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RESEARCH ARTICLE

Carbon dioxide is largely responsible for the acute inflammatory effects of tobacco smoke

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Abstract

Tobacco smoking is responsible for a vast array of diseases, particularly chronic bronchitis and lung cancer. It is still unclear which constituent(s) of the smoke is responsible for its toxicity. The authors decided to focus on carbon dioxide, since its level of concentration in mainstream cigarette smoke is about 200 times higher than in the atmosphere. The authors previously demonstrated that inhalation of carbon dioxide concentrations above 5% has a deleterious effect on lungs. In this study, the authors assessed the inflammatory potential of carbon dioxide contained in cigarette smoke. Mice were exposed to cigarette smoke containing a high or reduced CO_2 level by filtration through a potassium hydroxyde solution. The inflammatory response was evaluated by histological analysis, protein phosphatase 2 A (PP2A) and nuclear factor (NF)-κB activation, and proinflammatory cytokine secretion measurements. The data show that the toxicity of cigarette smoke may be largely due to its high level of CO_2. Pulmonary injuries consequent to tobacco smoke inhalation observed by histology were greatly diminished when CO_2 was removed. Cigarette smoke exposure causes an inflammatory response characterized by PP2A and NF-κB activation followed by proinflammatory cytokine secretion. This inflammatory response was reduced when the cigarette smoke was filtered through a potassium hydroxide column, and reestablished when CO_2 was injected downstream from the filtration column. Given that there is an extensive literature linking a chronic inflammatory response to the major smoking-related diseases, these data suggest that carbon dioxide may play a key role in the causation of these diseases by tobacco smoking.

Keywords: *Cigarette; lung; inflammation; PP2A; NFκB*

Introduction

Tobacco smoking is responsible for a vast array of diseases such as chronic bronchitis, cancer of the lung, cancer of the head and neck, esophageal cancer, and carcinoma of the bladder (Ezzati et al., 2005; Parkin et al., 2005). Smoking is also responsible of an increase in cardiovascular disease and stroke (Burns, 2003) as well as preterm deliveries and hypotrophy of the fetus (Jauniaux and Burton, 2007). Therefore, it is clear that both eradicating smoking and reducing the harm caused by smoking for those smokers who choose not to or cannot quit are health priorities. In order to make progress toward the second goal, there is a clear need to identify those smoke constituents responsible for tobacco smoke toxicity. However, despite the fact that numerous studies have been performed since the first demonstration of tobacco carcinogenicity by Doll and Hill (1950), no single smoke constituent has ever been established to be the cause.

Since the 1950s, cigarettes have undergone a progressive modification, which was envisaged to reduce the risks of smoking. In particular, filters were added, followed by the introduction of ventilated filters to produce low-delivery projects, leading to a reduction in the average machine-measured tar yield per cigarette by over 60% (Hoffmann and Hoffmann, 1997). Although there is evidence that filtered

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cigarettes are indeed a lower-risk product as compared to nonfiltered cigarettes (Harris et al., 2004), further reduction in tar has in fact provided limited benefit, if any, compared with higher-tar cigarettes based on population data (Kabat, 2003). On the other hand, a large number of epidemiological studies have been performed that suggest that lower-tar cigarettes may indeed have resulted in some reduction of smoking risk. Thirty-five studies of lung cancer are suggestive that smokers of low-tar cigarettes have a lower risk (by 20–30%) compared to smokers of higher-tar cigarettes. Only a minority of studies of heart disease provide evidence of a reduction in risk, on the order of 10%. Studies concerning chronic obstructive pulmonary disease (COPD) are inconsistent, but the majority suggests decreased risk in smokers of lower-tar cigarettes (Lee and Sanders, 2004). However, even if these results are correct, the reduction in risk is relatively small and of little significance from a public health perspective.

In the last 10 years a number of novel "tobacco harm reduction" (THR) products have been introduced to the market, although none has been particularly successful. To their purveyors, these new THR products represent an opportunity for inveterate smokers to reduce their risk of lung cancer and other diseases (Lee and Sanders, 2004). To health professionals, the new products pose a myriad of risks. As pointed out above, this new generation of THR products is not the first to promise reduced risk. In that neither filtered cigarettes or low-tar and nicotine cigarettes achieved the expected goal of reducing risks, primarily because smokers exhibit compensatory smoking behaviour (Kabat, 2003), it is an absolute requirement that any novel THR product be properly and thoroughly evaluated. Thus the importance of identifying smoke constituents that are causally related to disease is paramount.

Mainstream smoke can been divided in two parts: the particulate phase and the gas/vapor phase. Most published research has been focused on the particulate phase, which is primarily composed of tar, a complex mixture of thousands of different chemicals, including known carcinogens such as tobacco-specific nitrosamines or benzo[a]pyrene (Hammond and O'Connor, 2008; Hecht, 2006). The gas phase has been less well studied. In the gas phase, apart from nitrogen and oxygen (respectively 62% and 13% by weight of mainstream smoke), carbon dioxide prevails (about 12.5%), followed by carbon monoxide (4%) and water (1.3%) (Dube and Green, 1982; Norman, 1977). In the atmosphere, the partial pressure of carbon dioxide varies between 0.03% and 0.06% (Keeling, 2004). It is the most prominent greenhouse gas in the earth's atmosphere, and an isolated test at Mauna Loa in Hawaii reported a more than 12% (316 ppm in 1959 to 360 ppm in 1996) increase in its mean concentration. Carbon dioxide is mainly emitted from coal-fired power plants, refineries, chemical plants, cement firms, and vehicles. For instance, concentrations close to those measured in cigarette smoke have been detected in car exhausts or coal power plant emissions (Abolhassani et al., 2009).

