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Effects of Hypochlorite on Cultured Respiratory Epithelial Cells

C. KAMPF* and G.M. ROOMANS

Department of Medical Cell Biology, Uppsala University, Box 571, 751 23 Uppsala, Sweden

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Neutrophils and eosinophils are involved in the pathogenesis of many respiratory diseases. The enzymes myeloperoxidase and eosinophil peroxidase catalyze the reaction of H_2O_2 with Cl to produce the reactive oxygen species HOCl.

Normal human bronchial epithelial (NHBE) cells were exposed to 0.18–0.90 mM HOCl for 48 h, and studied with immunohistochemical, metabolic and morphological studies.

The ability of the cells to attach to each other and/or to the matrix was altered. Immunohistochemical studies showed a decreased amount of desmosomes and focal adhesion sites, although the morphology of the cells was not affected. The ability of the mitochondria to oxidize glucose was reduced. HOCl-exposed cells had an increased production of NO, probably by an increased activity of cNOS, due to increased intracellular Ca^{2+}. The antioxidant N-acetylcysteine inhibited both the NO production and the effects of HOCl on glucose oxidation. The cNOS-inhibitor N-propyl-L-arginine inhibited HOCl-induced NO production. X-ray microanalysis showed an increase in the intracellular Na^+/K^+ ratio, which indicates cell damage.

In conclusion, exposure to HOCl results in cell detachment and metabolic alterations in normal human bronchial epithelial cells. Oxygen radicals could in part mediate the effects. Oxygen radicals could hence contribute to the observed epithelial damage in respiratory diseases.

Keywords: normal human bronchial epithelial cells; hypochlorite; desmosomes; vinculin; nitric oxide

INTRODUCTION

Neutrophils are thought to be involved in the pathogenesis of various inflammatory lung disorders[1–5].

The enzyme myeloperoxidase (MPO), which is present in the granules of neutrophils, produces hypochlorous acid by the conversion of H_2O_2 and Cl to HOCl[6]. The ability of MPO to contribute to cell damage appears to be increased by its cationic nature[7]. This allows the enzyme to stick to the cell surfaces, thereby increasing the local concentration of hypohalous acid at the cell surface. Eosinophils, commonly found in asthmatic airways, contain lysosomal peroxidases e.g., eosinophil peroxidase (EPO) that can oxidize halides to generate reactive and toxic hypohalous acids[8], although other oxidants such as hypothiocyanate may also be produced[9]. HOCl is an extremely toxic oxidant that can react

* Correspondence: C. Kampf, Department of Medical Cell Biology, Uppsala University, Box 571, 751 23 Uppsala, Sweden. Tel. +46–18–4714292, Fax +46–18–551120, E-mail: caroline.kampf@medcellbiol.uu.se

with a variety of biological target molecules[10]. As an oxidant, HOCl has its primary effect on proteins. It reacts strongly with cell membrane proteins, but it also affects intracellular activities such as ATP synthesis via both enzymatic glucolysis and oxidative phosphorylation[11,12].

Damage to and shedding of the airway epithelium plays an important role in the development of airway diseases[13,14]. Desmosomes are important structural components that connect adjoining epithelial cells to each other. Vinculin is a component of the focal adhesion site, which is a cell-matrix complex that connects the internal actin filaments to the extracellular matrix. If HOCl has its primary effect on proteins, it is conceivable that HOCl could affect the number of desmosomes and focal adhesion sites, or the composition of the proteins forming these attachment sites. This could contribute to the damage to and the shedding of the epithelium observed in various lung disorders. Increased concentrations of HOCl have been shown to result in progressive loss in the adhesive properties of the extracellular matrix[15].

HOCl is known to induce generation of free radicals, such as hydroxyl radicals (.OH) and in the presence of amines HOCl produces N-chloramines (RNHCl)[16]. These agents can cause injury to cells, such as DNA damage, rise in intracellular Ca^{2+}, NAD(H) and glutathione depletion.

The current study was undertaken to define the effect of HOCl on normal human bronchial epithelial (NHBE) cells.

MATERIALS AND METHODS

Preparation of HOCl

Winterbourn[10] indicated that commercial sodium hypochlorite yielded results identical to those obtained with HOCl produced by the MPO system. Thus, diluting a stock solution of sodium hypochlorite (Sigma, St.Louis, MO, USA) and adjusting the pH to 6.85 with H_2SO_4 generated all concentrations of HOCl. The HOCl concentration was determined using 100 $M^{-1}cm^{-1}$ at 235 nm[17]. The HOCl was diluted in the culture medium and the experiments were performed in the culture medium.

Culture of Epithelial Cells

Normal human bronchial epithelial (NHBE) cells (Clonetics, San Diego, CA, USA) were cultured in plastic culture flasks (Costar, Corning Costar Corporation, Cambridge, MA, USA) in bronchial epithelial basal medium (BEGM) (Clonetics) supplemented according to the manufacturer's instructions with human recombinant epidermal growth factor (0.5 μg/ml), insulin (5 mg/ml), hydrocortisone (0.5 mg/ml), transferrin (10 mg/ml), epinephrine (0.5 mg/ml), triiodothyronine (6.5 μg/ml), bovine pituitary extract (13 mg/ml), retinoic acid (0.1 μg/ml), gentamicin (50 mg/ml) and amphotericin-B (50 mg/ml) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Although the cells are designated as bronchial cells, they may, in fact, according to the manufacturer's information, come from the trachea. The culture medium was exchanged every 48 hrs. Confluent cells were cultured on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA) or in Petri dishes (Becton Dickinson, Plymouth, UK) and to achieve desmosome formation the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM):Ham's F12 (1:1) (Gibco BRL/LifeTechnologies, Paisley, UK) supplemented with 5% fetal bovine serum (Gibco BRL/LifeTechnologies), penicillin (100 U/ml) (Sigma), streptomycin (100 μg/ml) (Sigma) and 1% non-essential amino acids (Sigma).

