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ORIGINAL RESEARCH

Effect of Inhibiting p38 on HuR Involving in β-AChR Post-transcriptional Mechanisms in Denervated Skeletal Muscle

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Abstract

Previous studies reported that RNA-binding protein human antigen R (HuR) mediates changes in the stability of AChR β-subunit mRNA after skeletal muscle denervation; also, p38 pathway regulated the stability of AChR β-subunit mRNA in C2C12 myotubes. However, the relationship between HuR and p38 in regulating the stability of AChR β-subunit mRNA have not been clarified. In this study, we wanted to examine the effect of inhibiting p38 on HuR in denervated skeletal muscle. Denervation model was built and 10% DMSO or SB203580 were administered respectively follow denervation. Tibialis muscles were collected in 10% DMSO-administered contralateral (undenervated) leg, 10% DMSO-administered denervated leg, SB203580-administered contralateral (undenervated) leg, and SB203580-administered denervated leg, respectively. P38 protein, β-AChR mRNA and protein, HuR protein, β-AChR mRNA stability, and HuR binding with AChR β-subunit mRNAs were measured. Results demonstrated that the administration of SB203580 can inhibit the increase of β-AChR protein expression and mRNA expression and stability, and RNA-binding protein human antigen R (HuR) expression, in cytoplasmic and nuclear fractions in skeletal muscle cells following denervation. Importantly, we observed that SB203580 also inhibited the increased level of binding activity between HuR and AChR β-subunit mRNAs following denervation. Collectively, these results suggested that inhibition of p38 can post-transcriptionally inhibit β-AChR upregulation via HuR in denervated skeletal muscle.

Keywords Acetylcholine receptors · p38 inhibitor · Human antigen R · Denervation · Post-transcriptional mechanisms

Introduction

Nerve transection caused skeletal muscle denervation, which resulted in AChRs changes (Ma et al. 2007; Kramer et al. 2017) and resistance to non-depolarizing muscle relaxants (NDMRs) (Wang et al. 2010a, b; Yang et al. 2013; Wang et al. 2018) in the skeletal muscle. Both transcriptional and post-transcriptional mechanisms were considered to be involved in regulating AChR expression in denervated skeletal muscle (Chakkalakal and Jasmin 2003; Joassard et al. 2015).

Previous report had demonstrated that skeletal muscle denervation increased the stability of the AChR β-subunit mRNA, which was attributed to increased interaction of RNA-binding protein (RBP), human antigen R (HuR), with the AU-rich element (ARE) of AChR β-subunit mRNA (Joassard et al. 2015). Also, denervation activated the p38 mitogen-activated protein kinase (p38 MAPK, p38) pathway (Evertsson et al. 2014). Furthermore, Joassard et al. (2015) found that activating or inhibiting the p38 MAPK pathway increased or decreased the expression and stability of AChR β-subunit transcripts in C2C12 myotubes. However, whether p38 MAPK pathway has effects on HuR expression or interaction of HuR with AChR β-subunit mRNA which contributes to AChR β-subunit mRNA stability in denervated skeletal muscle is not clarified.

Thus, this study wanted to investigate whether SB203580, p38 MAPK inhibitor, declined the stability of AChR β-subunit mRNA through restraining HuR expression and

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interaction of HuR with AChR β-subunit mRNA in denervated skeletal muscle, and clarify exact post-transcriptional mechanisms of increased AChR β-subunit mRNA after denervation in the skeletal muscle.

Materials and Methods

Ethics Statement

This study was approved by the Animal Care and Use Committee of Shanghai General Hospital Affilicated to Shanghai Jiaotong University School of Medicine (No, 201602017; Date, February, 17th, 2016) and was performed in compliance with the World Health Organization (WHO) International Guiding Principles for Animal Research.

Animals

Male SD rats (200–250 g) were allocated randomly into experimental groups. Rats were weighed daily for 2 weeks before denervation and 1 week after denervation, until the end of the study. After 2 weeks of acclimatization, rats were anesthetized with pentobarbital, 50 mg/kg intraperitoneal injection (IP).

Denervation Model

Rats were treated with pentobarbital (50 mg/kg ip). A small (7–10 mm) incision was made over the hip to excise a few millimeters of the right sciatic nerve. The incision was sutured by using a single stitch. The contralateral leg was considered as non-denervated control. Denervated rats were killed at 7 day after denervation with pentobarbital anesthesia and cervical dislocation.

Groups

Four groups were created including Group DS + C, Group DS + D, Group SB + C, and Group SB + D. Group DS + C was the contralateral (undenervated) leg of the animal which received 10% DMSO and not SB203580. Group DS + D was the denervated leg of the animal which received 10% DMSO and not SB203580. Group SB + C was the contralateral (undenervated) leg of the animal which received SB203580. Group SB + D was the denervated leg of the animal which received SB203580. SB203580 10 mg kg^{-1} or the same volume of 10% DMSO was respectively intraperitoneally injected into rats immediately after denervation once a day for 7 successive days. Fluid resuscitation was given to all rats via 10 mL of saline applied intraperitoneally. A heat lamp was used to keep rats warm until they recovered from anesthesia. A researcher in charge of grouping was not involved in following experiments and researchers participated in following experiments were blinded to the group assignment.

