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

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Abstract

Study examined 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 to control (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 exception 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 to control (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 on cold triggers molecular recruitment of skeletal muscle underlying the shift in its metabolism to more oxidative during prolonged cold acclimation. Our results suggest that nitric oxide has a role in maintaining skeletal muscle oxidative phenotype in late cold acclimation but question its role early on cold.

Abbreviations: ACADM, medium chain fatty acids acyl-CoA dehydrogenase; AMPKα, 5'-AMP-activated protein kinase α; GAPDH, glyceraldehyde-3-phosphate 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; PPARs, peroxisome proliferator-activated receptors; PYGB, glycogen phosphorylase; SCAS, succinyl-CoA synthetase.

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 study showed that structural and metabolic remodeling of brown (Petrovic 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 indicating 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: i) by recruitment of specific subpopulations of fibers within the same muscle; ii) by recruitment of muscles varying in fiber composition and iii) 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 in 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 acids oxidation (Jobgen et al., 2006), mitochondriogenesis (Puigserver et al., 1998), contractile function (Joneschild et al., 1999; Marechal and Gailly, 1999) and blood flow (Brevetti et al., 2003). L-arginine is the precursor for nitric oxide (NO) synthesis. In accordance, supplementation of L-arginine is widely used approach to examine metabolic effects of NO. Such studies are usually complemented with the use of N^{ω}-nitro-L-arginine-methyl ester (L-NAME), inhibitor of nitric oxide synthases in order to confirm 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.

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; 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; Petrovic et al., 2005; 2008). Rats were housed in individual plastic cages with drinking water and food *ad libitum.* The duration of cold-exposure ranged from one to 45 days (1, 3, 7, 12, 21, or 45 days), with six animals per experimental group. Body mass, food and fluid intake were recorded daily for each animal. The obtained data were previously reported (Petrovic et al., 2008).

At the end of the cold exposure period, animals were sacrificed by decapitation and the gastrocnemius 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 M sucrose, 0.1 mM EDTA and 50 mM Tris buffer, at pH 7.4 and sonicated.

SDS-PAGE and Western blotting

Western blots were conducted as described previously (Petrovic et al., 2010), using antibodies against: *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; 1:10000), succinyl-CoA synthetase (SCAS, 1:1000), beta actin (1:1000) (all purchased from Abcam, Cambridge, UK), phospho 5'-AMP-activated protein kinase α (AMPKα, 2 μg ml^{-1}) (Milipore International, Billerica, MA, USA) and for glycogen

phosphorylase (PYGB, 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). 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.

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). Analysis of variance (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 to the control, cold induced an increase in the protein level of PGC-1α from day 3 (A), PPARδ from day 7 (B) and both PPARα (C) and PPARγ (D) 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 day 1, 3, 7 and 45. Also, L-arginine increased the protein level of PPARα additionally during cold acclimation throughout

the entire 45-day period. PPARγ was increased compared to the untreated groups at day 12, 21 and 45 during cold acclimation. In contrast to L-arginine, the effects of L-NAME were different in the early and late period 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 to untreated groups: PGC-1α and PPARδ at day 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 (A) which showed no changes in expression during cold acclimation, cytochrome *c* protein content (B) was increased compared to the control throughout the period of cold acclimation, while after an slightly initial decrease in the protein level of ATP synthase (C) 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 to the control and untreated cold-acclimated animals throughout the entire cold acclimation period. In addition, treatment with L-arginine restored, and even increased the cold-induced slightly decrease in protein content of ATP synthase at day 1 and 45 compared with the control and 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 to 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 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 to untreated animals, while the expression of ATP synthase decreased compared to both untreated animals (day 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 (A, B, C) and the activity (D) of the enzymes involved in glucose metabolism. It can be seen that GAPDH protein level (A) was not affected by either cold, L-arginine or L-NAME treatment. Similarly, hexokinase II protein level (B) was unchanged during cold acclimation, while PYGB protein content (C) was decreased compared to the control during early cold acclimation (day 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 similar manner to L-arginine, L-NAME initially induced an increase in PYGB protein content compared to untreated animals from day 1 to day 12 of cold acclimation. In contrast, hexokinase II protein level was lower in L-NAME-treated rats compared to the control and untreated animals during early cold acclimation (day 1 and 3).

