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Salbutamol inhibits ubiquitin-mediated survival motor neuron protein degradation in spinal muscular atrophy cells

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ABSTRACT

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder that is currently incurable. SMA is caused by decreased levels of the survival motor neuron protein (SMN), as a result of loss or mutation of *SMN1*. Although the *SMN1* homolog *SMN2* also produces some SMN protein, it does not fully compensate for the loss or dysfunction of *SMN1*. Salbutamol, a β2-adrenergic receptor agonist and well-known bronchodilator used in asthma patients, has recently been shown to ameliorate symptoms in SMA patients. However, the precise mechanism of salbutamol action is unclear. We treated SMA fibroblast cells lacking *SMN1* and HeLa cells with salbutamol and analyzed *SMN2* mRNA and SMN protein levels in SMA fibroblasts, and changes in SMN protein ubiquitination in HeLa cells. Salbutamol increased SMN protein levels in a dose-dependent manner in SMA fibroblast cells lacking *SMN1*, though no significant changes in *SMN2* mRNA levels were observed. Notably, the salbutamol-induced increase in SMN was blocked by a protein kinase A (PKA) inhibitor and deubiquitinase inhibitor, respectively. Co-immunoprecipitation assay using HeLa cells showed that ubiquitinated SMN levels decreased in the presence of salbutamol, suggesting that salbutamol inhibited ubiquitination. The results of this study suggest that salbutamol may increase SMN protein levels in SMA by inhibiting ubiquitin-mediated SMN degradation via activating β2-adrenergic receptor-PKA pathways.

1. Introduction

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder characterized by degeneration of α-motor neurons in the spinal cord, leading to muscular atrophy of the limbs and trunk. Unfortunately, SMA remains incurable. SMA is classified into three groups, depending on the age of onset and the achievement of motor milestones: SMA type I (Werdnig–Hoffman disease; severe form), SMA type II (intermediate form) and SMA type III (Kugelberg–Welander disease; mild form) [1].

The survival motor neuron genes *SMN1* (telomeric *SMN*) and *SMN2* (centromeric *SMN*) have been identified as candidate SMA-causing genes at chromosome 5q11.2–13.3 [2]. *SMN1* and *SMN2* are largely identical, but nucleotide +6 in the coding region is a C in *SMN1* and a T in *SMN2*, though these are synonymous and do not result in an amino acid change. However, *SMN1*, but not *SMN2*, is now recognized as an SMA-causing gene, because *SMN1* is completely deleted in more than 90% of SMA patients [3] and deleteriously mutated in the remaining patients [4].

Although the *SMN1* homolog *SMN2* encodes the same protein, it does not fully compensate for the loss or dysfunction of *SMN1*. The C-to-T transition alters the splicing pattern in *SMN2* exon 7 [5,6]. *SMN1* exclusively produces full-length (FL) *SMN1* transcripts, while *SMN2* produces about 90% of exon 7-lacking (Δ7) *SMN2* transcripts and only about 10% of FL-*SMN2* transcript [7]. The low levels of functional SMN protein produced by the FL-*SMN2*

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transcript cannot fully compensate for the lack of functional *SMN1*-generated SMN protein. However, *SMN2* is never lacking in SMA patients [2] and its copy number correlates inversely with disease severity; a higher *SMN2* copy number may ameliorate the clinical phenotype [8]. *SMN2* may thus partly compensate for the loss of *SMN1* by modifying disease severity through the production of functional SMN protein.

Current treatment strategies for SMA have focused on enhanced production of SMN from *SMN2*, including by activation of *SMN2* transcription and by modulation of exon 7 skipping [9]. Valproic acid, a histone deacetylase inhibitor, is the representative drug for the first of these strategies [10,11], while ISIS-SMNRx, an antisense oligonucleotide masking a splicing suppressor in the *SMN2* intron 7 [12], is used for the second strategy.

However, the β2-adrenergic receptor agonist, salbutamol, which is widely used as a bronchodilator in asthma patients, has recently been reported to alleviate symptoms in SMA patients [13–15], and Angelozzi et al. reported that salbutamol increased SMN protein levels in SMA fibroblast cells [16]. However, the mechanism whereby salbutamol increases SMN protein levels remains unclear. Angelozzi et al. suggested that salbutamol may increase SMN levels by increasing *SMN2* transcripts [16]. Tiziano et al. also reported a significant and constant increase in FL-*SMN2* transcripts in peripheral blood cells from SMA patients treated with salbutamol [17]. However, Burnett et al. found that the SMN complex was stabilized by activation of cyclic AMP (cAMP) and protein kinase A (PKA), with consequent increases in SMN protein levels [18], suggesting that salbutamol enhances SMN protein levels through other pathways.