Western Blot Analysis

Tibialis muscles were homogenized at 4 °C in Lysis Buffer (Beyotime Biotech, China). Supernatants were collected from homogenates after centrifugation at 12,000×*g* for 15 min at 4 °C. Total protein content was assayed using the BCA protein assay kit (Beyotime Biotech, China) with bovine serum albumin as a standard. Protein extracts (30 μg) were then separated on SDS–polyacrylamide gels and transferred onto 0.2 μm nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were then blocked with 5% skimmed milk in Tris-buffered Saline with Tween 20 for 1 h at 37 °C, and then incubated overnight at 4 °C with primary antibodies. Blots were probed using antibodies (1:1000) directed against GAPDH or Actin (Kangchen Biotech, Inc., Shanghai, China), Histone (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HuR (Cell Signaling, Inc., USA), and p38, p-p38, β-AChR (Abcam, Inc., San Francisco, CA, USA). Chemiluminescent signals were visualized using ECL reagents (Millipore, Billerica, MA, USA) according to the manufacturer's protocol.

RNA Extraction and RT-PCR

Total RNA was extracted from tibialis muscles using TRIzol reagent (Invitrogen) as recommended by the manufacturer. TRIzol-extracted RNA was treated for 1 h with DNase I (Invitrogen) to eliminate possible DNA contamination. Approximately 1.0 μg of RNA was reverse transcribed using TaqMan™ Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). A real-time quantitative PCR was performed on an MX3005p real-time PCR system (Stratagene, La Jolla, CA, USA) using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies). The PCR reaction conditions were set as an initial denaturation step for 10 min at 95 °C to activate the polymerase, followed by 40 cycles of denaturation of 15 s at 95 °C, 30 s for annealing phase at 60 °C, and 30 s at 72 °C for extension. For these experiments, amplification of the β-AChR, 18S ribosomal subunit, and GAPDH was performed in triplicate with the following primer sequences: β-AChR, forward 5′-CATCAT CGCTCACCCCAC-3′ and reverse 5′-ACGGTCCACAAC CATGGC-3′, 18S Ribosomal, forward 5′-CGCCGCTAG AGGTGAAATC-3′ and reverse 5′-CCAGTCGGCATCGTT TATGG-3′, GAPDH, forward 5′-GGGTGTGAACCACGA GAAAT-3′ and reverse 5′-CCTTCCACAATGCCAAAG TT-3′.

In Vitro Stability Assays

In vitro stability assays were proceeded according to Joassard's description (Joassard et al. 2015). Proteins were extracted from tibialis muscles from DMSO- or SB203580-treated rats 7 days after denervation using 500 µL of precooling homogenization buffer [10 mmol L^{-1} Tris pH 8.0, 10 mmol L^{-1} KCl, 1.5 mmol L^{-1} $MgCl_2$, 2.5% IGEPAL CA-630, Protease Inhibitor Cock tail (25×) (Roche Diagnostics, USA)]. Protein extracts were centrifuged at 3500 g for 10 min after homogenization. Pellets were subsequently vortexed and incubated at 4 °C for 20 min in 100 µl extraction buffer [20 mmol L^{-1} Tris base pH8.0, 450 mmol L^{-1} NaCl, 10 mmol L^{-1} EDTA, Protease Inhibitor Cock tail (25×)]. After incubation, the pelleted fractions were centrifuged at 14,000 g for 10 min at 4 °C to remove cell debris. Supernatants enriched in cytoskeletal and nuclear fractions were collected and used for in vitro stability assays.

The RNA used in these assays was isolated from 3-day-old differentiated C2C12 myotubes (ATCC® CRL-1772™) using Tripure reagent (Roche Diagnostics, USA). Total RNA from C2C12 cells (0.2 µg/µl) and equal amount of tibialis muscles protein extracts (0.25 µg/µl) were incubated together in a degradation buffer (10 mmol L^{-1} Tris pH7.4, 10 mmol L^{-1} Potassium Acetate, 2 mmol L^{-1} Magnesium acetate, 2 mmol L^{-1} dithiothreitol (DTT), 0.1 mmol L^{-1} Spermin, 1 mmol L^{-1} adenosine Triphosphate (ATP), 0.4 mmol L^{-1} guanosine Triphosphate (GTP), 10 mmol L^{-1} Phosphocreatin, 1 µg creatine phosphokinase, 80 U SUPERNasin for 20 min at 37 °C. Time 0 was taken as RNA incubated in buffer without protein extracts for 1 min at 37 °C. The reactions were stopped at different time intervals by adding 250 µl of Trizol. The RNA was then precipitated with isopropanol in the presence of glycogen RNA grade (1 µl) (Thermo Fischer Scientific) as a carrier. The values of AChR β-subunit and 18S transcripts remaining at each time point were determined through RT-PCR analysis. Values were then plotted on a semi-logarithmic scale as a function of time. Four separate experiments were conducted, using four different muscle extracts. Half-life values were then determined relative to appropriate controls in each individual experiment. Relative half-life values were averaged and compared between samples.

