Human bronchial epithelial cell dysfunction following in vitro exposure to nitrogen dioxide

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Human bronchial epithelial cell dysfunction following in vitro exposure to nitrogen dioxide. J.L. Devalia, R.J. Sapsford, D.R. Cundell, C. Rusznak, A.M. Campbell, R.J. Davies. ©ERS Journals Ltd

ABSTRACT: Nitrogen dioxide (NO₂), is a major air pollutant, that causes bronchoconstriction and bronchial hyperreacivity, and may also lead to damage and inflammation of the airways epithelium. We have cultured human bronchial epithelial cells and investigated the effect of exposure to NO₂ for 20 min on epithelial cell membrane integrity and function in vitro.

Epithelial cell membrane damage and permeability were assessed by release of 51Cr from prelabelled cells, and movement of 14C-labelled bovine serum albumin (BSA) across the bronchial epithelial cell monolayers. Ciliary beat frequency (CBF) of the cells was monitored by the analogue contrast enhancement technique, and arachidonic acid (AA) metabolism was investigated by analysis of radio-labelled AA metabolites generated from cultures prelabelled by incubation with [3H]-arachidonic acid.

Exposure to 400 and 800 parts per billion (ppb) NO₂ significantly increased the release of 51Cr from 0.9±0.4%, in control cultures exposed to 5% CO₂ in air, to 9.7±3.2% and 13.9±3.5%, respectively. Similarly, NO₂ also significantly increased the movement of 14C-BSA across the epithelial monolayers from 1.3±0.2%, in control cultures, to 2.7±0.2%, 3.8±0.4% and 5.1±0.5%, respectively, in cultures exposed to 100, 400 and 800 ppb NO₂. Although NO₂ attenuated the CBF of the cells at all concentrations investigated, this was significant only at the concentration of 2,000 ppb NO₂. Studies of AA metabolism demonstrated that the epithelium cells synthesised prostaglandin E₂ (PGE₂), leukotriene C₄ (LTC₄) and 12- and 15-hydroxyeicosatetraenoic acid (HETE), constitutively, and that exposure to 400 and 800 ppb NO₂ led to significant generation of LTC₄ (range 7.0±2.1 to 43.7±6.4 fmol-μg⁻¹ cellular protein).

These studies suggest that acute exposure of human bronchial epithelial cells to NO₂ may lead to detrimental cell membrane and functional changes and, consequently, play a role in the aetiology of airways disease. Eur Respir J., 1993, 6, 1308-1316.

Pollutants, such as nitrogen dioxide, ozone and, to a lesser extent, sulphur dioxide, resulting from increased use of motor vehicles and increased combustion of fossil fuels, may play an important role in the development of airways disease. Epidemiological studies in children [1, 2], and adults [3], have demonstrated a strong link between cough and infections of the lower respiratory tract, and episodes of air pollution resulting from vehicle exhaust emissions and burning of liquid petroleum gas or kerosene.

Studies of nitrogen dioxide (NO₂) have suggested that it is a major pollutant, capable of inducing pulmonary damage. Although this agent does not produce significant alterations in airway function in healthy volunteers at low concentrations [4, 5], it induces bronchoconstriction and bronchial hyperreactivity at higher concentrations [6]. Asthmatics, however, have increased sensitivity to this agent, leading to bronchial constriction and hyperreactivity at concentrations as low as 400 ppb of NO₂ [5, 7].

The precise mechanism(s) underlying the effects of exposure to NO₂ have not been elucidated. It is possible, that NO₂-induced airway hyperreactivity may be mediated via oxidative free radicals, since hyperresponsiveness to methacholine in normal subjects can be prevented by pretreatment of these subjects with antioxidants, such as ascorbic acid [8]. Also, it has been proposed that short-term exposure to NO₂ may lead to an increase in bronchial tone as a result of release of mediators, such as histamine [9] and leukotriene B₄ [10]. More recently, Sandström and co-workers [11, 12] have suggested that NO₂ may induce an inflammatory cell response. Studies in animals have demonstrated that exposure to NO₂ affects the lung defence mechanisms, including effects on mucociliary clearance, the alveolar macrophage and the immune system [13], and that the terminal bronchioles are the major sites of NO₂-induced injury [14-16].

