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Up-regulation of the vitamin C transporter SVCT2 upon differentiation and depolarization of myotubes

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ABSTRACT

In addition to its role as a strong antioxidant, vitamin C regulates the differentiation of several cell lineages. In vertebrate skeletal muscle, the vitamin C transporter SVCT2 is preferentially expressed in slow muscle fibers. To gain insights into the possible involvement of intracellular vitamin C on early myogenesis, we investigated the regulation of SVCT2 expression in cultures of chick fetal myoblasts. SVCT2 expression increases in cultures of both, slow and fast muscle-derived myoblasts, as they fuse to form mainly fast myotubes. Interestingly, we found that SVCT2 could be positively modulated by potassium-induced depolarization of myotubes. These findings suggest that SVCT2-mediated uptake of vitamin C could play diverse roles on skeletal muscle development and physiology.

1. Introduction

The formation of skeletal muscle during development involves a series of sequential events, including the commitment of somite-derived precursor cells to mononucleated myoblasts, and their subsequent fusion into multinucleated myotubes [1]. Mature skeletal muscles consist of variable proportions of fast and slow myofibers which, ultimately, account for their contractile activity [2]. Fiber types can be classified according to various parameters, such as the expression of type-specific protein isoforms and metabolic properties. Indeed, whereas most fast fibers are glycolytic, slow myofibers rely on oxidative metabolism for a permanent supply of ATP [2].

Both mammalian and avian model systems have been employed to identify the intrinsic properties and extrinsic factors that determine the characteristics of mature fibers [2,3]. Whereas murine myoblasts seem preprogrammed to a particular phenotype [4], the definition of skeletal muscle fiber types in the chick relies on the patterning of muscle precursors, but also significantly on their innervation [5].

Vitamin C is the most important water-soluble antioxidant vitamin in the plasma and, as such, it has been characterized as one of the main non-enzymatic antioxidative systems in various tissues [6,7]. To accomplish intracellular functions, vitamin C must be efficiently incorporated into cells. Thus, whereas oxidized vitamin C, dehydroascorbic acid, is taken up by facilitative hexose transporters [8,9], two isoforms of high affinity sodium-dependent vitamin C co-transporters (SVCT1 and 2) mediate the uptake of ascorbic acid, the reduced form of vitamin C [10,11].

In addition to its role as a potent antioxidant, cumulative evidence supports that vitamin C positively regulates the differentiation of diverse cell lineages, such as osteoblasts, keratinocytes, and cells of the nervous system [12–15]. Interestingly, these effects of vitamin C are accompanied by dynamic expression patterns of their SVCT transporters [15–18], thus supporting the notion that transporter-mediated uptake of vitamin C is involved in cell differentiation.

We have recently conducted the first detailed characterization of sodium-vitamin C co-transporter 2 (SVCT2) expression in skeletal muscles [19]. Our data showed that SVCT2 is preferentially expressed in slow skeletal myofibers in both developing chicks and adult mammalian species, including human [19]. These findings suggest that the uptake of vitamin C may help to counteract oxidation in mature slow muscle fibers. However, a possible role for

Abbreviations: SVCT2, sodium-vitamin C co-transporter 2; PM, *pectoralis major*; MA, *medial adductor*; SERCA, slow sarco/endoplasmic reticulum calcium ATPase; ALD, *anterior latissimus dorsi*; PLD, *posterior latissimus dorsi*; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor κ of activated B cells

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vitamin C transport on early steps of myogenesis has not been addressed. Here, we have analyzed the expression of SVCT2, and its regulation by depolarization, in cultures of chick fetal myoblasts obtained from fast and slow muscles. Together with previous evidence, our data suggest that skeletal muscle development and physiology could be regulated by intracellular vitamin C.

2. Materials and methods

2.1. Animals

Fertilized chick eggs were incubated at 37.5 °C in a circulated air incubator. Chick embryos were staged according to Hamburger and Hamilton (HH stages) [20]. Experiments were conducted following the guidelines outlined in the Biosafety and Bioethics Manual of the National Commission of Scientific and Technological Research (CONICYT, Chilean Government). The Ethics Committee of University of Concepcion (Concepcion, Chile) approved all experimental procedures carried out during this study.

2.2. Cell cultures

Myoblasts were isolated from embryonic HH38 White Leghorn chick fast *pectoralis major* (PM) and slow *medial adductor* (MA) muscle tissue, as previously described [21]. The tissue was mechanically disrupted and then treated with 0.25% trypsin (HyClone, South Logan, UT, USA) for 15 min at 37 °C under mild agitation. Cells were suspended in growth medium (DMEM high glucose (Hyclone), 20% fetal bovine serum (Hyclone), 2% chick embryo extract and antibiotics) with repeated pipetting. The cell suspension was filtered through a triple nylon cloth filter and centrifuged 1500×*g* for 5 min. Cells were plated at a density of 50,000 cells per cm^2 of 0.5% gelatine-coated culture dishes in complete medium. To induce differentiation, growth medium was replaced with differentiation medium (DMEM high glucose, 10% horse serum plus 2.5% fetal bovine serum, 2% chick embryo extract and antibiotics) at day 2.

