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Early Detection of Ozone-induced Hydroperoxides in Epithelial Cells by a Novel Infrared Spectroscopic Method

ANJA HEMMINGSEN[a,*], JEREMY T. ALLEN[a], SIFU ZHANG[b], JOHN MORTENSEN[c] and MONICA A. SPITERI[a]

[a]*Lung injury and Inflammation Research Group, Department of Respiratory Medicine, North Staffordshire Hospital, Newcastle Road, Stoke-on-Trent, ST4 6QG, England;* [b]*Department of Biomedical Engineering and Medical Physics, Keele University, Stoke-on-Trent, England;* [c]*Department of Life Science and Chemistry, Roskilde University, Roskilde, Denmark*

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In the lower atmosphere ozone is a toxic and an unwanted oxidising pollutant causing injury to the airway epithelial cells by lipid peroxidation to yield products such as phospholipid hydroperoxides (PLHP). Measurements of PLHP, which are primary oxidation products, may reflect an early susceptibility of the target cell to oxidative stress. Biphasic cultures of bronchial epithelial cells (BEAS-2B) were exposed to ozone at environmentally relevant concentrations (0.1–1.0 ppm) for 4 and 12 h. Detection of PLHP was made using a novel technique based on fourier transform infrared spectroscopy (FTIR) in combination with high performance thin-layer chromatography (HPTLC). Six phospholipids were identified on the HPTLC plate; lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), lysophosphatidylethanolamine (LPE), phosphatidylinositol (PI), and phosphatidylethanolamine (PE). From the FTIR spectra, O–O stretching of hydroperoxides was identified in the range 890–820 cm^{-1}. Multivariate data analysis revealed a positive correlation ($r = 0.99$ for 4 h exposure and $r = 0.98$ for 12 h exposure) between ozone exposure levels and the region of the FTIR-spectrum comprising the main wavelengths for hydroperoxides. These data support this alternative, versatile and novel spectroscopic approach for the early detection of ozone-mediated damage in human airway epithelial cells.

Keywords: Ozone, lipid hydroperoxide, BEAS-2B cells, FTIR, HPTLC, oxidants

INTRODUCTION

The bronchial airways are constantly exposed to numerous atmospheric irritants including ozone that interact directly with lining epithelial cell and fluid components. Inhalation of ozone causes respiratory symptoms, probably through local formation of free radicals and their derived products.[1,2] Ideally, antioxidants in the epithelial lining fluid (ELF), such as reduced glutathione (GSH), ascorbate, urate, and alpha-tocopherol provide local protection of cells from ozone. The water-soluble antioxidants, ascorbic acid, uric acid and thiols, such as GSH, contribute to protection

*Corresponding author. Tel.: +44 1782 554765. Fax: +44 1782 552323. E-mail: med06@cc.keele.ac.uk.

by acting as target scavengers for ozone. The fat-soluble antioxidant, alpha-tocopherol is believed not to react directly with ozone but scavenges the peroxyl radicals derived from ozone-induced lipid peroxidation.[3,4] Ozone has been shown to react with macromolecules such as lipids, DNA, and proteins (amino acids).[5] Thus in the airway, ozone may induce its toxic effects through free radical reactions, either directly by oxidation of biomolecules in the ELF with production of free radical species such as hydroxyl radical, or by driving radical-dependent production of species such as hydroperoxides, aldehydes and ozonides which, though not radicals, are cytotoxic.[3,6]

Though many studies have examined oxidant damage to the airways, there are still uncertainties as to the connection between airway diseases and environmental irritants. Oxidant damage to the epithelium could lead to reduction in membrane fluidity, fall in membrane potential, increased cellular permeability and eventual cell rupture resulting in local inflammation.[7–9] The most frequent method used to measure ozone injury to the cell is by the thiobarbituric acid test.[3,10–13] Other methods include iodometric, and fluorescence detection of hydroperoxides and conjugated diene detection, but there are limitations and complications with all these methods. In particular, interference from other oxidising species such as hydrogen peroxide[11,14,15], haem protein, and purines and pyrimidines, which absorb strongly in the same range as conjugated dienes.[16]

Peroxidation of membrane lipids is a critical mechanism of ozone-induced injury. The peroxidation can be caused by Criegee ozonation, where ozone attacks the double bond in a fatty acid side chain[4] or by radicals produced from ozone's reaction with biomolecules, which can remove a H atom from a methylene group.[17] Measurements of primary oxidation products such as phospholipid hydroperoxides (PLHP) can reflect an early susceptibility of biological tissues to oxidative damage. Previously, chemical analyses of peroxidation products have focused on secondary oxidation products such as aldehydes,[11,12,18,19] often measured by gas–liquid chromatography with a mass spectrometer as the detector (GC-MS).[18,19] In this study we measured the primary products directly, to determine that peroxidation occurs in cell membrane lipids, rather than detection of their secondary breakdown products.