Glucose Oxidation Rate

The glucose oxidation rate was determined as previously described in detail[18].

Cell Viability and Attachment Experiments

The cell viability was determined by the trypan blue dye exclusion test (Sigma) and the fraction of dead cells (nuclei stained blue) was calculated after observation in a light microscope.

NHBE cells (about $5x10^6$ cells/ml) were plated in Petri dishes (Becton Dickinson) and the cells were grown for a few days. The total number of cells before and after HOCl exposure was calculated using a Bücker chamber (Tambro, Göteborg, Sweden). The difference between these numbers is an indication of the release of cells from the substrate.

Nitrite Measurement

Measurements of NO_2^- in NHBE cell culture supernatants by the Griess reagent[19] were taken as an indicator of NO generation.

X-ray Microanalysis

For X-ray microanalysis, cells (about $5x10^6$ cells/ml) were seeded out on 75 mesh titanium grids (Agar Scientific). The grids had been covered with a Formvar film (Merck, Darmstadt, Germany) and a thin carbon layer. Before use the grids were sterilized under ultraviolet light. The cells were allowed to grow at 37°C in an incubator with 5% CO_2/95% air atmosphere[20].

After 2–3 days in culture some of the cells were exposed to 0.18 mM, 0.45 mM or 0.90 mM sodium hypochlorite, and unexposed cells served as controls. After 48 hrs of culture the experiment was terminated by a quick rinse in distilled water (+4°C) and the cells were frozen in liquid propane, cooled by liquid nitrogen at –180°C and then freeze dried at –130°C under vacuum overnight. Finally the freeze-dried specimens were coated with a conductive carbon layer before analysis.

X-ray microanalysis was performed at 100 kV in the scanning-transmission electron microscopy (STEM) mode of a Hitachi H7100 with an Oxford Instruments (Oxford, UK) ISIS energy-dispersive spectrometer system. Quantitative analysis was carried out based on the peak-to-continuum after correction for extraneous background[21] and by comparing the spectra from the cells with those from a standard. Spectra were acquired for 50 sec and only one spectrum was obtained from each cell.

Preparation of Cultured Cells for Transmission Electron Microscopy (TEM)

NHBE cells, incubated with or without 0.18 mM, 0.45 mM or 0.90 mM HOCl for 48 hrs, grown in Petri dishes were fixed in 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (Agar Scientific, Stansted, UK) for 1 day. After being washed in 0.1 M cacodylate buffer the cells were post-fixed in 1% OsO_4 in cacodylate buffer for 20 min. A second washing in buffer was followed by dehydration in graded series of ethanol, and finally the cells were embedded in Agar 100 Resin (Agar Scientific). Sections were cut and contrasted with uranyl acetate/lead citrate and examined in a Hitachi (Tokyo, Japan) H7100 transmission electron microscope at 75 kV.

Immunohistochemistry of Desmosomes

Confluent NHBE cells grown on glass slides in DMEM:F12 medium were fixed in methanol at –20°C for 3 min, and rinsed with TBS (Tris-buffered saline; 0.05 M Tris-HCl, 0.15 M NaCl) pH 7.6 for 5 min. The cells were blocked with 10% normal human serum (NHS) (Sigma) in TBS for 30 min. After primary incubation with monoclonal anti-desmosomal cytokeratin (Sigma, cat no C-1041) 1:1500 in TBS for 1 hr at 37°C the cells were rinsed twice with TBS. Thereafter, cells were blocked in 10% NHS for 30 min and incubated with a biotinylated secondary antibody (Dako, Copenhagen, Denmark) for 1 hr. After rinsing with TBS FITC conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc,

West Grove, PA, USA) was bound to the biotinylated secondary antibody. The specimens were mounted in Vectashield (Vector, Burlingame, CA, USA) mounting medium for fluorescence and examined with a Leica (Rijswijk, Netherlands) microscope. Control slides were processed identically except that primary antibody was omitted.

Immunohistochemistry of Vinculin

NHBE cells grown on glass slides in BEGM medium were fixed in 4% paraformaldehyde for 3 min, and rinsed with TBS for 5 min. The cells were permeabilised in Triton X-100 diluted in TBS for 7 min. After a quick rinse in TBS, the cells were blocked with 5% normal rabbit serum (NRS) (Dako) in TBS for 10 min. After primary incubation with monoclonal anti-vinculin (Sigma, cat no V9131) 1:800 in TBS for 1 hr the cells were rinsed twice with TBS. Thereafter, cells were incubated with a FITC-conjugated secondary antibody (Dako) 1:40 for 1 hr. After rinsing with TBS the slides were mounted in Vectashield (Vector) mounting medium for fluorescence and examined with a Leica microscope. Control slides were processed identically except that primary antibody was omitted.

Nuclear Protein Extraction

Extraction of nuclear proteins for the electrophoretic mobility shift assay (EMSA) was performed by the method described by Dignam *et al.*[22] with minor modifications. Briefly, at various times after HOCl exposure, the cells were washed in 0.5 ml ice cold PBS and detached from the Petri dish. The cells were centrifuged at 12000 g for 10 sec, decanted and resuspended in 50 µl cold hypotonic buffer A (10 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM dithothreitol (DTT), 1 mM Pefabloc®SC (Pentapharm AG, Basel, Switzerland)), and left on ice for 10 min. Then the cells were centrifuged again at 12000 g for 10 sec. The supernatants were removed and the pellets were resuspended in the hypotonic buffer and homogenized. After the third centrifugation, the supernatant was removed and the pellet was resuspended in 50 µl of hypertonic buffer C (0.42 M KCl, 20 mM Tris pH 7.5, 20% (w/v) glycerol, 1.5 mM $MgCl_2$, 2 mM DTT, 1 mM Pefabloc®SC). The suspension was sonicated for 10 sec and left on ice for 30 min. After centrifugation, aliquots of the supernatant, containing nuclear proteins, were frozen in –70°C.