Carbon dioxide reacts with water to form bicarbonate and carbonic acid and these species are involved in multiple biochemical reactions. In a previous paper (Abolhassani et al., 2009), we demonstrated that the inhalation of concentrations of carbon dioxide above 5% has a deleterious effect on the lung. The inflammation caused by CO_2 is mediated by the methylation of protein phosphatase 2A (PP2A), which, in turn, activates the nuclear factor (NF)-κB pathway. The nuclear translocation of NF-κB results in the secretion of proinflammatory proteins such interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α (Schwartz et al., 2008).

This article describes a study designed to assess to what extent carbon dioxide might contribute to the toxicity of cigarette smoke.

Methods

Mouse cigarette smoke exposure

Ninety-five male BALB/c mice 6–7 weeks old (8/group) were obtained from the Centre d'Elevage Janvier and maintained in accordance with the European Community's guidelines concerning the care and use of laboratory animals. All aspects of the protocol conformed with the requirements of the laboratory's approval for animal research (1987 regulation). Mice were exposed for 60 min to cigarette smoke generated by a burning cigarette (Marlboro Light, Philip Morris) connected to a vacuum pump controlled by a tap. The smoke was passed through a filtration system consisting of two Plexiglas columns placed in series (100 × 0.97 cm Bio-Rad column + 48 × 1.96 cm Pharmacia column), half-filled with a 1 M potassium hydroxide (KOH) solution or acidified water (pH 5.5 with HCl). The mainstream cigarette smoke is passed over these solutions (slightly agitated to increase contact surface) at room temperature. Carbon dioxide is captured by the KOH solution but not by the acidified water. The mouse exposure chamber consisted of eight conic glass tubes fused to a glass chamber (total volume: 1.6 L), so that their muzzles were in contact with interior of chamber. Cigarette smoke is introduced into the chamber through a tube fitted to upper part of chamber after passing through the filtration system (Figure 1A). Two groups of eight mice each were exposed to cigarette smoke passed over acidified water, and two groups of eight mice each were exposed to cigarette smoke passed over the KOH solution. A third exposure condition was achieved by adjusting the CO_2 concentration of the mouse exposure chamber to between 9% and 13% by the injection of a pure CO_2 gas capsule (Carboxique) following filtration by the KOH solution, and two groups of eight mice were evaluated under those conditions. Partial CO_2 and O_2 pressures in the exposure chamber were measured using specific microelectrodes (Lazar Research Laboratories). The partial H_2O pressure was estimated to be about 50 mm Hg. The number of cigarette smoke particles present in each size range was counted in a 10-ml sample using a Met One 237A portable airborne particle counter (Hach Ultra). Thirty-two mice were used as controls—two groups of eight being exposed to air filtered by the acidified water and two groups of eight being exposed to air filtered by the KOH solution.