Cold acclimation induced an increase in LDH activity compared to the control (except at the 12-day time period) (Figure 3D). L-arginine treatment did not affect cold-induced changes in LDH activity, however, L-NAME treatment diminished the effects of cold acclimation on day 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 been seen from Fig. 4 that ACADM protein content (A) was increased at day 3, 7, 21 and 45 of cold acclimation, while the protein level of SCAS (B) was higher than that in the control throughout the entire cold acclimation period. 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 day 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 level compared to the control and untreated group. 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 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 to 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 (day 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 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 day 1 and 3 on cold. 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 day 1 and 3), a well-known energy sensor which when activated favors ATP producing pathways. Since LDH activity was markedly increased on day 1 it is likely that the energy demands of skeletal muscle at that time were dominantly 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 level of ATP synthase was only slightly decreased at day 1 of cold acclimation suggests that oxidative phosphorylation also take a part in ATP production initially on cold.

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 acids 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 which 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; Jager 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 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, this is the first data to show the effects of cold exposure on PPARα and PPARγ expression in skeletal muscle, suggesting their role in respect to skeletal muscle metabolic remodeling during cold acclimation. Activation of different PPAR isoforms seems to have overlapping effects on fatty acids metabolism with similar molecular targets (including key regulatory enzymes in glucose and lipid metabolism, the tricarboxylic acid cycle and electron transport chain) (Ferre, 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 oxidative phosphorylation 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. (2009). Lipids are the preferred metabolic fuel during periods of sustained submaximal exercise (McClelland et al., 2004) and shivering thermogenesis (Vaillancourt et al., 2009) due to 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 due to increased β-oxidation (Petrovic 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 (Jankovic 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 on cold, recruitment of lipid metabolism in skeletal muscle is coordinated with a significant

reduction in white adipose tissue mass (Jankovic 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 on cold). It has been well documented that the regulatory role of adiponectin on lipid and glucose metabolism in skeletal muscle involves the above-mentioned 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/PPARs 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 on cold (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/PPARs 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 (Mayer 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; Marechal 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 (Petrovic et al., 2008). The present study extends the data on the role of L-arginine in the regulation of skeletal muscle response

to metabolic demand during cold acclimation. L-arginine induced additional upregulation of OXPHOS complexes, i.e. increased protein level 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 on cold. In addition, L-arginine induced an increase in the protein level of hexokinase II on day 1, suggesting its involvement in the regulation of skeletal muscle response to shivering-related energy demand (mainly based on glucose) early on cold. It is likely that L-arginine improves the recruitment of stored glucose to support glucose utilization throughout cold acclimation, since 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 since phospho-AMPKα protein level was 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., 2012) 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 one on cold, included upregulation of co-transcriptional/transcriptional machinery based on PGC-1α and PPARs, since L-arginine accelerated and intensified the increase in PGC-1α protein level and additionally increased PPARα and PPARγ protein level 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 NO synthase, (NOS) endothelial (e), neuronal (n) and inducible (i), are expressed in skeletal muscle. Furthermore, Peralta et al. (2003) reported that mitochondrial NOS (mtNOS) is the major isoform in skeletal muscle which participates in the response to cold acclimation. Recently, 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, during early cold acclimation the effects of

L-arginine and L-NAME on the examined regulatory proteins and metabolism-related enzymes were not consistent, 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 skeletal muscle oxidative phenotype, established during early cold acclimation, after the period of intensive shivering. This hypothesis is supported by our previous results (Petrovic et al., 2008), which showed a clear opposite effect of L-arginine and L-NAME on catalase and glutathione peroxidase activities on day 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 in various (patho)physiological conditions (Henningsson et al., 2000; Vasilijevic et al., 2007). 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 (Petrovic et al., 2009; Vucetic et al., 2011), pancreas (Vasilijevic et al., 2007a, b) and white adipose tissue (Jankovic 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 on cold was maintained until the end of cold acclimation suggesting that the role of skeletal muscle in adaptation to cold is more that shivering and includes regulation of whole-body

energy homeostasis. Importantly, the L-arginine/NO producing pathway takes part in establishing 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 which may improve the metabolic profile in skeletal muscle in obesity and type II diabetes.