Subcellular Fractionation

According to Dimauro's previous report (Dimauro et al. 2012), Tibialis muscles in rats were ground to power in liquid nitrogen with a mortar and pestle. Samples were resuspended in 300–500 µl of STM buffer containing 250 mM sucrose, 50 mM Tri-HCl pH 7.4, 5 mM $MgCl_2$ protease, and phosphatase inhibitor cocktails (Sigma-Aldrich, St.Louis, MO, USA) and homogenized for 1 min on ice in a Dounce homogenizer. After vortexed and centrifuged, the nuclei were lysed with 10–20 passages through sonication. The lysate was centrifuged at 9000 rpm for 30 min (4 °C), the resultant supernatant was nuclear fraction. The cytosolic fraction was extracted from supernatant after first centrifugation and centrifuged at 800 rpm for 10 min.

Immunofluorescence

Tibialis muscle was cross cut using a microtome and 10 µm cross-sections were obtained at − 20 °C. Sections were fixed using acetone for 1 min at room temperature. After be permeabilized for 45 min in 0.5% Triton X-100, the sections were incubated with a 1:200 dilution of mouse anti-HuR antibody (sc-5261, Santa Cruz Biotechnology) at 4 °C overnight. After three washes with phosphate-buffered saline (PBS), the preparations were incubated with m-IgGκBP-CFL 555 (sc-516177, Santa Cruz Biotechnology) used as secondary antibody for 1 h at room temperature in the dark. Immunofluorescence images were visualized and processed using an Leica DM5500 B microscope (Leica, Wetzlar, Germany).

RIP Assays

RNA immunoprecipitation assay was performed as previous report (Joassard et al. 2015). Tibialis muscles in rats were minced and mixed with 1% formaldehyde in phosphate buffer saline (PBS) for 1 h at room temperature. The reaction was stopped by washing with precooled PBS. Tissues was suspended in radioimmunoprecipitation assay (RIPA) buffer supplemented with ribonuclease (RNase) inhibitor (Applied Biosystems, Foster City, CA, USA) and homogenized using a polytron homogenizer. Equal amounts of whole cell extracts were immunoprecipitated with Protein A agarose-bound anti-HuR (Abcam, Cambrige, UK) or IgG as control. The breads were washed using modified RIPA buffer supplemented with 1 mol L^{-1} NaCl, 1% sodium deoxycholate, 2 mmol L^{-1} EDTA, and 2 mol L^{-1} urea; suspended with 50 mmol L^{-1} Tris pH 7.5, 5 mmol L^{-1} EDTA, 10 mmol L^{-1} dithiothreitol (DTT), and 1% SDS; and heated to 70 °C for 1 h to reverse cross-linking. RNA was extracted using Tri Reagent (Invitrogen, Carlsbad, CA, USA) and analyzed by Real-time PCR.

Statistical Analysis

Figure 2 was plotted on a semi-logarithmic scale as a function of time. Data were expressed as mean ± standard deviation (SD). Using One-way analysis of variance (ANOVA) with Tukey tests or unpaired *t* test compared whether differences in different groups were statistically significant. Statistically difference was defined as $P < 0.05$.

Results

SB203580 Inhibited Activation of p38 MAPK Following Skeletal Muscle Denervation

Denervation or application of SB203580 had no effects on protein expression of total p38 MAPK. Ratio of p-p38 to total p38 protein content increased 2.6 fold in the denervated side of the group treated with 10% DMSO compared with the contralateral side (0.18 ± 0.05 vs 0.40 ± 0.06, $P < 0.0001$). Compared with the contralateral side, ratio of p-p38 to total p38 protein content in the denervated skeletal muscle after the administration of SB203580 had no significant changes (0.17 ± 0.04 vs 0.22 ± 0.03, $P > 0.05$). However, compared with the denervated side of the group treated with 10% DMSO, ratio of p-p38 to total p38 protein content in the denervated side of the group treated with SB203580 significantly declined (0.40 ± 0.06 vs 0.22 ± 0.03, $P < 0.0001$) (Fig. 1). Above results suggested that SB203580 can suppress p38 MAPK activation induced by denervation in skeletal muscle.

