M.M. and I.G. critically revised the manuscript. B.K. designed the experiments, interpreted the data, drafted the paper and critically revised the manuscript. All authors were involved in writing the paper and had final approval of the submitted and published versions.

COMPETING INTERESTS

No competing interests declared.

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