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Figure legends:

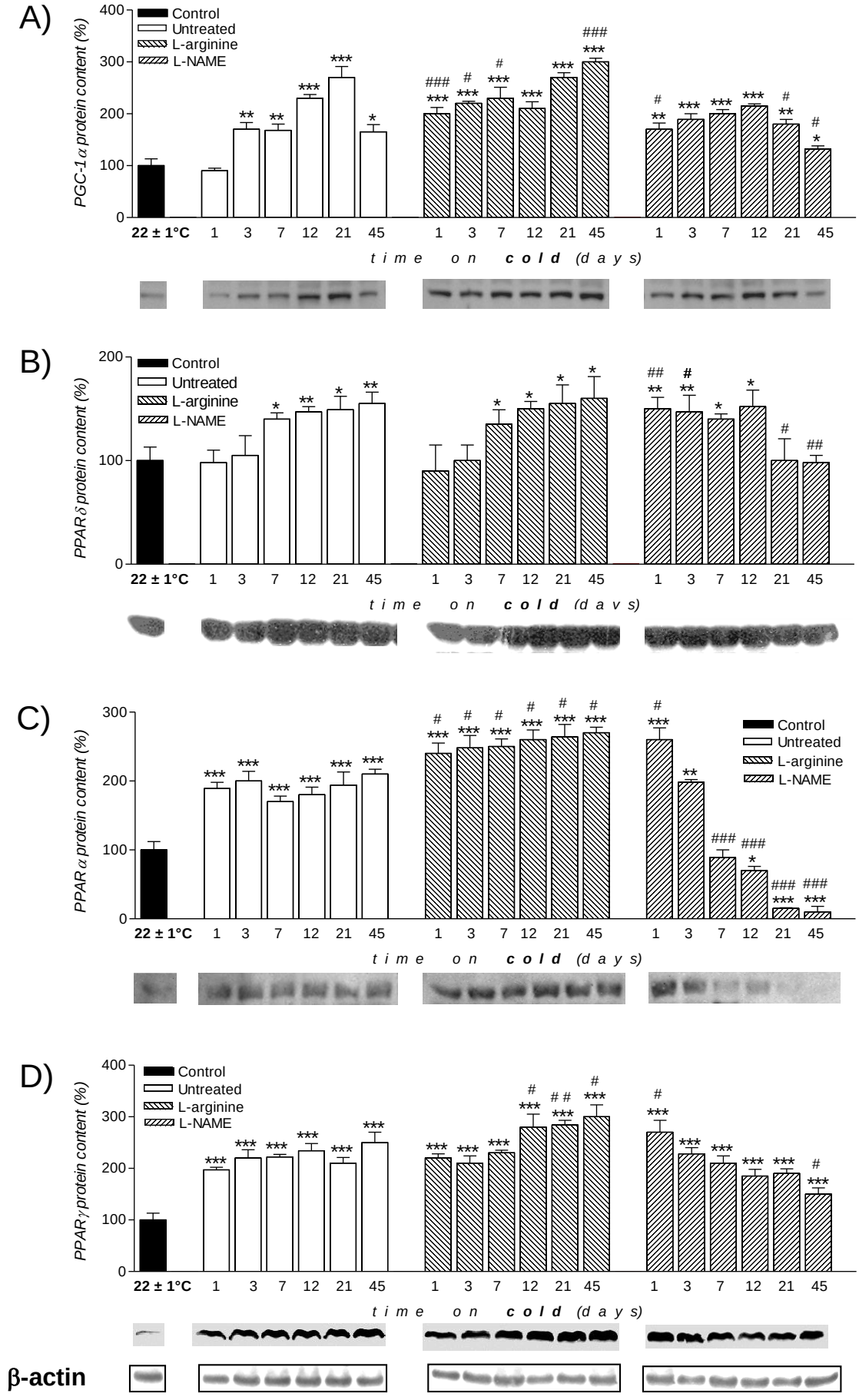
Fig. 1. Changes in the expression of PGC-1α (A), PPARδ (B), PPARα (C) and PPARγ (D) in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated animals during cold acclimation. Data showing protein levels, expressed related to a control acclimated to room temperature taken as 100%, represents the mean ± S.E.M. of three independent experiments. *Compared to control, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; #Compared to untreated rats during the same period of cold acclimation, # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$.