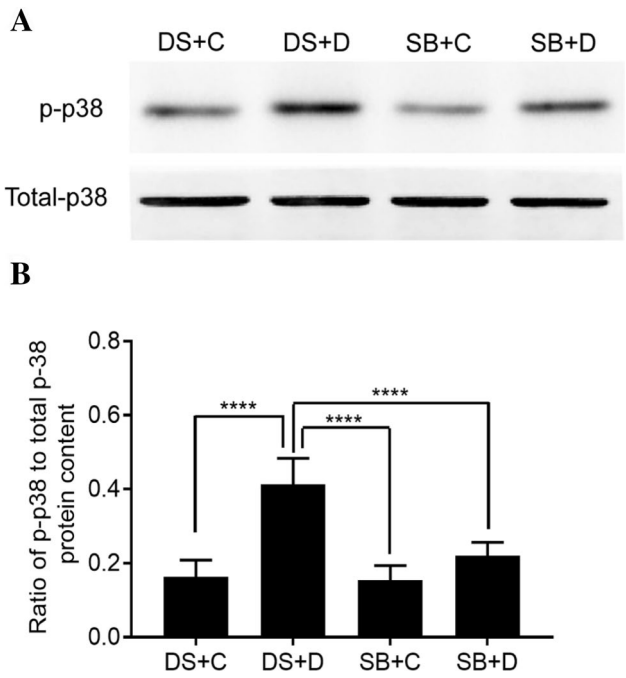

Fig. 1 Effects of SB203580 on p-p38 after denervation in skeletal muscle. **a** Representive immunoblots of p38 phosphorylation (p-p38) and total p38 protein content in different groups. **b** Quantification of ratio p-p38/total p38 in different groups. n = 5 per group, ****$P < 0.001$. *DS + C* DMSO + Control, *DS + D* DMSO + Denervation, *SB + C* SB203580 + Control, *SB + D* SB203580 + Denervation

SB203580 Antagonizes the Increased mRNA and Protein Expression of β-AChR Following Leg Denervation

mRNA and protein expression of β-AChR approximately increased 2.7- ($P < 0.001$) and 2.6-fold ($P < 0.001$), respectively, in the denervated side of the group treated with DMSO compared with the contralateral side. After the administration of SB203580, mRNA and protein expression level of β-AChR in the denervated skeletal muscle was 1.6-($P > 0.05$) and 1.3-fold greater ($P > 0.05$), respectively, compared with the contralateral side. Compared with DMSO administration, the administration of SB203580 respectively led to an approximate 38% and 40% reduction of β-AChR mRNA and protein expression (both $P < 0.01$) in the denervated skeletal muscle. Thus, we identified that the administration of SB203580 significantly inhibited the denervation-induced increase in mRNA and protein expression of β-AChR in rat skeletal muscle (Fig. 2).

In Vitro Stability Assays Showed that SB203580 Exerts Effect upon Stability of the AChR β-Subunit mRNAs Induced by Denervation

To test whether the alterations in protein expression of β-AChR described in the previous section were due to a change in the stability of AChR β-subunit mRNAs, we performed in vitro stability assays. Compared with the contralateral side, denervation significantly increased the half-life of the AChR β-subunit mRNA in the group treated with DMSO ($P < 0.0001$). The administration of SB203580 markedly reduced the half-life of AChR β-subunit mRNA ($P < 0.0001$) in the denervated side of the group treated with SB203580 compared to that in the denervated side of the group treated with DMSO (Fig. 3, Table 1). Collectively, these results suggested that SB203580 can antagonize the increased stability of AChR β-subunit mRNA induced by denervation.

SB203580 Inhibits HuR Protein Expression Induced by Skeletal Muscle Denervation

Denervation led to significantly increased expression of HuR protein in skeletal muscle ($P < 0.001$). SB203580, p38 MAPK inhibitor, can inhibit this effect induced by denervation ($P < 0.01$), but have no effect on innervated skeletal muscle ($P > 0.05$). Experiments also showed that HuR protein levels exerted a greater increase in expression in both nuclear and cytoplasmic fractions of the denervated side than the contralateral side in the group treated with DMSO ($P < 0.0001$). The administration of SB203580 had no effect upon HuR protein levels in the innervated tibialis muscles ($P > 0.05$), but significantly attenuated the increased protein