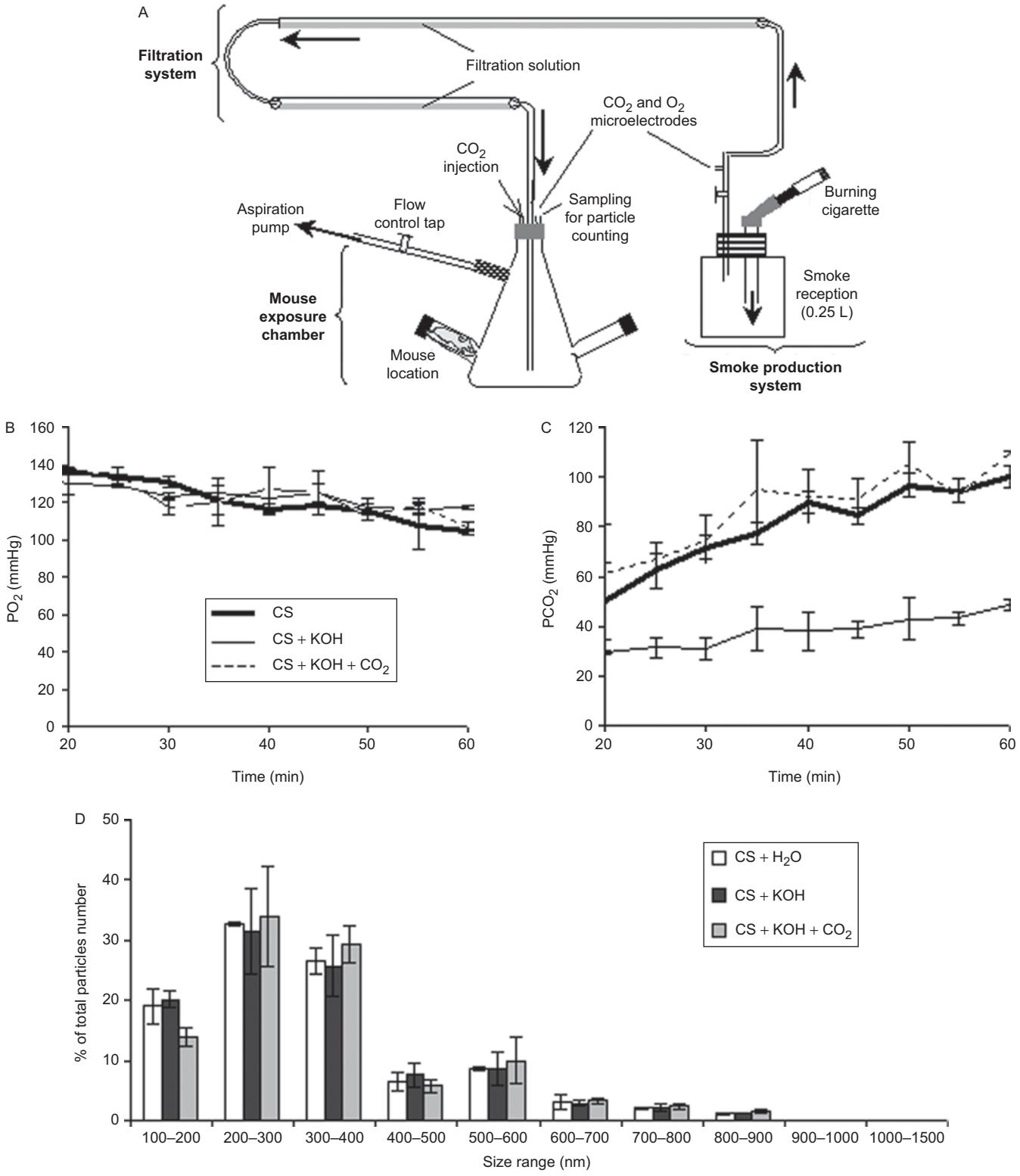

Figure 1. Experimental setup used for cigarette smoke exposure and control values during each experiment. (A) Diagram of the experimental setup. Mice were exposed to cigarette smoke generated by a burning cigarette connected to a vacuum pump. The filtration system consisted of two Plexiglas columns placed in series, half-filled with 1 M potassium hydroxide (KOH) solution or acidified water (pH 5.5 with HCl). The flow of smoke passes (dark arrow) over this solution, and carbon dioxide is captured by the KOH solution. The mouse exposure chamber consisted of eight conic glass tubes fused to a glass chamber, so that the muzzles were in contact with the interior of chamber. Cigarette smoke exits from a tube fitted to upper part of chamber after passing through the filtration system. In two experiments the CO_2 was directly injected into the mouse exposure chamber. (B, C) Partial O_2 (B) and CO_2 (C) pressures (mm Hg) in the exposure chamber were measured (see B for legend of B and C). The graphs display mean ± *SEM* from two experiments. (D) Cigarette smoke particle number (expressed in percentage of total particle number) present in each size range (from 100 to 1500 nm) was counted in a sample withdrawn at the 30th min. The graphs shown are representative of two experiments under each exposure condition with *SD*.

Lung histology

Samples for histology were obtained independently, based on one group of seven mice exposed to normal cigarette smoke and one group of eight mice exposed to cigarette smoke where the CO_2 had been filtered out by the KOH solution. Twenty-eight hours after exposure to cigarette smoke, mice were anesthetized with a urethane injection and the lungs were fixed by intratracheal injection of Hydrosafe (LABOnord). The lungs were removed, treated with Hydrosafe solution, embedded in paraffin, and cut into 5-µm sections. Evaluation of overall histologic lung inflammation was performed using a semiquantitative and reproducible blinded scoring system (Curtis et al., 1991; Van Hove et al., 2008). For each animal, six lung sections stained with either hematoxylin, periodic acid–Schiff, or Masson's trichrome were observed. A value between 0 and 3 was attributed to the tissue sections for each grading criterion (see Table 1): inflammation in alveolar wall, periarteriolar, perivenous, and peribronchial regions, and pleural area.

Proinflammatory cytokine secretion measurement

Lungs from mice exposed to cigarette smoke were carefully deblooded (by severing of the inferior vena cava) to discard most of the leukocytes, and washed in situ with phosphate-buffered saline (PBS) before removal. A mixture of primary lung cells was prepared from whole lung by collagenase type 4 and DNase I digestion. These cells were then cultivated to allow measurement of different cytokines (see Abolhassani et al., 2009, for detailed description). Levels of the following cytokines were measured by enzyme-linked immunosorbent assay (ELISA; DuoSet ELISA development kit, R&D Systems), as already described (Abolhassani et al., 2008): RANTES (regulated upon activation, normal T cell expressed and secreted; CCL5), IL-6, macrophage inflammatory protein (MIP)-2, and TNF-α.

Table 1. Histologic grading criteria.

Alveolar wall inflammation (Alv):

1. Increased number of inflammatory cells in alveolar walls.
2. As above, plus 1–3 foci per section showing cellular alveolar exudate and atelectasis.
3. As above, plus more than 3 foci per section showing cellular alveolar exudate and atelectasis.

