

Formaldehyde interferes with airway epithelium integrity and functions in a dose- and time-dependent manner

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ABSTRACT

Formaldehyde (HCHO) is a common indoor air pollutant. To assess its potential role and mechanism of action in asthma, we exposed the bronchial epithelial cell lines Calu-3 and 16HBE to HCHO (70–7000 μM) according to two exposure schedules (30 min and 24 h), before measuring cell viability, necrosis and apoptosis, reactive oxygen species production, cytokine release, as well as trans-epithelial electrical resistance (TEER) of cell monolayers. Whereas exposure to HCHO for 30 min had a limited effect on cell viability, exposure for 24 h to 1400–7000 μM HCHO induced a pronounced dose-dependent cell death. The important decrease in cell viability observed after 24 h exposure to the highest concentrations of HCHO (1400–7000 μM) was accompanied by important LDH release and ROS production, whereas a 4 h exposure to lower HCHO concentrations (350 μM) induced cell apoptosis. Also, exposure to HCHO for 30 min dose-dependently inhibited basal and lipopolysaccharide-induced interleukin-6 (IL-6) and IL-8 production by bronchial epithelial cells. As well, HCHO triggered a dose- and time-dependent decrease in TEER of Calu-3 cell monolayers. The present work demonstrates that HCHO interferes with airway epithelium integrity and functions, and may thus modulate the onset and the severity of asthma. However, importantly, conditions of exposure to HCHO, e.g. level and duration, are determinant in the nature of the effects triggered by the pollutant.

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1. Introduction

Formaldehyde (HCHO) is a chemical compound that is used in industries for the manufacturing of a variety of consumer products including wood-based furnishing materials or glass whole insulations made with adhesives containing formaldehyde resins, paints, flooring materials, disinfectants, preservatives as well as cosmetics (de Groot et al., 2009; Kelly et al., 1999; Salthammer et al., 2010). HCHO is also produced by combustion processes, including smoking (Li et al., 2002). Due to its wide use and the large variety of its sources, HCHO is a very common pollutant of occupational and domestic environments, where it is responsible for human exposure by the airway route (Bruinen de Bruin et al., 2008; Hodgson et al., 2002; Jurvelin et al., 2003). HCHO concentrations measured indoors (average concentrations from 8.0 to 94.9 $\mu\text{g m}^{-3}$ with maximal values up to 224 $\mu\text{g m}^{-3}$) often exceed those found outdoors (average concentrations from 4.7 to 15.7 $\mu\text{g m}^{-3}$ with maximal values up to 23 $\mu\text{g m}^{-3}$) (Ferrari et al., 1998; Hodgson et al., 2002; Marchand et al., 2006; Rumchev et al., 2002; Smedje and Norback,

2001; Zhang et al., 1994). Therefore, HCHO is considered as a major indoor air pollutant.

Asthma is a disorder of the conducting airways that manifested by a variable degree of airflow obstruction and hyperresponsiveness to a wide range of exogenous and endogenous stimuli (Bousquet et al., 2000). In allergic asthma, these features result from an aberrant immune response to common environmental allergens that leads to production of allergen-specific immunoglobulin E and airway inflammation. The main immune/inflammatory cells involved in asthma include dendritic cells as antigen-processing and presenting cells, T-helper 2 cells as orchestrators of the disease, and mast cells, eosinophils and neutrophils as effectors. These cells infiltrate the airways and contribute to the pathogenesis and chronicity of the disease by responding to and producing a variety of mediators, including pro-inflammatory cytokines and chemokines.

Atmospheric pollution is a factor of pathogenesis and severity of asthma (Penard-Morand et al., 2010; Rage et al., 2009). Among pollutants, indoor air pollutants are of concern, since nowadays town dwellers spend up to 70–80% of their life indoors (Bernstein et al., 2008; Burr, 1999). Thus, it has been suggested that exposure to HCHO could play a role in the increased incidence and gravity of asthma observed in developed countries over the last decades (Britton, 1992; Burr et al., 1989). In agreement with this

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hypothesis, epidemiological studies suggested a possible association between HCHO exposure and asthma exacerbation (Smedje and Norback, 2001; Wieslander et al., 1997). As well, Rumchev and collaborators (2002) observed an increased risk of asthma in children exposed to HCHO. In a human exposure study, Krakowiak et al. (1998) reported higher symptom scores and an increase in leukocytes and eosinophils in nasal washes recovered from asthmatic and healthy subjects exposed to HCHO. More recently, house dust mite asthmatics were shown to exhibit an exacerbation of lung function after inhalation of allergen when pre-exposed to HCHO compared to ambient air (Casset et al., 2006). However, some clinical studies did not find any effect of HCHO on lung function and airway response to allergen in asthmatic subjects, suggesting that more investigations are needed to conclude on the role of HCHO in asthma (Ezratty et al., 2007; Harving et al., 1990).

The airway epithelium represents the interface between the environment and the respiratory tract and is thus the first defense mechanism against atmospheric pollutants (Mills et al., 1999). It also plays a critical role in the pathogenesis of asthma (Holgate, 2008). Indeed, the airway epithelium is the place where the sensitization to allergens and the asthmatic reaction are initiated. Thus, by damaging the epithelial barrier or altering its permeability, pollutants like HCHO may favor the encounter between allergens and dendritic cells, or between allergens and mast cells, and thus facilitate the sensitization or the asthmatic reaction, respectively (Pons-Lebeau, 2007). Damage and loss of integrity of the airway epithelium are recognized patterns of asthma severity (Holgate, 2008). Epithelial cells are also important regulators of the immune response (Schleimer et al., 2007). Indeed, inflammatory cell recruitment and differentiation, as well as antigen sensitization are under the influence of epithelial-derived factors, which may be produced in increased amount upon airway exposure to air pollutants (Mills et al., 1999). Among these factors, interleukin-8 (IL-8) is a neutrophil chemo-attractant that is associated with asthma severity (Devalia et al., 1993; Rusznak et al., 1996). As well, IL-6 is a pleiotropic mediator that plays a role in leukocyte activation and is present at increased levels in the airways and blood of asthmatic patients (Auger et al., 2006). Thus, the trigger of epithelial factors may be another mechanism by which HCHO could contribute to increase the onset or severity of asthma. This pollutant may also be responsible for the production of reactive oxygen species (ROS) (Teng et al., 2001), which may activate inflammatory cells, interfere with the release of mediators, damage airway epithelial cells and increase epithelium permeability (Henricks and Nijkamp, 2001).