The effects at the epithelium include ciliary loss, loss of tight junctions, hyperplasia, epithelial flattening, damage to surface membranes and severe disruption of interepithelial tight junctions.
In view of the likely occurrence of airway epithelial cell damage following inhalation of NO₂, our aim was to investigate the effect of exposure to NO₂ for 20 min, on human bronchial epithelial cell membrane integrity and permeability, ciliary activity and the metabolism of arachidonic acid by these cells \textit{in vitro}.

\textbf{Materials and methods}

All chemicals and reagents were of tissue culture grade and, unless otherwise stated, were obtained from the Sigma Chemical Co. (Poole, UK).

\textbf{Bronchial tissue}

Bronchial tissue was obtained from 12 patients (eight male and four female, mean age 67 yrs, range 53–80 yrs), all of whom were smokers and undergoing lobectomy or pneumonectomy for squamous cell carcinoma of the lung, at St Bartholomew's Hospital, London. Following resection, tissue which appeared macroscopically free of tumour and deemed to be "normal" by the operating surgeon was collected and processed, within 0.5 to 1 h of resection, for tissue culture.

\textbf{Isolation, culture and identification of bronchial epithelial cells}

Bronchial epithelial cells were cultured by an explant cell culture technique, developed in our laboratory and described in detail previously [17]. Briefly, 2–3 sections were explanted into either untreated 35 mm diameter Falcon® "Primaria®" plastic culture dishes (Becton Dickinson Ltd, Oxford, UK) or into 9 mm diameter Falcon® cell culture inserts, with 0.45 µm pore size microporous membranes (Becton Dickinson Ltd, Oxford, UK) and incubated, at 37°C in a 5% CO₂ in air atmosphere, in freshly prepared and micropore filter-sterilized culture medium; containing 2.5 ml Na-serum fiv (Flow Laboratories, Scotland, UK), 250 µg bovine pancreatic insulin, 250 µg human transferrin, 36 µg hydrocortisone, 3 mg L-glutamine and 1 ml of the antibiotics/antimycotic solution, in 100 ml Medium 199. Explants cultured in dishes and cell culture inserts were incubated in 2.5 and 0.4 ml medium, respectively, and observed for cell outgrowth over 2–3 weeks, until the cells had grown to confluency.

The purity of the cultures and the identity of the cells was confirmed by: 1) light microscopy; 2) electron microscopy; and 3) indirect immunoperoxidase staining techniques, using specific monoclonal antibodies directed towards cytokeratin and specific ciliated epithelial cell antigens [17].

\textbf{Exposure of epithelial cell cultures to NO₂}

Exposure of cells to NO₂ was carried out in an airtight polycarbonate chamber, with a capacity of 5.5 l. Gas mixtures containing different concentrations of NO₂ were generated by diluting a 20 parts per million (ppm) NO₂ in air "Spectrascel" grade stock gas mix (BOC Special Gases, London, UK) with a 5% CO₂ in air gas mix, using a Quantitech Model QC 101 gas blender (Quantitech Ltd, Milton Keynes, UK). The concentration of NO₂ in the final gas mix to be investigated was assessed and confirmed by the use of a CEA Instruments Inc. Model TGM 555 NO₂ monitor (Quantitech Ltd, Milton Keynes, UK). The cultures were exposed to 0 to 2,000 parts per billion (ppb) NO₂ for 20 min at room temperature (23–25°C), at a flow rate of 1.5 l/min⁻¹. Efficient exposure to the final gas mix was achieved by gently tilting the exposure chamber, alternately, at intervals of 2.5 s, to an angle of 10° from the horizontal, every 90° in the horizontal plane on a Luckham 4RT rocking table (Luckham Ltd, Burgess Hill, UK) (fig. 1), thereby momentarily displacing approximately half the medium covering the surface of the culture plate during each tilt. Following exposure, the cells were incubated further for up to 24 h, at 37°C in humidified 5% CO₂ in air, and during this incubation each culture was assessed for changes in either: 1) release of ³¹Cr from cells labelled with sodium chromate; 2) movement of radiolabelled bovine serum albumin (³¹C-BSA) across the epithelial cell monolayer; 3) ciliary beat frequency (CBF); or 4) generation of arachidonic acid metabolites.