Lipid hydroperoxides have been measured using high performance liquid chromatography (HPLC) with chemiluminescent,[20,21] UV,[22] and electrochemical assays.[23,24] However, quantification remains problematic, because of a lack of appropriate standards.[25] These chromatographic methods possess the advantage of sensitivity (ng range), and are practical and efficient when handling many samples, with acceptable reproducibility, but can be laborious. Fourier transform infrared spectroscopy (FTIR) has the advantages of HPLC methods; it is relatively simple and versatile and importantly, other oxidising species will not interfere as with the iodometric and fluorescence methods. Furthermore, if sample material is only available from cells, lipid amount is invariably limited; it is thus necessary to use a method with high sensitivity such as FTIR. Accordingly, we have developed a novel technique utilising FTIR in combination with high performance thin-layer chromatography (HPTLC) for the detection of PLHP formation in ozone-exposed human bronchial epithelial cells cultured at an air–liquid interface. The method was validated by demonstrating a clear positive correlation ($r = 0.99$ for 4 h exposure and $r = 0.98$ for 12 h exposure) between ozone exposure levels and the region of the FTIR-spectrum comprising the main wavelengths for hydroperoxides.

MATERIALS AND METHODS

Cell Culture

The human bronchial epithelial cell line BEAS-2B (American Type Culture Collection, Rockville,

Maryland, USA), derived by transformation of healthy human bronchial epithelial cells with an Ad12-SV40 construct was cultured biphasically. Cultures were initiated by plating 5×10^4 cells/cm^2 onto 30 mm Millicell-CM^{TM} inserts (Millipore, Bedford, Maryland), coated with a 0.1% collagen film (Sigma Chem. Co., Poole, Dorset, UK) (1 : 4 dilution in 60% ethanol), in serum-free medium. The inserts were used with standard 6-well cell culture plates (Labtech International, Framfield, UK). The serum-free medium consisted of Ham's F12 medium supplemented with six growth factors; hydrocortisone (0.1 μM), endothelial cell growth supplement (3.75 μg/ml), cholera toxin (10 ng/ml), insulin (5 μg/ml), epidermal growth factor (12.5 ng/ml) and triiodo-L-thyronine (3 nM)[26] (Sigma). Antibiotic and antimycotic (Sigma) (10 000 U penicillin/10 mg streptomycin/25 μg amphoteracin B per ml) were added to the medium. The cells were incubated at 37°C in a humidified 95% air and 5% CO_2 atmosphere. Cells were fed basally with 1.8 ml of medium and apically with 1.5 ml. Media changes were performed every second day, and the inserts kept immersed in the culture medium until they reached near-confluency.

Cell Exposure System

A 316 stainless steel cell exposure chamber (volume = 1 l, 5 cm high and 16 cm in diameter) was specifically constructed to contain one standard 6-well cell culture plate, each well holding one Millicell insert. The exposure chamber was pre-warmed to 37°C. The chamber comprises a lid with inlet and outlet gas ports, and a thermocouple sensor to monitor the temperature, that seals onto a base unit containing the Millicells (Figure 1). The outlet gas port is of a larger diameter (0.95 cm) than the inlet (0.63 cm) to ensure no internal pressure. The chamber is gastight with viton rings sealing the two halves, ports and all other components being constructed from 316 stainless steel.

Gas enters the chamber through the inlet port tangentially to promote swirling and even exposure across the top of the culture wells and is removed close to the floor opposite the entry port. All surfaces in contact with ozone were designed to be inert to its oxidising effects. All the gases, air, CO_2, and oxygen were fitted with a 0.2 μ filter (Polytetrafluoroethylene in-line) immediately after leaving their gas cylinders (Figure 1). In addition, prior to analysis, gas mixtures passed through a Teflon particulate filter attached to the inlet of the chamber. Ozone was produced by passing pure medical-grade oxygen through an ultraviolet ozone generator (Dasibi 1008-PC, Quantitech, Milton Keynes, UK) at a flow rate of 4 l/min. Ozone exits the instrument and its flow is adjusted to 1–2 l/min, excess gas being vented by use of a bypass valve. Ozone was mixed with filtered air (1.8–2.8 l/min) and CO_2 (0.2 l/min) so the flow rate of the gas mixture through the chamber in total was 4 l/min. The two gases, air and ozone, meet each other at 90° to ensure mixing. Prior to entry into the chamber the gas stream enters an incubator, maintained at 37°C ± 1°C monitored by the exposure chamber thermocouple, where it is heated and humidified by bubbling through H_2O in a gas washing bottle, to permit prolonged cell exposures (Figure 1). Ozone concentration was monitored with an ultraviolet ozone analyser (Dasibi 1008-PC).