To assess the role and the mechanism of action of HCHO in asthma, some studies investigated the effects of HCHO on airway epithelial cell integrity and functions. However, these works gave disparate results. Indeed, whereas one study did not found any significant cell necrosis and IL-8 release in response to HCHO (Pariselli et al., 2009), other ones reported a decrease in cell viability, a cell death by apoptosis or an increase in IL-8 secretion (Balharay et al., 2008; Lim et al., 2010; Persoz et al., 2010). This disparity could result from differences in experimental conditions among studies, e.g. type of cell line used (bronchial or alveolar), mode of cell exposure to HCHO (gaseous or solubilized HCHO), duration of exposure (30 min for gaseous exposure compared to 24 h for exposure to solubilized HCHO), as well as concentrations of the pollutant. In this context, the aim of the present work was to further characterize the effects of HCHO on bronchial epithelial cells in culture, in order to provide a better understanding of the epithelial functions that may be impaired by this pollutant. This work was conducted in two bronchial epithelial cell lines, Calu-3 and 16HBE, exposed to solubilized HCHO. This mode of exposure was chosen, since it allowed us to expose cells for short (30 min) as well as long periods of time (24 h) to a wide range of HCHO concentrations before the analysis of cell integrity and functions. This analysis

included the measurements of cell viability, necrosis and apoptosis, reactive oxygen species production, cytokine release, as well as trans-epithelial electrical resistance (TEER) of cell monolayers.

2. Materials and methods

2.1. Chemicals and reagents

Formaldehyde (HCHO, 37% in water), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR), hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), camptothecin (CAM) and lipopolysaccharide (LPS, *Escherichia coli* 055:B5) were purchased from Sigma-Aldrich. DMEM/F-12 culture medium, fetal bovine serum (FBS), fungizone, penicillin-streptomycin mix ($10,000 \text{ U mL}^{-1}$ and $10,000 \text{ µg mL}^{-1}$, respectively), trypsin, Hepes (1 M) and phosphate buffered saline (PBS) were manufactured by Gibco and obtained from Invitrogen Corp. Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes.

2.2. Cell lines and culture

The Calu-3 cells were obtained from the American Type Culture Collection (ATCC) and the 16HBE14o- (16HBE) cells were a generous gift from Dr. D. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA, USA). The two cell lines were grown in culture flasks (Becton-Dickinson) with DMEM/F-12 culture medium supplemented with 10% FBS, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 5 mM Hepes. Culture was carried out at 37°C , in a 5% CO_2 and 100% humidified atmosphere. At confluence, cells were released with trypsin (0.5% in PBS), counted and transferred to culture plates (Becton-Dickinson) or inserts (Thin-cert PET cell culture inserts with transparent membrane and 0.4 µm pores, Greiner Bio-One). When cells were cultured on inserts, 1 µg mL^{-1} fungizone was added to the culture medium. Medium was changed every other day. For all experiments, cells were from passages 21–31 and 47–54 for Calu-3 and 16HBE, respectively.

2.3. Cell exposure to HCHO

Exposure in culture plates – cells were seeded in plates at a density of $10^5 \text{ cells cm}^{-2}$ and cultured until confluence (5–6 days after seeding). Concentrations of interest in HCHO (70–7000 µM), and H_2O_2 as positive control, were obtained by dilution of stock commercial solutions into the culture medium. Exposures to toxicants lasted for 30 min or 24 h, and biological assays were performed at 24 h after the beginning of exposure in both cases. For the 30 min exposures, medium containing the toxicant was changed to fresh culture medium or culture medium containing LPS (100 µg/mL) up to 24 h.

Exposure in inserts – cells were seeded on inserts at a density of $5 \times 10^5 \text{ cells cm}^{-2}$ and cultured for 10–12 days before exposure to HCHO. At this time point, cultures had a TEER of at least 1400 ohm cm^2 . Cells were exposed to HCHO by replacing the apical medium by medium containing HCHO, for 30 min or 24 h, as described above.

2.4. Cell viability assessment

Viability of cells cultured in plates or inserts was assessed by the MTT assay according to Mosmann (1983) or by the NR assay according to Borenfreund et al. (1990). Cells were exposed to HCHO as described above. The culture medium present in plate wells or in upper compartments of inserts was removed and cells were washed once with 200 (plates) or 350 (inserts) µL PBS. Then, fresh culture medium containing 1 mg mL^{-1} MTT or 100 µg mL^{-1} NR was added allowing living cells to metabolize MTT or incorporate NR. After dye incubation, cells were either lysed with DMSO (MTT assay) or washed with a fixative (1% formaldehyde–1% CaCl_2) followed by the addition of a 1% acetic acid solution containing 50% ethanol (NR assay). Absorbance of the resulting solution was read at 570 nm or 540 nm using a Multiskan FC reader (Thermo Scientific). Cell viability was expressed as the percentage of the absorbance of treated cells relative to the absorbance of control cells.

