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SHORT COMMUNICATION

Cold adaptation overrides developmental regulation of sarcolipin expression in mice skeletal muscle: SOS for muscle-based thermogenesis?

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ABSTRACT

Neonatal mice have a greater thermogenic need than adult mice and may require additional means of heat production, other than the established mechanism of brown adipose tissue (BAT). We and others recently discovered a novel mediator of skeletal muscle-based thermogenesis called sarcolipin (SLN) that acts by uncoupling sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA). In addition, we have shown that SLN expression is downregulated during neonatal development in rats. In this study we probed two questions: (1) is SLN expression developmentally regulated in neonatal mice?; and (2) if so, will cold adaptation override this? Our data show that SLN expression is higher during early neonatal stages and is gradually downregulated in fast twitch skeletal muscles. Interestingly, we demonstrate that cold acclimation of neonatal mice can prevent downregulation of SLN expression. This observation suggests that SLN-mediated thermogenesis can be recruited to a greater extent during extreme physiological need, in addition to BAT.

KEY WORDS: Neonatal development, Cold acclimatization, SERCA, Thermogenic mechanisms

INTRODUCTION

Cold is a powerful environmental stimulus, profoundly impacting animal behavior, which programs whole-body metabolic and physiological changes and prepares vertebrates for long-term behavioral adaptations to reduce the energy cost of survival. However, adaptation to cold among mammals (including mice) varies significantly and depends on their ability to recruit thermogenic mechanisms including shivering and non-shivering thermogenesis (NST) to maintain a constant body temperature. Mice have been extensively used to understand thermogenic mechanisms. It has been shown that mice are very resilient to long-term cold (≤4°C) exposure and can adapt by increasing their metabolic rate as well as by reallocation of whole-body energy budget to fuel the sites of NST. In mice, brown adipose tissue (BAT) has been implicated as a major contributor to NST (Cannon and Nedergaard, 2004). However, recent studies by others and by us have shown that apart from shivering, skeletal muscle is also an important site of NST (Bal et al., 2012; Rowland et al., 2015; McKay et al., 2013; Louzada et al., 2014). We have demonstrated that skeletal muscle-based NST is largely mediated by a sarcoplasmic reticulum (SR) membrane protein called sarcolipin (SLN) (Bal et al., 2012). SLN is a small single transmembrane peptide composed of 31 amino acids localized in the SR membrane and its expression is tightly regulated and predominantly restricted to striated muscles (Odermatt et al., 1998). Our studies showed that mice lacking SLN were sensitive to cold challenge when BAT function was minimized and that reintroduction of SLN rescued the cold-sensitive phenotype, indicating thermogenic function of SLN (Bal et al., 2012). *In vitro* studies have shown that SLN interacts with sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Odermatt et al., 1998; Sahoo et al., 2015) and causes uncoupling of Ca^{2+} transport from ATP hydrolysis, leading to heat production (Mall et al., 2006). Interestingly, SLN expression is developmentally regulated in skeletal muscle of rats, with the expression being relatively high in all skeletal muscles during fetal development and reaching the highest level on the day of birth (Babu et al., 2007). Thereafter, SLN expression is gradually downregulated during neonatal development and becomes restricted mainly to slow-oxidative muscles (Babu et al., 2007). However, the physiological relevance of having high levels of SLN during neonatal stages has not been studied.

It is well known that neonates of all species are extremely cold sensitive, and the thermogenic need is the highest during early neonatal development (Barnett and Neil, 1972). Cold adaptation by neonates (including mice pups) is very different from that of adults in the relative extent of recruitment of various physiological responses such as thermogenesis, heterothermy and behavioral adaptations. It would be evolutionarily advantageous during neonatal development to have multiple mechanisms of thermogenesis in addition to BAT. Therefore, in this study we wanted to understand whether SLN-mediated muscle-based NST is one of the thermogenic mechanisms recruited during neonatal development of mice. We show that the SLN expression pattern during neonatal stages in mice is similar to that shown previously for rats. We further tested whether increasing thermogenic demand (by cold exposure) during neonatal development can alter the SLN expression pattern in skeletal muscles. Our data show that cold stimulus can override developmental signals and prevent the downregulation of SLN expression.

