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Brief Communication

Neuronal NOS localises to human airway cilia

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ABSTRACT

Background: Airway NO synthase (NOS) isoenzymes are responsible for rapid and localised nitric oxide (NO) production and are expressed in airway epithelium. We sought to determine the localisation of neuronal NOS (nNOS) in airway epithelium due to the paucity of evidence.
Methods and results: Sections of healthy human bronchial tissue in glycol methacrylate resin and human nasal polyps in paraffin wax were immunohistochemically labelled and reproducibly demonstrated nNOS immunoreactivity, particularly at the proximal portion of cilia; this immunoreactivity was blocked by a specific nNOS peptide fragment. Healthy human epithelial cells differentiated at an air–liquid interface (ALI) confirmed the presence of all three NOS isoenzymes by immunofluorescence labelling. Only nNOS immunoreactivity was specific to the ciliary axonemeand co-localised with the cilia marker β-tubulin in the proximal part of the ciliary axoneme.
Conclusions: We report a novel localisation of nNOS at the proximal portion of cilia in airway epithelium and conclude that its independent and local regulation of NO levels is crucial for normal cilia function.

1. Background

Nitric oxide (NO) is a mobile, reactive and ubiquitous signalling molecule within the airway, regulating vascular and bronchial tone, airway permeability [1] and ciliary beat frequency (CBF) [2–5]. It is pro-inflammatory with anti-bacterial action [6,7] and tumouricidal activity [8]. NO is generated from L-arginine by the catalytic activity of nitric oxide synthase (NOS) isoenzymes. The NOS isoenzymes, encoded by NOS_{1-3} genes on different chromosomes, include neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) respectively. NOS activity is dependent on the presence of cofactors including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide, and heme [6,9–12].

NADPH reactivity, iNOS and eNOS are localised in the epithelial cell layer of the healthy and allergic human nasal mucosa [5,13–16]. Bronchial epithelium biopsied from atopic asthmatic subjects demonstrated immunoreactivity for nNOS, iNOS and eNOS throughout the columnar and basal cells, with prominent staining for each at the apical surface of columnar cells [17]. Neuronal NOS is also reported in nervous, vascular, gut epithelium but there is little data from nasal or bronchial epithelium, with no reports of localisation in cilia [14,16–19].

The aim of our study was to determine the expression of nNOS protein in healthy human airway ciliated epithelium.

2. Methods

2.1. Ethics statement

Our research received approval from the National Research Ethics Service (NRES), South Central Committee (06/Q1702/109) and locally at the University Hospital Southampton NHS Foundation Trust Research and Development Department (RHMCH10395). All subjects gave written informed consent.

2.2. Immunohistochemical labelling of nitric oxide synthases

Human nasal polyp ($n = 7$ subjects) in paraffin wax sections and healthy human bronchial tissue ($n = 3$ subjects) in glycol

Abbreviations: ALI, air–liquid interface; BEGM, bronchial epithelial cell growth medium; CBF, ciliary beat frequency; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; DAB, 3,3′-diaminobenzidine; GMA, glycol methacrylate; IFT, intraflagella transport; IgG, immunoglobulin G; IL-1β, interleukin-1 beta; NADPH, nicotinamide adenine dinucleotide phosphate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NOS_{1-3}, neuronal nitric oxide synthase 1–3; PBS, phosphate-buffered saline; PKA, protein kinase A; PKIs, protein kinase A inhibitors; PIN, protein inhibitors of neuronal nitric oxide synthase; TBS, tris-buffered saline.

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methacrylate (GMA) resin sections were immunostained using standard protocols. For paraffin wax sections, antibodies were applied following heat based antigen retrieval in 10 mM citrate buffer (pH 6.0). The Calbiochem (Merck, UK) primary rabbit polyclonal anti-nNOS (1414–1434) antibody (1 mg/ml stock) was used at 1:1500 for paraffin wax sections and 1:100 for nNOS in GMA resin sections and incubated overnight at 4 °C. A primary mouse monoclonal β-tubulin antibody (Sigma) (ascites fluid) used at 1:15,000 was used as a positive control for cilia staining in GMA resin sections. Equivalent concentrations of an anti-IgG isotype antibody were used as a negative control. The synthetic peptide fragment CRSESIAFIEESKKDTDEVFSS corresponding to amino acids 1414–1434 of nNOS (4 mg/ml stock) (Calbiochem, Merck, UK) was applied as a specific blocking peptide when co-incubated 4:1 (v/v) with the anti-nNOS antibody. The 21 amino acid sequence only predicted nNOS by NCBI Blast protein analysis [20]. Alzheimer's brain tissue was used as a positive control for specific nNOS immunoreactivity [21]. Immunolabelling was visualised by streptavidin biotin-peroxidase labelling with a 3,3′-diaminobenzidine (DAB) chromogen and sections counterstained with Mayer's haematoxylin.

2.3. Immunofluorescent labelling of nitric oxide synthases in primary epithelial cell culture

Otherwise healthy epithelial cells were acquired from nasal brushing biopsies from patients attending a primary ciliary dyskinesia (PCD) diagnostic clinic, and PCD diagnosis had been excluded. Confluent basal epithelial cells were cultured on 12 mm Costar Transwell membranes coated with 300 μg/ml Purecol (Nutacon, The Netherlands) at an air–liquid interface (ALI) for up to 21 days in specialised medium (CC3170, Clonetics, Lonza UK 1:1 with DMEM-high glucose 4.5 g/l 41966, Invitrogen, UK) with additional 100 nM all-trans retinoic acid (Sigma, UK) until differentiated and ciliated [22].