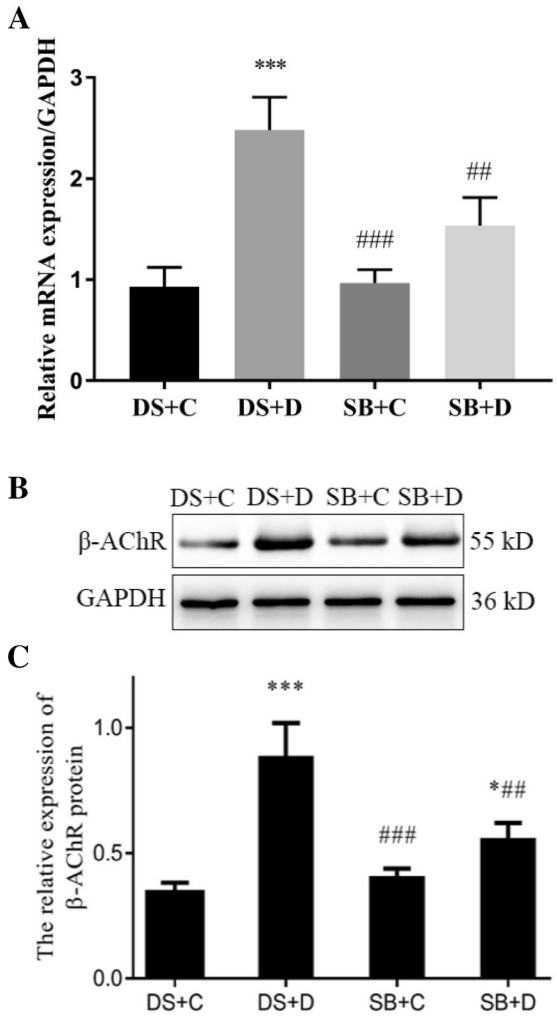

Fig. 2 SB203580 inhibited the denervation-induced increased mRNA and protein expression of β-AChR. **a** mRNA levels of β-AChR. **b** Representive immunoblots of β-AChR. **c** Quantification of β-AChR/GAPDH. Data are expressed as mean ± SD, n = 5 per group. $^{*}P < 0.05$, $^{***}P < 0.001$ versus Group DS + C; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Group DS + D. *DS + C* DMSO + Control, *DS + D* DMSO + Denervation, *SB + C* SB203580 + Control, *SB + D* SB203580 + Denervation

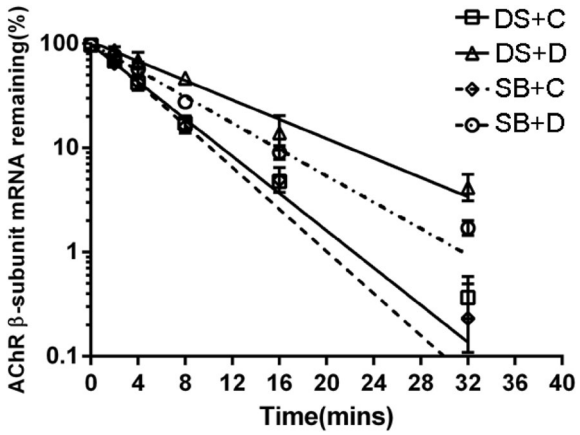

Fig. 3 Effects of SB203580 on AChR β-subunit mRNA stability in denervated skeletal muscles. Quantification of the amounts of AChR β-subunit mRNA at each time point was plotted on a semi-log scale as the percentage of RNA measured at time 0. The 18S transcript was used as a control. n = 5 per group. *DS + C* DMSO + Control, *DS + D* DMSO + Denervation, *SB + C* SB203580 + Control, *SB + D* SB203580 + Denervation

Table 1 AChR β-subunit mRNA half-lives across different groups

Groups	AChR β-subunit mRNA half-life (mins)
DS + C	3.30 ± 0.24
DS + D	$6.69 \pm 0.37^{****}$
SB + C	$3.27 \pm 0.18^{\#\#\#\#}$
SB + D	$4.54 \pm 0.24^{****\#\#\#\#\&\&\&\&}$

$^{****}P < 0.0001$ versus Group DS + C; $^{\#\#\#\#}P < 0.0001$ versus Group DS + D; $^{\&\&\&\&}P < 0.0001$ versus Group SB + C. *DS + C* DMSO + Control, *DS + D* DMSO + Denervation, *SB + C* SB203580 + Control, *SB + D* SB203580 + Denervation

expression of HuR induced by denervation in both nuclear and cytoplasmic fractions ($P < 0.0001$ and $P < 0.001$, respectively) (Fig. 4a–e).

In these experiments, we noted that the expression of HuR protein in the nuclear fraction was 2.0-fold higher than that in the cytoplasmic fraction in DS + C group. Following denervation, the ratio of cytoplasmic to nuclear fractions was almost 1 (Fig. 4f). HuR protein levels increased approximately three times, and six times, in nuclear and cytoplasmic fractions, respectively, after denervation in skeletal muscle administrated by DMSO. Furthermore, increased amounts of HuR in cytoplasmic fractions were significantly higher than that in nuclear fractions ($P < 0.0001$) (Fig. 4g). These data indicated that denervation accelerated the nucleo-cytoplasmic shuttling of HuR.

The administration of SB203580 inhibited the increased expression of HuR protein in both nuclear and cytoplasmic fractions in the denervated legs (both $P < 0.0001$) (Fig. 4d, e). However, the extent of inhibition upon HuR expression in the cytoplasmic fraction was no more intense than that in the nuclear fraction (Fig. 4h).

To verify above results, we undertook immunofluorescence experiments with antibody against HuR in tibialis muscle cross-sections. The results validated that denervation caused HuR increase both nuclear and cytoplasmic fractions as compared to innervation and SB23580 effectively suppressed HuR expression both nuclear and cytoplasmic fractions (Fig. 5).

