Supplementary Material

NOX4 over-expression mediates intrinsic airway smooth muscle hyper-contractility in asthma

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Material and Methods

Subjects

Asthmatic subjects and healthy controls were recruited from Leicester, UK. Asthmatic subjects had a consistent history and objective evidence of asthma. Asthma severity was defined by Global Initiative for asthma treatment steps (mild-moderate GINA 1-3, severe GINA 4-5) (2). Subjects underwent extensive clinical characterization including sputum induction and video-assisted fibreoptic bronchoscopic examination. The study was approved by the Leicestershire Ethics Committees and all patients gave their written informed consent.

Cell isolation and culture

Pure airway smooth muscle bundles were isolated from bronchoscopic samples (n=23 asthma, n=19 non-asthma) and from lung resection from subjects with normal lung function (n=6). Airway smooth muscle was cultured in DMEM with Glutamax-1 supplemented with 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin, 0.25µg/mL amphotericin, 100µM non-essential amino-acids and 1mM sodium pyruvate. airway smooth muscle ASM cell characteristics were determined by immunofluorescence and light microscopy with α-smooth muscle actin-FITC direct conjugate (Sigma, Gillingham, Dorset, UK) as previously described[8].

Immunohistochemistry

Sequential 2µm sections were cut from glycomethacrylate embedded bronchial biopsies and stained using a monoclonal anti-human 8-oxodg antibody (Abcam, Cambridge, UK), and appropriate isotype control mouse IgG1 (DAKO, Cambridge, UK). Positive 8-oxodG staining was observed within the nuclei. 8-oxodG staining by the airway smooth muscle was

assessed using a semi-quantitative score of no staining=0, very low=1, low=2, moderate=3, high=4, and very high=5.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and immunoblotting

Airway smooth muscle cells from normal and asthma donors were grown to about 90% confluence. The cells were washed once with ice-cold PBS and then scraped into 1xSDS-sample buffer containing 1% SDS, 62.5mM Tris-HCl pH 6.8, 2.5% β-mercaptoethanol, 10% glycerol and 0.0005% bromophenol blue. The lysates were sonicated, and SDS-PAGE and western-blotting were performed as described (23). Briefly, samples were subjected to electrophoresis on SDS-polyacrylamide gels and gels were transferred to Polyvinylidene fluoride membrane (Millipore). Rabbit polyclonal anti-human SOD2 and SOD1 were purchased from Abcam and detection used horse anti-rabbit IgG HRP-conjugated secondary antibody (Cell Signaling Technology). Mouse anti-actin antibody was purchased from Santa Cruz as a HRP conjugate. Detection was by enhanced chemiluminescence (GE healthcare).

Human 8-oxoguanine DNA glycosylase 1 (hOGG1)-modified comet assay (hOGG1 comet)

DNA damage was assessed using the human 8-oxoguanine glycosylase 1 (hOGG1)-modified comet assay (hOGG1 comet), as previously described (24). Airway smooth muscle cells were incubated in the presence or absence of 100μM H_2O_2 for 30min and then suspended in 0.6% low melting point agarose. Eighty microlitres of the agarose gel (containing approximately 2×10^4 cells) were dispensed onto glass microscope slides, coated previously with 1% normal melting point agarose. The agarose was allowed to set on ice under a coverslip and the slides left overnight in ice-cold lysis buffer (100mM disodium EDTA, 2.5M NaCl, 10mM Tris-HCl, pH 10, containing 1% triton X-100, which was added freshly). Slides were washed once with