The exhaust from the exposure chamber was removed through the outlet and vented through a 2% neutral buffered potassium iodide solution (0.06 M KI containing 0.067 M Na_2HPO_4, 0.067 M KH_2PO_4).[27] The system is able to regulate exposure to within ±1% of target ozone parts per million (ppm).

Testing the Uniformity of the Exposure

The uniformity of the exposure was tested to make sure that each culture well received equal amounts of ozone. Four ml of 2% neutral buffered potassium iodide, was pipetted into each well. The vial contents were exposed to three ozone

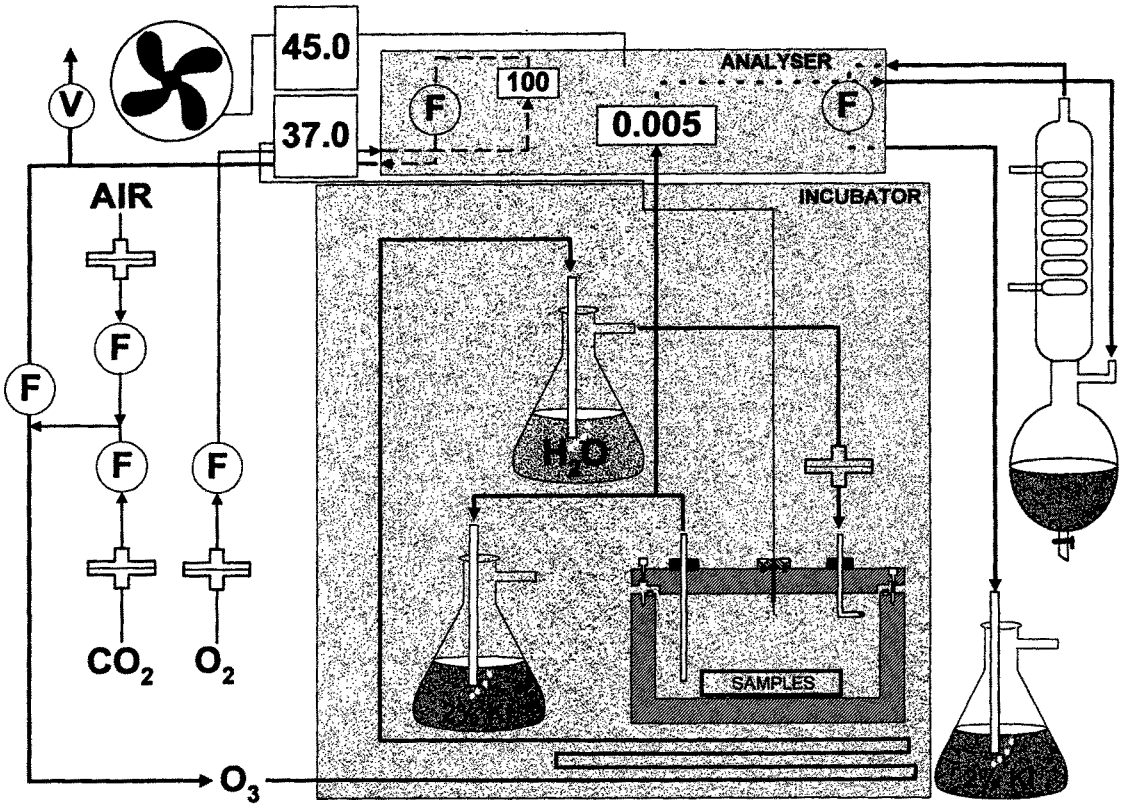

FIGURE 1 *In vitro* cell exposure system. V: bypass valve, F: flowmeter. The cell exposure chamber was constructed to house six Millicell units in a microtitre plate. Oxygen was used as feed gas for ozone generation at a flow rate of 4 l/min. Ozone exits the instrument and its flow is adjusted to 1–2 l/min. Ozone was then mixed with filtered air (1.8–2.8 l/min) and CO_2 (0.2 l/min), producing a gas mixture flow rate of 4 l/min. Prior to entry into the chamber the gas stream enters an incubator maintained at 37°C, where it is heated and humidified by bubbling through H_2O in a gas washing bottle. Ozone concentration was monitored with an ultraviolet ozone analyser (Dasibi 1008-PC).

concentrations between 0.1 and 1.0 ppm of ozone for up to 60 min. The resulting colour development was measured with a spectrophotometer set at 352 nm.

Ozone Exposure

In urban air, concentrations of ozone range from a few parts per billion (ppb) in less polluted areas to more than 1 ppm in areas polluted by industrial activity.[28] The cells were therefore exposed to low (0.1 ppm), medium (0.5 ppm), and high (1.0 ppm) ozone concentrations in respect to environmentally relevant concentrations for 4 and 12 h. Each experiment had a control, where cells in Millicell inserts were exposed to the same gas mixture, minus ozone. For each data point cells from three different inserts were exposed.