Elecrophoretic Mobility Shift Assay (EMSA)

The EMSA method used is that described by Baeuerle *et al*[23]. Briefly, a double stranded 26 mer oligonucleotide containing the κβ binding site (5'AGCTTCAGAGGGGACTTTCCGAGAGG-3'), was labeled with [α-^{32}P]d CTP (Megaprime labeling kit, Amersham International). Nuclear proteins were denatured with formamide (27%) and added to a binding solution (10 mM Tris pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.2% deoxycholic acid, 2 mg polydeoxyinosinic acid, 4% glycerol) containing the oligonucleotide and incubated for 30 min. As a negative control, a 100–1000 excess non-labeled oligonucleotide was used. The DNA-protein complexes were then separated on a non-denaturating polyacrylamide gel (5%) for 75 min in 0.5 × TBE (Trisborate EDTA). The gel was then dried and autoradiographed on an X-ray film (Amersham International) at –70°C.

Western Blot Analysis

Cells were rinsed in ice-cold PBS and removed from the Petri dish. The cells were sonicated for 10 seconds in TE buffer (10 mM Tris, 1 mM EDTA). The protein content in the samples was determined by the method of Bradford[24]. Proteins were percipitated in two volumes of ice-cold acetone and pelleted by centrifugation. After removal of the supernatant, samples were

dissolved in SDS-gel sample buffer (2% SDS, 100 mM Tris pH 6.8, 100 mM β-mercaptomethanol, 0.01% bromophenol blue, 10% glycerol) by boiling for 5 min. Equal amounts of protein (10–20 μg) were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose filters. The filters were pre-blocked for one hour in PBS containing 5% fat-free milk powder, followed by incubation with a monoclonal anti-iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:400 in PBS +1% bovine serum albumin (BSA) +0.1% Tween-20. The filters were incubated with a horseradish-peroxidase conjugated secondary antibody (Amersham) and detected using an enhanced chemiluminescence system (ECL-kit, Amersham) according to the manufacturer's instructions.

Measurements of Cytosolic Free Ca^{2+}

Cells (about $5x10^6$ cells/ml) grown on coverslips were loaded with 5 μM fura-2/acetoxymethyl ester (fura-2/AM) (Molecular Probes) in BEGM for 30 min at 37°C in 5% CO_2/95% air. The cells were rinsed with Ringer's Standard solution (140 mM NaCl, 5 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, and 5 mM glucose) pH 7.4 before measuring. The excitation wavelengths used were 340 and 380 nm, respectively. The emission wavelength was 510 nm.

Calibration of $[Ca^{2+}]_i$ was performed in Ca^{2+}free Ringer's Standard solution by subsequently adding 4 mM EGTA-20 mM Tris and 30μM of the ionophore ionomycin to obtain the limiting ratio for the unbound form (R_{min}) of fura-2. The limiting ratio (R_{max}) for the Ca^{2+}-saturated form of fura-2 was obtained by adding $CaCl_2$. Twenty mM $MnCl_2$ was finally added to obtain the autofluorescence. The ratio was converted to $[Ca^{2+}]_i$ by the method of Grynkiewicz *et al.*[25] using the equation

$$[Ca^{2+}]_i = (R - R_{min})/(R_{max} - R) * K_d * S$$

R was the experimentally determined fura-2 fluorescence ratio and the dissociation constant (K_d) used was 224 nM. S was the ratio of fluorescence at 380 nm in a Ca^{2+} free solution to that of a Ca^{2+} containing solution in the presence of ionomycin. The measuring procedure was carried out at room temperature.

Statistical Analysis

Data are presented as the mean ± SEM, and statistical analysis was performed using analysis of variance (ANOVA) or, for the X-ray microanalysis experiments and for the Ca^{2+}experiments, Student's *t*-test. Significance was attributed to probability values of less than 0.05. When experiments were performed in triplicate, such as glucose oxidation rate and nitrite measurements, a mean was calculated and considered as one separate observation.

RESULTS

Glucose Oxidation Rate

The effects of exposure of NHBE cells to different concentrations of HOCl on the glucose oxidation rate were investigated. 0.18 mM HOCl decreased the glucose oxidation with 18%, 0.45 mM HOCl decreased the oxidation significant with 38%, and when the cells were exposed to 0.90 mM the decrease was almost 100% (not shown). Treatment with the antioxidant N-acetylcysteine (NAC) significantly decreased the toxic effect of 0.45 mM HOCl.

Cell Viability and Cell Attachment

The effects of exposure of NHBE cells to different concentrations of HOCl on cell viability were investigated. 0.18 mM HOCl reduced the viability with 5%, 0.45 mM HOCl reduced the viability significant with 24%, and 78% of the 0.90 mM