Perivenous regions (Ven):

1. Occasional veins cuffed by inflammatory cells.
2. Most veins surrounded by a thin layer (1–5 cells thick) of inflammatory cells.
3. Most veins surrounded by a thick layer (>5 cells thick) of inflammatory cells.

Periarteriolar (Art):

Same scoring criteria as perivenous regions.

Peribronchial (Bro):

Same scoring criteria as perivenous regions.

Pleural inflammation (Pl):

1. Scattered inflammatory cells in pleural region.
2. Accumulation of a thin layer (1-5 cells thick) of inflammatory cells.
3. Accumulation of a thick layer (> 5 cells thick) of inflammatory cells.

Note. Following the method described by Curtis et al. with a few adaptations (Curtis et al., 1991; Van Hove et al., 2008), a value between 0 and 3 was attributed to the tissue sections for each grading criterion.

PP2A activity assay

PP2A activity was measured in fresh cells as already described by Abolhassani et al. (2008) using the R&D systems PP2A DuoSet IC activity assay kit according to the manufacturer's description. An immobilized capture antibody specific for the catalytic subunit of PP2A binds both active and inactive PP2A. After washing away unbound material, a synthetic phosphopeptide substrate was added that is dephosphorylated by active PP2A to generate free phosphate, which is detected by a sensitive dye-binding assay using malachite green and molybdic acid.

NF-κB p65 activation

Nuclear extraction was performed on cells using a nuclear extraction kit (Active Motif, Rixensart, Belgium) (Abolhassani et al., 2008). Five micrograms of proteic extracts were tested for the NF-κB activation by using the NF-κB p65 TransAM transcription factor assay kit (Active Motif), an ELISA-based transcription factor detection assay, according to the manufacturer's instructions.

PP2Ac semiquantitative RT-PCR

Total RNA of right lung from mice was prepared by RNeasy mini kit (Qiagen). cDNAs were generated by incubating 2 µg of total RNA, 2000 pmol oligo dT, 1.0 mM dNTP, 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase, and 5× reverse transcriptase (RT) buffer (Promega). Polymerase chain reaction (PCR) primers for PP2Ac and glyceraldehydes-3-pphosphate dehydrogenase (GAPDH; IBA) were the following: PP2Ac sense (5'-AGACACACTGGATCACATCC-3') and antisense (5'-CCATGATTGCAGCTTGGTTA-3') and GAPDH sense (5'-AATGGTGAAGGTCGGTGTGAAC-3') and antisense (5'- GAAGATGGTGATGGGCTTCC-3'). The size of amplified PCR products was obtained by subjecting them to electrophoresis at 100 V using 2% agarose gel (Invitrogen) for about 30 min and subsequent staining with 0.5 mg/ml ethidium bromide tris/borate/EDTA buffer.

Statistical analysis

Discrete ordinal data were analyzed by the Mann-Whitney *U* test. A statistically significant difference was defined at $p < .05$. The nonparametric distribution-free Kruskal-Wallis test was used to compare three or more independent groups of sampled data and screen significant differences between the groups. When $p < .05$, Mann-Whitney *U* test with Bonferroni's conservative corrections were applied to compare individual groups (significant when $p < .0083$). Statistical calculations were performed with the statistical packet SPSS 16.0 (SPSS, Chicago, IL).

Results

The experiments described in Methods were designed to assess the contribution of carbon dioxide in cigarette

smoke to cigarette smoke–induced acute inflammation. In order to accomplish this goal, an appropriate experimental setup was constructed and healthy mice were exposed to cigarette smoke for 1 h (Figure 1A). Briefly, this setting was composed of a smoke producing system, a carbon dioxide filtration system, and a mouse exposure chamber. The cigarette smoke exits from a tube fitted to the upper part of the exposure chamber after passing through the filtration system. Carbon dioxide reacts with water to undergo hydration and form carbonic acid and bicarbonate ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$). Trapping depends on the CO_2 concentration and pH: $pH = pK + \log [HCO_3^-/CO_2]$ (Henderson-Hasselbalch equation). Increased pH drives the equilibrium to the right, since the hydrogen ion formed is converted to water. Therefore, carbon dioxide is trapped by alkaline water. In order to selectively capture carbon dioxide contained in cigarette smoke, we introduced a filter containing a potassium hydroxide (KOH) solution between the cigarette and the mouse chamber. On the contrary, acidified water (pH 5.5) does not modify the concentration of carbon dioxide reaching the mice.

During these experiments, we measured the CO_2 and O_2 levels (see Tables S1 and S2). Because of instability of the gas pressures during the first 20 min, we present data based on the last 40 min of the experiment. The O_2 concentration in the mouse exposure chamber was measured at between 74.77 and 143.73 mm Hg (Figure 1C) in the smoke produced (CO_2-filtered or not). When filtered with the control acidified water solution, the CO_2 concentration was measured at between 34.78 and 109.84 mm Hg (average partial pressure 84.52 mm Hg or 12.7%) (Figure 1B). When the smoke is filtered with a potassium hydroxide solution, the CO_2 level is reduced to levels between 23.07 and 55.22 mm Hg (average 38.89 mm Hg or 5.8%), which confirms that a significant portion of the CO_2 is indeed trapped. In a last experiment, the CO_2 concentration of the filtered cigarette smoke in the exposure chamber was raised by direct injection of the pure gas resulting in a CO_2 level between 41.71 and 119.72 mm Hg (average 89.3 mm Hg or 13.4%). We analyzed the particle size distribution profile between cigarette smoke filtered with H_2O or KOH and cigarette smoke filtered with KOH with addition of CO_2. In all experiments, the total particle number varies from $3.17\ 10^8$ ($\pm\ 6.27\ 10^7$) to $4.66\ 10^8$ ($\pm\ 9.58\ 10^7$) in 10 ml of cigarette smoke. The small variability observed in between groups in the same experiment was of the same order as in between independent experiments. As shown on Figure 1D, there is no apparent difference under these experimental conditions in the particles size distribution (0.1 to 1.5 µm) of a sample withdrawn at the 30th min of the experiment.