2.3. Reverse transcription – polymerase chain reaction (RT-PCR)

Total RNA from PM and MA myoblasts cultures was reverse transcribed as previously described [19]. For amplification, a cDNA aliquot in a volume of 12.5 μl containing (in mM) 20 Tris buffer pH 8.4, 50 KCl, 1.6 $MgCl_2$, 0.4 dNTPs, plus 0.04U Taq polymerase (Fermentas, ON, Canada) was incubated 95 °C for 5 min, 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s for 32 cycles. Primers were designed based on the chick *SVCT2* sequence, as described [19]. PCR products were separated by 1.2% agarose gel electrophoresis and visualized following ethidium bromide staining.

2.4. Western blot

Total proteins were obtained at different culture days by scraping culture dishes in a 0.3 M sucrose solution containing a protease inhibitor cocktail (80 μM aprotinin, 1.5 mM pepstatin A, 2 mM leupeptin, 104 mM AEBSF, 4 mM Bestatin, 1.4 mM E-64) (Sigma, St. Louis, MO, USA), and extensive passing myotube extracts through a 1 ml syringe. Supernatants containing the total protein extracts were obtained after centrifuging at 6000×*g* for 10 min at 4 °C. Proteins from fast and slow muscles were extracted as described [19]. For immunoblotting, 100 μg (muscle tissue) or 30 μg (cultured myotubes) were loaded in each lane and fractionated by SDS–PAGE, transferred to nitrocellulose membranes, and probed against goat anti rat SVCT2 1/200 (G-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti chick fast C-protein [22] 1/200, mouse anti chick slow sarco/endoplasmic reticulum calcium ATPase (SERCA) [23] 1/200, or goat anti human β-actin 1/5000 (Santa Cruz Biotechnology) antibodies overnight at 4 °C. Antibodies against chick muscle proteins were obtained from the Developmental Studies Hybridoma Bank at The University of Iowa, IA, USA. Peroxidase-conjugated secondary antibodies 1/2000 (Jackson Immuno Research, West Grove, PA, USA) were incubated for 2 h at RT. Reactions were developed with enhanced chemiluminescence according to the ECL Western blotting analysis system (Perkin–Elmer, Waltham, MA, USA).

2.5. Immunochemistry

Chick embryo forelimbs were cryosectioned and immunostained with anti SVCT2 along with anti chick fast SERCA [23], as

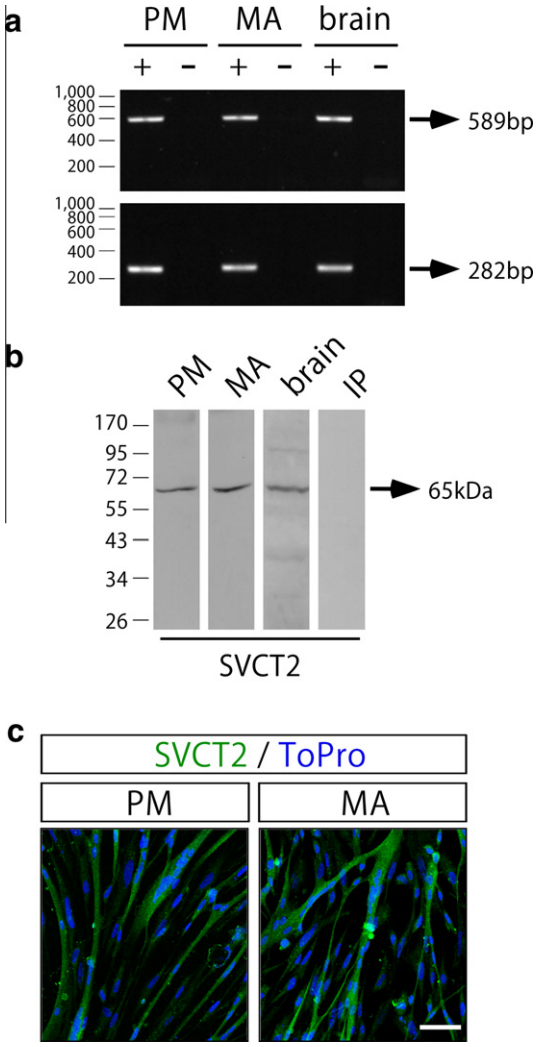

Fig. 1. SVCT2 is expressed in chick myotubes. (a) Total RNAs from PM and MA day 5 myotubes were subjected to RT-PCR analyses to detect chick *SVCT2* (*upper gel*), and the housekeeping control β-*actin* (*lower gel*). RNA from HH42 chick brain and reverse transcriptase negative reactions (RT-) were used as positive and negative controls, respectively. Molecular size standard (in bp) are shown on the left. Arrows on the right indicate the molecular size of the observed bands. (b) Total proteins from day 5 PM and MA myotubes were analyzed by Western blot using a polyclonal anti rat SVCT2 antibody. Proteins samples from HH38 chick brain were used as positive control, whereas pre-incubation of anti SVCT2 with the inhibitory peptide (IP) was used as negative control. Mass standard markers (in kDa) are shown on the left. Arrows on the right show the mass of the observed bands. (c) PM and MA myoblasts were differentiated for 5 days, fixed, and subsequently stained with an anti SVCT2 antibody (green). ToPro (blue) was used to counterstain nuclei. Bar, 50 μm.