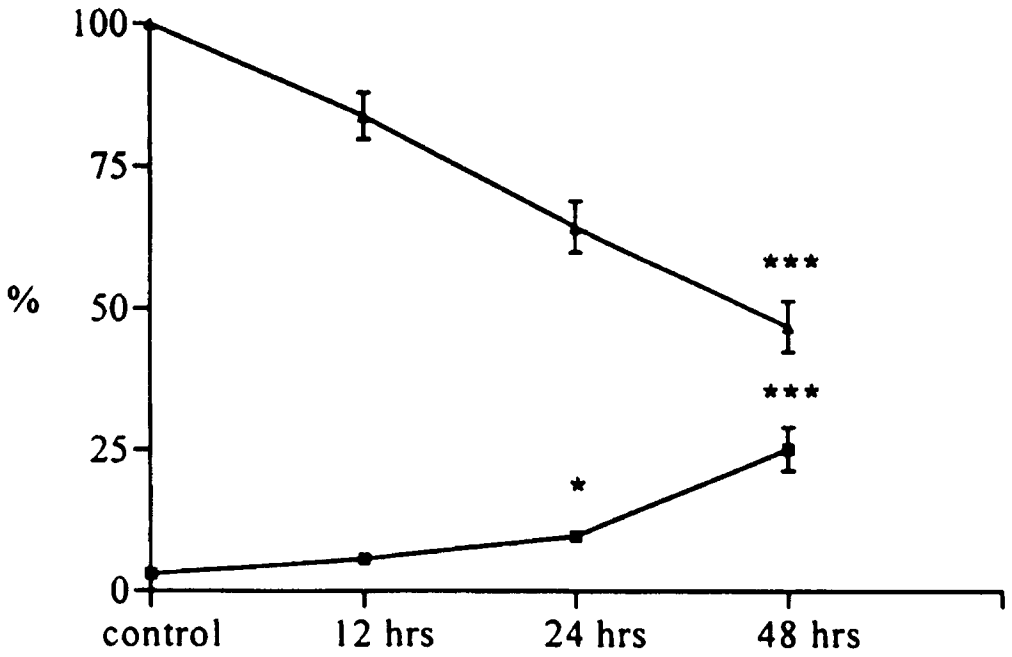

FIGURE 1 Time-course of cell viability and attachment experiments for NHBE cells exposed to 0.45 mM HOCl. Values are means ± SEM for 5–11 experiments. *: $p < 0.05$; ***: $p < 0.001$, compared to the control. Squares indicate % dead cells and triangles indicate % attached cells

exposed cells were dead. 1.0 mM NAC reduced the effects of 0.45 mM HOCl on cell viability.

Time course experiments show that 0.45 mM HOCl starts to affect the cell viability after 24 hrs. However, the cell viability was even more extensively altered after 48 hrs (Fig. 1). A significant reduction of the ability of the cells to attach to the matrix was observed after 48 hrs (Fig. 1).

Nitrite Measurements

Nitrite accumulation in the medium was increased when the cells were exposed to 0.18 mM HOCl and 0.45 mM HOCl, with the latter concentration increasing the nitrite accumulation ten-fold compared to the control (Fig.2). Due to low glucose oxidation rate and high cell death the effect of 0.90 mM HOCl was not analyzed. When cells were treated with 0.45 mM HOCl in the presence of 1.0 mM NAC, the increase in nitrite accumulation was markedly reduced (Fig.2). When the cells were treated with 0.45 mM HOCl in the presence of 100 μM N-propyl-L-arginine, a cNOS inhibitor, the increase in nitrite accumulation was also reduced (Fig.2).

X-ray Microanalysis

In cells exposed to 0.18 mM and 0.45 mM HOCl the Na^+, S^{2-} and Cl^- contents were increased compared to the control cells (Fig.3). The concentration of the other elements measured did not change significantly. The (total) Ca concentration in the cells was generally below the detection limit of the instrument. However, in cells exposed to 0.90 mM, calcium was detectable. Moreover, the Na^+ content in cells exposed to this concentration of HOCl had also increased whereas the Mg^{2+}, S^{2-}, Cl^- and the K^+ content had decreased (Fig.3).

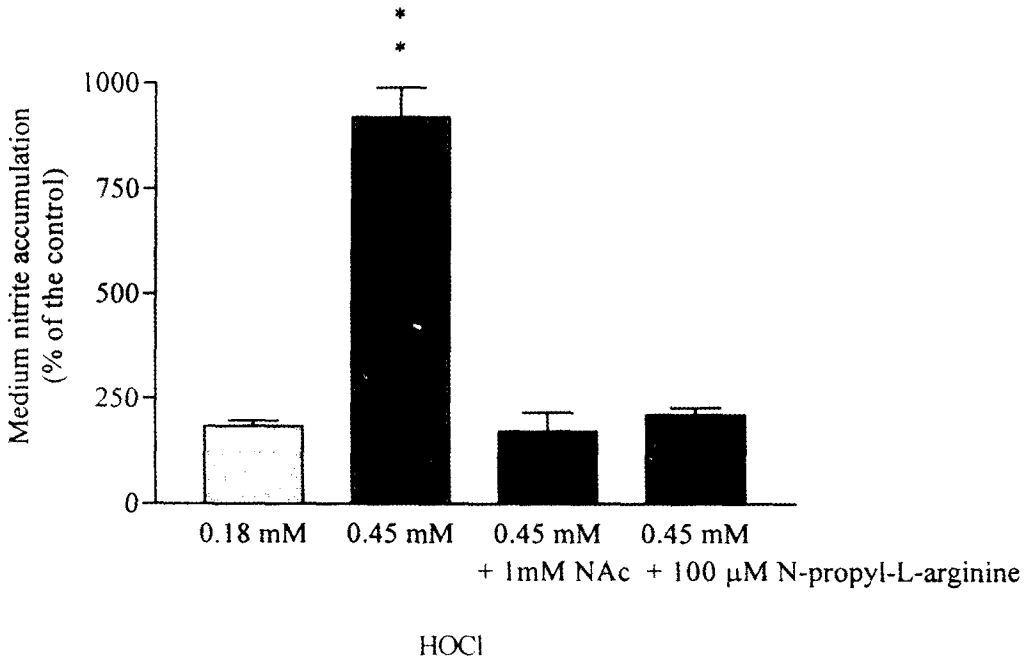

FIGURE 2 Nitrite accumulation in the medium of NHBE cells exposed to 0.18 mM HOCl, 0.45 mM HOC1, 0.45 mM HOCl + 1 mM NAC and 0.45 mM HOCl + 100 μM N-propyl-L-arginine. The data are expressed as percentage of the control value. Values are means ± *SEM for 6–10 experiments.* **: $p < 0.01$, compared to the control