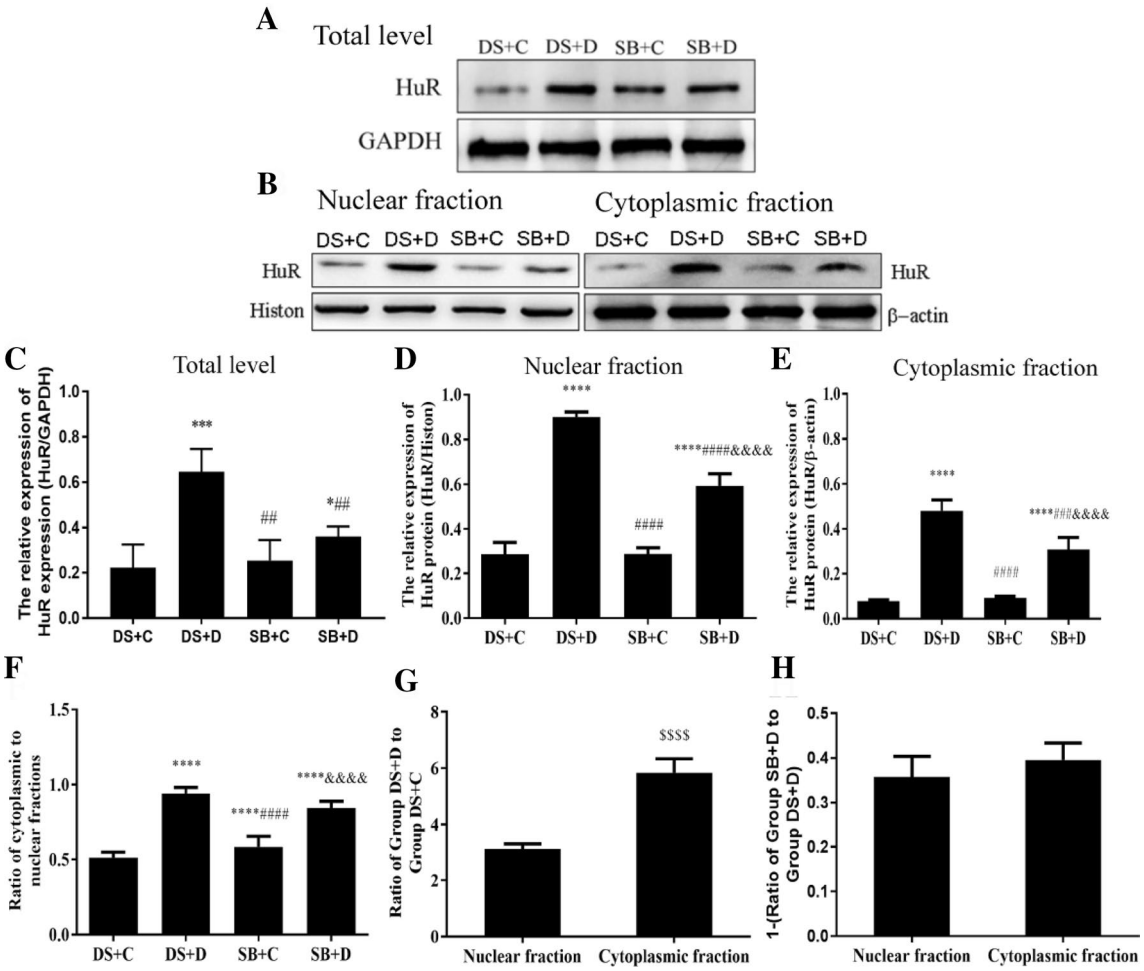

Fig. 4 Representative western blots (**a** and **b**) and their quantification (**c**–**h**) for HuR protein expression from total level and subcellular fractions of DMSO-administered and SB203580-administered innervated and denervated tibialis muscles. Data are expressed as mean ± SD, n = 5 per group. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus Group DS + C; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ versus Group DS + D; $^{\&\&\&\&}P < 0.0001$ versus Group SB + C; $^{\$\$\$\$}P < 0.0001$ versus Group nuclear fraction. *DS + C* DMSO + Control, *DS + D* DMSO + Denervation, *SB + C* SB203580 + Control, *SB + D* SB203580 + Denervation

The Effect of SB203580 upon HuR Binding with AChR β-Subunit mRNAs in Denervated Skeletal Muscle

Next, we wanted to examine whether there was an interaction between HuR and AChR β-subunit mRNAs and what effects SB203580 might exert upon such an interaction. As shown in Fig. 5, HuR did indeed bind to AChR β-subunit mRNAs in DS + C group. The binding of HuR to AChR β-subunit mRNAs was significantly increased in denervated skeletal muscle when compared to contralateral skeletal muscle after treated by DMSO ($P < 0.001$). Compared with DMSO administration, the administration of SB203580 significantly reduced the extent of binding between HuR and AChR β-subunit mRNAs in denervated skeletal muscles ($P < 0.05$) (Fig. 6).

Discussion

Our study indicated that SB203580, a p38 MAPK inhibitor, can effectively inhibit denervation-induced increased expression of β-AChR protein, which was associated with changes in the stability of AChR β-subunit mRNA by regulating the expression of HuR and binding of HuR to AChR β-subunit mRNAs following denervation.