We first analyzed the consequences of exposure to acidified water–filtered cigarette smoke versus potassium hydroxide–filtered cigarette smoke on mouse lung histology. About 28 h after cigarette smoke exposure, lung histology was characterized by inflammation, with numerous lymphocytes, macrophages, and few polymorphonuclear neutrophils (Figure 2A). This inflammation was alveolar, perivenular, subpleural, peribronchial, and periarteriolar

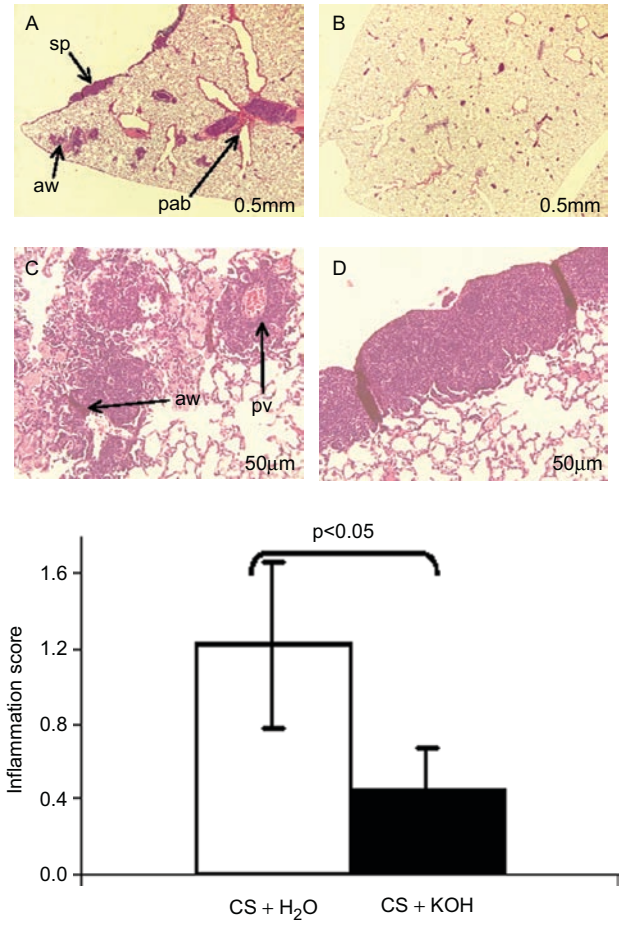

Figure 2. Consequences on mouse lung histology of exposure to cigarette smoke with/without potassium hydroxide solution filter. (A) Lung exposed to cigarette smoke showing an inflammation of the alveolar walls (aw), visceral pleura and subpleura (sp), and periarteriolar and peribronchial regions (pab). (B) Lung exposed to cigarette smoke filtered with potassium hydroxide most frequently showing a normal architecture with no inflammation. (C, D) Lung exposed to cigarette smoke (high magnification) showing an inflammation in foci of alveolar walls associated with hyperplasia of alveolar macrophages (aw) and perivenular inflammation (pv) (C) as well as strong inflammation (grade 3) of pleural and subpleura areas (D). (E) Evaluation of overall histologic lung inflammation (white columns: cigarette smoke, $n = 8$; dark columns: KOH-filtered cigarette smoke, $n = 7$) using a semiquantitative and reproducible scoring system. Data are expressed as mean ± *SEM*.

(Figures 2C and D). The presence of inflammation around peribronchovascular and subpleural areas means that there is diffusion along the lymphatic network. Interestingly, in the cases where carbon dioxide is captured, inflammation is significantly decreased (Figure 2B). We established an inflammatory score for each lung sample analyzed using a validated grading system (Curtis et al., 1991; Van Hove et al., 2008) and determined that the lungs exposed to cigarette smoke were highly inflamed. When carbon dioxide is partially trapped, the overall lung inflammation score dropped by two thirds (Figure 2E).

These observations were confirmed by the analysis of proinflammatory cytokine secretion. Normal lung cells were retrieved from the mice exposed to cigarette smoke and cultured for 20 h in order to perform ELISA proinflammatory

cytokine (MIP-2, TNF-α, RANTES, and IL-6) measurements (Figure 3) (Abolhassaniet al., 2008, 2009). As expected, there is an increase in cytokine secretion in response to cigarette smoke exposure as compared with normal air (Figures 3A–D): for RANTES, IL-6, MIP-2, and TNF-α, $p < .001$ for the difference between ambient air filtered with acidified H_2O and cigarette smoke filtered with acidified H_2O.