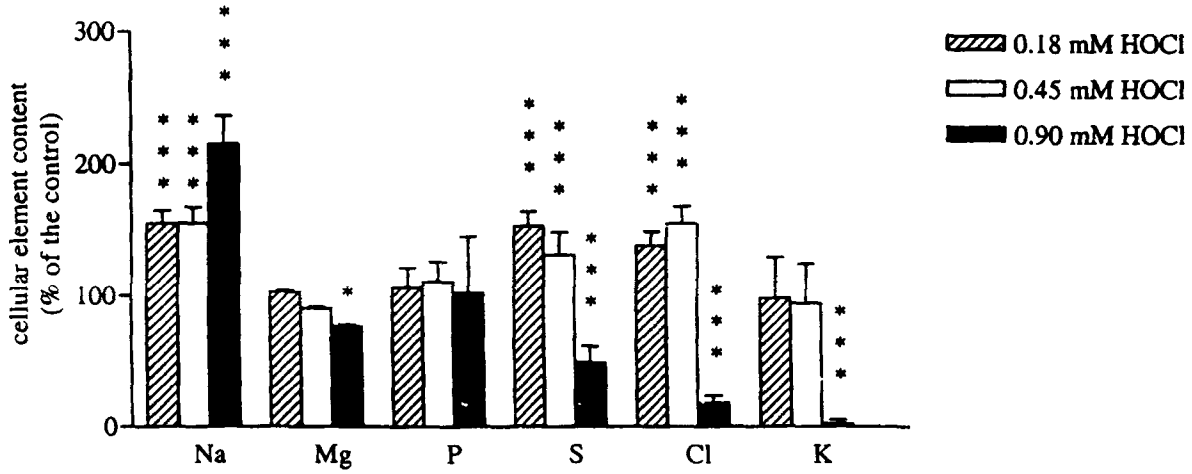

FIGURE 3 Effects of 0.18 mM, 0.45 mM, and 0.90 mM HOCl on the cellular elemental content in NHBE cells. The data are expressed as percentage of the control value. Values are means ± SEM. The data are based on four experiments. In each experiment, about 20 cells were analyzed. *: $p < 0.05$; ***: $p < 0.001$, compared to the control

TEM

Cells exposed to 0.18 mM and the 0.45 mM HOCl showed a similar morphology as control cells. However, the morphology of the cells exposed to 0.90 mM HOCl was severely affected. The cell membrane was not intact and the cytoplasmic matrix had been lost from the cell (not shown). Moreover, hardly any organelles, such as mitochondria, could be detected.

Immunohistochemistry of Desmosomes

In the control cells many desmosomes lined the cell borders (Fig.4a). There were difficulties in investigating the desmosome formation of the cells exposed to HOCl for 48 hrs. The cell volume seemed to change and the cells detached from each other. Even though there was no cell contact, spot-like immunoreactivity was observed on the cell membrane (Fig.4b). These spots were not as numerous as when the cells were in contact. Cells exposed to 0.45 mM HOCl for 24 hrs did not show any differences compared to the control.

Immunohistochemistry of Vinculin

Vinculin was distributed around the control cells (Fig.5a). The ability of the cells to attach to the matrix was altered when the cells were exposed to HOCl for 48 hrs and there was a decrease in the amount of vinculin observed. Some of the cells lacked vinculin completely, whereas other cells showed a marked decrease in the amount of this protein (Fig.5b). Cells exposed to 0.45 mM HOCl for 24 hrs did not show any differences compared to the control.

EMSA and Western Blot Analysis

EMSA did not show any induction of NF-κB, and Western blot analysis did not show any induction of iNOS in NHBE cells after treatment with 0.45 mM HOCl (data not shown).

Measurements of Cytosolic Free Ca^{2+}

HOCl caused a significant increase of the $[Ca^{2+}]_i$ in NHBE cells from 90 nM to 120 nM (not shown).

DISCUSSION

HOCl produced by myeloperoxidase is likely to be the major oxidant from neutrophils and may be an important contributor to inflammatory tissue damage. Likewise, HOCl can be produced by eosinophils. In this study we have shown that the ability of the cells to attach to each other or to the matrix is decreased by HOCl. HOCl induces nitrite formation, reduces the glucose oxidation rate and the cell viability. Exposure to HOCl results in an increased Na^+/K^+ ratio, which indicates cell damage. TEM did not show any morphological changes, except when the highest concentration of HOCl was used. The HOCl concentrations present in the airways of cystic fibrosis patients have been estimated to be in the range of 3–8 mM[26], which is considerably higher than the concentrations used in the present study.

Because the cells had to be exposed to HOCl under a long period, exposure had to be performed in culture medium. Under these conditions, HOCl may react with amino groups in the medium to form chloramines and other chlorination/oxidation products. Hence, the observed effects may be due in part to chloramines rather than directly to HOCl. However, also in tissue HOCl is in the presence of organic substances and can form chloramines.

Although the type of adhesion plaques in the NHBE cells in vitro may not be identical to the adhesive cell contacts in bronchial epithelial cells in vivo, the observed changes in vinculin point to a realistic possibility of cytoskeletal/adhesive cell contact modifications also in vivo. It is unclear if detachment is due only to modulation of the adhesive proteins of the extracellular