Appropriate controls were also prepared by exposing cultures to 5% CO₂ in air in the exposure chamber for 20 min, and then treating further as for the test cultures.

\textbf{Estimation of nitrogen dioxide solubilized in culture medium}

The solubility of NO₂ in the medium was assessed by measurement of the azo-dye formed as a result of the reaction between NO₂ and Lyshkow's reagent [18], at 550 nm. At this wavelength, the intensity of the azo-dye formed is directly proportional to the concentration of the NO₂ solubilized in the medium.

Culture dishes containing 2.0 ml of phenol red-free Medium 199 (Gibco BRL, Paisley, Scotland, UK), containing 1% SF-1 serum free supplement (Northumbria Biologicals Ltd, Cramlington, UK) and 1.7% Lyshkow reagent, were exposed to 100–2,000 ppb NO₂ for 20 min, in the airtight polycarbonate exposure chamber, as above. Following exposure, the culture dishes were equilibrated at 37°C in a 5% CO₂ in air for 1 h, and the medium from each dish was analysed for pH, osmolality and absorbance at 550 nm. Osmolarity of the medium was monitored by use of a Roehling Type 11 automatic micro-osmometer (Camlab Ltd, Cambridge, UK), and the absorbance at 550 nm was measured by injecting the medium directly into the sample cell of the Model TGM 555 NO₂ monitor. The amount of NO₂ dissolved in the medium was estimated from a standard curve, prepared by determining the absolute absorbance produced by the direct reaction of Lyshkow's reagent and gas mixtures of known concentrations of NO₂, in the NO₂ monitor.

\textbf{Estimation of epithelial cell membrane damage}

Epithelial cell membrane damage was assessed by estimating the release of ³¹Cr from cells with radiolabelled
sodium chromate (Amersham International plc, Amersham, UK). Prior to exposure to NO₂, explants were removed from 2-3 week old confluent cultures and the cells incubated overnight in the presence of 2.0 μCi [³⁵Cl] sodium chromate. Following this incubation the culture medium was decanted and the cells were washed gently with Medium 199, to remove any radiolabel not incorporated into the cells. Immediately prior to exposure to NO₂, 200 μl aliquots of culture medium were collected from each culture and stored on ice until analysis. The cultures were then exposed to NO₂ for 20 min, as described above, and further incubated for 1 h at 37°C in 5% CO₂ in air. At the end of incubation, the medium was collected and the cells were gently washed with 1.0 ml fresh ice-cold medium. The culture medium and the wash were pooled and the adherent cells were scraped off into 3.0 ml Medium 199. Two hundred micro-litre aliquots of all samples were mixed with 5.0 ml of Ready-Solv® CP scintillation cocktail (Beckman-RiIC Ltd, High Wycombe, UK) and monitored for radioactivity, in a Beckman LS1800 scintillation counter (Beckman-RiIC Ltd, High Wycombe, UK). Radioactivity released into the medium was calculated as a percentage of total in the culture, according to the formula:

\[
\text{Total CPM in medium} - \frac{\text{Total CPM in medium}}{\text{Total CPM in medium} + \text{Total CPM in cell pellet}} \times 100
\]

All results were expressed as the percentage radioactivity released into the medium, above baseline levels.