To clarify the mechanism responsible for the salbutamol-induced increase in SMN protein levels, we treated SMA fibroblast cells lacking *SMN1* and HeLa cells with salbutamol. We analyzed *SMN2* transcript and SMN protein levels in SMA fibroblast cells, and assessed the effects of salbutamol on SMN protein ubiquitination in HeLa cells.

2. Materials and methods

2.1. Establishment of fibroblast cells and cell cultures

Skin tissue for primary culture of fibroblast cells was obtained from a 13-year-old girl with SMA type 3, who had complete absence of *SMN1* but carried three copies of *SMN2*. This study was approved by the ethical committee of Kobe University, and informed consent was obtained from the patient and her parents.

Alteration of *SMN2* transcript and SMN protein levels were investigated in SMA fibroblast cells. The fibroblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-low glucose (Sigma-Aldrich, St. Louis, MO, USA) containing 10,000 U/ml penicillin, 10 mg/ml streptomycin, 25 μg/ml amphotericin B (Fungizone; Life Technologies, Carlsbad, CA, USA), and 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich) in a 5% humidified CO_2 atmosphere at 37 °C. Fibroblast cells were analyzed at 5–15 passages.

Ubiquitinated SMN was investigated in HeLa cells carrying two copies of *SMN1* and two copies of *SMN2*. HeLa cells were used for co-immunoprecipitation (co-IP) experiments because of their ability to produce more SMN protein than fibroblast cells. HeLa cells were maintained in DMEM–high glucose (Sigma-Aldrich) containing 10,000 U/ml penicillin, 10,000 μg/ml streptomycin, 25 μg/mL amphotericin B (Life Technologies) and 8% heat-inactivated FBS in a 5% CO_2 atmosphere at 37 °C.

2.2. Salbutamol treatment of fibroblast cells

Salbutamol sulfate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Salbutamol solution 1 mM was freshly prepared in phosphate-buffered saline (PBS) before each use. To clarify if *SMN2* transcript or SMN protein was induced by salbutamol, SMA fibroblast cells were incubated with final concentrations of salbutamol sulfate of 0.005, 0.05, and 0.5 μM, respectively. *SMN2* transcript levels were measured at 0 m (pretreatment), 5 m, 15 m, 30 m, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 36 h. SMN protein levels were measured at 0 h (pretreatment), 1 h, 6 h, 8 h, 12 h, 24 h and 36 h.

The mechanism whereby salbutamol increased SMN protein levels was further investigated by adding PKA inhibitor 14–22 amide cell-permeable, myristoylated (Calbiochem; Darmstadt, Germany) (final concentration; 1 μM) and DUB inhibitor PR-619 (LifeSensors, Malvern, PA, USA) (final concentration; 10 μM), respectively, to SMA fibroblast cells with salbutamol (final concentration; 0.5 μM), and incubating for 36 h.

2.3. Reverse-transcription PCR analysis of salbutamol-treated fibroblast cells

Total RNA extraction and cDNA synthesis were conducted as described previously [19]. To evaluate transcript levels of the *SMN* genes, we amplified FL- and Δ7-*SMN* transcripts from cDNA of SMA fibroblast cells. Real-time quantitative reverse-transcription PCR was performed using a LightCycler 1.5 (Roche Diagnostics GmbH, Mannheim, Germany). cDNA amplification was carried out using FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH).

The cDNA fragments containing exons 7 and 8 and exons 5 and 6/8 (flanking sequences of the exon boundary) represented FL- and Δ7-*SMN*, respectively. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous reference gene and the relative denominator for the *SMN2* transcript. The amount of *SMN2* transcript (relative to *GAPDH*) at 0 min (pretreatment time) was normalized to 1.0 in each dose group. The primer sequences and PCR conditions are listed in Table 1 (Supplementary material).

2.4. Western blotting analysis of salbutamol-treated fibroblast cells

Protein samples were prepared as described previously [19]. The protein samples were homogenized and subjected to western blotting. Briefly, the homogenized protein samples were electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Cleveland, OH, USA) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, CA, USA). Immunostaining of the membranes was performed using the iBind Western System (Life Technologies). The following combinations of antibodies were used to detect SMN and tubulin: mouse anti-SMN antibody (BD Transduction Laboratories, Franklin Lakes, NJ, USA), ECL horseradish peroxidase (HP)-linked sheep anti-mouse IgG (GE Healthcare), rabbit polyclonal anti-beta tubulin antibody (Abcam, Cambridge, MA, USA), and ECL HP-linked donkey anti-rabbit IgG (GE Healthcare).

Chemiluminescent signals produced using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) were detected using a luminescent image analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan). The signal intensity of the membrane was determined using Multi Gauge Version 3.0 software (Fujifilm). Western blotting experiments and signal-intensity analysis were repeated at least three times. Tubulin was used as an endogenous reference protein and as the relative denominator for the SMN