To characterize the mechanism of action of cigarette smoke, we measured the PP2A and p65 NF-κB activities (Figure 4). The data show that tobacco smoke stimulates the transcription and phosphatase activity of PP2A (Figure 4A and B) (phosphate released: $p < .001$ for the difference between ambient air filtered with acidified H_2O and cigarette smoke filtered with acidified H_2O). There also is a subsequent translocation of p65 NF-κB from the cytoplasm to the nucleus (Figure 4C) (NF-κB translocation: $p < .001$ for the difference between ambient air filtered with acidified H_2O and cigarette smoke filtered with acidified H_2O).

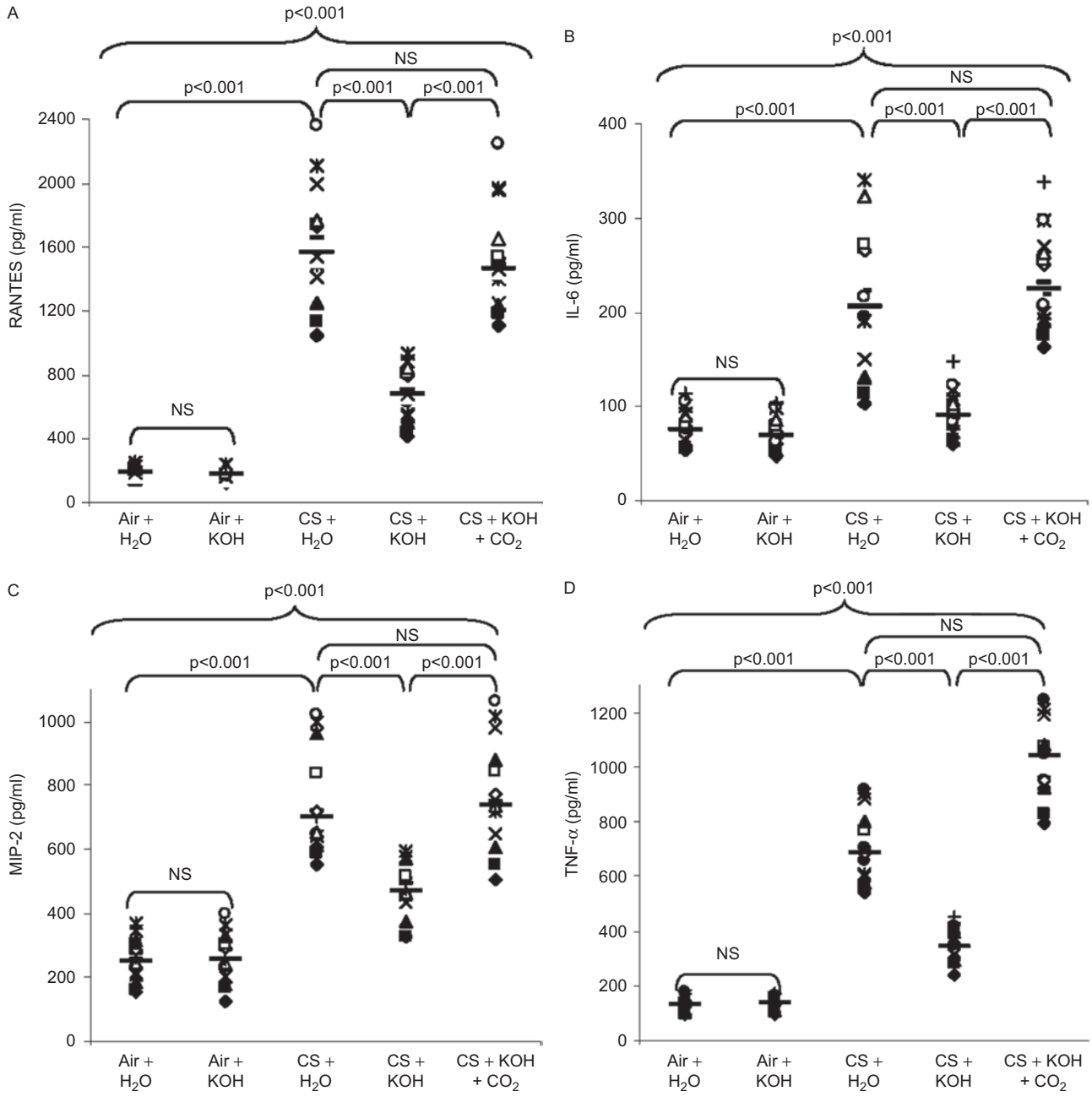

Figure 3. Cigarette smoke exposure induces the secretion of proinflammatory cytokines, strongly reduced in the presence of a potassium hydroxide solution filter and recovered with CO_2 addition. Mouse pulmonary cells were prepared from mice exposed to cigarette smoke filtered with water (CS + H_2O) or potassium hydroxide solution (CS + KOH) or with addition of CO_2 (CS + KOH + CO_2), as compared with ambient air (Air + acidified H_2O or Air + KOH). Retrieved cells were then cultivated to allow measurement of RANTES (A), IL-6 (B), MIP-2 (C), and TNF-α (D) secretion by ELISA (pg/ml). For each group, the horizontal bar is the median ($n = 15$). Groups were compared using the Kruskall-Wallis test to screen significant differences between the groups (upper values). When $p < .05$, Mann-Whitney U test with Bonferroni's conservative corrections were applied to compare individual groups (values above a pair of strip charts, significant when $p < .0083$). NS: nonsignificant.