AChRs are well-characterized heteromeric transmembrane proteins made up of four constitutive subunits (2α, 1β, and 1δ) and one variable γ subunit which switches to an ε isoform during muscle development in order to form adult AChRs (Wang et al. 2010a, b). Studies have demonstrated that the AChR β-subunit is essential for AChR assembly (Quiram et al. 1999) and becomes a rate-limiting factor in the assembly of AChRs (Saedi et al. 1991). Furthermore,

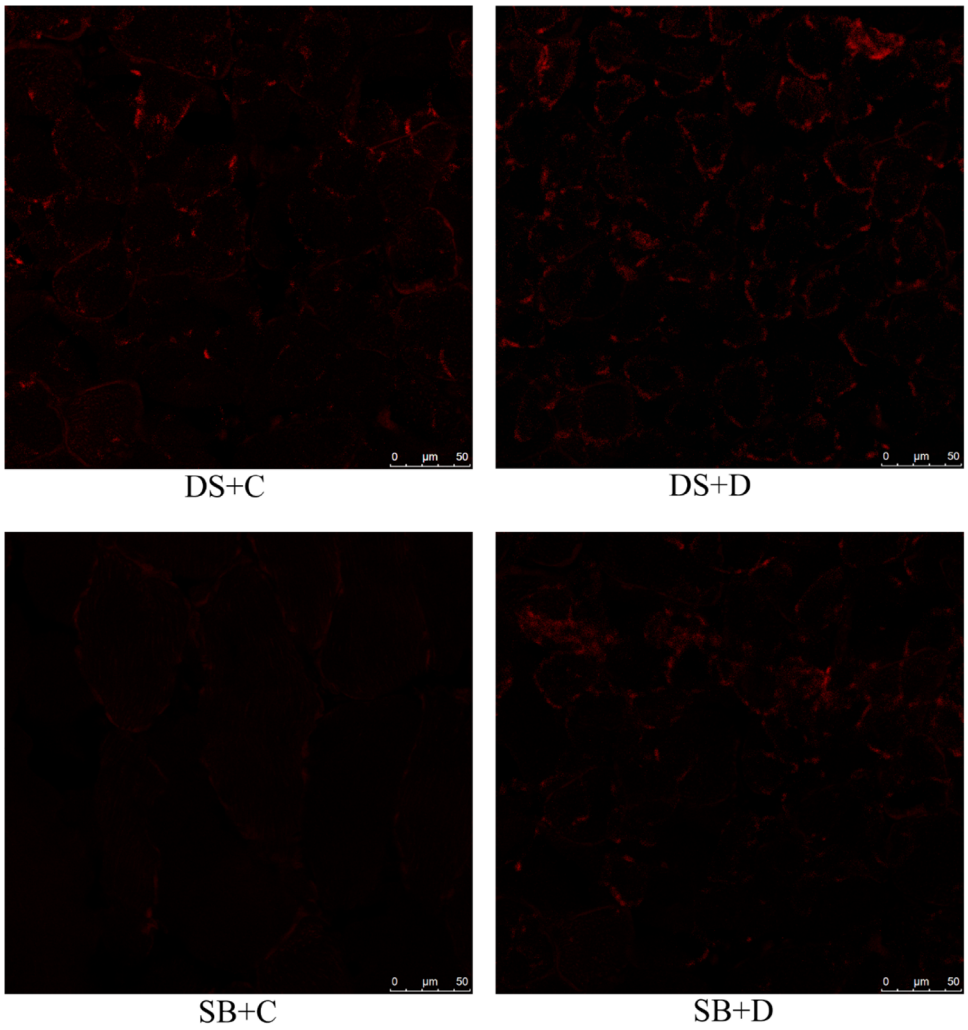

Fig. 5 The immunofluorescent staining for HuR protein expression from DMSO-administered and SB203580-administered innervated and denervated tibialis muscles. *DS* + *C* DMSO + Control, *DS* + *D* DMSO + Denervation, *SB* + *C* SB203580 + Control, *SB* + *D* SB203580 + Denervation

sequence comparison across human, orangutan, cow, rat, and mouse revealed that one ARE in the 3′-untranslated region (3′UTR) of the β-subunit of AChR mRNA was conserved across multiple species (Joassard et al. 2015). Consequently, in this study we investigated the AChR β-subunit and observed changes of its protein expression and mRNA stability following denervation.

HuR is a well-characterized stabilizing RBP, and a ubiquitously expressed protein of the ELAV-1 family (embryonic lethal abnormal vision in Drosophila) (von Roretz et al. 2011). HuR is predominantly located within the nucleus and is exported to the cytoplasm when stimulated. This stimulus-dependent transport between the nucleus and the cytoplasm is referred to as 'HuR shuttling'. By binding to ARE-containing mRNAs in the nucleus, HuR transports mRNAs through nuclear pores and protects them during and after export to the cytoplasm, thereby enhancing mRNA stability (Pan et al. 2005; David et al. 2007; Doller et al. 2008). In the present study we observed that there were a few expression of HuR in the innervated skeletal muscle, but a significant increase of HuR in the nucleus and the cytoplasm, especially in the cytoplasm, in the denervated skeletal muscle. Furthermore, the amount of AChR β-subunit mRNA was found to parallel the expression of HuR in the innervated and denervated skeletal muscle by RNA immunoprecipitation (RIP) assays which showed that denervation induced the expression of AChR β-subunit mRNA by regulating binding of HuR to AChR β-subunit mRNA. Above results suggested that denervation induced increased HuR expression and binding of HuR to AChR β-subunit mRNA which caused an increase of AChR β-subunit mRNA expression.