Estimation of epithelial cell permeability

Bronchial epithelial cell permeability was assessed by estimating the movement of [¹⁴C]-BSA (Amersham International plc, Amersham, UK) across epithelial cell monolayers established in cell culture inserts. Explants were removed from confluent cultures, and the cells in the inserts were then allowed to overgrow the area of the culture membrane left barren on removal of the explant. Prior to exposure to NO₂, fully confluent cultures were washed gently with fresh culture medium and then incubated in the presence of 0.025 μCi [¹⁴C]-BSA. After incubation for 30 min, 200 μl aliquots of medium in each insert well were monitored for radioactivity, as above, and if this was found to be less than 0.5% of the total added into the insert, the culture was exposed to a specific concentration of NO₂, as described above. In this manner, at least six separate cultures each were exposed to each concentration of NO₂ for 20 min, and then further incubated for 1 h at 37°C in 5% CO₂ in air. At the end of incubation, the medium in each well was collected and again analysed for radioactivity, as above.

Estimation of epithelial cell ciliary beat frequency (CBF)

In contrast to cultures used in investigations of the effect of NO₂ on epithelial permeability, 2-3 week old cultures established on tissue culture plates were used for these studies. CBF of the cells was measured by a modification of the analogue contrast enhancement technique, as described previously [17].

Prior to treatment, each culture was equilibrated for 2 min at room temperature, and monitored for the "baseline" CBF in at least six randomly chosen areas of ciliated cells, the positions of which, in the dish, were accurately noted with a horizontal and a vertical Vernier scale on the microscope stage. At least six separate cultures were exposed to each concentration of NO₂ or 5% CO₂ in air for 20 min, and then further incubated for 1 h, at 37°C in 5% CO₂ in air. At the end of this incubation, the cultures were equilibrated again for 2 min at room temperature, and monitored for CBF in the same areas as at the beginning of the experiment.

The CBF in each culture was calculated as the mean over six areas within the culture, at baseline and after 1 h of incubation, and expressed as % attenuation, from baseline, after 1 h.

Estimation of arachidonic acid metabolites

Prior to exposure to NO₂, cultures established in culture dishes were incubated for 24 h in culture medium further...
supplemented with 1% SF-1 serum-free supplement (Northumbria Biologicals Ltd, Cramlington, UK), and to be referred to as SF-1 medium from now on. Preliminary experiments suggested that preincubation of the cells in medium containing the SF-1 supplement, which provided a free fatty acids pool (linoleic acid, oleic acid and palmitic acid), was necessary for the synthesis of any metabolites of arachidonic acid, in the absence of any exogenously added arachidonic acid. Following this initial incubation, the explants were removed and the cells were incubated for a further 24 h in SF-1 medium, supplemented additionally with 1 μCi/ml [3H]-arachidonic acid (Amersham International plc, Amersham, UK). Prior to exposure, the cells were washed three times with prewarmed and pregassed SF-1 medium, and then exposed to either NO2 or 5% CO2 in air for 20 min. Following exposure, the medium was collected from each culture plate and the incubation terminated, after either 1, 2, 4, 6 or 24 h. The cells were washed with 1.0 ml fresh ice-cold Medium 199, and the wash was pooled with the culture medium. The adherent cells were scraped off into 1.0 ml Medium 199. All the samples were stored at -70°C until required for analysis of arachidonic acid metabolites, by reversed-phase-high performance liquid chromatography (RP-HPLC) and cellular protein, according to the method of Lowry et al. [19]. All results were expressed as fmol·μg-1 cellular protein.

Sample processing. Aliquots of 1.5 ml of methanol were added to each sample, and the samples vortexed for 15 min. Following centrifugation at 3,000×g for 10 min, supernatants were collected by aspiration from all samples and the cell pellets washed twice, by resuspension in 1.0 ml diethyl ether, vortexing and then centrifugation as above. Following each wash, the appropriate supernatants were pooled, mixed with the original culture medium and then blown to dryness under nitrogen. The concentrated medium samples were resuspended in 250 μl aliquots of 70% methanol, and finally analysed by RP-HPLC. The cell pellets were resuspended in 1.0 ml aliquots of 1 M NaOH and incubated at 56°C for 2 h, prior to assay for total cellular protein.