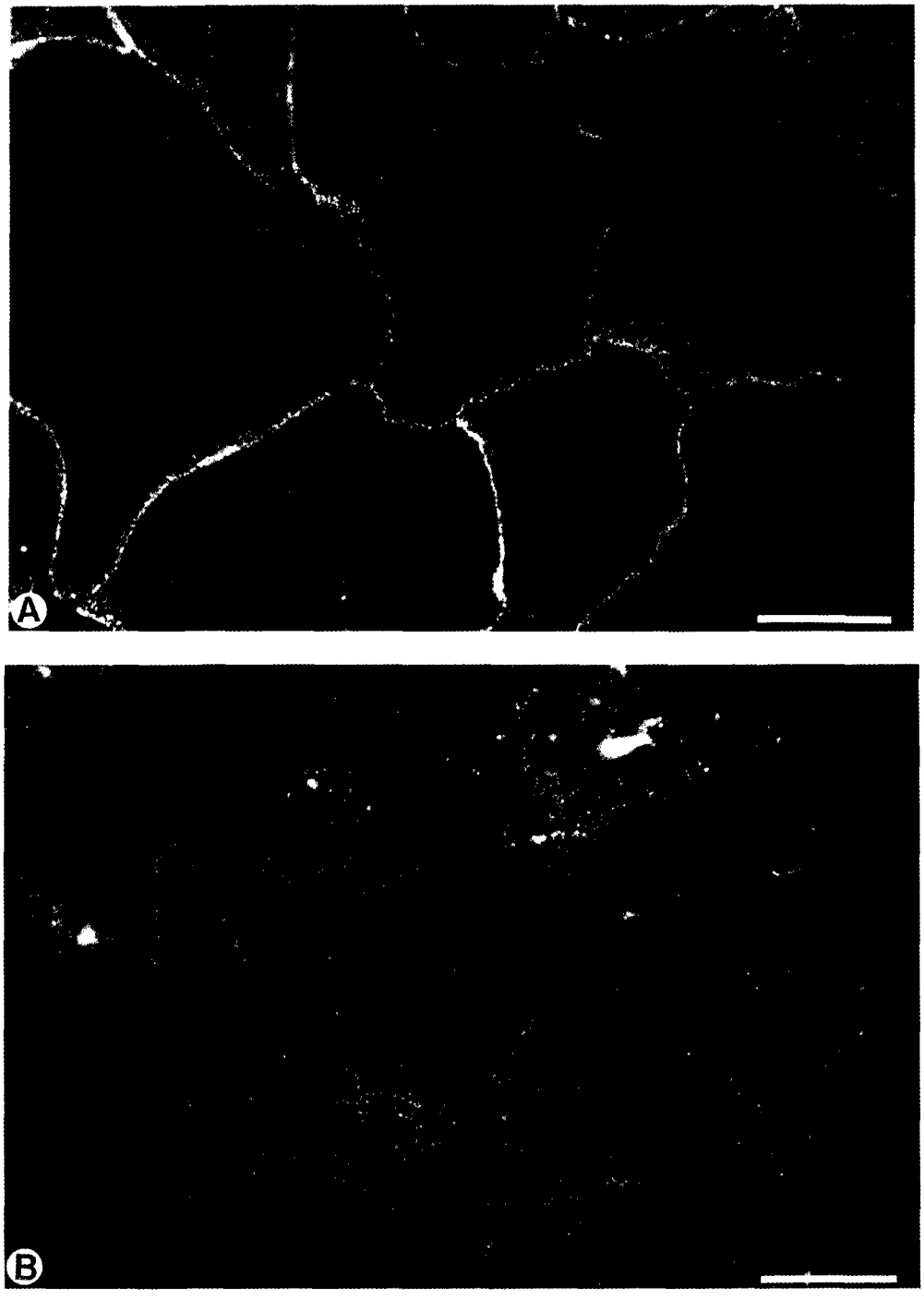

FIGURE 4 Desmosome formation in NHBE cells grown in DMEM:F12 medium. a) Control cells with many desmosomes. b) HOCl-exposed cells show a decrease in the number of desmosomes and attachment sites. A light staining is present throughout the cells, presumably to immunoreaction with precursor molecules. For this experiment, the cells were first grown to confluence, and then HOCl was added. Bar 80 μm