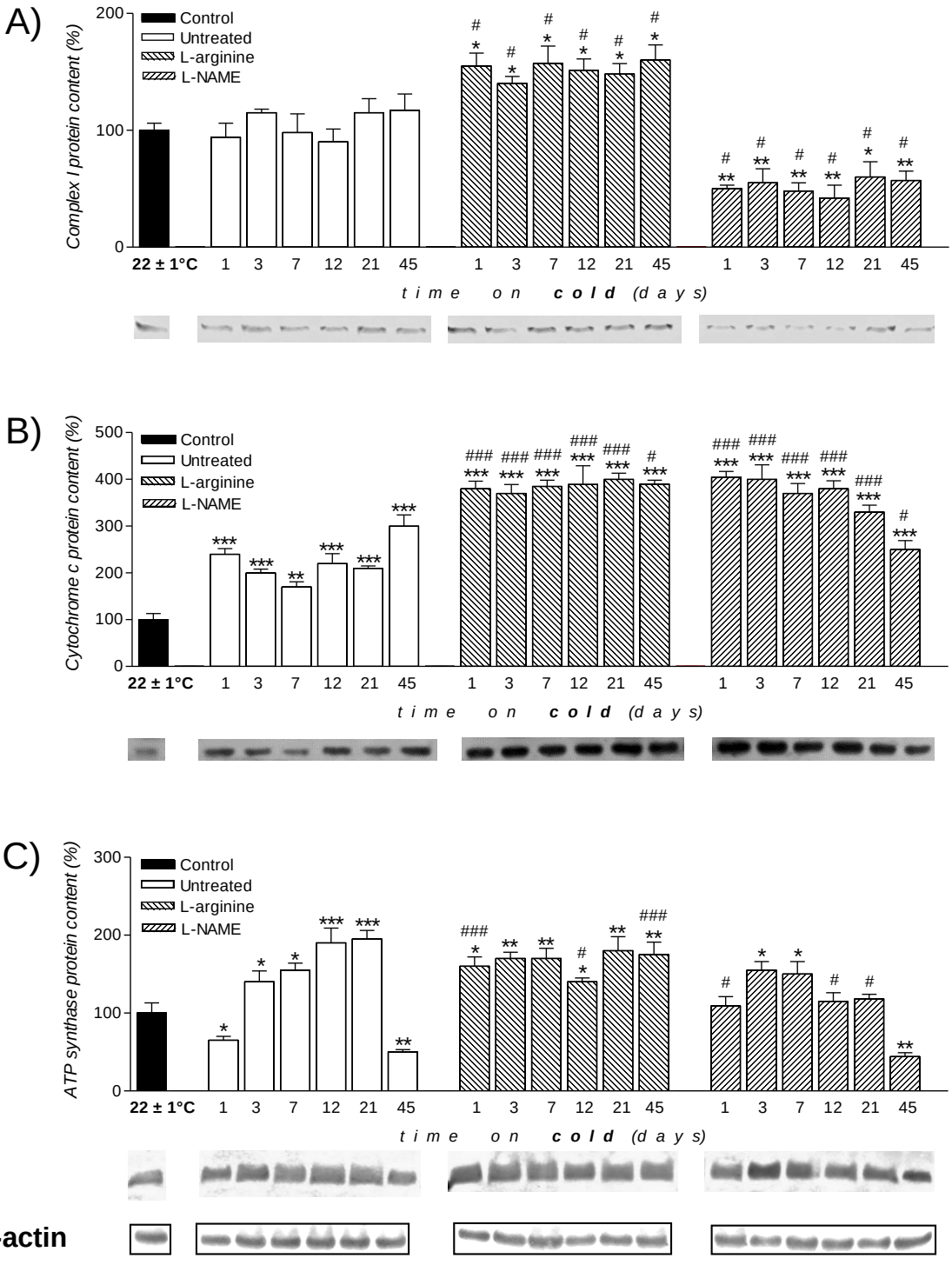
Fig. 2. Time course of changes in the expression of complex I (A), cytochrome *c* (B) and ATP synthase (C) in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated animals during cold acclimation. Protein content is expressed relative to a control acclimated to room temperature, which was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