RESULTS AND DISCUSSION

It is well known that neonates are more susceptible to cold than adult mice and their thermogenic demand is high because they have less insulation (their fur is not developed and their skin is thin). Also, they lose more heat to the surroundings because of their large surface area to volume ratio and their muscles are not well developed for shivering as the muscle fibers are undergoing maturation. Therefore, activation of more than one mechanism for heat generation is essential in neonates. BAT is the dominant mechanism of thermogenesis in rodents and neonates of all mammalian species (Cannon and Nedergaard, 2004). In addition to BAT, we and others have shown that SLN-mediated muscle-based NST is an important mechanism of

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heat production. In this study, we tested whether SLN expression follows a similar expression pattern in neonatal mice to that shown for neonatal rats previously. Our findings confirm that when reared at normal housing temperature (23±1°C), SLN expression is developmentally regulated in mice. We observed high expression of SLN in early neonatal stages at both protein (Fig. 1A, B) and RNA levels (Fig. 1C, D). As previously reported, the SERCA isoforms switch from slow (SERCA2a) to fast (SERCA1a) during the development process, as observed in both quadriceps and gastrocnemius (Fig. 1A, B), which contributes to the fast twitch contraction–relaxation properties (Babu et al., 2007). The expression levels of calsequestrin, the calcium-buffering protein inside the SR, gradually increase during development (Fig. 1A, B) as SR volume increases and higher buffering capacity becomes necessary to cope with the increasing SR Ca^{2+} load.

As SLN has been shown to play a thermogenic role in adult mice, we wanted to test whether cold challenge can influence this developmental pattern of SLN expression. To this effect, we acclimated 7 day old neonatal mice to cold (4°C) by gradually lowering ambient temperature over a period of 10 days (Fig. 2A). Our data show that the neonates are able to maintain temperature (M. Pant, N. Bal and M. Periasamy, unpublished observations) at the cost of reduced body mass (Fig. 2B). The mice reared at normal housing temperature (control) showed a significant gain in body mass between 14 and 21 days of age (14 days: 6.302 g and 21 days: 8.895 g, N=6 each). In contrast, 21 day old cold adapted (CA) neonates did not gain significant mass in that one week (14 days: 4.707 g and 21 days: 5.668 g, N=6 each). In addition, the difference in mass between CA mice and their controls was more significant at the end of cold adaptation when the mice were 21 days old (control: 8.895 g, CA: 5.668 g, N=6) than at 14 days of age (control: 6.302 g, CA: 4.707 g, N=6). It is known that neonatal mice reared under cold are lower in body mass compared with their controls (Barnett and Neil, 1972). Therefore, these data indicate that the neonates subjected to cold adaptation maintain body temperature at the cost of a gain in body mass.

Interestingly, cold acclimation prevented the downregulation of SLN protein expression in the 21 day old mice quadriceps (Fig. 2C) and gastrocnemius (Fig. 2D), compared with their controls reared at 23±1°C. We found that SLN was significantly upregulated in quadriceps (Fig. 2C; control: 0.1721±0.03955, CA: 2.198±0.09261, N=4, P<0.0001) and gastrocnemius (Fig. 2D; control: 0.4759±0.08642, CA: 1.377±0.04747, N=4, P<0.0001) of cold-reared mice. This study demonstrates for the first time that SLN-mediated muscle-based thermogenesis can be recruited in neonates and that cold acclimation supersedes the developmental signals of SLN expression. In addition, we observed that SERCA2a expression was higher upon cold acclimation (Fig. 2E), as has been previously reported for ducklings (Dumonteil et al., 1995). In contrast, SERCA1a and calsequestrin expression were unaffected in cold-reared mice (Fig. 2E). It has been suggested by previous studies that cold acclimation increases the percentage of slow fibers and oxidative capacity in the skeletal muscles (Bruton et al., 2010; Mineo et al., 2012) and, therefore, a higher SERCA2a level may support the physiological needs of such muscles. We propose that as SLN-mediated thermogenesis demands more ATP, muscles having a high oxidative capacity are better suited to recruit muscle-based NST. Moreover, the lack of an effect of cold acclimation on developmental regulation of