distilled water and immersed in two changes of enzyme digestion buffer [40mM HEPES, 0.1M KCl, 0.5mM EDTA and 0.2mg/ml BSA (pH 8.0)], for 5 min each time, at room temperature. hOGG1 was added to the gel (50µL/gel) diluted in enzyme reaction buffer to a final concentration of 4U/mL. Gels were covered with a cover slip and incubated in a humidified chamber for 45 min at 37 ºC. The cover slips were removed and the slides were placed in a horizontal electrophoresis tank, covered with cold alkaline electrophoresis buffer (300mM NaOH, 1mM disodium EDTA, pH ≥ 13) for 20 min and electrophoresed at 27V (0.7V/cm) and 300mA for 20 min. Slides were neutralised with 0.4M Tris-HCl, pH 7.5 for 20 min, washed with distilled water and then allowed to dry. All procedures were carried out under subdued light to minimise adventitious DNA damage. For staining, slides were re-hydrated in distilled water, incubated with a freshly made solution of 2.5µg/mL propidium iodide (PI) for 20 min, washed again for 30 min and allowed to dry. Comets were visualised by fluorescence microscopy at ×200 magnification. Images were captured by an on-line CCD camera and analysed with the Perceptive Instruments Comet Assay software version IV (Perceptive Instruments, Haverhill, UK). A total of 100 cells were analysed per sample, 50 per duplicate slide. The tail moment was calculated for each cell by Perceptive Instruments Comet Assay IV software.

Flow cytometry

Confluent (80-90%) airway smooth muscle cells were growth arrested in insulin/transferrin-sodium selenite (ITS) media (ITS+3; Sigma-Aldrich, Gillingham, Dorset, UK) for 24h. Airway smooth muscle were fixed with 4% paraformaldehyde with or without saponin 0.1% for extracellular or intracellular staining; re-suspended and permeabilised in PBS/0.5% BSA with or without 0.1% saponin (1×10^6cells/mL) as previously described (20). Cells were stained with antibodies to rabbit polyclonal NOX4 (Abcam, Cambridge, UK), NOX 1-3 and

5 (Insight, Biotechnology, Wembly, UK) or mouse monoclonal bradykinin B2 receptor indirectly labelled with FITC (BD) or their appropriate isotype controls indirectly labelled with fluorescein isothiocyanate (FITC; Dako), and cells were stained with α-smooth muscle actin FITC direct conjugate (Sigma) or appropriate isotype control (Dako). Analysis was performed by single colour flow cytometry (FACScan, CellQuest software Becton-Dickinson, Oxford, UK) and was quantified as percentage positive compared to control.

Immunofluorescence

Cells were stained with polyclonal antibodies for NOX1-5 or SOD2 appropriate isotype controls, indirectly labeled with FITC and counterstained with 4',6'-diamidino-2 phenylindole (DAPI; Sigma).

Gene array and analysis

RNA expression levels from the airway smooth muscle were examined using the Human Genome U133A probe array (GeneChip, Affymetrix, Santa Clara, CA, USA). Hybridized biotinylated cRNA was stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA), scanned with a HP Gene Array Scanner. All Affymetrix U133 plus 2.0 GeneChip® arrays passed the quality control characteristics. Image data from each microarray were individually scaled to an intensity of 200 using GCOS (Affymetrix). Scaled average difference and absolute call data were exported to text files and used for analysis implemented by DNA Chip Analyzer (D-CHIP) to determine genes that demonstrated significant differential expression between health and disease by >2-fold up or down-regulated following 1000 permutations or were present in ≥5 asthmatic/healthy compared to ≤1 healthy/asthmatics.

Real-Time Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated using Peq Gold total RNA kit (Peq Lab). The concentration and purity of each sample were determined by analyzing spectrophotometric absorption at 260/280nm. Real-Time Reverse Transcription–Polymerase Chain Reaction was performed using SuperScript Vilo cDNA synthesis kit and amplification performed with Express SYBR GreenER qPCR Supermix Universal (Invitrogen). The internal normaliser gene used was 18S RNA, and was carried out with 18S forward (GTTGGTTTTCGGAACTGAGG) and 18S reverse (GCATCGTTTATGGTCGGAAC) primers, while amplification of NOX4 was carried out with NOX4 forward (TGGCTGCCCATCTGGTGAATG) and NOX4 reverse (GCATCGTTTATGGTCGGAAC) primers as previously described (25). The relative quantification was done using the comparative deltadeltact method ($2^{-\Delta\Delta Ct}$) method and expressed as fold change as previously described (25).