Samples of fresh SF-1 medium "spiked" with known amounts of [3H]-arachidonic acid and several commercially available [3H]-arachidonic acid metabolites (Du Pont (UK) Ltd, Stevenage, UK) were also processed, in parallel with the test samples, to correct for losses and estimate final recovery of compounds of interest. Radioactivity in these samples was monitored in the Beckman LS1800 scintillation counter (Beckman-RIC Ltd, High Wycombe, UK), and expressed as a percentage of the total added at the beginning of processing.

RP-HPLC of arachidonic acid metabolites. A Gilson Model 305 master HPLC pump (Anachem Ltd, Luton, UK), regulating a Gilson Model 302 auxiliary HPLC pump (Anachem Ltd, Luton, UK) and connected to a Rheodyne 8125 injection valve (with a 20 μl injection sample loop) via a Gilson Model 811B Dynamic mixer (Anachem Ltd, Luton, UK), was used as the solvent delivery system. Chromatography was performed on a 5.0 cm×4.6 mm (internal diameter) Dynamax-300@ C-18 column (Anachem Ltd, Luton, UK), using a mobile phase consisting of 1% glacial acetic acid in water (adjusted to pH 5.4 with ammonium hydroxide) and methanol, pumped at a constant flow rate of 1 ml·min-1. The radioactive samples were eluted by use of a step-up methanol gradient, according to the method of Hinson et al. [20], and detected by a Berthold LB 506 C-1 HPLC radioactivity monitor (Berthold, St Albans, UK), fitted with a 2 ml flow through cell. The radioactivity in each sample was evaluated by an integral Motorola 68B09 CPU microprocessor, and the signals generated were digitized by an IBM AT computer, linked to and controlling the entire system.

Standardization. Standardization of the assay was performed by the external standardization method, using commercially available preparations of [3H]-arachidonic acid metabolites, including prostaglandin E2 (PGE2), leukotrienes B4 and C4 (LTB4 and LTC4) and 5-, 12- and 15-hydroxyeicosatetraenoic acids (5-HETE, 12-HETE and 15-HETE) (Du Pont (UK) Ltd, Stevenage, UK).

Confirmation of LTC4 generation, by radioimmunoassay. On the basis of results obtained by RP-HPLC, the ability of NO2 to induce significant generation of LTC4 by the epithelial cells in vitro was confirmed by radioimmunoassay (RIA). Epithelial cultures were treated in all respects as described previously, except that these were incubated in the absence of radiolabelled or exogenously added arachidonic acid. Following exposure to 400 or 800 ppb NO2, samples were collected 1 h, 6 h and 24 h after treatment, and processed as described above. All samples were then assessed for LTC4, using commercially available radioimmunoassay kits (Amersham International plc, Aylesbury, UK), which detected LTC4 with high reproducibility, by means of monoclonal antibody recognition, and at concentrations between 5–500 pg. The samples were also analysed for total protein, as described above, and the results were expressed as fmol LTC4·μg-1 cellular protein.

Statistical analysis

Individual data from each treatment group were analysed for skewness, and then expressed as mean±SEM. Means of all the treatment groups were compared by one way analysis of variance (ANOVA) to test for significance of any differences, and then followed-up by further analysis with Scheffé's test, to assess differences between individual treatment groups. All values of p<0.05 were considered to be significant.

Results

We have demonstrated that human bronchial epithelial cells could be cultured to confluence, as monolayers of tightly associated polygonal cells in vitro, and confirmed our previous findings [17].
Solubility of NO₂

Under these conditions, 40.0±0.8% (range 38.0±2.4% to 42.5±3.5%) of the total NO₂ in the gas mix was taken up into the culture medium, and this was found to be the case for all concentrations between 100–800 ppb (2.0 ppm) NO₂ studied. The pH and osmolarity of the culture medium, following exposure to NO₂ for 20 min were not affected.