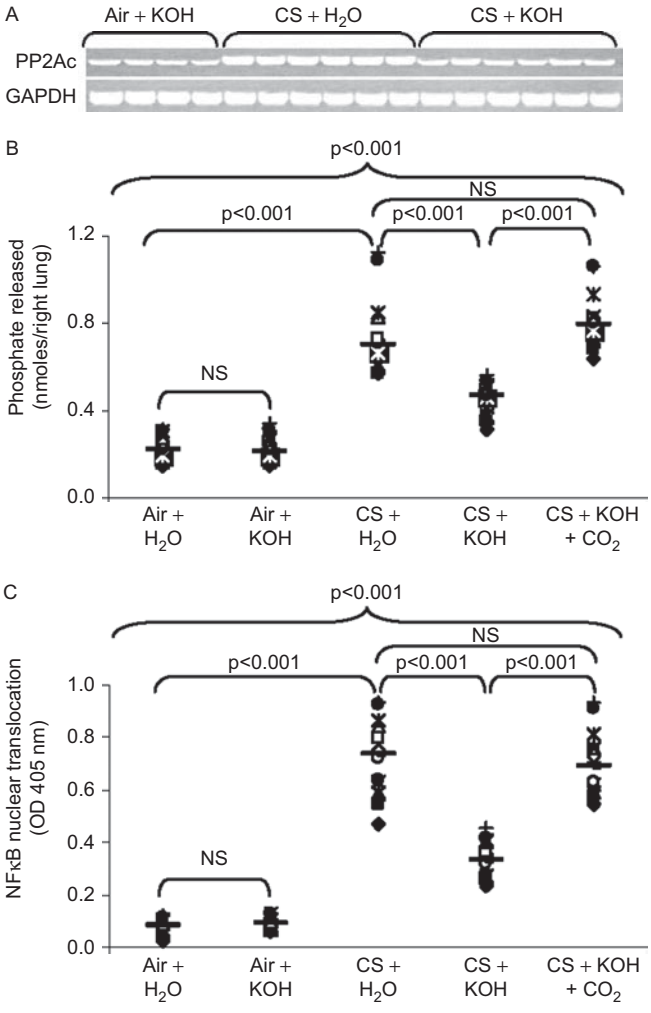

Figure 4. Cigarette smoke exposure causes PP2A and NF-κB activation, strongly reduced in the presence of potassium hydroxide and recovered with CO_2 addition. Mouse pulmonary cells were prepared from mice exposed to cigarette smoke filtered with acidified water (CS + acidified H_2O) or potassium hydroxide solution (CS + KOH) or with addition of CO_2 (CS + KOH + CO_2), as compared with ambient air (Air + acidified H_2O or Air + KOH). (A) PP2Ac transcription was measured by semiquantitative RT-PCR as compared with GAPDH control. PP2A activity (B) and NF-κB transcription (C) were measured. For each group, the horizontal bar is the median ($n = 15$). Statistical analyses were performed as described in Figure 3.

In order to show the contribution of CO_2 to the proinflammatory response, we demonstrated that the enhanced cytokine secretion induced by cigarette smoke is clearly reduced by potassium hydroxide filtration (Figure 3A–D): for RANTES, IL-6, MIP-2, and TNF-α, $p < .001$ for the difference between cigarette smoke filtered with acidified H_2O and cigarette smoke filtered with potassium hydroxide solution. Similarly, the PP2A and NF-κB inductions caused by cigarette smoke are reduced in the presence of the potassium hydroxide filter (Figure 4A–C): phosphate released and NF-κB translocation; $p < .001$ for the difference between cigarette smoke filtered with acidified H_2O and cigarette smoke filtered with potassium hydroxide.

Finally, in order to confirm the specificity of this inflammation response, we injected CO_2 into the mouse exposure chamber, after the cigarette smoke had been filtered by potassium hydroxide (Figure 1A and B). In this condition, we observed an increase in PP2A activity (phosphate released: $p < .001$ for the difference between cigarette smoke filtered with KOH and cigarette smoke filtered with KOH with addition of CO_2) (Figure 4B), NF-κB nuclear translocation (NF-κB translocation; $p < .001$ for the difference between cigarette smoke filtered with KOH and cigarette smoke filtered with KOH followed by addition of CO_2) (Figure 4C), and proinflammatory cytokine secretion (for RANTES, IL-6, MIP-2, and TNF-α, $p < .001$ for the difference between cigarette smoke filtered with KOH and cigarette smoke filtered with KOH with addition of CO_2) (Figure 3). Thus, it appears that carbon dioxide is a major proinflammatory component of cigarette smoke.

Discussion

Carbon dioxide is currently receiving considerable attention because of its known role in global warming. Its effect on human health, however, has not been extensively studied. We have assessed the lung inflammatory potential of carbon dioxide contained in cigarette smoke, since its level of concentration in mainstream smoke is about 200 times higher than in the atmosphere.