Assessment of airway smooth muscle contraction by Collagen Gel Analysis

Airway smooth muscle cells were harvested either post-treatment with diphenyleneiodonium (DPI) (1, 5µM for 6h; Sigma) apocynin (10µM for 30min;), or the SOD mimetic manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) (10, 50, 100µg/mL for 6h; Enzo Life Sciences), or post-transfection with anti NOX-4 or anti SOD-2 siRNA, and their contractile properties assessed using collagen gel contraction assay.

Collagen gels (299µL of collagen [Inamed Biomaterials], 37µL of 10× DMEM [Invitrogen], 20µl of sodium bicarbonate [Invitrogen]) were impregnated with 144µL airway smooth muscle cells resuspended in DMEM with Glutamax-1 supplemented with penicillin (100U/mL), streptomycin (100µg/mL), amphotericin (0.25µg/mL), non-essential amino-acids (100µM) (Invitrogen), sodium pyruvate (1mM) and insulin-transferrin-selenium (1%)

(Sigma) at 1.735×10^6 cells/mL, as previously described (20). 500µL of gel mixture was added to each well of a PBS 2% BSA pre-coated 24-well plate and allowed to polymerize at 37°C for 90min. After polymerization, 500µL DMEM with Glutamax-1 (supplemented as above) was added to each well and the gel was detached from the plastic surface to allow free contraction. Bradykinin was then added to each well (final concentration 1nM) in an equal volume of the above media. The collagen gels were photographed at specified time points over a 1h period and the gel size as a percentage of the well area was calculated by measuring the collagen gel area versus the well area at specific time points using ImageJ software (National Institutes of Health, USA). All gel conditions were performed in duplicate. Cell viability in the gels was mean (SEM) 87 (3)% assessed by trypan blue exclusion in cells recovered from the gels by collagenase digestion (Sigma).

Silencing of NOX4 and SOD2 expression using siRNAs

NOX4 and SOD2 expression were specifically silenced in airway smooth muscle cells using two 25 nucleotide prevalidated siRNA duplexes (StealthTM Select RNAi purchased from Invitrogen) directed against human NOX4 (siRNA14: sense 5'CCUCAUGAUCACAGCCUCUACAUAU3' and antisense 5'AUAUGUAGAGGCUGUGAUCAUGAGG3'and against human SOD2 (siRNA34: sense 5'AAUCAACUGGGAGAAUGUAACUGAA3' and antisense 5'UUCAGUUACAUUCUCCCAGUUGAUU3'. A siRNA that showed no significant homology to any known protein was used as a negative control (Stealth™ RNAi negative control; Invitrogen). siRNAs were introduced into airway smooth muscle cells by nucleofection (Lonza AG, Cologne, Germany). Transfections were performed according to the manufacturer's instructions. Transfection efficiency was >90% as assessed by monitoring the transfection efficiency of fluorescent siRNA (Block-itTM Fluorescent Oligo from

Invitrogen) and knockdown of NOX4 and SOD2 gene expression was about 80-90% and 90-95% respectively as determined by qPCR.

Intra-cellular reactive oxygen species (DCFDA assay)

Viable cells (10^4/well) were plated into 96-well collagen, allowed to adhere overnight and serum starved for 24h. The cells were washed with PBS and incubated with 10μM 2′,7′-dichlorofluorescin diacetate (DCFDA) in 5% CO_2/95% air at 37°C for 30min, as described previously (26). Cells were then washedand incubated with PBS or 10mM H_2O_2 Fluorescence was measured using a Novostar plate reader (BMG Labtech, Germany); temperature was maintained at 37°C, and read at excitation 485nm and emission 520nm after 1h.

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Figures Legends

Figure E1 (A) Quantitative RT-PCR analysis of SOD2 mRNA in normal and asthmatic ASM cells (mean±SEM; n=18). (B) SOD2 expression by ASM by immunofluroscence left panel rabbit IgG isotype control with 4',6-diamidino-2-phenylindole (DAPI) stained nuclei and right panel SOD2 expression in green counterstained with DAPI . (C) Representative western blots for SOD1 and 2 protein expression by ASM from asthmatics and healthy controls and (D) their intensity of expression (n=12). Between group comparisons were made by unpaired t-tests.