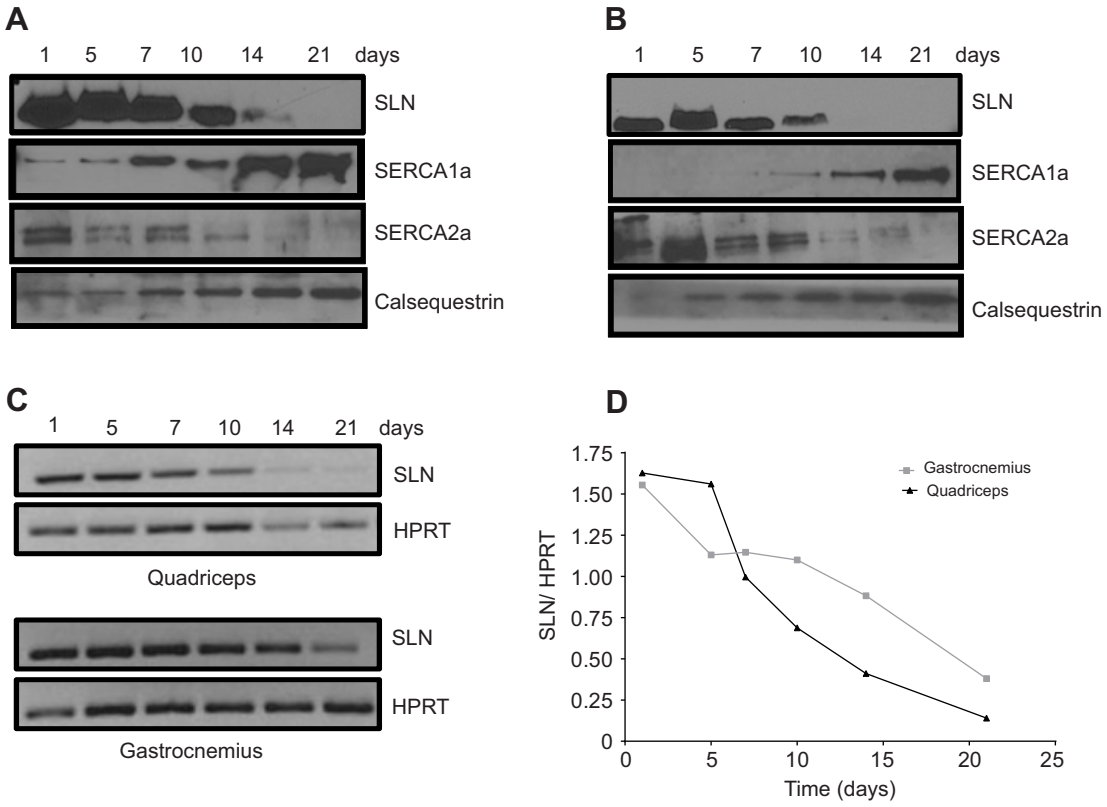

Fig. 1. Sarcolipin expression is significantly higher during early neonatal stages but is gradually downregulated in fast twitch skeletal muscle. (A,B) Western blot showing expression levels of sarcolipin (SLN), SERCA1a, SERCA2a and calsequestrin in quadriceps (A) and gastrocnemius (B) muscles from neonatal mice. SLN levels are gradually downregulated in fast twitch muscles by postnatal day 21. (C) SLN mRNA expression in quadriceps and gastrocnemius muscle of neonatal mice correlates well with protein expression. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression is included as a control. (D) Quantification of SLN mRNA expression normalized to HPRT, showing transcriptional downregulation of SLN during muscle development.