A range of external stimuli are known to activate the p38 pathway, including UV light, osmotic shock, heat, growth factors and inflammatory cytokines. The p38 pathway regulates a number of cellular responses including cell differentiation and apoptosis. P38 has been implicated as a critical participant in modulating the stability of mRNAs containing the AU-rich element (ARE) (Wu et al. 2013). Subbaramaiah et al. (2003) reported the direct involvement of p38-MAPK in the HuR-mediated stabilization of cyclooxygenase-2

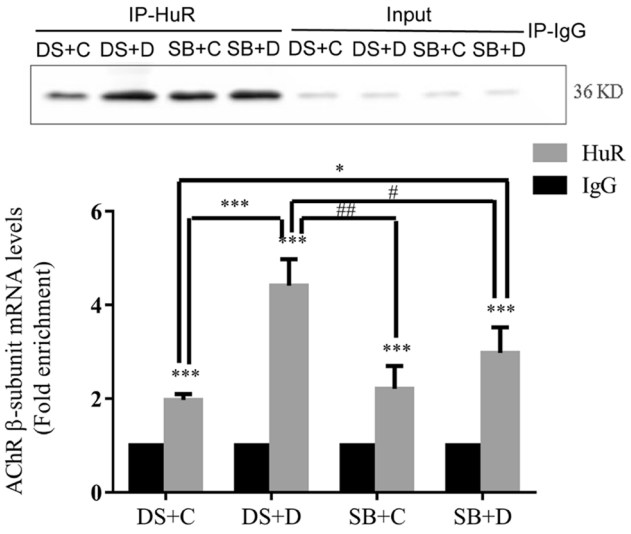

Fig. 6 RNA immunoprecipitation (RIP) experiments were performed in respective groups. **a** a representative western blot confirming the quantity of immunoprecipitated HuR (Input and IP). **b** The relative amount of AChR β-subunit mRNA in each immunoprecipitate. Data are expressed as mean ± SD, n = 5 per group. $^{***}P < 0.001$ versus Group DS + C and corresponding Group IgG, respectively; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Group DS + D. *DS + C* DMSO + Control, *DS + D* DMSO + Denervation, *SB + C* SB203580 + Control, *SB + D* SB203580 + Denervation

(COX-2) mRNA in human mammary epithelial cells in response to taxanes. Furthermore, the angiogenesis inhibiting drug, thalidomide, was reported to destabilize COX-2 mRNA by inhibiting p38 activity, a process which was accompanied by reduced HuR shuttling (Jin et al. 2007). Lin's study also showed that endotoxin induced the stabilization of toll-like receptor 4 mRNA relying upon activation of the p38-MAPK pathway (Lin et al. 2006). Joassard et al. (Joassard et al. 2015) demonstrated that p38 pathway regulated expression and stability of AChR β-subunit transcripts in vitro experiment. Consistent with Joassard's study, we certified that SB203580, an inhibitor of p38, inhibited the increased stability of β-AChR mRNA induced by denervation in vivo.

Denervation induced the upregulated protein expression of AChR, which was attributable to an increased amount of mRNA. These changes in the amount of mRNA available may arise from the levels of transcriptional mRNA and post-transcriptional mRNA stability. Ma's study have demonstrated that denervation activated transcription of AChR mRNA (Ma et al. 2007). Similarly, Joassard et al. (2015) verified that a post-transcriptional mechanism was involved in regulating AChR protein expression. Though Joassard's report showed that p38 pathway regulated expression and stability of AChR β-subunit transcripts in vitro, results obtained from in vitro experiments were not always in accordance with its in vivo. Furthermore, it did not explore whether HuR involved in the interaction between p38 pathway and stability of AChR β-subunit mRNA. So, in this study, we wanted to see whether p38 pathway regulated HuR expression and binding of HuR to AChR β-subunit mRNA which changed the stability of AChR β-subunit mRNA in vivo experiments. Results manifested that inhibiting activation of the p38 pathway reduced AChR β-subunit mRNA and protein expression, the stability of AChR β-subunit mRNA and HuR expression. Furthermore, decreased extent of AChR β-subunit mRNA paralleled with expression of HuR after inhibiting p38 owing to reduced interaction between HuR and AChR β-subunit mRNA. Thus, our data indicated that p38 pathway post-transcriptionally regulated the expression of AChR via HuR in skeletal muscle after denervation.

In summary, our data suggested that denervation led to an increase in the expression of AChRs partly from a post-transcriptional mechanism. Blockade of the p38 pathway can effectively reduce the AChR expression via reduced expression of HuR and interaction between HuR and AChR β-subunit mRNA.

Author Contributions HW and S-tL designed the study. HW carried out the study and drafted the manuscript. XZ collected important background information and performed the statistical analysis. HW, XZ, and YW carried out the molecular biology experiments. L-HC carried out literature, data acquisition, and manuscript editing. All authors reviewed the manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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