Figure E2 (A) TGF-β release by ASM from asthmatics and healthy controls over 72 h (n=15). (B) Example flow cytometric analysis of ASM α-smooth muscle actin (SMA) expression basally. Grey line corresponds to IgG isotype control, black line α-SMA. (C) α-SMA in ASM from subjects with asthma and healthy controls (n=34). Between group comparisons were made by unpaired t-tests.

Figure E3 (A) Example flow cytometric analysis of ASM α-SMA expression following transfection with negative control siRNA or NOX4 siRNA. Grey line corresponds to IgG isotype control, black line α-SMA and (B) α-SMA in ASM transfected with negative control siRNA or NOX4 siRNA (n=5). (C) Example flow cytometric analysis of ASM α-SMA expression following transfection with negative control siRNA or SOD2 siRNA. Grey line corresponds to IgG isotype control, black line α-SMA and (D) α-SMA in ASM transfected with negative control siRNA or SOD2 siRNA (n=3). Between group comparisons were made by paired t-tests.

Table E1 Clinical characteristics of primary airway smooth muscle donors

	Normal	Asthma
Number	19	23
Age [#]	49 (5)	45 (3)
Male/ Female	11/8	12/11
Never/current/ex-smokers	13/0/4	16/1/5
Atopy n (%)	8	50*
FEV_1% predicted[#]	95 (4)	83 (5)*
Pre-BD FEV_1/FVC %[#]	79 (2)	69 (2)*
BD response (%)[#]	0 (0)	4 (2)*

[#]mean (SEM), ^ geometric mean (95% CI), ~median (IQR), BD-bronchodilator, *p<0.05 compared to control.

Table E2a. Genes differentially expressed in ASM cells by microarray analysis in subjects with asthma compared with healthy control subjects. ≥2 fold, p≤0.05 with presence calls ≥100%. False discovery rate of 1000 permutations 29.7%.

Affymetrix ID	*Gene Symbol*	*Gene Name*	*Entrez Gene ID*	*Fold Difference*	*P value*
202016_at	MEST	mesoderm specific transcript homolog (mouse)	4232	6.72	0.035
201310_s_at	C5orf13	chromosome 5 open reading frame 13	9315	3.82	0.005
201309_x_at	C5orf13	chromosome 5 open reading frame 13	9315	3.50	0.018
205047_s_at	ASNS	asparagine synthetase	440	3.18	0.017
220892_s_at	PSAT1	phosphoserine aminotransferase 1	29968	3.01	0.031
204203_at	CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	1054	2.70	0.008
200862_at	DHCR24	24-dehydrocholesterol reductase	1718	2.53	0.004
234994_at	KIAA1913	KIAA1913	114801	2.53	0.001
223062_s_at	PSAT1	phosphoserine aminotransferase 1	29968	2.50	0.037
202887_s_at	DDIT4	DNA-damage-inducible transcript 4	54541	2.37	0.024
217430_x_at	COL1A1	collagen, type I, alpha 1	1277	2.31	0.044
214927_at	ITGBL1	integrin, beta-like 1 (with EGF-like repeat domains)	9358	2.21	0.033
216037_x_at	TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	6934	2.17	0.004
224516_s_at	CXXC5	CXXC finger 5	51523	2.17	0.049
227400_at	NFIX	nuclear factor I/X (CCAAT-binding transcription factor)	4784	2.02	0.004
207071_s_at	ACO1	aconitase 1, soluble	48	-2.01	0.003
209652_s_at	PGF	placental growth factor, vascular endothelial growth factor-related protein	5228	-2.07	0.011
202422_s_at	ACSL4	acyl-CoA synthetase long-chain family member 4	2182	-2.08	0.020
203414_at	MMD	monocyte to macrophage differentiation-associated	23531	-2.10	0.011
201272_at	AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	231	-2.18	0.022
202464_s_at	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	5209	-2.18	0.040
202450_s_at	CTSK	cathepsin K	1513	-2.21	0.005
226632_at	CYGB:	cytoglobin	114757	-2.23	0.029
227705_at	TCEAL7	transcription elongation factor A (SII)-like 7	56849	-2.23	0.010
225532_at	CABLES1	Cdk5 and Abl enzyme substrate 1	91768	-2.26	0.005
204142_at	ENOSF1	enolase superfamily member 1	55556	-2.30	0.030
217739_s_at	PBEF1	pre-B-cell colony enhancing factor 1	10135	-2.30	0.007
226218_at	IL7R	interleukin 7 receptor	3575	-2.39	0.035
215223_s_at	SOD2	superoxide dismutase 2, mitochondrial	6648	-2.40	0.023
209631_s_at	GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	2861	-2.47	0.037
230869_at	FAM155A	family with sequence similarity 155, member A	728215	-2.68	0.027
205404_at	HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	3290	-2.69	0.018
203908_at	SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4	8671	-2.92	0.015
216841_s_at	SOD2	superoxide dismutase 2, mitochondrial	6648	-3.35	0.001
217590_s_at	TRPA1	transient receptor potential cation channel, subfamily A, member 1	8989	-4.43	0.018