Extraction of Lipids and Lipid Hydroperoxides

The method to extract total lipids is based on a procedure originally described by Folch *et al.*[29] for the extraction of lipids from tissues. Following each exposure, cells were immediately trypsinised on their inserts,[26] washed and spun down, to retrieve the cells. Trypsinised cells were resuspended in 250 µl methanol, and transferred to a 1.5 ml microcentrifuge tube. Five hundred µl

chloroform with 150 mg/l butylated hydroxytoluene (BTH),[30] were added to the resuspension. BTH is an antioxidant that is added to chloroform to minimise further oxidative damage to unsaturated fatty acids.[30]

The chloroform–methanol solution contains the lipids together with nonlipid contaminants. To separate these, 200 µl of an aqueous solution of 50 mM $CaCl_2$ was added and mixed thoroughly for 1 min.[29] The proportion of the solvents were 0.8 : 1.0 : 2.0; aqueous : methanol : chloroform. The tubes were centrifuged at 500*g* for 5 min to separate into two distinct phases. The chloroform phase was removed to a fresh tube on ice. A second extraction was performed on the remaining aqueous/methanol phase with an additional two volumes of chloroform. The chloroform phases were pooled and evaporated to a volume of 40 µl in a 37°C heating block under a stream of nitrogen. The air in the tube was flushed out with nitrogen, resealed and stored at −70°C until further analysis by HPTLC.

High Performance Thin-layer Chromatography

A 10 × 20 cm HPTLC pre-coated silica gel plate with concentrating zone, 2.5 × 20 cm (Merck No 13749) was used to separate total lipids extracted from BEAS-2B cells. Of the 40 µl sample, 5 µl sequential applications were made to give a spot diameter of about 7 mm. In between each application the spot was dried under a stream of nitrogen. To separate phospholipids and PLHP in a total lipid sample, the plate was first eluted with hexane/Et_2O (6 : 4, v/v) a fast migrating mobile phase of low polarity, to remove neutral lipids. A second polar mobile phase $CHCl_3$/MeOH/ethanol/triethylamine/H_2O (30 : 10 : 25 : 35 : 8, v/v/v/v/v) is used to separate the major phospholipid classes.[31] The plate was developed 15 min in the first solvent system to a distance of 90 mm, dried and developed in the second solvent system for about 45 min.

The plates were visualised with a 1% solution of N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (TMP) in 50% aqueous methanol containing 1 ml of glacial acetic acid per 100 ml of reagent. The chromatogram was immersed for 30 s in the TMP-reagent, and scanned after ~5 min in transmittance mode at $\lambda = 400$–700 nm using an imaging densitometer (Model 670, Biorad, Hemel Hempstead, UK). Alternatively, plates were visualised by immersing the chromatogram for 15 s in a solution of cupric sulphate pentahydrate (100 g) and orthophosphoric acid (80 ml) diluted to 1000 ml with water,[25] charred for 30 min at 185°C, and scanned in reflectance mode at $\lambda = 400$–700 nm. (Dioleoyl)-phosphatidylcholine (PC), (dioleoyl)-phosphatidylethanolamine (PE), phosphatidylinositol (PI), sphingomyelin (SM), (oleyl)-lysophosphatidylcholine (LPC), and (palmitoyl)-lysophosphatidylethanolamine (LPE) (all applied as 5 µg) (Sigma), were used as external standards, and cumene hydroperoxide (CHP) (98–1470 ng) as a post run external standard.

Fourier Transform Infrared Spectroscopy

Infrared spectra were obtained with a System 2000 FTIR spectrophotometer (Perkin-Elmer. Beaconsfield, Bucks, UK) equipped with a horizontal attenuated total reflection (ATR) unit. The ATR unit consists of a horizontal stage equipped with mirrors. A ZnSe crystal with a 75° face angle and a size of 60 mm long and 20 mm wide, large enough to cover the entire sample run on the HPTLC plate, was used in which total 12 inter-reflections can take place. The transmission range of ZnSe was from 17 000 to 600 cm^1 and this covers all possible absorption peaks of our sample. Prior to visualisation with TMP the HPTLC plate was placed on the ZnSe crystal and pressure was applied on the plate by a pressure clamp to enable the sample to come into close contact with the crystal surface. The sample plates from the 4 and 12 h exposures to ozone were scanned in the range 3000–700 cm^{-1}. In addition a plate with the

standard, CHP (49 ng/μl) was used as a control. The background spectrum (air) was initially recorded and automatically subtracted from every sample spectrum.

Data Analysis

The spectroscopic data in the range 900–700 cm^{-1} were analysed by multivariate data analysis (The Unscrambler® Camo ASA, Oslo, Norway) with the use of Principal Component Analysis (PCA). The purpose of the analysis is to decompose the spectroscopic data into a "structure part" and a "noise part" to detect "hidden phenomena". The "hidden phenomena" are expressed in principal components, which are variance-scaled vectors in variable space.

