Glucocorticoid receptor beta and Histone Deacetylase1 & 2 expression in the airways of severe asthma

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ONLINE DATA SUPPLEMENT

METHODS

Ethical approval

The study was approved by the Research Ethics Committees of each Institution (Belfast - Office of Research and Ethics Committee of Northern Ireland reference 06/NIR02/114, Leicester - Leicestershire, Northamptonshire, & Rutland Research Ethics Committee reference 04/Q2502/74). Written informed consent was gained from all participants prior to their involvement.

Subjects

Subjects with asthma had a clinical diagnosis of asthma with a current history of recurrent wheezing and response to asthma medication. Healthy volunteers had no history of asthma or persistent respiratory symptoms and normal lung function. All participants had an FEV_1 of greater than 60% predicted.

Asthmatic subjects in Leicester underwent further formal assessment and demonstrated one or more of the following: FEV1 bronchodilator response >12% in response to salbutamol, PC_{20} methacholine <8 mg/ml or PEF % amplitude of the mean >20%. All participants were current non-smokers with no upper or lower respiratory tract infection in the 6 weeks prior to their bronchoscopy. All asthmatic participants were clinically stable on their usual medication at the time of bronchoscopy. Written informed consent was gained from all participants prior to their involvement.

Bronchoscopy

Bronchoscopy was performed using standard technique. In brief, after intravenous sedation and local anaesthesia with topical lignocaine, bronchial biopsies and bronchial bushings were obtained from lobar / segmental airways.

Gene expression analysis

RNA samples were quantified with a Nanodrop ND-1000 UV-spectrophotometer (Thermo Scientific, West Palm Beach, FL), and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only RNA samples with a RNA Integrity Number RIN value greater or equal to 5 were used for subsequent gene expression analyses. RNA was converted to cDNA by whole transcriptome amplification (Qiagen, Valencia, CA) for subsequent qPCR analyses.

C_T values were averaged among technical quadruplicates. Delta C_T was calculated as target gene expression (C_T) minus internal housekeeping control, GAPDH gene expression (C_T). Human Universal Reference RNA (Clontech, P/N 639654) was prepared as cDNA and utilised as plate calibrators. DeltaDelta C_T was calculated as sample Delta C_T minus plate calibrator Delta C_T.

We utilized several filtering criteria to ensure high quality, interpretable qPCR results for target and housekeeping gene analyses. Each of the following criteria were required for all qPCR data for acceptability:

1. C_T values for negative control samples, mock RT (healthy control biopsy sample) and no template control were > 40.
2. C_T values for samples were > 15 and < 40.
3. COV for replicate wells for a sample < 10%.

GAPDH C_T values were within the range of 16.7 and 30.0 C_T.

Bronchial biopsy immunohistochemistry

Following infiltration and embedding, two μm sections were cut from each biopsy. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide. In Belfast, antigen retrieval was performed on all sections (pressure cooked in 0.01M Tris-EDTA pH 9.0 for 3 minutes). Sections were incubated with primary antibody overnight at 4°C, then primary antibodies were detected by incubation for 30 min at RT with peroxidase-labelled EnVision anti-mouse or anti-rabbit secondary antibodies (both Dako, Ely, UK) using 3,3'-diaminobenzidine (DAB; Dako, Ely, UK) as chromagen. Matched biopsies from the Leicester cohort were processed for immunohistochemistry in a similar fashion and stained as described previously using 3-Amino-9-ethylcarbazole, which gave a red reaction product.

A 4-point scoring system was used: 0 - no detectable expression in epithelium or submucosa; 1 - patchy or focal areas of low intensity epithelial expression, not all cells expressing positivity for protein, scattered positivity in submucosa; 2 - moderate / majority of epithelial and submucosal cell expression; 3 - widespread and strong epithelial and submucosal cell expression.

For confocal analysis, primary antibodies were incubated on sections for 1 h at 37°C, washed in PBS, and detected by incubating in goat anti-rabbit Alexa 568 (Molecular Probes, 1:500) for 1 h at 37°C. All sections were mounted in propidium iodide (Sigma, 1:5000) for examination under a ×40 oil-immersion objective on a Leica TCS/NT confocal microscope equipped with a krypton–argon laser.

Epithelial cell culture and Western blotting

Bronchial brushings were cultured in Promocell® medium (Promocell, UK) and at Passage 1, cell lysates produced (NE-PER lysis kit, Pierce Biotechnology, MSC, Dublin). Lysates were boiled in reducing buffer for 5 min then 20µg of protein was loaded per well and 8% gels were run at 100V for 45 min followed by transfer onto a nitrocellulose membrane for 2 h. Blots were blocked with 5% non-fat milk in Tris-buffered saline (TBS) to reduce non-specific antibody binding followed by incubation with primary antibody overnight at 4°C (antibodies as above), then incubation with a HRP-linked secondary antibody for 1 h at room temperature (ab6721, Abcam).

Immunocytochemistry

Primary bronchial epithelial cells were grown onto collagen-coated Thermanox coverslips (Cat no. 174950, NUNC) and cultured at 37°C and 5%CO_2 until 80% confluent. Coverslips were washed twice with PBS and then fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature. In order to achieve nuclear staining, cells were permeabilised for 15 minutes with PBS containing 0.01% saponin (Cat no. S4521, Sigma-Aldrich, UK), 0.1% BSA (Cat no. A-2153, Sigma-Aldrich,

UK), and 1% Tween-20 (Cat no. 11322465001, Roche, UK). Following two washes with PBS containing 0.1% Tween-20, non-specific binding was blocked with 1% BSA in PBS, and primary antibody (in PBS) was applied overnight. The following day, coverslips were washed twice with PBS/0.1% Tween-20 and then incubated for 1 hour at room temperature with Alexoflour-488-conjugated secondary antibody. Five minutes before the end of this incubation, nuclear stain was added and following two further washes in PBS/0.1% Tween-20 coverslips were mounted on slides with Vectashield (Vectorshield Laboratories, Dublin) and assessed with on the Fluorescence microscope.

Immunoprecipitation

Briefly, 50μg cell lysate was supplemented with 450ul RIPA buffer containing protease inhibitors followed by addition of anti- GRβ antibody (1:10 dilution). Samples were rocked on ice for 2 h to allow antibody binding then 25ul of pre-washed magnetic beads were added to each lysate mixture and rocked overnight at +4°C. Beads were washed three times with wash buffer (50mM Tris pH7.5, 150mM NaCl, 0.1% Triton X diluted in sterile water), with the supernatant removed following each wash. Sample reducing buffer was added to the magnetic bead-antibody-protein immunoprecipitate and boiled (5 min, 95°C) to separate the beads from the antibody and protein. The supernatant was loaded onto gels which were run as described above.

ADDITIONAL RESULTS

Study population

Patients in the severe asthmatic group were significantly older than the other two study groups and had significantly lower and more obstructive lung function (Table 1). Duration of asthma and both dose of inhaled steroid and number of add-on therapies were greater in the severe asthma group compared to the mild/ moderate asthmatics; 15 of 31 severe asthmatics were on maintenance systemic steroids. More of the severe asthma participants had previously smoked, though the pack-year history was low with a median of 2.5 years.

Figure 5. Western blots of nuclear (A) and cytoplasmic (B) extracts from primary bronchial epithelial cell lysates stained with anti-GRβ antibody (ab3581) demonstrating the 90kDa and 180kDa band in both compartments.