Table E2b: Genes present in ≥5 asthmatic compared to ≤1 healthy controls

Affymetrix ID	*Gene Symbol*	*Gene Name*	*Entrez Gene ID*
230424_at	C5orf13	Chromosome 5 open reading frame 13	9315
214188_at	HEXIM1	Hexamethylene bis-acetamide inducible 1	10614
224339_s_at	ANGPTL1	Angiopoetin-like 1	9068
236442_at	DPF3	D4, zinc znc double PHD fingers, family 5	8110
213789_at	TBC1D25	TBC1 domain family, member 25	4943
214954_at	SUSD5	Sushi domain containing 5	26032
213493_at	SNED1	Sushi, nidogen and EGF-like domains	25992
214806_at	BICD1	Bicaudal D homolog 1 (Drosophila)	636
242851_at	KIAA1919	KIAA1919	91749
204262_s_at	PSEN2	Presenilin 2 (Alzheimer disease 4)	5664
234921_at	ZNF470	Zinc finger protein 470	388566
205214_at	STK17B	Serine/threonine kinase 17b	9262
204662_at	CP110	CP110 protein	9738
206565_x_at	SMA4	Glucuronidase, beta pseudogene	11039
209053_s_at	WHSC1	Wolf-Hischhorn syndrome candidate 1	7468
1558044_s_at	EXOSC6	Exosome component 6	118460
203881_s_at	DMD	Dystrophin (muscular dystrophy, Duchenne and Becker types)	1756
203441_s_at	CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	1000
219773_at	NOX4	NADPH oxidase 4	50507
219875_s_at	PPPDE1	PPPDE peptidase domain containing 1	51029
230872_s_at	TTLL3	Tubulin tyrosine ligase-like family, member 3	26140
209068_at	HNRPDL	Heterogeneous nuclear ribonucleoprotin D-like	9987
212776_s_at	OBSL1	Obscurin-like 1	23363

Table E2c: Genes present in ≤5 asthmatic compared to ≥1 healthy controls

Affymetrix ID	*Gene Symbol*	*Gene Name*	*Entrez Gene ID*
231923_at	TMEM150C	Transmembrane protein 150C	441027
229412_at	TAF8	TAF8 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 43kDa	129685
228000_at	ADC	Arginine decarboxylase	113451
210375_at	PTGER3	Prostaglandin E receptor 3 (subtype EP3)	5733
215779_s_at	HIST1H2BG	Histone cluster 1, H2bg	8339
230054_at	PRRT1	Proline-rich transmembrane protein 1	80863
229584_at	LRRK2	Lucine-rich repeat kinase 2	120892
203661_s_at	TMOD1	Tropomodulin 1	7111
213303_x_at	ZBTB7A	Zinc finger and BTB domain containing 7A	51341
209829_at	C6orf32	Chromosome 6 open reading frame 32	9750
204385_at	KYNU	Kynureninase (L-kynurenine hyrdolase)	8942