2.5. Cell necrosis assessment

Cell necrosis was assessed by measuring the release of lactate dehydrogenase (LDH) in the culture medium using the Cytotoxicity Detection Kit Plus (Roche Applied Science) according to the manufacturer's instructions. LDH activity was expressed as the percentage of the maximal LDH activity, obtained by cell lysis.

2.6. Intracellular ROS production assessment

ROS generation was measured using the method described by Wang and Joseph (1999). Just before exposure to HCHO or H_2O_2 , cells were incubated with culture medium containing 20 µM DCFH-DA, for 30 min at 37°C and 5% CO_2 . DCFH-DA was then removed, cells were washed with PBS and exposed to HCHO or H_2O_2 as described above. After exposure, fluorescence of each well was read immediately using a Spectra Max Gemini XPS microplate reader (Molecular Devices) with an excitation filter set at 485 nm and an emission filter set at 530 nm. Data were expressed as fold induction of the fluorescence intensity relative to the intensity of the non-exposed control cells.

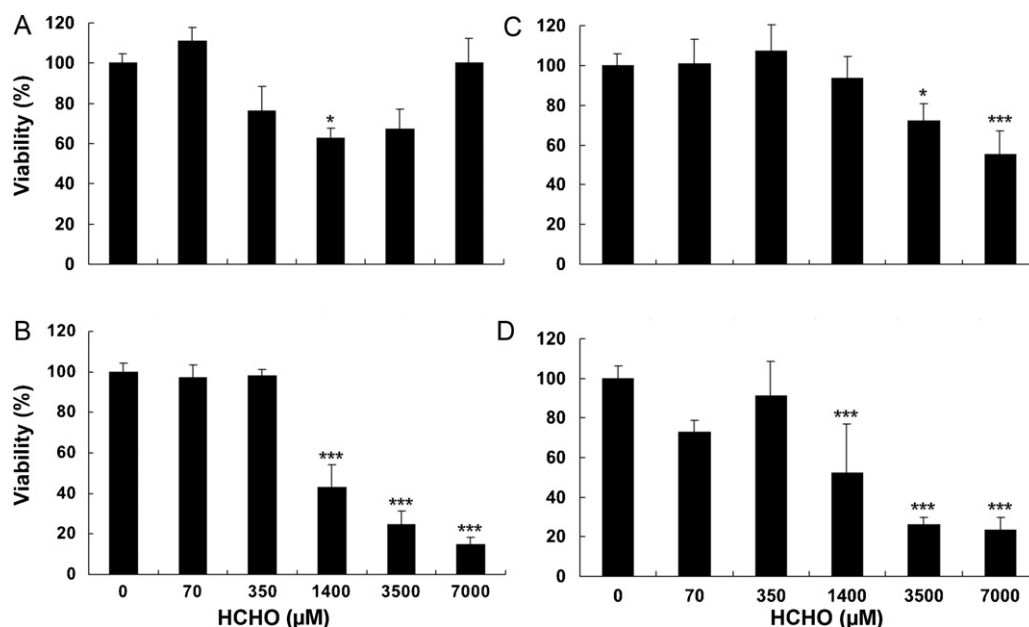


Fig. 1. Dose- and time-dependent effects of HCHO on cell viability. Calu-3 cells were exposed for 30 min (A and C) or 24 h (B and D) to increasing concentrations of HCHO and cell viability was assessed 24 h after the beginning of exposure, using the MTT (A and B) and NR (C and D) assays, as described in Section 2. Data are mean $\pm$ SEM of 6 or 9 values from 2 to 3 experiments. * $p < 0.05$ and *** $p < 0.001$ vs control cells.

2.7. Apoptosis assessment

Apoptosis was assessed by measuring the induction of caspase-3 activity and cytoplasmic nucleosomes. Cells were exposed to HCHO, or camptothecin (CAM) as a positive control, for 4 h and lysed directly after exposure for apoptosis assays. The Caspase-3 Activity Assay Kit and the Cell Death Detection ELISA Kit (Roche Applied Science) were used according to the manufacturer's instructions. Data were expressed as fold induction of the fluorescence (Caspase-3 Activity assay) or the colorimetric (Cell Death Detection assay) intensity of treated cells, relative to the intensity of the non-exposed control cells.

2.8. Cytokine assay

Culture media were collected at the end of cell exposure to toxicants, centrifuged at $3000 \times g$ for 5 min and stored at -20°C until use. The cytokines IL-6 and IL-8 were quantified using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences), according to the manufacturer's instructions. Absorbance was read at 450 nm with a correction at 570 nm using a Multiskan FC reader (Thermo Scientific). Calibration curves were used to calculate the cytokine concentrations, expressed in ng mL^{-1} .

2.9. TEER measurement

The development or the disruption of a tight epithelial cell monolayer was determined by measuring the TEER during cell culture or HCHO exposure, with an ohmmeter and chopstick electrodes (World Precision Instruments, Inc.). During cell culture, TEER measurements were performed every other day and resistance values were expressed in ohm cm^2 . During exposure to HCHO, TEER was measured at 0 h, 0.5 h, 1.5 h, 4 h, 8 h and 24 h and data were expressed as percentage of the initial TEER ($t = 0$). In both cases, the TEER value of an unseeded insert was subtracted from each measurement.

2.10. Statistical analysis

Results were expressed as arithmetic means $\pm$ standard error of the mean (SEM). Statistical differences were analyzed from raw data by one-way analysis of variance (ANOVA) followed by the Bonferroni's test using the GraphPad Prism 5 software. Data were considered as statistically different when $p < 0.05$.