Transwell membranes containing ALI differentiated ciliated epithelial cells ($n = 5$ subjects) were excised and fixed in ice cold methanol for 10 minutes. All subsequent steps were carried out at room temperature. Membranes were blocked for 30 minutes in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.05% triton X-100 (blocking buffer) before dissection into segments. Between steps, membrane segments were washed three times in blocking buffer for 5 minutes. All antibodies were diluted with blocking buffer and membrane segments incubated for 90 minutes. The Calbiochem (Merck, UK) primary rabbit polyclonal antibodies verified by published Western Blot: anti-nNOS (1414–1434) [14,23], anti-iNOS (1131–1144) [24,25], anti-eNOS (599–913) [25] (1 mg/ml stock) were used at 1:200. The synthetic peptide fragment CRSESIAFIEESKKDTDEVFSS corresponding to amino acids 1414–1434 of nNOS (4 mg/ml stock) (Calbiochem, Merck, UK) was applied as a specific and irrelevant blocking peptide. It was co-incubated 4:1 (v/v) with each anti-NOS antibody.

A secondary Alexa488 conjugated goat anti-rabbit antibody (Invitrogen, UK) (2 mg/ml stock) was used at 1:500 in the dark. For co-localisation experiments ($n = 3$) a mouse monoclonal anti-β-tubulin antibody (ascites fluid) (Sigma, UK), as a marker of cilia [26], was used at 1:500 followed by a secondary Alexa594 conjugated chicken anti-mouse antibody (Invitrogen, UK) (2 mg/ml stock) used at 1:500 in the dark. Membrane segments were finally incubated for 15 minutes in the dark with 5 μM Sytox orange (Invitrogen, UK) or DAPI (Sigma, UK) at 1:500, diluted in PBS, washed and mounted in 100 μl Mowiol between two large glass coverslips to allow easy orientation of membrane segments. Fluorescence was imaged using a laser scanning SP5 confocal microscope (Leica Microsystems, UK).

3. Results

3.1. nNOS localisation in nasal polyp and healthy bronchial tissues by immunohistochemistry

In nasal polyp tissue sections ($n = 7$ subjects) and healthy human bronchial tissue sections ($n = 3$ subjects) nNOS staining was clearly seen in cilia (Fig. 1b and e). The nNOS immunoreactivity was similar to that of cilia specific β-tubulin staining (Fig. 1g) but was evidently absent from the distal third portion of the cilia (Fig. 1b and e). The nNOS immunoreactivity in the proximal portion of the cilia of both the nasal polyp and healthy bronchial tissue was consistently blocked by the addition of the peptide fragment corresponding to the sequence used to raise the anti-nNOS antibody (Fig. 1c and f). The specificity of the nNOS antibody was verified in paraffin wax embedded Alzheimer's brain tissue sections, by its appropriate immunolabelling of cell bodies, neurites and blood vessels concentrated at Alzheimer's plaques (Fig. 1h and i) [21]. Control sections substituting the primary antibody for a primary isotype matched control antibody (Fig. 1a and d) or omitting antibodies (not shown) demonstrated no immunoreactivity.

3.2. nNOS localisation in primary nasal epithelial cell cultures by immunofluorescence labelling

Healthy cultures of human primary nasal epithelial cells differentiated and ciliated at ALI and cilia were perpendicular to the epithelial cell layer by confocal microscopy (Fig. 2) and also illustrated by a representative scanning electron microscope image (supplementary Fig. S1a). Neuronal NOS consistently localised to the ciliary compartment of ciliated ALI-cultured epithelial cells ($n = 5$ subjects) (Fig. 2b) and co-localised with the cilia marker β-tubulin [26], in the proximal part of the ciliary axoneme but not towards the distal portion (Fig. 2c; and supplementary Fig. S1j and l). The immunoreactivities of iNOS and eNOS were demonstrated: diffuse and compartmentalised cytoplasmic iNOS (Fig. 2d–f), and diffuse cytoplasmic eNOS (Fig. 2g–i), with neither localised to the ciliary axoneme as depicted by confocal microscopy orthogonal sectioning (f and i) ($n = 5$ subjects). The iNOS showed some punctate immunostaining near to the base of the cilia (Fig. 2d–f).

The nNOS blocking peptide was co-incubated with the anti-nNOS antibody and specifically blocked its reactivity (supplementary Fig. S1m–o). The nNOS blocking peptide applied at the same concentration had no blocking or modifying effects on the immunoreactivities of the anti-iNOS antibody (supplementary Fig. S1d–f) or the anti-eNOS antibody (supplementary Fig. S1g–i).

Negative controls had counterstained nuclei with primary antibodies omitted and demonstrated no specific immunoreactivity with both secondary antibodies combined (Fig. 2a). Further negative controls included incubating samples with rabbit polyclonal anti-nNOS antibody with the inappropriate chicken anti-mouse A594 secondary antibody (supplementary Fig. S1b), or mouse monoclonal anti-β-tubulin antibody with the inappropriate goat anti-rabbit A488 secondary antibody (supplementary Fig. S1c) to show absence of cross-reactivity.

4. Discussion

We have demonstrated a novel localisation for nNOS consistently to the proximal portion of cilia in human nasal polyp and healthy bronchial tissue, which co-localised with the cilia marker β-tubulin in healthy bronchial tissue sections. The immunolocalisation of nNOS to cilia was confirmed by its consistent abolition using a blocking peptide, and the antibody specificity was corroborated by demonstrating relevant nNOS