RESULTS

Uniformity of Ozone Exposure

The uniformity of the exposure was tested to ensure that each culture well received an equivalent amount of ozone. These results are presented in Table I. The absorbance increased with increasing concentration and time and the exposures of the different wells in the exposure chamber were consistent, with standard errors all beneath 10%.

TABLE I Exposure of tissue culture plates to ozone

Ozone conc. (ppm)	Time (min)	Mean absorbance	Standard error (%)
0.10	15	0.105	0.65
0.10	60	0.277	0.98
0.50	15	0.580	4.00
0.50	60	1.247	6.41
1.00	15	1.120	6.57

Experiments were performed at three ozone concentrations, for 15 and 60 min. For each exposure six replicate culture wells were used containing 4 ml 2% neutral buffered KI. Results are presented as mean values for absorbance reading ±% standard error.

HPTLC Detection of Phospholipid Hydroperoxides from Cells Exposed to Ozone

CHP (98–1470 ng) external standard was effectively visualised (Figure 2). For the ozone-exposed cell samples, six lipid bands were identifiable in each case on the HPTLC plate (result not shown). The chromatograms showed good reproducibility, with standard errors < 10% for the retardation factors (R_F). In our experiment the mean R_F values lay between 0.3 and 0.8. From the phospholipid standards it was possible to assign the sample bands to the major phospholipid classes (Figure 2) which resolve in the following order, LPC ($R_F = 0.25$), SM ($R_F = 0.28$), PC ($R_F = 0.32$), LPE ($R_F = 0.38$), PI ($R_F = 0.43$), and PE ($R_F = 0.50$). Other studies that have used the same solvent system have obtained similar results.[25,31] There was no identifiable PLHP band present which would have been apparent as an additional band between PC and PE.

FTIR Spectra of Phospholipid Hydroperoxides

Hydroperoxides are all found in the range 890–820 cm^{-1},[32] which corresponds with the region of O–O stretching. FTIR-spectra are shown for a CHP standard (Figure 3) and ozone-exposed BEAS-2B cells compared to un-exposed cells (Figure 4). From the CHP spectrum a number of hydroperoxide peaks were identified within this region, namely, 892, 882, 871, 864, 855, 839, 846 and 829 cm^{-1}. The same peaks could be identified in the sample spectra with the addition of a peak at 820 cm^{-1} and the peak at 855 cm^{-1} has shifted to 850 cm^{-1}. It is worth noting that use of FTIR plus HPTLC results in a clear increase in sensitivity over HPTLC alone since no detectable PLHP band was found on the HPTLC plate.

Utilising the PCA (The Unscrambler®) the optimum number of principal components was selected with care from a total residual variance plot. Too few components run the risk of relating

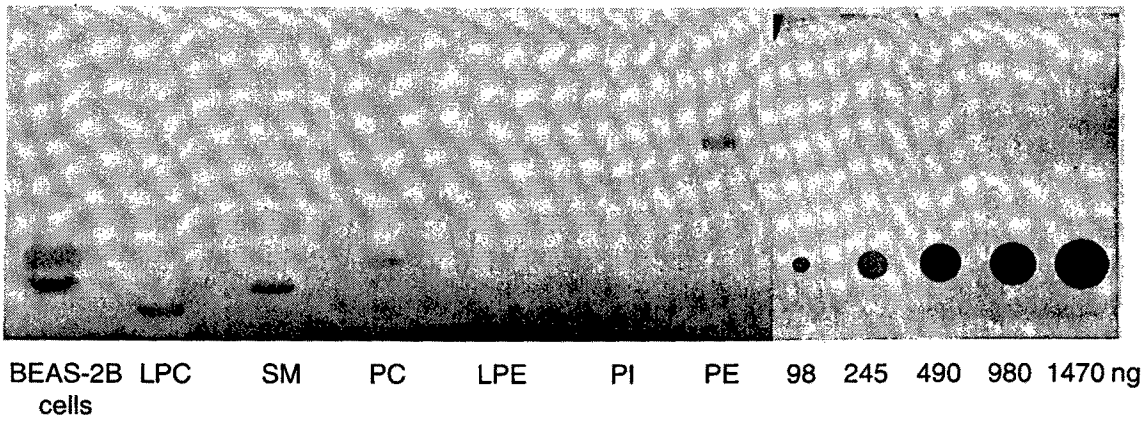

FIGURE 2 HPTLC separation of lipids extracted from BEAS-2B cells, phospholipid standards and CHP standards. Lane 1: Lipids extracted from BEAS-2B cells (440 µg), lane 2: 20 µg LPC, lane 3: 5 µg SM, lane 4: 5 µg PC, lane 5: 5 µg LPE, lane 6: 5 µg PI, lane 7: 10 µg PE, lane 8: 98 ng CHP, lane 9: 245 ng CHP, lane 10: 490 ng CHP, lane 11: 980 ng CHP, lane 12: 1470 ng CHP. Visualised in cupric sulphate pentahydrate and orthophosphoric acid and then charred for 30 min at 185°C.