3. Results

3.1. Dose- and time-dependent effect of HCHO on cell viability

To investigate the dose- and time-dependent effect of HCHO on bronchial epithelial cell viability, Calu-3 cells were exposed in culture plates to increasing concentrations of HCHO (70–7000 μM)

for 30 min or 24 h and cell viability was assessed at 24 h. Tetrazolium salt-based assays have been widely used to investigate the effect of HCHO on airway epithelial cell viability (Balharry et al., 2008; Lim et al., 2010; Persoz et al., 2010). Therefore, in the present study, the MTT assay was used in the first place. As shown on Fig. 1 (panels A and B), HCHO decreased viability of Calu-3 cells in a dose- and time-dependent manner. A decrease in cell viability of 24–38% was observed after a 30 min exposure to 350 μM , 1400 μM and 3500 μM HCHO, but not for higher or lower concentrations (Fig. 1A). However, these decreases reached statistical significance for 1400 μM HCHO only (38% decrease, $p < 0.05$). In contrast, after a 24 h exposure, cell viability was markedly decreased by 57–85% at 1400 ($p < 0.001$), 3500 ($p < 0.001$) and 7000 μM ($p < 0.001$) HCHO, with a maximal decrease of 85% at the concentration of 7000 μM (Fig. 1B). In a study on human dental pulp fibroblasts, buccal epithelial cells and HeLa cervix cancer cells exposed to HCHO, Lovschall et al. (2002) found however that the NR assay is more sensitive than the MTT assay in some cell types. Therefore, in the present study, the NR assay was used to verify the dose- and time-dependent effect of HCHO observed with the MTT assay. As shown on panels C and D of Fig. 1, the NR assay gave similar results, except when cells were exposed to 7000 μM HCHO for 30 min. In this condition, indeed, whereas the MTT assay did not indicate a significant change in cell viability, the NR assay showed a significant decrease (45%, $p < 0.001$).

3.2. Effect of HCHO on cell viability, necrosis and ROS production

Induction of oxidative stress and necrosis is major mechanism of cell death evoked by toxicants. In order to investigate whether the decrease in cell viability induced by HCHO is accompanied by oxidative stress and/or cell necrosis, Calu-3 cells were exposed in culture plates to HCHO (70–7000 μM) for 30 min or 24 h, and cell necrosis and ROS production were assessed at 24 h. No induction of LDH release or ROS production was observed after cell exposure for 30 min to HCHO, whatever its concentration (data not shown). In contrast, as shown on Fig. 2, significant cell necrosis (panel A) and increases in ROS production (panel B) were observed after a 24 h

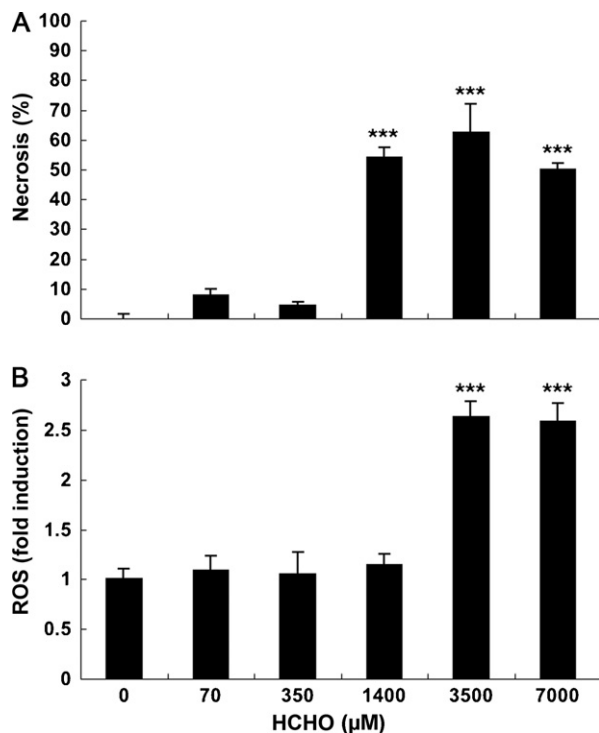


Fig. 2. Effect of HCHO on cell necrosis and ROS production. Calu-3 cells were exposed for 24 h to increasing concentrations of HCHO, and cell necrosis (A) and intracellular ROS production (B) were assessed at the end of exposure, as described in Section 2. Data are mean $\pm$ SEM of 9 values from 3 experiments. *** p < 0.001 vs control cells.

exposure of cells to the highest HCHO concentrations (e.g. 1400 μ M, 3500 μ M and/or 7000 μ M).

In order to consolidate these results in regards to the data previously reported in the literature, the effect of HCHO on airway epithelial cell viability, necrosis and ROS production was assessed in a second cell line, the 16HBE cell line. In a comparative experiment, Calu-3 and 16HBE cells were exposed in culture plates to 7000 μ M HCHO or 4 mM H_2O_2 as a positive control, for 30 min or 24 h, and viability (Fig. 3A and B), necrosis (Fig. 3C and D) and ROS production (Fig. 3E and F) were assessed at 24 h. Significant decreases in cell viability and increases in necrosis and ROS production were observed after a 24 h exposure to H_2O_2 for both cell lines, as a control (Fig. 3B, D and F). No significant changes in viability of Calu-3 and 16HBE cells were observed after a 30 min exposure to 7000 μ M HCHO (Fig. 3A, white bars), as already described in Fig. 1. In the same conditions, there was no increase in ROS production for the two cell lines (Fig. 3E, white bars), and a significant increase in LDH release for 16HBE cells only (Fig. 3C, white bars). However, a significant decrease in cell viability was observed for Calu-3 and 16HBE cells after a 24 h exposure to HCHO (Fig. 3A, black bars) and this decrease was associated with a significant necrosis (Fig. 3C, black bars) and an important ROS production (Fig. 3E, black bars) for the two cell lines.