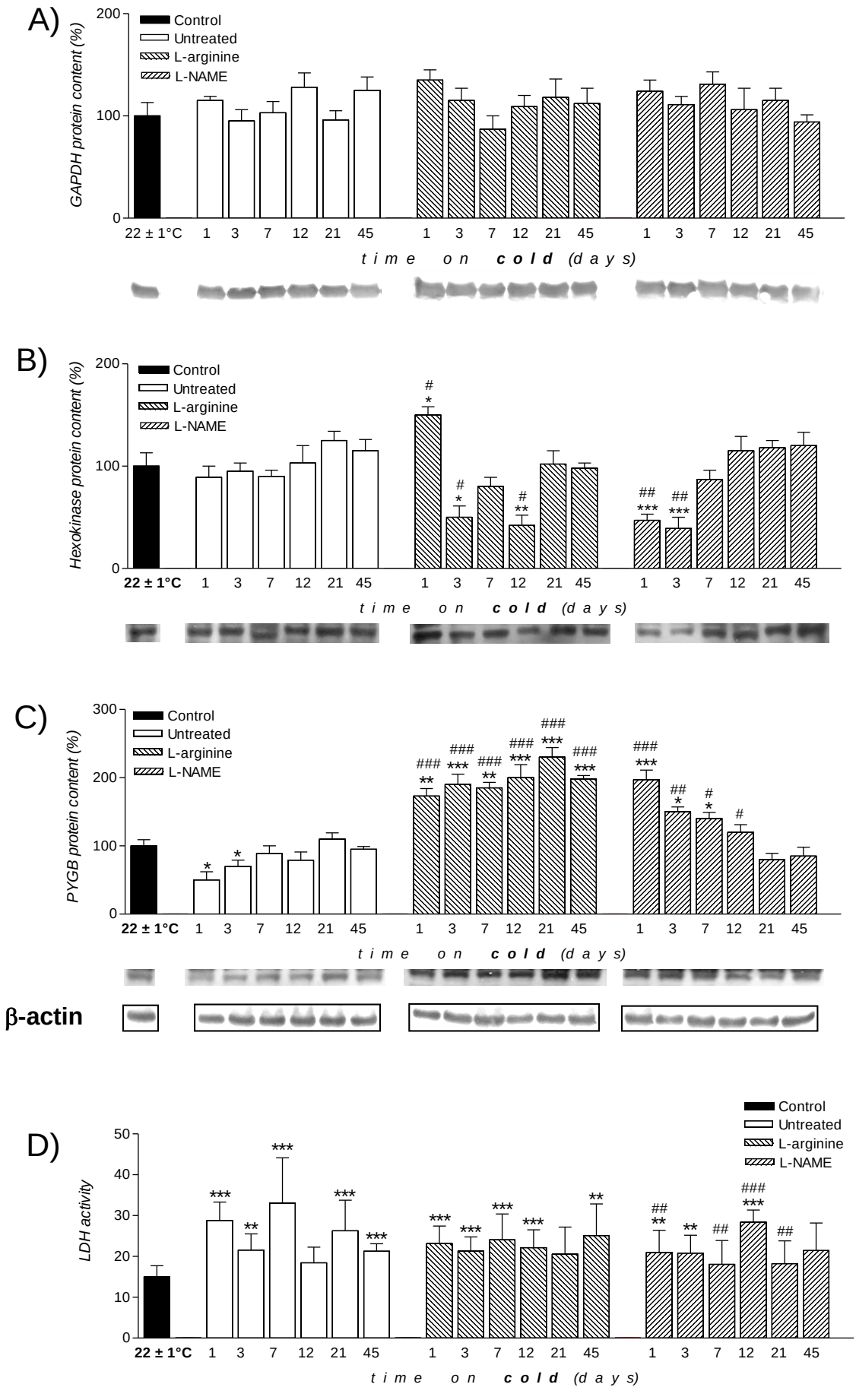
Fig. 3. Time-dependent changes in the protein expression of GAPDH (A), hexokinase II (B), PYGB (C) and activity of LDH (D) in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated animals during cold acclimation. The results of the representative experiment and densitometric analysis are shown. The obtained data for protein content was expressed as a % of the control taken as 100% and represents the mean ± S.E.M. of three independent experiments. *Compared to control, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; #Compared to untreated rats during the same period of cold acclimation, # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$.