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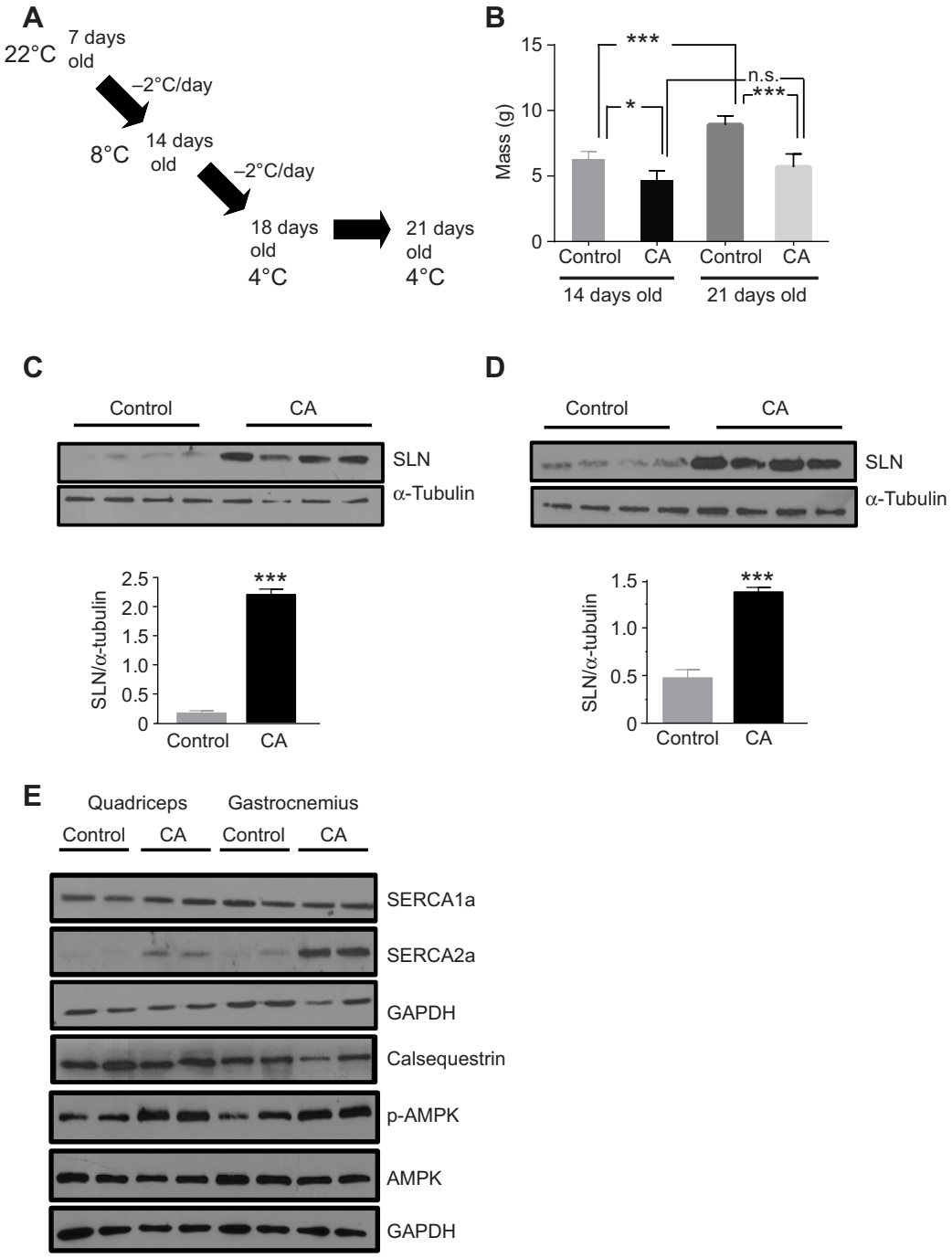

Fig. 2. Cold stress overrides developmental downregulation of SLN expression. (A) Flow chart showing the gradual cold adaptation protocol for the neonates. (B) Body mass of neonates reared at 23°C (control) and gradually adapted to cold (cold adapted, CA) at 14 and 21 days post-natal age. The mice reared under gradual cold gained less body mass compared with the controls. (C,D) Top, western blot depicting SLN protein expression in quadriceps (C) and gastrocnemius (D); bottom, quantification of SLN levels normalized to α-tubulin shows a significant increase in quadriceps (C, $P<0.0001$, $N=4$) and in gastrocnemius (D, $P<0.0001$, $N=4$) of cold-reared mice. (E) Western blots depicting the expression levels of calcium-handling proteins SERCA1a and SERCA2a and a key regulator of energy metabolism AMP-activated kinase (AMPK). SERCA2a and phosphorylated (p-)AMPK expression are upregulated in cold-adapted mice. SERCA1a and calsequestrin expression are not affected by cold adaptation. *$P<0.05$, ***$P<0.001$.

SERCA1a and calsequestrin suggests that the contractile maturation of the muscles is not affected. In addition, we saw an upregulation of phosphorylated AMP-activated kinase (p-AMPK) protein levels in cold-adapted muscles (Fig. 2E). AMPK is a major regulator of skeletal muscle metabolism and has been shown to be activated to the phosphorylated form (p-AMPK) during conditions of increased energy demand including cold challenge (Koh et al., 2008; Kus et al., 2008; Oliveira et al., 2004). Upregulation of p-AMPK levels along with increased SERCA2a levels further indicate that muscle-based thermogenesis is increasingly recruited in the neonates in response to cold challenge.

