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ABSTRACT

Spinal muscular atrophy (SMA) is an inherited neuromuscular disorder caused by homozygous absence of the survival motor neuron gene (*SMN1*). All patients have at least one, usually two to four copies of the related *SMN2* gene which, however, produce insufficient levels of functional SMN protein due to the exclusion of exon 7 in the majority of *SMN2* transcripts. Here, we show that salbutamol, a β2-adrenoceptor agonist, determines a rapid and significant increase in *SMN2*-full length mRNA and SMN protein in SMA fibroblasts, predominantly by promoting exon 7 inclusion in *SMN2* transcripts. These data, together with previous clinical findings, provide a strong rationale to investigate further the clinical efficacy of salbutamol in SMA patients.

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disease which presents with variable phenotype ranging from very severe to mild (type I to III). SMA I is a leading genetic cause of infant mortality. The disease is caused by homozygous loss of the survival motor neuron (*SMN1*) gene,[1] although patients retain at least one copy of the closely related *SMN2* gene. Both genes encode the SMN protein, but *SMN2* produces an insufficient level of functional protein due to a C-T transition in exon 7 which determines the exclusion of this exon in the majority of mature transcripts.[2] At present, no treatment for SMA is available. Recently, several drugs have been studied for their effect on *SMN2* expression in vitro and/or clinical efficacy in vivo.[3–14] Among these, albuterol (salbutamol), a β2-adrenoceptor agonist (β2-agonist), has been shown to improve muscle strength in SMA patients in a pilot trial.[15] In the present study we have investigated the effect of salbutamol on *SMN2* mRNA and protein levels in cell lines from SMA patients.

METHODS

Fibroblast cell lines were established from a type I and type II SMA patient, both with 2 *SMN2* genes, and a SMA type III patient with three copies of the *SMN2* gene. The cell cultures were treated with different concentrations of salbutamol (Sigma Aldrich, St Louis, Missouri, USA), prepared in phosphate buffered saline (PBS), within the therapeutic concentration range of 0.01–0.05 μM.[16] Since transcript analysis showed similar data at both concentrations (data not shown), all subsequent studies were performed using the concentration of 0.05 μM. *SMN2* full length (*SMN2*-fl) and *SMN2* exon 7-deleted (*SMN2*-del7) transcript levels, gem number and SMN protein levels were measured using semiquantitative real time polymerase chain reaction (PCR), immunocytofluorescence and western blot, respectively, as previously described.[5] Total *SMN2* mRNA was measured using the same probe and forward primer as for *SMN2*-del7 quantification and a reverse primer (5′-CATACTTCCCAAAGCATCAGCA-3′) in exon 6. Densitometry of western blots was performed using Quantity One version 4.6.0 software (Biorad, Hercules, California, USA). Statistical analysis was done using Statsoft software (Statsoft Inc., Tulsa, Oklahoma, USA). T test for independent variables and one way analysis of variance (ANOVA) were used to compare the data of each treated to the respective untreated culture.

RESULTS

Salbutamol increases SMN2 transcript levels

SMA fibroblast cell lines were treated with 0.05 μM salbutamol for time periods ranging from 5 min to 4 h. In all three cell lines a significant increase in *SMN2*-full length (*SMN*-fl) transcript levels ($p \leqslant 0.0005$) was found relative to the respective untreated cultures (fig 1). Surprisingly, an approximately twofold increase in *SMN2*-fl transcript levels was detected already after 5 min of treatment. The maximum increase in *SMN2*-fl transcripts (⩾200%) was observed after 30–60 min, followed by a slow decline of the *SMN2* mRNA levels.

The rapid increase in *SMN2*-fl transcript levels led us to hypothesise that salbutamol may affect exon 7 inclusion during *SMN2* mRNA maturation rather than promoter activation. Therefore, we measured both *SMN2*-fl and exon7-deleted (*SMN2*-del7) transcript levels simultaneously. Indeed, we found in parallel to the increase in *SMN2*-fl mRNA, a significant decrease ($p \leqslant 0.02$) in *SMN*-del7 transcript levels (fig 1). After 5 min of treatment, *SMN2*-del7 transcript levels were reduced by 16.5–23% relative to the untreated cultures. After 15 min of salbutamol exposure, all three cultures showed the maximum decrease in *SMN2*-del7 transcripts, ranging from 30–40%; thereafter transcript levels slowly returned towards baseline.

To determine whether salbutamol may also activate transcription the total *SMN2* transcript levels were measured in the three cell lines. A slight increase in total *SMN2* transcript levels of up to a maximum of 50% at 15–30 min of treatment was observed, suggesting an effect of salbutamol also on promoter activation (fig 1).