3.3. Induction of apoptosis by HCHO

Recently, Lim et al. (2010) reported the capacity of HCHO to induce apoptosis in the alveolar epithelial cell line A549. Therefore, we intended to investigate the capacity of HCHO to induce apoptosis in bronchial epithelial cells. Calu-3 cells were exposed in culture plates to increasing concentrations of HCHO for 4 h followed by assessment of apoptosis according to previous studies (Liu et al., 2010). Fig. 4 shows the effect of increasing concentrations of HCHO on caspase-3 activity (A) and cytoplasmic nucleosomes (B)

in Calu-3 cells. Exposure to 350 μ M HCHO led to a 5-fold induction of caspase-3 activity (p < 0.001) and a 2-fold induction of cytoplasmic nucleosome quantity (p < 0.01) compared to non-exposed cells, whereas other HCHO concentrations were without significant effect on the two apoptosis-associated events. Exposure to the apoptosis inducer camptothecin (CAM) in the same conditions led to a 4-fold induction of caspase-3 activity (p < 0.001) and a 2-fold induction of cytoplasmic nucleosome amount (p < 0.01). In these test conditions (4 h exposure), no significant increase in LDH activity was observed whatever the HCHO concentration (data not shown).

3.4. Impact of HCHO on IL-6 and IL-8 production

In order to assess the impact of HCHO on the immunoregulatory functions of the bronchial epithelium, IL-6 and IL-8 levels were measured in the culture supernatants of Calu-3 and 16HBE cells pre-exposed for 30 min to increasing concentrations of HCHO and further cultured for 24 h in the presence of medium alone (basal cytokine production) or of medium containing *E. coli* LPS (evoked cytokine production). As shown on Fig. 5, Calu-3 cell pre-exposure to HCHO resulted in a dose-dependent decrease in both basal and LPS-evoked IL-6 (Fig. 5A) and IL-8 (Fig. 5B) production. Basal IL-6 and IL-8 release, as well as LPS-induced IL-8 production were decreased by pre-exposures to 3500 μ M (64% decrease, p < 0.001) and 7000 μ M (97% decrease, p < 0.001) HCHO, and LPS-induced IL-6 secretion was significantly decreased by a pre-exposure to 7000 μ M HCHO (75%, p < 0.001). Likewise, pre-exposure to HCHO markedly decreased both basal- and LPS-induced cytokine production in 16HBE cells. Thus, in cells pre-exposed to 3500 μ M HCHO, inhibition of basal IL-6 and IL-8 production reached 91% and 93% and reduction of LPS-evoked IL-6 and IL-8 release reached 95% and 63%, respectively. In these experiments, inhibition of cytokine production induced by pre-exposure to HCHO was not associated with decreased cell viability (data not shown).

3.5. Effect of HCHO on the epithelial monolayer permeability

In order to assess the effect of HCHO on the epithelial permeability by TEER measurements, we first investigated which cell line from Calu-3 and 16HBE is the best adapted for this kind of measurements. Thus, we measured TEER values of Calu-3 and 16HBE monolayers cultured on inserts as a function of time from seeding up to 17 days of culture. As illustrated on Fig. 6, TEER values increased from the first days of culture for both cell lines. They further increased regularly for Calu-3, reaching 1360 ± 159 ohm cm^2 at 14 days of culture, before decreasing. In contrast, TEER values measured on 16HBE cultures stopped growing a few days only after seeding, and reached a plateau between 424 ± 39 ohm cm^2 and 661 ± 77 ohm cm^2 during the 13 following days. Therefore, the effect of HCHO on the epithelial permeability was assessed on Calu-3 cell cultures.

Calu-3 monolayers were exposed to increasing concentrations of HCHO during 24 h and TEER values were measured before and at different times during exposure. As well, cell viability was assessed at the end of exposure. Fig. 7 shows that TEER values (A) and viability (B) of Calu-3 cell monolayers were not affected by exposure to 70 μ M or 350 μ M HCHO compared to control cells. The TEER of the monolayers exposed to 1400 μ M HCHO significantly decreased from 1.5 h (32% decrease, p < 0.01) to 24 h (86% decrease, p < 0.001) of exposure suggesting an increased permeability (Fig. 7A), whereas interestingly, no significant modification of cell viability was observed at this HCHO concentration (Fig. 7B). The TEER of Calu-3 cell monolayers exposed to 3500 μ M HCHO also significantly decreased from 1.5 h of exposure (Fig. 7A), but