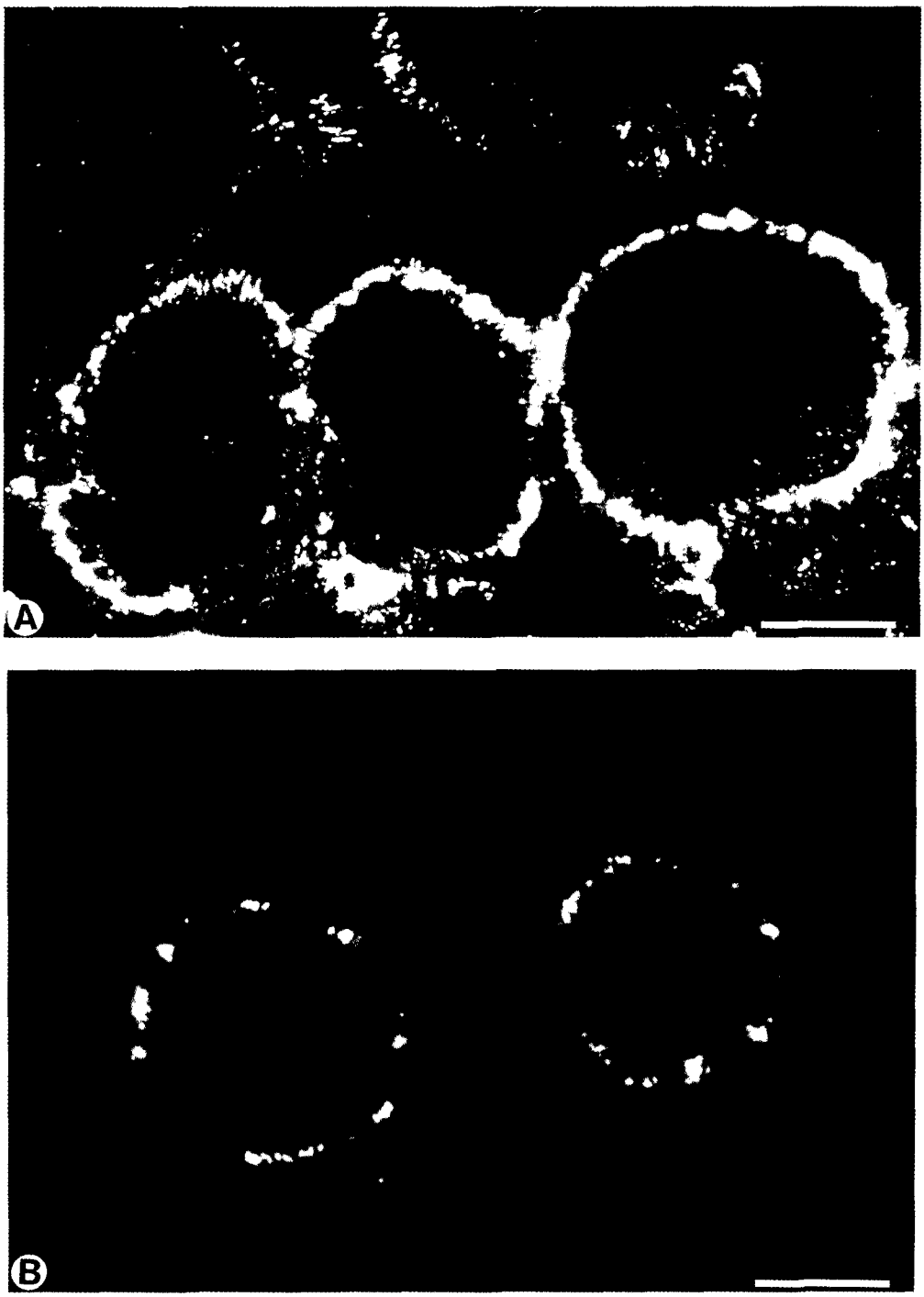

FIGURE 5 Vinculin formation in NHBE cells grown in BEGM. a) Control cells with vinculin around the cells. b) HOCl-exposed cells show a decrease in the amount of vinculin and focal adhesion sites. The cells grown in the serum-free BEGM medium have a more rounded shape, compared to the cells grown in the DMEM:F12 medium (Fig. 4). Bar 60 μm

matrix, or also due to cell damage. Guo *et al.*[26] reported that a non-cytotoxic dose of HOCl did not change the number of tight junctions or cell morphology in rabbit tracheal epithelium, even though the electrical resistance was altered. The experiment of Fig. 1 shows that the decrease in attachment of the cells caused by HOCl is more extensive than the cell death caused under the same conditions. Therefore, the decreased attachment can not purely be explained by cell death. Also, X-ray microanalysis shows an increase in S after treatment with up to 0.45 mM HOCl. Since the S content of the cells is representative for proteins, the decrease in desmosomal and adhesive proteins is not simply due to a general affect on protein synthesis.

Schraufstätter *et al.*[11] reported that HOCl caused a disturbance of various plasma membrane functions resulting in loss of cellular K^+ and an increase in cell volume. The perturbation of the plasma membrane integrity explains the reduced cell viability observed by the trypan blue exclusion test, which indicate necrosis. Apoptosis was not studied due to the observed morphological effects of 0.9 mM HOCl. The cells were lysed and there was almost no cytoplasmic material present. Like Schraufstätter *et al.*[11] we have observed changes in Na^+, K^+ and Ca^{2+}. However, we only observed changes in K^+ and Ca^{2+} when the cells were exposed to such a high concentration of HOCl that the cells more or less collapsed and the diffusible ions leaked out of the cell. At lower concentrations of HOCl the K^+ and Ca^{2+} content of the cells was unaffected. Although there was no significant change in the morphology of the mitochondria at 0.45 mM HOCl, there was a decrease in the glucose oxidation rate. This could be explained by perturbation of the respiratory chain by the free radicals produced by HOCl, and could result in a decrease in the production of ATP. To investigate by which mechanism HOCl induces damage to the NHBE cells, we used NAC[16], which is generally regarded as an antioxidant. It has been proposed that NAC protects the cells from the effects of HOCl by several mechanisms, among which is scavenging of HOCl and free radicals such as ·OH (hydroxyl radicals)[16], although its effect in this system may be more complicated. In addition to oxidant scavenger functions of NAC, there is evidence showing that NAC promotes cellular glutathione production, and in this way NAC could reduce or even prevent oxidant mediated damage to cell culture or animals[16]. However, NAC may also directly react with HOCl or its products and in this way protect the cell. N-propyl-L-arginine reduces the NO production induced by HOCl, which could be explained by an inhibition of one or more of the three isoforms of endothelial, inducible and neuronal nitric oxide synthase (eNOS, iNOS or nNOS). Two of the three NOSs are constitutively (cNOS) expressed whereas one is inducible (iNOS).

The nitrite accumulation and the generation of NO could be induced by two different mechanisms. First, HOCl induces free radicals, which in turn activate nuclear factor-κB (NF-κB). NF-κB is an essential transcription factor that controls the gene expression of cytokines, chemokines, growth factors, and cell adhesion molecules[27]. Inappropriate activation of NF-κB has been linked to inflammatory events associated with asthma and lung fibrosis[27]. Second, the membrane permeability and the cellular metabolism are affected by HOCl, which results in an increased level of intracellular Ca^{2+}, which in turn gives rise to an increased activity of cNOS. Western blot analysis and EMSA did not show any induction of iNOS or NF-κB, respectivly. N-propyl-L-arginine is a rather specific inhibitor of cNOS[28], and inhibits the HOCl-induced NO production. Taken together, these experiments imply that the increased NO production by the NHBE cells is caused mainly by an increased activity of cNOS, due to an increased level of intracellular Ca^{2+}. Although HOCl increases the production of NO by airway epithelial cells, this does not necessarily imply that an inflammation process in which neu-

trophils or eosinophils dominate results in increased levels of NO in the airways. There can be numerous explanations to the absence of elevated levels of NO in the exhaled air of patients with inflammatory respiratory diseases e.g., a decrease in the diffusion of NO across the membrane due to mucus and oedema and also failure of upregulation of iNOS[29,30]. Individual variations and experimental differences may have an impact on the mean result in studies on small groups of patients,

Curran *et al.*[31] report an increased NO level in the exhaled air of asthmatics and also in vitro evidence for NO production in the airways. In CF, on the other hand, there is no observed increase in exhaled NO[29]. In COPD, recent reports[32,33] provide conflicting data.

In summary, our results suggests that HOCl can react with membrane proteins, such as desmosomal and vinculin proteins, which results in cell detachment and shedding of the respiratory epithelial cells. This could be a contributing factor to observed epithelial losses in respiratory diseases.