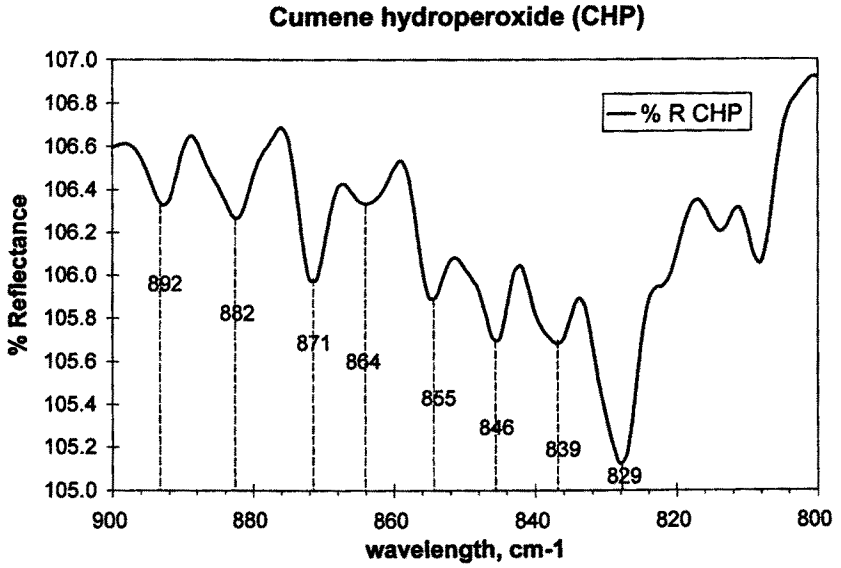

FIGURE 3 FTIR spectra of the standard, CHP (49 ng/µl). O–O stretching of hydroperoxides was identified in the range 890–820 cm^{-1}, namely; 892, 882, 871, 864, 855, 846, 839, 829 cm^{-1}. FTIR conditions are described in the Methods section.

only to a superficial data structure. On the other hand, use of too many components can lead to interpretation of parts of the noise structure. The optimum number of principal components for our spectroscopic data was shown to be two; first principal component (PC1) and second principal component (PC2). PC1 represents the PHLP, and PC2 may reflect the double bonds of the *cis*-configuration in the fatty acid side chains of lipids, as the alkene C–H band of the *cis*-configuration are found in the range 730–675 cm^{-1}.[33]

For the 4 h exposure, principal component regression (PCR) with two components reveals

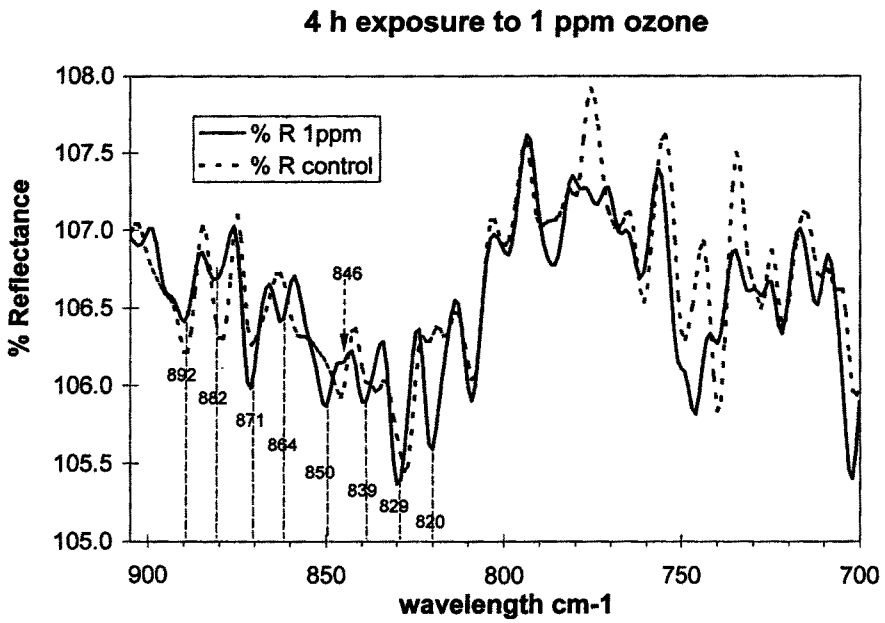

FIGURE 4 FTIR spectra of BEAS-2B cells exposed to 1 ppm ozone for 4 h compared to the control. O–O stretching of hydroperoxides was identified in the range 890–820 cm^{-1}, namely; 892, 882, 871, 864, 850, 839, 829, 820 cm^{-1}. FTIR conditions are described in the Methods section.