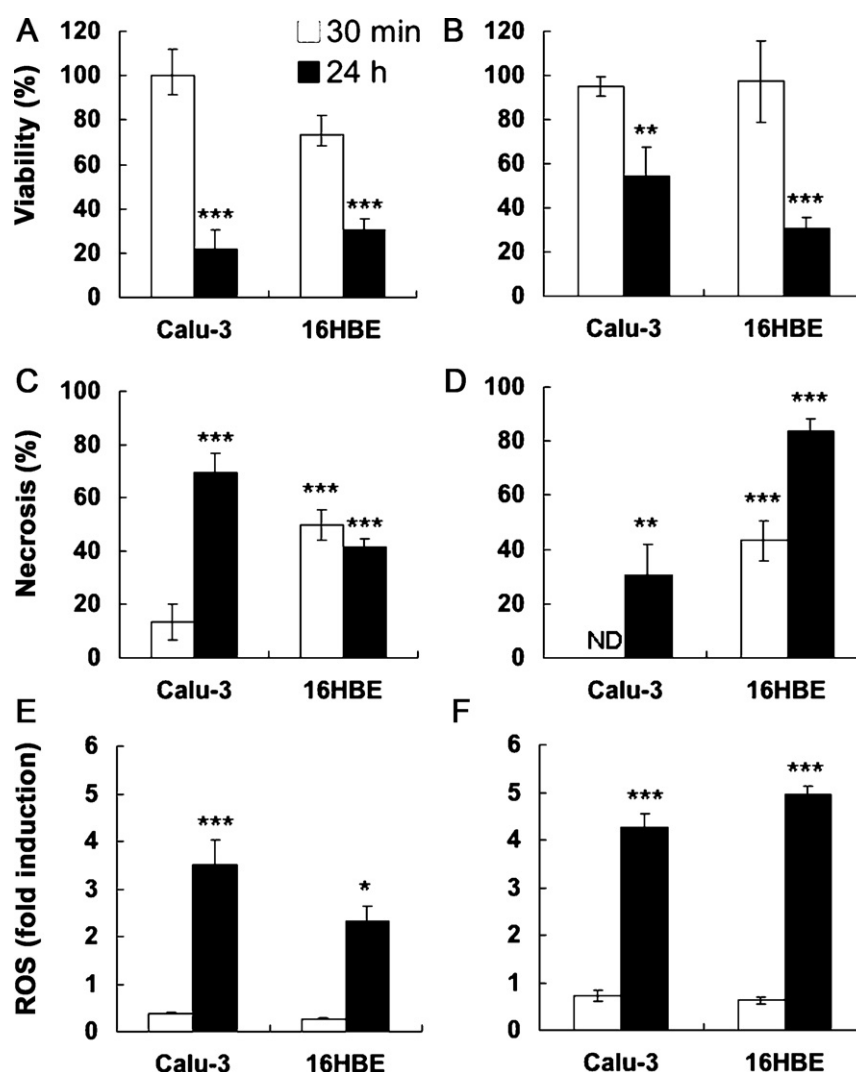


Fig. 3. Effect of HCHO on cell viability, necrosis and ROS production. Calu-3 and 16HBE cells were exposed to 7000 μM HCHO (A, C and E) or to 4 mM H_2O_2 as a positive control (B, D and F), for 30 min (white bars) or 24 h (black bars), and cell viability as measured by the MTT assay (A and B), necrosis (C and D) and intracellular ROS production (E and F) were assessed at 24 h, as described in Section 2. Data are mean $\pm$ SEM of 9 or 12 values from 3 to 4 experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs control cells. ND, non detected.

the increase in permeability was then associated with a significant decrease (55% decrease, $p < 0.01$) in cell viability (Fig. 7B).

4. Discussion

Atmospheric pollutants are known to be responsible for harmful health effects towards the respiratory tract. Among them, HCHO is a common indoor air pollutant, which is suspected to play a role in the development and the severity of asthma. While exposures of lung epithelial cells to HCHO have been reported in the literature, the mechanisms by which HCHO could interact with the bronchial epithelium in the context of asthma remain unclear. Thus, the aim of this study was to further investigate the effects of HCHO on bronchial epithelial cell integrity and functions. In this work, the two well-documented bronchial epithelial cell lines, Calu-3 and 16HBE, were exposed to a range of HCHO concentrations according to two exposure schedules. Assessment of the effects triggered by HCHO included cell viability, necrosis and apoptosis, as well as ROS production, cytokine and chemokine release, and changes in epithelial monolayer permeability.

The airway epithelium is at the interface between the environment and the respiratory tract. Maintenance of its integrity is therefore of prime importance in the defense of the lung against

atmospheric pollutants. Investigating the effect of HCHO concentrations and time of exposure on cell viability using the Calu-3 cell line, our results showed that HCHO is capable of decreasing bronchial epithelial cell viability in a time- and dose-dependent manner. Indeed, whereas a very short-term exposure (30 min) to HCHO (70–7000 μM) resulted in a limited effect, exposure for 24 h to 1400–7000 μM HCHO induced a pronounced dose-dependent cell death. These observations were made by means of two viability assays, the MTT assay which has been previously used to investigate the effect of HCHO on airway epithelial cell viability (Balharry et al., 2008; Lim et al., 2010) and the NR assay which has been found to be a more sensitive assay in some cell types (Lovschall et al., 2002). Both assays gave similar results, except when cells were exposed to 7000 μM HCHO for 30 min. In this condition, indeed, whereas the MTT assay did not indicate a significant change in cell viability, the NR assay showed a significant decrease. Taking into account that the MTT assay measures an enzymatic activity, the absence of clear dose-response effect after the 30 min exposure using this assay suggests that a short exposure to high concentrations of HCHO may stimulate cell metabolic activity. Taken together, our viability results suggest that only long-term exposure to high concentrations of HCHO – at least 200 times higher than the physiological concentrations (Heck et al., 1985; Luo et al., 2001) – can lead to a loss

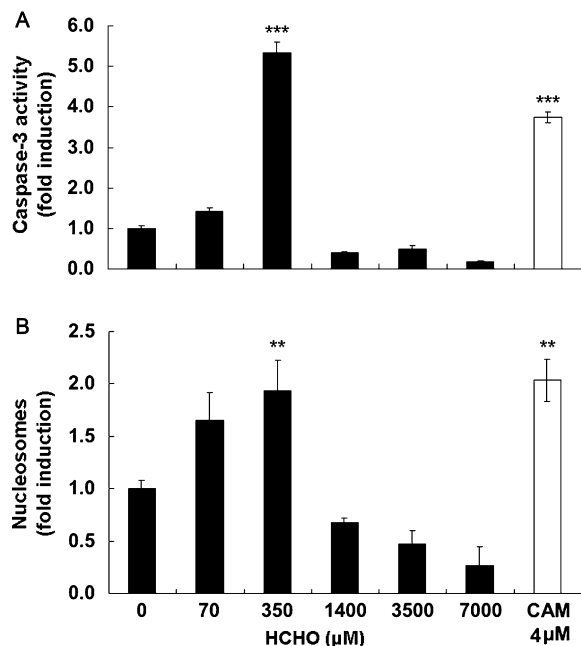


Fig. 4. Induction of apoptosis by HCHO. Calu-3 cells were exposed to increasing concentrations of HCHO (black bars) or to 4 μM camptothecin (CAM, white bar) as a positive control of apoptosis for 4 h, and caspase-3 activity (A) and cytoplasmic nucleosomes (B) were assessed in cell lysates directly after exposure. Data are mean $\pm$ SEM of 9 values from 3 experiments. ** p < 0.01 and *** p < 0.001 vs controls.