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References

1. K.S. Jung and H.S. Park (1999) Evidence for neutrophil activation in occupational asthma. *Respirology*, 4, 303–306.
2. C. Marguet, F. Jouen-Boedes, T.P. Dean, and J.O. Warner (1999) Bronchoalveolar cell profiles in children with asthma, infantile wheeze, chronic cough, or cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, 159, 1533–1540.
3. R.E. Nocker, T.A. Out, F.R. Weller, E.P. Mul, H.M. Jansen, and J.S. van der Zee (1999) Influx of neutrophils into the airway lumen at 4 h after segmental allergen challenge in asthma. *International Archives of Allergy and Immunology*, 119, 45–53.
4. P.S. Hiemstra, S. van Wetering, and J. Stolk (1998) Neutrophil serine proteinases and defensins in chronic obstructive pulmonary disease: effects on pulmonary epithelium. *European Respiratory Journal*, 12, 1200–1208.
5. J.O. Warner (1992) Immunology of cystic fibrosis. *British Medical Bulletin*, 48, 893–911.
6. J.E. Harrison and J. Schultz (1976) Studies on the chlorinating activity of myeloperoxidase. *Journal of Biological Chemistry*, 251, 1371–1374.
7. R.J. Selvaraj, J.M. Zgliczynski, B.B. Paul, and A.J. Sbarra (1978) Enhanced killing of myeloperoxidase-coated bacteria in the myeloperoxidase-H2O2-Cl-system. *Journal of Infectious Diseases*, 137, 481–485.
8. S.J. Weiss, S.T. Test, C.M. Eckmann, D. Roos, and S. Regiani (1986) Brominating oxidants generated by human eosinophils. *Science*, 234, 200–203.
9. A. Slungaard and J.R.J. Mahoney (1991) Thiocyanate is the major substrate for eosinophil peroxidase in physiologic fluids. Implications for cytotoxicity. *Journal of Biological Chemistry*, 266, 4903–4910.
10. C.C. Winterbourn (1985) Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. *Biochimica et Biophysica Acta*, 840, 204–210.
11. I.U. Schräufstatter, K. Browne, A. Hams, P.A. Hyslop, J.H. Jackson, O. Quehenberger, and C.G. Cochrane (1990) Mechanisms of hypochlorite injury of target cells. *Journal of Clinical Investigation*, 85, 554–562.
12. C.G. Cochrane (1991) Cellular injury by oxidants. *American Journal of Medicine*, 91, 23S-30S.
13. R.J. Davies and J.L. Devalia (1992) Asthma. Epithelial cells. *British Medical Bulletin*, 48, 85–96.
14. L.A. Laitinen and A. Laitinen (1994) Structural and cellular changes in asthma. *European Respiratory Review*, 4, 348–351.
15. M.C. Vissers and C. Thomas (1997) Hypochlorous acid disrupts the adhesive properties of subendothelial matrix. *Free Radical Biology and Medicine*, 23, 401–411.
16. A. Gillissen and D. Nowak (1998) Characterization of N-acetylcysteine and ambroxol in anti-oxidant therapy. *Respiratory Medicine*, 92, 609–623.
17. J. Morris (1966) The acid ionization constant of HOCl from 5 to 35. *Journal of Physical Chemistry*, 70, 3798–3805.
18. S. Sandler and D.L. Eizirik (1996) Culture of human pancreatic islet cells. In Methods in Molecular Medicine: Human cell culture protocols (ed. G.E. Jones), Human Press Inc., Totowa, NJ, USA, pp. 391–407.
19. L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, and S.R. Tannenbaum (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry*, 126, 131–138.
20. A. von Euler and G.M. Roomans (1992) Ion transport in colon cancer cell cultures studied by X-ray microanalysis. *Cell Biology International Reports*, 16, 293–306.
21. G.M. Roomans (1988) Quantitative X-ray microanalysis of biological specimens. *Journal of Electron Microscopic Technique*, 9, 19–43.

22. J.D. Dignam, R.M. Lebovitz, and R.G. Roeder (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Research*, 11, 1475–1489.
23. P.A. Baeuerle and D. Baltimore (1988) Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. *Cell*, 53, 211–217.
24. M.M. Bradford (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72:248–54, 248–254.
25. G. Grynkiewicz, M. Poenie, and R.Y. Tsien (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry*, 260, 3440–3450.
26. Y. Guo, M. Krumwiede, J.G. White, and O.D. Wangensteen (1996) HOCl effects on the tight junctions of rabbit tracheal epithelium. *American Journal of Physiology*, 270, L224-L231.
27. P.J. Barnes and M. Karin (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *New England Journal of Medicine*, 336, 1066–1071.
28. H.Q. Zhang, W. Fast, M.A. Marletta, P. Martasek, and R.B. Silverman (1997) Potent and selective inhibition of neuronal nitric oxide synthase by N omega-propyl-L-arginine. *Journal of Medical Chemistry*, 40, 3869–3870.
29. L.P. Ho, J.A. Innes, and A.P. Greening (1998) Exhaled nitric oxide is not elevated in the inflammatory airways diseases of cystic fibrosis and bronchiectasis. *European Respiratory Journal*, 12, 1290–1294.
30. G. Hedenstierna and M. Högman (1998) Can exhaled NO be used as a marker of airway inflammation? *European Respiratory Journal*, 12, 1248–1249.
31. A.D. Curran (1996) The role of nitric oxide in the development of asthma. *Int International Archives of Allergy and Immunology*, 111, 1–4.
32. S.R. Rutgers, T.W. van der Mark, W. Coers, H. Moshage, W. Timens, H.F. Kauffman, G.H. Koeter, and D.S. Postma (1999) Markers of nitric oxide metabolism in sputum and exhaled air are not increased in chronic obstructive pulmonary disease. *Thorax*, 54, 576–580.
33. M. Corradi, M. Majori, G.C. Cacciani, G.F. Consigli, E. deMunari, and A. Pesci (1999) Increased exhaled nitric oxide in patients with stable chronic obstructive pulmonary disease. *Thorax*, 54, 572–575.