TABLE II Explained variances of spectroscopic data (X-expl) and of ozone concentration (Y-expl)

Principal components	4 h exposure		12 h exposure	
	X-expl (%)	Y-expl (%)	X-expl (%)	Y-expl (%)
PC1	28	76	87	11
PC2	23	21	8	75
Total	51	97	95	86

that 97% of the ozone variables (PC1: 76%, PC2: 21% see Table II) can be correlated to 51% of the spectral variance leaving 49% of the spectra as noise components. After 12 h exposure, PCR reveals that 84% of the ozone variable (PC1: 11%, PC2: 75%, see Table II) can be correlated to 95% of the spectral variance leaving only 5% of the spectroscopic data as noise components. Multivariate data analysis with two principal components (PC1 and PC2) revealed a linear positive correlation ($r = 0.99$ for 4 h exposure and $r = 0.98$ for 12 h exposure) between ozone exposure levels and the region of the FTIR-spectrum comprising the main wavelengths for hydroperoxides (see Figures 5 and 6), with a root mean square error of calibration (RMSEC) of 0.04 ppm for 4 h exposure and 0.08 ppm for 12 h exposure. The value of RMSEC for 4 h exposure indicates a detection limit of approximately 0.12 ppm.

DISCUSSION

We have described an innovative technique based on FTIR combined with HPTLC for the detection of PHLP generated through early damage in epithelial cells exposed to ozone, at environmentally relevant concentrations.

Six phospholipids were identified on the HPTLC plate; LPC, SM, PC, LPE, PI and PE. It was possible to detect 100 ng of CHP, with charring and the TMP dye. However no distinct bands of lipid hydroperoxide produced from ozone exposed cells were identified using HPTLC alone. It is probable that failure to see bands is related to the minimal amounts of PLHP formed.

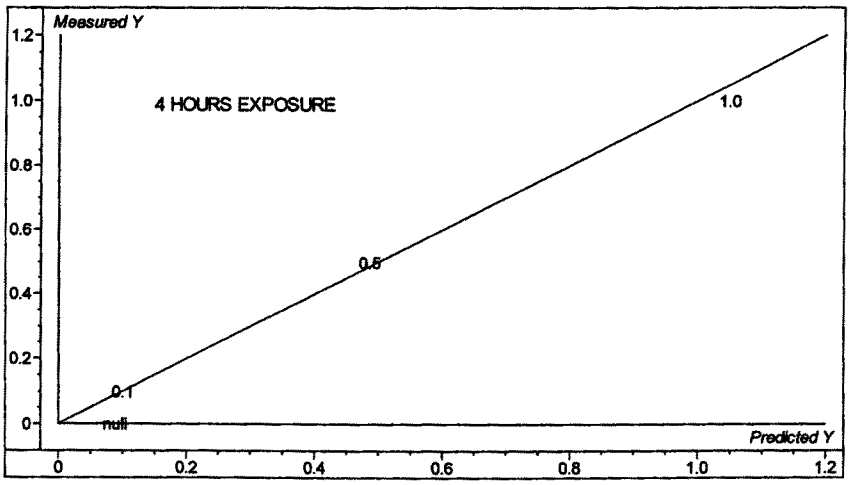

FIGURE 5 A comparison of predicted and measured Y-values (ozone exposure levels) for the 4 h exposure. The ozone exposure levels are predicted from the spectroscopic data. Multivariate data analysis with two principal components (PC1 and PC2) revealed a linear positive correlation ($r = 0.99$) between ozone exposure levels and the region of the FTIR-spectrum comprising the main wavelengths for hydroperoxides, with a RMSEC of 0.04 ppm.

