INHALED CORTICOSTEROIDS MODULATE THE (+)INSERT SMOOTH MUSCLE MYOSIN HEAVY CHAIN IN THE EQUINE ASTHMATIC AIRWAYS

Roxane Boivin, Amandine Vargas, Josiane Lefebvre-Lavoie, Anne-Marie Lauzon, Jean-Pierre Lavoie

Online Data Supplement

METHODS

Reverse transcription

For each sample, either 1 µg or 500 ng (endobronchial biopsies) of total RNA was reverse transcribed in a total reaction volume of 20 µL with 1 µL of RNAsin 10 000 U (Promega, Madison, WI, USA), 1 µL of oligo $(dT)_{12–18}$ primer 0.5 µg/µL, 1 µL of Superscript III 200 U/µL, 1 µL of dNTP Mix 10 mM, 4 µL of Buffer First-Strand 5X, and 1 µL of DTT 0.1 M (Invitrogen, Carlsbad, CA, USA). Reverse transcription reactions were done at 65 °C for 5 minutes, 50 °C for 60 minutes and 70 °C for 15 minutes with appropriate no RT control. All reactions were purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions.

Quantitative PCR (qPCR)

Two microliter of cDNA template were added to obtain a 20 µL final reaction volume containing 0.5 µM forward and reverse primers. The 21 bp (+)insert SMMHC sequence itself served as the insert reverse primer. qPCR conditions were optimized for all primer sets. Amplification conditions included a denaturation step of 10 min at 95 °C followed by 45 cycles of denaturation, annealing and elongation steps, and one melting curve. All concentrations of target gene cDNA were calculated relatively to their respective standard curves. Each primer set generated only one PCR product, and the identity and integrity of these products were confirmed by sequencing. Relative expression of (+)insert was therefore quantified by reporting the content of this isoform to total SMMHC.

(+)insert relative protein analysis (mass spectrometry)

Protein in-gel digestion

Bands were placed in 96-well plates and then washed with water. Tryptic digestion was performed on a MassPrep liquid handling robot (Waters, Milford, USA) according to the manufacturer's specifications and to the protocol of Shevchenko *et al*(1) with the modifications suggested by Havlis *et al*(2). Briefly, proteins were reduced with 10 mM DTT and alkylated with 55mM iodoacetamide. Trypsin digestion was performed using 126 nM of modified porcine trypsin (Promega, Madison, WI, USA) at 58 °C for 1 h. Digestion products were extracted using 1% formic acid, 2% acetonitrile followed by 1% formic acid, 50% acetonitrile. The recovered extracts were pooled, vacuum centrifuge dried and then resuspended into 15 µL of 0.1% formic acid and 5 µL were analyzed by mass spectrometry.

SIS peptides

Purified synthetic peptides containing [$^{13}C_6$ $^{15}N_2$]Lys and [$^{13}C_6$, $^{15}N_4$]Arg (Thermo Fisher Scientific, Ulm, Germany) were reconstituted in 0.1% formic acid to a final concentration of 50 pmol/µL. A solution containing 20 pmol/µL of DTSITQGPPLAYGELE [Lys($^{13}C_6$; $^{15}N_2$)] ((+)insert SMMHC) and 2.5 pmol/uL of NADLTSEL [Arg($^{13}C_6$; $^{15}N_4$)] (total SMMHC) was prepared from the stock solutions and used to reconstitute the samples after tryptic digestion for relative quantification.

LC-MRM analysis

Samples were analyzed on a ABSciex 5500QTRAPTM hybrid triple quadrupole/linear ion trap mass spectrometer equipped with an Eksigent nanoLC AS2 cHiPLC nanoflexcontrolled by Analyst 1.6TM and with a nanospray ionization source. MS analysis was conducted in positive

ion mode with an ion spray voltage of 2300 V. For each samples, 5 µL were injected. Peptides were desalted on a 200 µm x 6 mm chip trap column packed with ChromXP C18, 3 um, (Eksigent part of ABSciex, Dublin, CA, USA) at 2 µL/min of Solvent A (formic 0.1%) then switched in line at a flow rate of 1000 nL/min on a 200 um x 15 cm chip column packed with ChromXP C18, 3 µm (Eksigent part of ABSciex, Dublin, CA, USA) with a 16 min linear gradient from 5 to 25% of solvent B (ACN 0.1% FA), then a 2 min linear gradient from 25 to 80% B, followed by a 2 min linear gradient. Cycle times was 2.24 sec. Nebulizer gas was set to 8 (Gas1), curtain gas to 20 and heater to 150 °C. Preset values for declustering potential(3) and collision energy (CE) were used. LC-MRM/MS analyses were performed using all the transitions from y and b ions with m/z greater than the precursor for each peptide. Quantification was done with MultiQuant 2.1 and based on the relative areas of the SIS and endogenous peptides. The MRM transition that gave the highest area counts was used for the quantitation, with the other transitions acting as qualifier transitions to confirm peptide retention times and the fragment ion ratios. A blank solvent injection was run between biological samples to prevent sample carryover on the HPLC column and the samples were injected in random order. Samples were analyzed in duplicate, i.e., two repeats of the same protein sample.

REFERENCES

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