of airway epithelium integrity. Supporting this hypothesis, Persoz et al. (2010) reported a limited loss of viability only and Pariselli et al. (2009) did not observe any LDH release in airway epithelial cells exposed for a very short period of time (30 min and 1 h, respectively) to 75 $\mu\text{g}/\text{m}^3$ (concentration classically found indoors) and 750 $\mu\text{g}/\text{m}^3$ (concentration far beyond the ones found indoors)

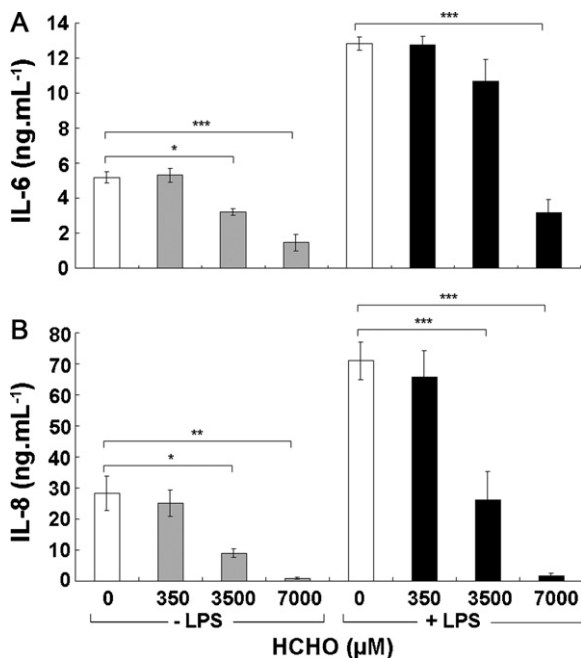


Fig. 5. Impact of HCHO on IL-6 (A) and IL-8 (B) production. Calu-3 cells were pre-exposed to increasing concentrations of HCHO (grey and black bars) or medium alone (white bars) for 30 min and further cultured in the presence of medium alone (-LPS) or of medium containing *E. coli* LPS (+LPS) for 24 h. IL-6 and IL-8 concentrations were measured in the culture medium by ELISA. Data are mean $\pm$ SEM of 9 values from 3 experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs controls.

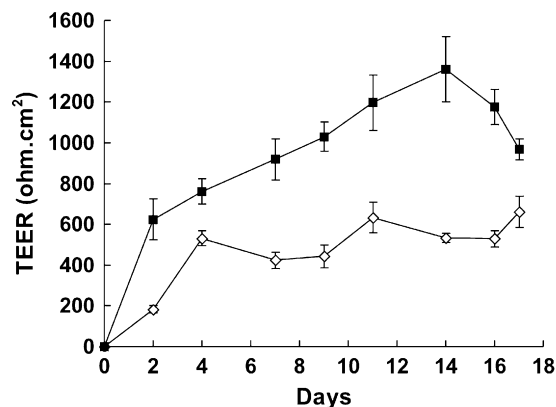


Fig. 6. Trans-epithelial electrical resistance (TEER) of Calu-3 (■) and 16HBE (◇) cell monolayers as a function of days of culture after seeding. Cells were seeded on inserts and TEER was measured every other day up to 17 days of culture. Data are mean $\pm$ SEM of TEER measurements performed on 3 different inserts (n = 3).

HCHO, respectively. As well, our results at 24 h are in clear accordance with the data of Balharry et al. (2008) showing a decrease in cell numbers after a 24 h exposure to 5000 μM HCHO and more.

Induction of oxidative stress and necrosis is major mechanism of cell death evoked by toxicants. We therefore investigated the effect of HCHO on LDH release and ROS production in our experimental set-up. We then found that the important decrease in cell viability observed after a 24 h exposure to the highest HCHO concentrations was accompanied by important LDH release and ROS production in the two cell lines used. This result indicates that necrosis is involved in the decrease of airway epithelial cell viability.

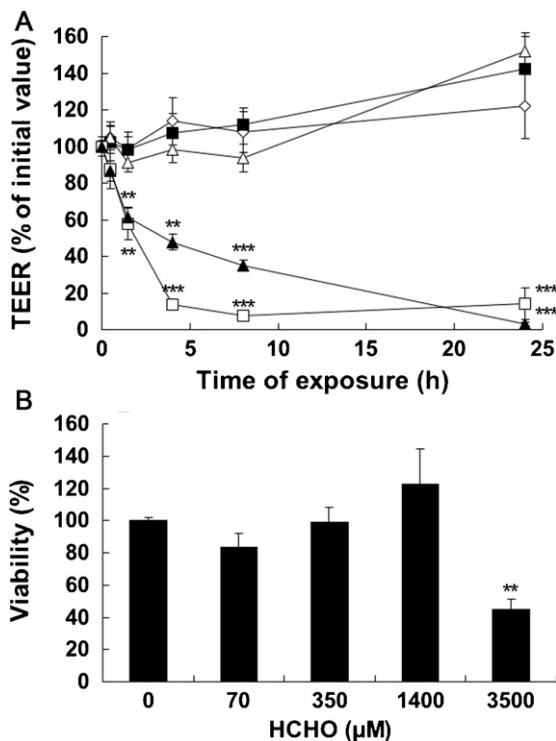


Fig. 7. Effect of HCHO on cell monolayer integrity (A) and viability (B). Calu-3 cells were cultured on inserts until maximal TEER values were reached, and 0 (◇), 70 (■), 350 (△), 1400 (□) or 3500 μM (▲) HCHO was added on the apical side of the inserts. TEER was monitored at the beginning of the exposure (t = 0 h), and at t = 0.5, 1.5, 4, 8 and 24 h. At 24 h, cell viability was assessed by the MTT assay as described in Section 2. Data are mean $\pm$ SEM of 9 values from 3 experiments. ** p < 0.01 and *** p < 0.001 vs controls.