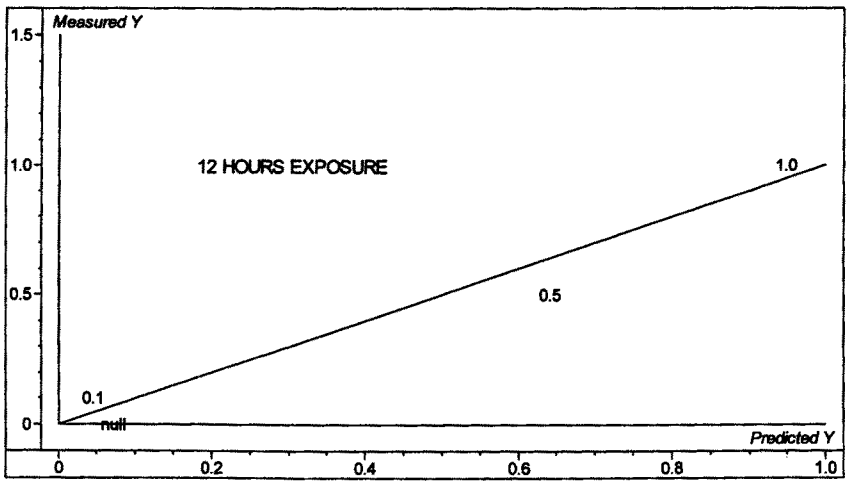

FIGURE 6 A comparison of predicted and measured Y-values (ozone exposure levels) for the 12 h exposure. The ozone exposure levels are predicted from the spectroscopic data. Multivariate data analysis with two principal components (PC1 and PC2) revealed a linear positive correlation ($r = 0.98$) between ozone exposure levels and the region of the FTIR-spectrum comprising the main wavelengths for hydroperoxides, with a RMSEC of 0.08 ppm.

For example, it has been shown that from 1 mg lipids extracted from 1 g salmon, 3.5% were phospholipids (35 mg), of which only 50–250 ng were hydroperoxides.[31] In this study, given that the number of BEAS-2B cells retrieved from one insert is approximately 6.67×10^5 cells then assuming the lipid composition from the salmon applies, from three inserts ($\sim$20 mg cells [H.C. Bisgaard, Roskilde University, personal communication]), 20 µg total lipids would have been extracted containing $\sim$1–5 ng hydroperoxides, below the detection limit of the Wurster dye. This supports the adjunct use of FTIR, which enabled detection of < 5 ng hydroperoxides.

In recent years FTIR is increasingly applied as a biodiagnostic tool.[34–38] FTIR has also been used for detection of drug-induced changes in mouse lung tissue.[37] The advantage of using FTIR together with HPTLC is its ability to focus on specific components of interest in single cell systems, in contrast to FTIR alone of whole tissue, which will give a complex spectrum of a multicomponent system. FTIR to our knowledge has not yet been used to detect lipid peroxidation in human lung cell cultures. Our method has the advantages of being technically simple, requires little sample material (less than 5 ng) when ATR is used, with an assay time of approximately 2 h. It does not need to be accompanied by a detection system such as chemiluminescent,[21] UV,[22] and iron-thiocyanate[39] assay as required with HPLC. FTIR is versatile, as it allows detection of other structural changes in lipids such as the disappearance of the *cis*-double bond in the fatty acid side chains. Importantly the method we describe (FTIR with HPTLC) measures a primary oxidation product, PHLP in ozone-damaged cells. Other methods such as GC-MS would only have detected aldehydes, which are secondary breakdown products.[18,19] In addition, many aldehydes have low boiling point (bp) and can therefore be difficult to detect on GC-MS, as the retention time will be very short, albeit that this can partially be overcome by using 2,4-dinitrophenylhydrazones.[40,42]

From the FTIR spectra in our study, O–O stretching of hydroperoxides was identified in the range 890–820 cm^{-1}. The O–O stretching peaks identified in the FTIR spectra for the ozone-exposed cells differed slightly from those identified in the spectra for the CHP standard. If the functional groups in a chemical compound are isolated and do not interfere with each other, then the vibration frequency of the same group in a different compound should be identical. However, other parts in the molecule affect the vibration of any group in a complex compound. Therefore, the absorption peaks in different compounds are always shifted within a narrow range. The O–O stretching peak at 855 cm^{-1} in CHP shifted to 850 cm^{-1} in the sample spectra for the ozone exposed cells, because the bond force between the two O in the sample spectra was reduced slightly compared to that in CHP. The peak at 846 cm^{-1} in the spectra of ozone-exposed cells was reduced by the peak shifting from 855 to 850 cm^{-1}.

The multivariate data analysis is a model system, which uses the spectroscopic data from the different exposure experiments to predict ozone concentrations in relation to one another. When plotting the predicted versus measured ozone levels, it will reflect the accuracy of the prediction (correlation). The model in this case showed a clear linear positive correlation between the measured and predicted ozone levels (Figures 5 and 6), indicating that the peak areas in the FTIR-spectrum comprising the main wavelengths for hydroperoxides increases linearly with increasing ozone concentration. The correlation for the 4 h exposure ($r = 0.99$) was better than for the 12 h exposure ($r = 0.98$), presumably because after 12 h the hydroperoxides are starting to break down to secondary reaction products.

Our data have shown that epithelial cell production of hydroperoxides occurs in a concentration-dependent fashion with ozone levels of 0.1–1.0 ppm. Other studies support similar ozone-induced membrane cytotoxicity.[43] The model presented in this current study supports the applicability of a combined HPTLC and FTIR approach for early detection of ozone-mediated damage in airway lining cells. The method shows considerable promise in being able to detect primary products of peroxidation and offers the major advantage of relative simplicity.

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