Effect of NO₂ on epithelial cell membrane integrity

Light microscopic examination of cultures exposed to 100–800 ppb NO₂ for 20 min demonstrated that this agent did not lead to any gross cellular damage or detachment of the epithelial monolayers, at any concentration studied, under these conditions (fig. 2).

Fig. 2. – Light micrograph of a 2–3 week old culture of human bronchial epithelial cells exposed to 800 ppb NO₂ for 20 min. Following exposure to NO₂, the cells were further incubated for 1 h at 37°C in 5% CO₂ in air, and then observed by Hoffman Modulation Contrast optics. (Magnification x300; bar=10 μm).

Investigations of the effect of NO₂ exposure on the release of 51Cr from the bronchial cells, however, demonstrated that this agent induced a small but significant release of 51Cr, from cells "loaded" with 51Cr overnight. Exposure of cells to 400 and 800 ppb NO₂, respectively, led to release of 9.7±3.2% (p<0.05) and 13.9±3.5% (p<0.01) 51Cr above baseline levels, compared with 9±0.4% 51Cr released above baseline, in control cells exposed to 5% CO₂ in air (fig. 3). Although exposure of the cells to 100 ppb NO₂ also led to slightly increased release of 1.6±0.2% 51Cr, this was not significant when compared to release from control cells.

Similarly, investigations of the effects of NO₂ on cell permeability demonstrated that this was also increased significantly, as indicated by increased movement of 14C-BSA across the epithelial cell monolayers (fig. 4). NO₂ was effective at all concentrations between 100–800 ppb studied, and induced migration of 2.7±0.2% to 5.1±0.5% of total 14C-BSA, after 1 h of incubation, as compared to migration of 1.3±0.2% of total 14C-BSA, observed in control cells exposed to 5% CO₂ in air.

Effect of NO₂ on CBF

Measurement of CBF demonstrated that NO₂ attenuated the CBF of the bronchial epithelial cells in vitro (fig. 5). Although 100 and 400 ppb NO₂ decreased the CBF by 2.3±1.7% and 3.0±2.5%, respectively, this was not found to be significantly different from the 1.0±0.5% attenuation in CBF noted in the control cultures, exposed to 5% CO₂ in air. Exposure of the cells to 800 ppb NO₂, however, led to a greater attenuation of 6.8±1.7% in the CBF, which when compared to control exposures just failed to reach significance. In contrast, exposure to 2,000 ppb NO₂ led to significant attenuation of 14.2±2.5% (p<0.01) in the CBF, in comparison with control exposures with 5% CO₂ in air.
Effect of NO₂ on generation of AA metabolites

RP-HPLC of the radiolabelled metabolites of arachidonic acid, demonstrated that this was a highly specific and reproducible technique for the simultaneous analysis of several metabolites (fig. 6a). Inter- and intra-assay coefficients of variation, for commercially available standards, were found to be 7% and 5%, with the limits of detection for: i) PGE₂, and LTC₄; and ii) 5-HETE, 12-HETE and 15-HETE, respectively, being 0.1 and 0.4 pg·20 μl⁻¹ of injected sample, at a signal to noise ratio of 10:1.

Similar RP-HPLC analysis of the arachidonic acid metabolites generated by epithelial cell cultures demonstrated that these cells constitutively synthesized PGE₂, 12-HETE and 15-HETE under normal circumstances, and that exposure to NO₂ did not significantly alter the synthesis of these metabolites (fig. 6b and 7).

In comparison with control cells exposed to 5% CO₂ in air, exposure of cells to both 400 and 800 ppb NO₂ resulted in a significant time-dependent generation of LTC₄ between 7.6±2.5 to 43.7±6.4 fmol·μg⁻¹ cellular protein, over a period of 24 h incubation, as assessed by HPLC (fig. 8a). Generation of LTC₄ observed in cultures exposed to 400 ppb, however, was not significantly different from that observed in cultures exposed to 800 ppb NO₂. Analysis of elution times from the HPLC column, of the radiolabelled LTC₄ standard and the LTC₄ in the culture samples, following application onto the column demonstrated that these were similar, and ranged between 13.3±13.6 min (fig. 6 a and b). In comparison, LTB₄ eluted from the column after 14.5±14.8 min following application onto the column, and was not detected by this method, under these conditions.