ity triggered by high concentrations of HCHO, and that an oxidative stress could be responsible for this cell death, as suggested by Lim et al. (2010). No ROS induction was observed after a 30 min exposure to the same concentrations of HCHO. This is in accordance with the results of Teng et al. (2001) who reported a time-dependent increase in oxidative stress in response to HCHO in rat hepatocytes. Recently, Lim et al. (2010) reported the capacity of HCHO to induce apoptosis in the A549 alveolar epithelial cell line. As well, an increased number of apoptotic cells has been observed in bronchial and bronchiolar tissue of rats exposed to HCHO (Sandikci et al., 2009). Moreover, Liu et al. (2010) described the activation of caspases by crotonaldehyde in BEAS-2B cells, indicating that this capacity is shared by other aldehydes. In agreement with these studies, we observed a significant induction of apoptosis after a 4 h exposure to HCHO, as assessed by the measurement of caspase-3 activity and cytoplasmic nucleosomes. These responses occurred at 350 μ M HCHO, but not for lower or higher concentrations. These data combined with our other results on cell viability and necrosis, suggest that exposure to moderate concentrations of HCHO may induce apoptosis in bronchial epithelial cells, whereas more important exposures – to higher concentrations and for longer periods of time – lead to cell necrosis. This kind of toxic feature has been already described for H₂O₂ on human lung fibroblasts (Teramoto et al., 1999).

Inflammation of the airways is an important feature of asthma (Bousquet et al., 2000). In this context, we investigated the effect of HCHO on the production of the pro-inflammatory cytokine IL-6 and the neutrophil chemo-attractant IL-8 by bronchial epithelial cells, at both basal and LPS-induced levels. A dose-dependent decrease in both basal and LPS-induced production of these mediators was observed after a 30 min cell exposure to HCHO, suggesting that HCHO interferes with the cytokine production machinery and that a 30 min exposure is enough to induce a mechanism of inhibition that is not reversed by a following 24 h incubation without HCHO. Importantly, cell viability was not significantly altered in these experiments, demonstrating that the cytokine inhibition evoked by HCHO is not due to cell death. IL-6 is a pleiotropic cytokine that plays a role in leukocyte recruitment, whereas IL-8 is a chemokine that is responsible for neutrophil attraction (Mukaida, 2003). Inhibition of these two mediators suggests that HCHO may exhibit a protective effect in asthma. This hypothesis may be surprising in consideration of the pro-inflammatory effect of HCHO that was reported in a recent *in vitro* study (Persoz et al., 2010). In this study, indeed, an increase in IL-8 release (IL-6 production remaining unchanged) was observed in cells pre-sensitized with TNF- α and exposed to gaseous HCHO. However, in agreement with our data, different aldehydes have been reported to inhibit cytokine production evoked by LPS *in vitro* (Kim et al., 2010; Li et al., 1997). Importantly, this inhibitory activity resulted from inhibition of activation of the primary transcription factor involved in pro-inflammatory cytokine expression, NF- κ B, (Kim et al., 2010; Li et al., 1999). At last, supporting a possible inhibition of asthma-associated inflammation by HCHO, an impaired inflammatory response to allergen was observed in rats repeatedly exposed to HCHO (Franco et al., 2009).

The role of physical barrier is a major property of the bronchial epithelium. This property can be assessed on cell monolayers by measuring the TEER, as an indicator of the epithelial permeability (Florea et al., 2003). TEER values of Calu-3 and 16HBE cells cultured on inserts were assessed to compare the ability of the two cell lines to acquire barrier property. Our results clearly demonstrated that Calu-3 cells are able to produce higher TEER values than 16HBE cells, and confirmed that this line is a good candidate for permeability studies (Geys et al., 2007). Calu-3 cells were thus used to investigate the effects of HCHO on airway epithelium permeability. The absence of modification of the TEER during a 24 h

exposure to 70 μ M or 350 μ M HCHO compared to control indicated that these concentrations are not able to harm the epithelial barrier, whereas exposure to 1400 μ M or 3500 μ M provoked a rapid loss of integrity that was already detectable at 2 h. Interestingly, the MTT assay performed in this experiment indicated that there was no decrease in cell viability after exposure to 1400 μ M HCHO, whereas the 3500 μ M exposure induced a significant cell death. This result demonstrates that HCHO is able to alter the barrier property of the bronchial epithelium without necessarily inducing cell death. Alteration of the junctional complexes that allow epithelial cells to produce a TEER could explain this increase in monolayer permeability induced by HCHO in the absence of cell death (Arican et al., 2009; Rotoli et al., 2009).

The airway epithelium is one of the first potential targets for inhaled HCHO. The present work demonstrates that this indoor air pollutant exhibits pleiotropic effects on the bronchial epithelium, depending on its concentration and the duration of exposure. These effects may modulate the onset and the severity of asthma. By inducing cell death by apoptosis or necrosis or by altering the barrier property of the airway epithelium, HCHO could facilitate or aggravate the chronic wounding of the airways or the epithelial loss that occur in severe asthma, and thus promote the response to allergen. On the contrary, by inhibiting pro-inflammatory cytokine and/or chemokine release, HCHO may exhibit a protective effect in asthma. Importantly, our results demonstrate that conditions of exposure to HCHO, e.g. level and duration, are determinant in the nature of the effects triggered by the pollutant.

Conflict of interest statement

None declared.

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