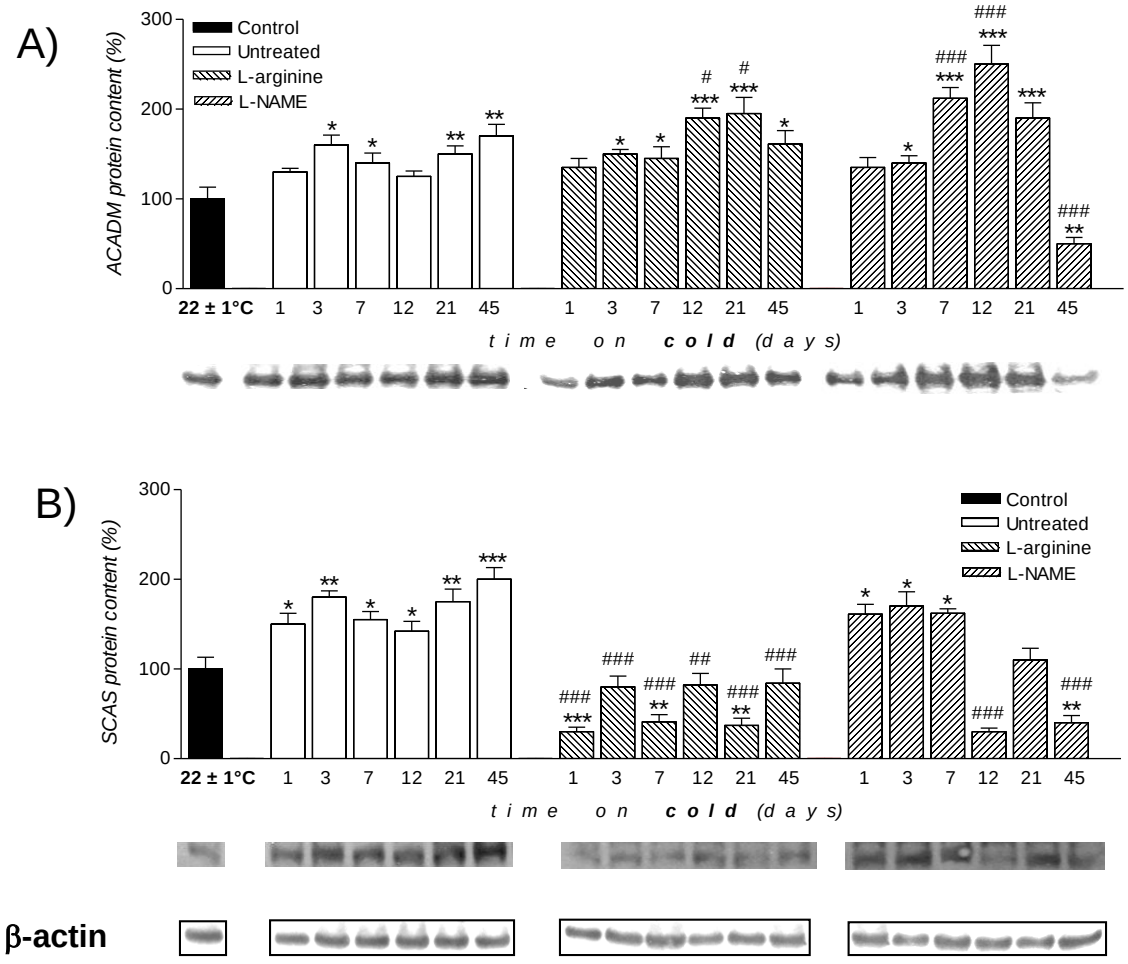
Fig. 4. Expression profiles of ACADM (A) and SCAS (B) in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated animals during cold acclimation. Data showing protein levels, expressed related to a control acclimated to room temperature taken as 100%, represents the mean ± S.E.M. of three independent experiments. *Compared to control, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; #Compared to untreated rats during the same period of cold-acclimation, # $P < 0.05$; ### $P < 0.001$.