As shown in fig 1, the time curves were strikingly similar for the three cell lines. Moreover, the increase in *SMN2*-fl transcript levels triggered by salbutamol was almost exactly

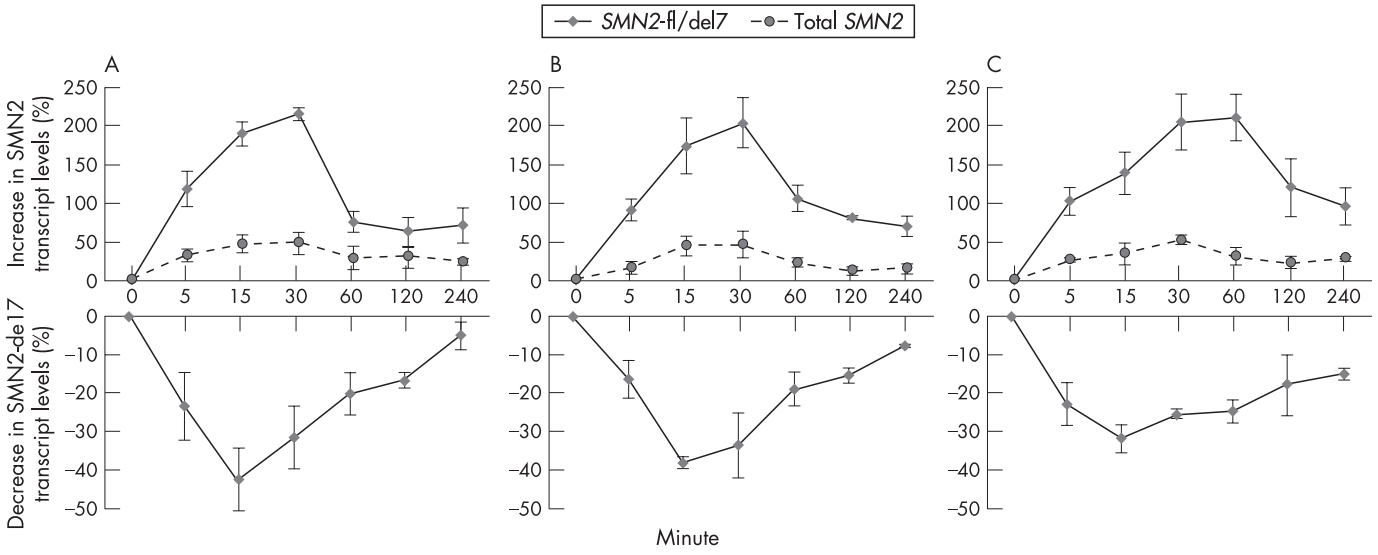

Figure 1 Effect of salbutamol treatment on *SMN2* full-length (*SMN2*-fl), exon 7-deleted (*SMN2*–del7) and total *SMN2* transcript levels in fibroblast cell lines from a type I (A), a type II (B) and a type III (C) SMA patient. Each data point represents the average percentage increase/decrease in transcript levels in treated versus untreated cultures. Values represent averages of three separate experiments; for each experiment, real time PCR reactions were performed in triplicate and repeated at least two times. Error bars represent standard deviation.

concomitant to a decrease in *SMN2*-del7 levels, with the exception that the maximum decrease in *SMN2*-del7 transcripts slightly anticipated the peak increase in *SMN2*-fl mRNA (at 15 and 30 min, respectively). These data, together with the finding of a relatively modest increase in total *SMN* mRNA, strongly suggest that salbutamol enhances *SMN2*-fl transcript levels mainly by stimulating inclusion of exon 7 into the mature transcripts.

Salbutamol increases gem number and SMN protein levels

The SMN protein is localised in the cytoplasm and the nucleus where it is concentrated in dot-like structures, designated gems. The number of gems is notably reduced in SMA patients, therefore gem number count is considered a suitable test of the efficacy of a drug in enhancing SMN protein expression. For SMA type I and type II cultures, we found a 2.9-fold increase ($p \leqslant 0.002$) in the number of nuclei with gems after 1–2 h of salbutamol treatment and a maximum of 3.7- to 4.6-fold increase in the number of gems/100 nuclei after 2–4 h ($p \leqslant 4 \times 10^{-4}$). The SMA type III cell line, which had higher baseline gem numbers, reached a maximum of 2.3- and 2.6-fold ($p \leqslant 1.3 \times 10^{-4}$) increase in positive nuclei and gem number, respectively (fig 2A,B). An increase in both positive nuclei and gem numbers was already detectable within 30 min of treatment in all three cell lines. Western blot analysis showed a significant increase in SMN protein levels in the salbutamol