We designed a specific experimental setup in order to expose mice to cigarette smoke with normal or reduced levels of carbon dioxide. Several types of carbon dioxide filtration systems have been developed for use in confined environments such as a submarine or space ship (Warkander et al., 2000) (lithium-, monoethanolamine-, or lime-based), or by the petrol, gas, or cement industries (Siriwardane et al., 2001) (activated carbon or zeolites). However, none of these techniques could be used for this application because of their complexity or due to the lack of selectivity of pores, which would also trap the particles from the particulate phase (activated carbon and zeolites). Thus we trapped carbon dioxide by using a filter between the cigarette and the mouse chamber containing a potassium hydroxide solution. This experimental device has its own limitations, since we were not able to decrease the level of carbon dioxide below 5.8%. However, as measured in the exposure chamber, the filtration system does not appear to reduce the level of particles contained in the smoke.

Pulmonary damage observed by histology and an inflammatory response following tobacco smoke exposure is consistent with previous studies assessing the acute effects of smoke exposure, which have demonstrated the development of inflammation and oxidative stress in vivo (Van der Vaart et al., 2004). In the first hours following the stress, an inflammatory response was observed, characterized by the recruitment of alveolar macrophages and neutrophils and by an increased epithelial permeability (Van der Vaart et al., 2004). Similar effects have also been observed in vitro. For example, the release of inflammatory mediators, IL-1β and monocyte chemoattractant protein-1 (MCP-1), was shown 1 h after the exposure of alveolar macrophages to cigarette smoke (Castro et al., 2004). Our results demonstrate that both

the lung inflammation score and proinflammatory cytokine secretion were greatly diminished when carbon dioxide was partially removed by a potassium hydroxide solution filter. Additionnaly, the injection of CO_2 in the smoke downstream from the filtration column resulted in the recovery of the full inflammatory response. Thus we demonstrate that the lung acute toxicity of cigarette smoke may be a result of carbon dioxide exposure.

We also demonstrated that exposure to carbon dioxide causes NF-κB to translocate from the cytosol to the nucleus, thereby acting as a transcription factor for a number of proinflammatory proteins. This is consistent with the literature, as 1 h after the exposure of alveolar macrophages to cigarette smoke, the release of IL-1β and MCP-1 was shown to be subsequent to the nuclear translocation of NF-κB (Castro et al., 2004). In previous papers (Abolhassani et al., 2008, 2009; Schwartz et al., 2008), we reported that different proinflammatory stimuli (hyperosmolarity and hypercapnia) are transduced by PP2A, which appears to control the translocation of NF-κB from the cytoplasm to the nucleus. This noncanonical role of PP2A, as a positive activator of NF-κB nuclear translocation, has been described by others (Bhattacharyya et al., 2008); however, it is still controversial (Barisic et al., 2008; Marasa et al., 2008).

There are several reports in the literature suggesting that exposure to increased concentrations of CO_2 can be deleterious to the lungs. The reason for these, mostly old, studies was to assess the effect of a confined environment such as a submarine or space ship (Niemoeller and Schaefer, 1962; Schaefer et al., 1963), and, as noted above, filters have been developed to remove CO_2 from such environments. Guinea pigs and rats exposed to different CO_2 concentrations (1.5%, 3%, and 15% CO_2) had extensive lung inflammation, with loss of surfactant, hyaline membrane formation, and atelectasis, and respiratory distress syndrome. In a follow-up study (Schaefer et al., 1964), guinea pigs were exposed to up to 15% CO_2. The initial phase (6 h) was marked by respiratory acidosis accompanied by pulmonary inflammation (edema, congestion, atelectasis, and hemorrhage) and changes in the lamellar bodies (intracellular stores of surfactant) of the granular pneumocytes (type II). We recently confirmed and extended these earlier findings (Abolhassani et al., 2009). The exposure of cells and whole mice to CO_2 concentrations higher than 5% results in the secretion of multiple proinflammatory cytokines mediated by the nuclear p65 NF-κB translocation, itself a consequence of PP2A activation. Small interfering RNAs (siRNAs) targeted toward PP2Ac reversed the effect of carbon dioxide both on NF-κB activation and cytokine secretion.

Conclusion

Thus, the results of our study suggest that the carbon dioxide contained in cigarette smoke is responsible for a large part of tobacco-related pulmonary proinflammatory effects. Furthermore, taking into account the close link between smoking-induced pulmonary inflammation and cancer (Engels, 2008), and more generally the concept of cancer as an inflammation-based disease (Coussens and Werb, 2002; Israël and Schwartz, 2006) (Mantovani et al., 2008), as well as the old literature describing the potential carcinogenic consequences of the exposure of cell cultures to carbon dioxide (Bauer, 1925; Mottram, 1928), the presence of carbon dioxide in cigarette smoke may be of major importance with respect to smoking-related disease.

Given the relatively small size of this study, it should be regarded as a pilot study, and independent confirmation is important. In addition, only two doses of CO_2 were utilized, 12% and 5.8%. The shape of the dose-response curve should be assessed in a further study. Lastly, although we obtained some mechanistic information, detailed information as to how carbon dioxide causes a pulmonary inflammatory response is still lacking. Nevertheless, the results that we report are quite striking and are completely consistent with previous literature.

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Declaration of interest

This work was funded by Biorébus company. Biorébus company is applying for a patent relating to the content of this paper. The other authors declare that they have no competing interest.

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