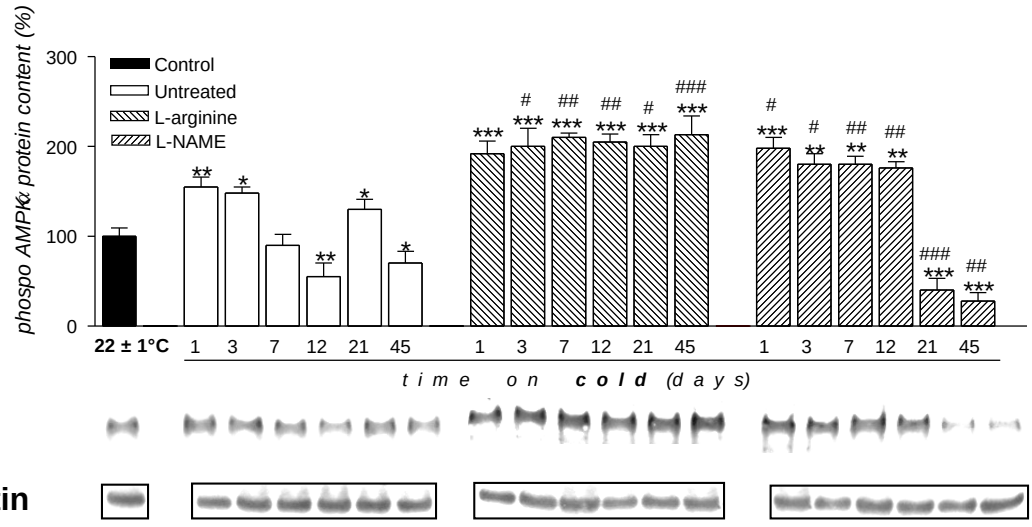
Fig. 5. Changes in the expression of AMPKα protein in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated animals during cold acclimation. Data obtained after quantification of AMPKα bands and expressed as a % of the control taken as 100%, represents the mean ± S.E.M. of three independent experiments. *Compared to control, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; #Compared to untreated rats during the same period of cold-acclimation, # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$.


A)
PGC-1α protein content (%)
Control
Untreated
L-arginine
L-NAME
22 ± 1°C
time on cold (days)
B)
PPARδ protein content (%)
C)
PPARα protein content (%)
D)
PPARγ protein content (%)
β-actin


A)
Complex I protein content (%)
Control
Untreated
L-arginine
L-NAME
22 ± 1°C
1 3 7 12 21 45 1 3 7 12 21 45 1 3 7 12 21 45
time on cold (days)
B)
Cytochrome c protein content (%)
Control
Untreated
L-arginine
L-NAME
22 ± 1°C
1 3 7 12 21 45 1 3 7 12 21 45 1 3 7 12 21 45
time on cold (days)
C)
ATP synthase protein content (%)
Control
Untreated
L-arginine
L-NAME
22 ± 1°C
1 3 7 12 21 45 1 3 7 12 21 45 1 3 7 12 21 45
time on cold (days)
β-actin


A)
GAPDH protein content (%)
Control
Untreated
L-arginine
L-NAME
22 ± 1°C
time on cold (days)
B)
Hexokinase protein content (%)
C)
PYGB protein content (%)
β-actin
D)
LDH activity


A)
ACADM protein content (%)
Control
Untreated
L-arginine
L-NAME
22 ± 1°C
time on cold (days)
B)
SCAS protein content (%)
β-actin


phospo AMPKα protein content (%)
300
200
100
0
Control
Untreated
L-arginine
L-NAME
22 ± 1°C
1 3 7 12 21 45 1 3 7 12 21 45 1 3 7 12 21 45
time on cold (days)
β-actin