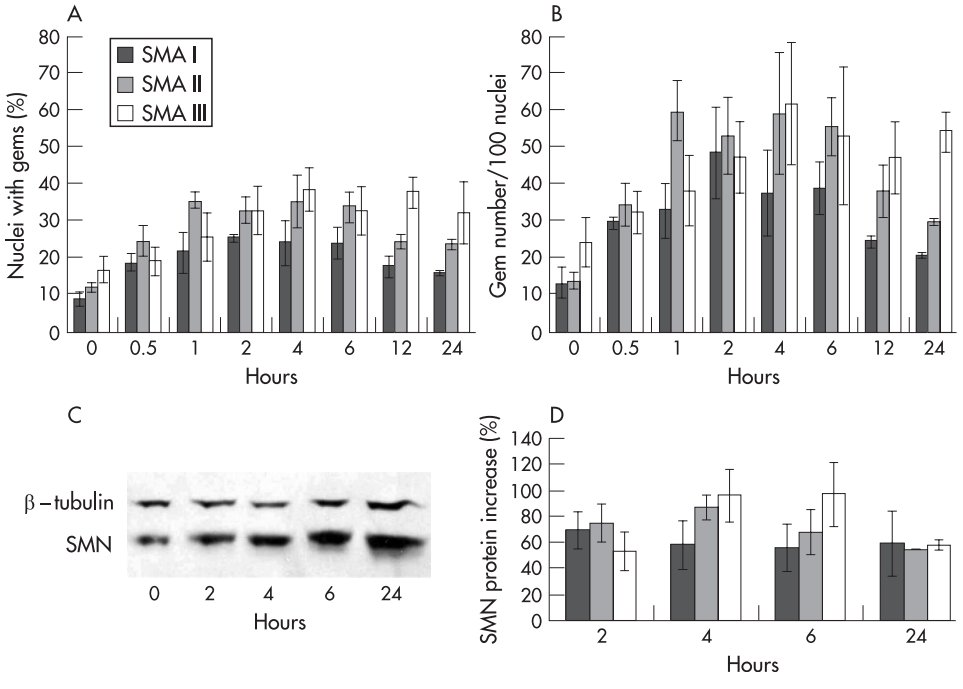

Figure 2 Effect of salbutamol treatment on gem number and SMN protein levels in the three cell cultures. (A) Percentage of nuclei with gems and (B) number of gems/100 nuclei in salbutamol treated and untreated cell lines. (C) Representative western blot of SMN and β-tubulin. (D) SMN protein levels in salbutamol treated versus untreated cell cultures, quantified by densitometric analysis and normalised to β-tubulin used as loading control. Values represent the average (±SD) of at least three independent experiments for each cell line.

treated cultures ($p \leqslant 0.02$). Similar to the results of gem counts, the SMN protein levels were increased after 2 h of treatment. The three cell lines reached an average increase of 80% in SMN protein levels at 4 h and maintained elevated levels for at least 24 h (fig 2C,D).

DISCUSSION

All SMA patients have at least one, usually two to four *SMN2* genes, being higher copy numbers generally associated with milder phenotypes. The levels of full length *SMN* mRNA and functional protein produced by these genes are insufficient to prevent disease onset and progression due to alternative splicing of the majority of *SMN2* transcripts. Thus, increasing the synthesis of full length mRNA from the *SMN2* genes could be of invaluable therapeutic importance. Evidence has recently been provided that *SMN2* gene expression can be enhanced by pharmacological treatment with different compounds. We and others have shown that the histone deacetylase inhibitors phenylbutyrate and valproic acid increase *SMN2* levels both in vitro and in vivo, but their clinical efficacy remains unclear.[3–8 14] No clinical data are yet available on other drugs that have been shown to affect *SMN2* expression in vitro including aclarubicin, hydroxyurea, sodium vanadate, indoprofen and a quinazoline compound, and the side effects of some of them may preclude their use for SMA treatment.[9–12]

β2-agonists are widely used drugs, mainly for the treatment of asthma, and are well tolerated. Besides their known anabolic effects on skeletal muscle, several studies have documented also a neuroprotective effect of these drugs. For example, clenbuterol has been shown to cross the blood–brain barrier and to enhance regeneration of motor neuron axons in motor neuron degeneration (mnd) mice,[17] to promote regeneration of peripheral nerves,[18] and to ameliorate denervation-induced atrophy in rat models.[19] Preliminary evidence for a clinical effect of β2-agonists in SMA patients has previously been reported and awaits confirmation by placebo controlled trials.[15] We show here that salbutamol induces a significant and almost immediate increase in *SMN2*-fl mRNA in SMA fibroblasts and also a significant increase in apparently functional SMN protein, as demonstrated by a 2.5- to >4-fold increase in gem numbers within 1–2 h of treatment, as well as a significant increase in protein levels.

The observed concomitant decrease in *SMN2*-del7 transcript levels suggests that salbutamol stimulates inclusion of exon 7 in *SMN2* mRNA. It is known that the majority of *SMN2* transcripts lack exon 7, but the percentage of full length and del7 transcripts may vary in different cell types. We and others have previously shown that in primary SMA fibroblasts between 50–80% of transcripts are del7.[3 20] Thus, the observed 30–40% reduction of the level of *SMN2*-del7 transcripts in fibroblast cultures following salbutamol treatment is likely to account, at least in part, for the high increase (≥200%) in *SMN2*-fl transcripts. A fraction of the increase in *SMN2*-fl transcript levels may be ascribed to an effect of salbutamol on transcription activation, which may be related to the previously reported cAMP-responsive elements in the *SMN* promoter.[21]

To our knowledge this is the first evidence that a β2-agonist may influence the splicing pattern of a gene, which raises the possibility that splicing of other target genes could also be affected. How salbutamol mediates correct splicing of *SMN2* transcripts is unknown. One possible mechanism could be the activation of specific SR-proteins which play a key role in splicing regulation. Efforts to elucidate the pathway involved in salbutamol-mediated *SMN2* splicing are currently in progress.

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