The ability to adapt to cold has a significant value for the evolutionary success of endotherms. Cold stress during neonatal development (when they are not exposed to cold *in utero*) poses a

major threat to the survival of newborn animals and often can result in death when they are not protected, because their NST mechanisms are not fully proficient. Therefore, having more than one mechanism of heat production would help in minimizing the energy cost (primarily met by food/lactation) for maintaining constant body temperature and allow energy investment in other productive activities. SERCA-based ATP hydrolysis as a means of heat production has been recruited in many species during evolution including the 'heater organ' of endothermic fishes (Morrissette et al., 2003). Moreover, the ability to recruit SLN/SERCA-mediated muscle-based NST without significantly affecting muscle maturation and function will be favorably selected for, specifically in non-tropical climates where temperature and food availability dramatically fluctuate seasonally. Recent studies from our lab further show that SLN increases fatigue resistance of muscle while increasing energy expenditure and contributes to thermogenesis (Maurya et al., 2015; Rowland et al., 2015; Sopariwala et al., 2015). Collectively, these findings along with our data suggest that SLN is an important marker of muscle-based thermogenesis. Interestingly, SLN is expressed abundantly in adult skeletal muscles of large mammals including dog, rabbit and pig, which contain only negligible amounts of or non-functional BAT (Rowland et al., 2014), indicating that muscle-based NST may play a greater role in such animals. Therefore, determining the physiological relevance of high levels of SLN in these species will be instrumental in better understanding muscle-based NST.

MATERIALS AND METHODS

Ethical statement

All study protocols (2009A0149) were approved by the Ohio State University Institutional Animal Care and Use Committee (OSU-IACUC). All of the animal procedures were conducted in accordance with the American Veterinary Medical Association Guide for the Care and Use of Laboratory Animals.

Mice

The mice were housed at 23±1°C on 12 h:12 h light:dark cycle and had access to food and water *ad libitum*. The wild-type (WT) neonates were killed at 1, 5, 7, 10, 14 and 21 days, and tissues were collected. For gradual cold acclimation, the 7 day old WT litter and their mother were kept in a cage inside a temperature-controlled cabinet, starting at 23±1°C. The temperature was dropped by 2°C every day at 10:00 h until it reached 4°C; mice were housed at this temperature until they were killed at 21 days of age (Fig. 2A). Age-matched WT neonates reared at normal housing temperature (23±1°C) were used as controls. Mice were weighed at 14 and 21 days of age.

Western blotting

Tissue homogenates were separated using 10% SDS-PAGE gels or 16% Tris-Tricine gels for SLN, transferred to nitrocellulose membrane and immunoprobed with specific primary antibodies: sarcolipin (Millipore), SERCA1a and SERCA2a (custom made), calsequestrin (custom made), GAPDH (Fitzgerald), α-tubulin (Cell Signaling), and AMPK and p-AMPK (Cell Signaling); followed by horseradish peroxidase-conjugated secondary antibody. The antibody dilutions were as per the manufacturer's protocol. Signals were detected by WestDura substrate (Pierce) and quantified by densitometry (ImageJ 1.41o).

Reverse transcriptase PCR

Muscle tissues were homogenized in Trizol reagent (Life Technologies) and RNA was isolated as per the manufacturer's instructions. RNA (1 µg) was reverse transcribed using Thermoscript reverse transcriptase (Invitrogen). The following primers were used for SLN: F-GCTCCTCTT-CAGGAAGTGAAG, R-TGGCCCCTCAGTATTGGTAGG. Primers for hypoxanthine-guanine phosphoribosyltransferase (HPRT), used as loading control, were: F-CAGTCCCAGCGTCGTGATTAGCGA, R-GCCACAATGTGATGGCCTCCCAT.

Statistics

Data are presented as means±s.e.m. Statistical analysis was performed using Prism 3.0 software. Student's unpaired *t*-test or one-way ANOVA was used to determine statistically significant differences. $P<0.05$ was considered significant.

Competing interests

The authors declare no competing or financial interests.

Author contributions

M. Pant, N.C.B. and M. Periasamy contributed to conception, design, interpretation of data and manuscript writing. M. Pant and N.C.B. acquired and analyzed the data.

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