Analysis of LTC₄ by RIA confirmed the findings by HPLC. In contrast to HPLC, however, RIA demonstrated that control cultures exposed to 5% CO₂ in air, also generated LTC₄ between 13.3±3.1 and 16.2±1.8 fmol·μg⁻¹ cellular protein and that, exposure to 400 and 800 ppb NO₂ led to significantly greater synthesis of LTC₄, ranging between 26.7±4.5 and 44.0±9.9 fmol·μg⁻¹ cellular protein (fig. 8b). As demonstrated before, differences in LTC₄ generated in cultures exposed to either 400 or 800 ppb NO₂ were not found to be significant.
Generation of epithelial cells exposed to described phase high performance liquid chromatography; RIA: radioimmunoassay.

p<0.01 for up to 24 h Fig. 8.

Fig. 7. - Constitutive synthesis of a) 15-HETE, b) PGE, and c) 12-HETE by 2-3 week old human bronchial epithelial cells incubated over a period of 24 h at 37°C in 5% CO₂ in air, assayed by RP-HPLC. Results are expressed as mean±SEM (n=6 separate cultures at each time point). RP-HPLC: reverse phase high performance liquid chromatography. For further abbreviations see legend to figure 6.

Fig. 8. - Generation of LTC₄ by 2-3 week old human bronchial epithelial cells exposed to NO₂ for 20 min and then further incubated for up to 24 h at 37°C in 5% CO₂ in air. All samples were processed as described in the text and analysed by: a) RP-HPLC; and b) RIA. Results are expressed as mean±SEM (n=6 at each time point). *: p<0.05; **: p<0.01 vs 0 ppb NO₂ (control). LTC₄: leukotriene C₄; RP-HPLC: reverse phase high performance liquid chromatography; RIA: radioimmunoassay.

Discussion

Our studies have demonstrated that exposure of human bronchial epithelial cells to 100-2,000 ppb NO₂ for 20 min, leads to an increase in epithelial cell membrane damage and epithelial permeability, attenuation of ciliary activity and increased synthesis of inflammatory mediators, such as LTC₄, in vitro. This suggests that short-term peak exposure to NO₂, at concentrations often found at the kerbside in heavy traffic [21, 22] or indoors in households with gas cooking stoves [23], may lead to irreversible cellular changes, and ultimately cell damage/death in the airway epithelium.

The finding that the NO₂-induced effects appear to be dose-dependent, and that high concentrations (up to 2,000 ppb) of the agent are required to elicit a significant effect on the ciliary activity of the epithelial cells, suggests that the cilia are less susceptible, and that any adverse effects are likely to be a consequence of cytotoxicity, rather than a direct effect on the cilia. These findings are consistent with the findings from animal studies, which have demonstrated that NO₂ leads to severe disruption of the interepithelial tight junctions, and have suggested that this disruption results in loss of both the barrier properties and ciliary function of the epithelium [15, 24].

Although the precise mechanism(s) underlying morphological changes in the epithelium, following exposure to NO₂, have not been elucidated, several studies have suggested that this agent may potentiate its toxic effects at the cell membrane via direct and/or indirect oxidative mechanisms, which generate free radicals and lead to biochemical and metabolic changes, including lipid peroxidation, sulphodyl oxidation and decreased enzyme activity [25]. It has been suggested that damage to the cell membrane lipids results in the activation of phospholipase A and subsequent release of arachidonic acid, which may itself be metabolised via both the cyclo-oxygenase and lipoxygenase pathways to generate potent inflammatory compounds [26].

The present studies have also demonstrated that human bronchial epithelial cells can metabolize arachidonic acid constitutively to 15-HETE, PGE₂, 12-HETE and LTC₄ and that exposure to NO₂ significantly enhances the synthesis of LTC₄. It is of interest, however, that the LTC₄ synthesized constitutively by control cultures was detected by RIA and not by HPLC. Whilst it is possible that this discrepancy may be a consequence of RIA specificity resulting from ~5% and 0.5% cross reactivity of the LTC₄ antiserum with LTD₄ and LTE₄, respectively, it is more likely to be a result of major differences, such as the final sample size analysed (20 μl in HPLC compared to 100 μl in RIA), and the mode of detection (radiochemical in HPLC versus immunocytochemical in RIA), in the assay procedures employed for analysis of LTC₄.

Overall, however, our finding that human bronchial epithelial cells can metabolize arachidonic acid to PGE₂, 12-HETE and 15-HETE in vitro is in agreement with the findings of Churchill et al. [27], and Hunter et al. [28], who, respectively, have also demonstrated that cultured human tracheal epithelial cells are capable of generating cyclo-oxygenase and 12(15)-lipoxygenase metabolites of arachidonic acid, in vitro.
Although neither of these groups have investigated the generation of LTC₄ in their models, studies with animal tracheal epithelial cells have shown that these generate LTC₄ and LTD₄ in addition to prostaglandins D₂, E₂, F₂α, and 6-keto-F₂α (PGD₂, PGE₂, PGF₂α and PG6-keto-F₂α) [29, 30]. The animal studies, however, suggest that generation of such mediators is dependent on the initial stimulus and the species of the animal under investigation and, additionally, may differ depending on both the concentration and the prime source (exogenous or endogenous) of arachidonic acid under investigation [29, 31].

Although our studies have primarily investigated the metabolism of exogenously added arachidonic acid, the final concentration of 2.5 pmoles used was in the order of magnitude ten to a hundred fold lower than that used by other investigators [28-30], and was, therefore, unlikely to influence the metabolic profile of arachidonic acid observed in our system. Indeed, our studies have demonstrated that the bronchial epithelial cells can generate comparable amounts of LTC₄ after 6 to 24 h, both in the presence or absence of exogenous arachidonic acid, and suggest that the small amount of exogenously added radiolabelled arachidonic acid did not influence its metabolism by these cells. The finding, however, that exposure to NO₂ increased synthesis of LTC₄ but not LTD₄, suggests that this agent may contribute to airway pathogenesis, particularly bronchoconstriction, via generation of LTC₄ and, possibly, other inflammatory mediators.

Prior to investigating the effect of NO₂ on these cells, however, we evaluated the suitability of our model for uptake and solubilization of this agent into the culture medium covering the epithelial cell monolayers, and consequently demonstrating some analogy with the epithelial lining fluid covering the epithelium in vivo. Although data of NO₂ solubility in vivo are not presently available, we have demonstrated that approximately 40% of the NO₂ in the atmosphere generated is solubilized into the culture medium in vitro, and that this is independent of the concentration of NO₂ used. These findings are similar to the findings of Postlethwaite and Mustapa [32], who have investigated the "fractional uptake" of NO₂ in an isolated perfused rat lung model and have demonstrated that approximately 65% of this agent was solubilized in their system. Additionally, these authors have also demonstrated that the fractional uptake of NO₂ was not altered even at concentrations in the range 4,000–20,000 ppb (4.0–20.0 ppm), which are in the order of up to tenfold greater than those employed in our studies.

In conclusion, our studies have demonstrated that despite kinetic limitations and the lack of naturally occurring defence mechanisms, such as the presence of a "protective" epithelial lining fluid (ELF) [33], and endogenous "reducing" agents, which may limit the potential oxidant-induced cell damaging effect of NO₂ [25], cultured human airway epithelial cells offer a suitable in vitro model to study the effects and the mechanism(s) underlying the effects of direct exposure to this agent. Using this model, we have shown that NO₂, at concentrations found at the kerbside in heavy traffic and in confined areas where gas fuels are being used for cooking, affects human bronchial epithelial cell membrane integrity and function, and suggest that this agent may alter the patency of airways of especially susceptible individuals, such as asthmatics.

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