# Carbon Dioxide is Responsible for the Acute Toxicity of Tobacco Smoke

Schwartz Laurent[1], Guais Adeline[2], Chaumet-Riffaud Philippe[3,4], Grévillot Georges[5], Sasco Annie J[6], Molina Thierry Jo[7], Abolhassani Mohammad[8].

[1] AP-HP Hôpital Pitié-Salpétrière, Service de radiothérapie, Paris, France.

[2] Biorébus, Paris, France.

[3] Université Paris-Sud, EA4046, UFR de Bicêtre, Le Kremlin-Bicêtre, France.

[4] AP-HP, CHU de Bicêtre, Service de Biophysique et de Médecine nucléaire, France.

[5] Laboratoire des Sciences du Génie Chimique CNRS – ENSIC, Nancy, France.

[6] Epidemiology for Cancer Prevention, INSERM U897, ISPED, Victor Segalen Bordeaux 2 University, Bordeaux, France.

[7] Université Paris Descartes, AP-HP Hôtel-Dieu, Paris, France.

[8] Pasteur Institute of Iran, Tehran, Iran.

Running Head : Tobacco Smoke-Induced Acute Toxicity is caused by CO2.

Contact information :

Laurent Schwartz, AP-HP Hôpital Pitié-Salpétrière, Service de radiothérapie, bd. de l'Hôpital 75013 Paris France. e-mail: laurent.schwartz@polytechnique.edu

Phone : +33681899030

Fax : +33140700130

**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. We took interest in carbon dioxide, since its level of concentration in mainstream cigarette smoke (about 12.5% by weight) is about 200 times higher than in the atmosphere. We previously demonstrated that inhalation of carbon dioxide concentrations above 5% have a deleterious effect on lungs. However, the inflammatory potential of carbon dioxide contained in cigarette smoke has never been assessed.

Here, we show that the toxicity of cigarette smoke is largely due to the considerable concentration of carbon dioxide. We found that pulmonary injuries consequent to tobacco smoke inhalation observed by histology were greatly diminished when withdrawing carbon dioxide through a potassium hydroxide filter. We demonstrate that cigarette smoke exposure causes an inflammatory response characterized by Protein Phosphatase 2 A (PP2A) and NF-κB activation followed by Macrophage Inflammatory Protein 2 (MIP-2), Regulated upon Activation, Normal T-cell Expressed, and presumably, Secreted (RANTES), IL-6 and TNF-α secretion. This inflammatory response was reduced when the cigarette smoke was filtered through a potassium hydroxide column, and retrieved when CO2 was injected downstream the filtration column. This inflammation is thus specific of carbon dioxide inhalation.

Our data suggest that the role of carbon dioxide in inflammation has been overlooked. Taking into account the tight links between inflammation and cancer, it also raises the issue of its potential implication in carcinogenic processes.

**Keywords**

Cigarette filter – 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, oesophagus and carcinoma of the bladder (13, 27). Smoking is also responsible of an increase in cardiovascular disease and stroke (6) as well as pre-term deliveries and hypotrophy of the foetus (18). Therefore, it is clear that eradicating smoking is a health priority.

Although numerous studies have been performed since the first assumption of tobacco toxicity by Doll and Hill (10), no compound has ever been established to be the cause. The mainstream smoke has been divided in two parts: the particulate phase and the gas phase. Most research has been focused on the particulate phase which contains the tar, a complex mixture of thousands of different chemicals such as tobacco-specific nitrosamines or benzo[a]pyrene (14,15).

Since the 1950s, cigarettes have undergone a progressive modification, including the addition of filters and a reduction in the average machine-measured tar yield per cigarette by over 60% (16). Yet, these newer products have in fact provided limited benefit if any, compared with higher tar cigarettes (19). The apparent reduction in risk and the relative benefits of low tar cigarettes appears limited and can not be established with certainty because of the large number and heterogeneity of studies performed. 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.

To their purveyors, these new "Tobacco Hharm Reduction" (THR) products represent an opportunity for inveterate smokers to reduce their risk of lung cancer and other diseases (21). To health professionals, the new products pose a myriad of risks. This new generation of THR products is not the first to promise reduced risk, however. Both filtered cigarettes and low tar and nicotine cigarettes were marketed with explicit health themes, ultimately with disastrous results for public health: over the period when the average tar level of cigarettes declined markedly (1954-1993), lung cancer rates actually increased in the United States and and the United Kingdom (19).

The gas phase has been less studied. In the gas phase, apart from nitrogen and oxygen (respectively 62 and 13% by weight of mainstream smoke) carbon dioxide is prevailing (about 12.5%) followed by carbon monoxide (4%) and water (1.3%) (11, 26). In the atmosphere, the partial pressure of carbon dioxide varies between 0.03 and 0.06% (20). It is the most prominent greenhouse gas in earth atmosphere and an isolated test at Mauna Loa in Hawaii revealed more than a 12% (316 ppm in 1959 to 360 ppm in 1996) increase in its mean concentration. Carbon dioxide is mainly emitted from coal-fire power-plants, refineries, chemical plants, cement firms and vehicles. For instance, concentrations close to those measured in cigarette smoke are detected in car exhausts or coal power-plant emissions (2).

In organisms, carbon dioxide reacts with water to form carbonic acid and is involved in multiple biochemical reactions. In a previous paper (2), we demonstrated that the inhalation of concentrations of carbon dioxide above 5% have a deleterious effect on the lung. The inflammation caused by the CO2 is mediated by the methylation of the protein phosphatase 2A (PP2A) which, in turn, activates the NF-κB pathway. The nuclear

translocation of NF-κB results in the secretion of the pro-inflammatory proteins such IL-6, IL-8 and TNF-α (30).

This article describes a study aiming to determine if the toxicity of cigarette smoke is, at least partially, due to its considerable concentration of carbon dioxide.

## Materials and Methods

**Mouse cigarette smoke exposure.** Thirty-two 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) and were approved by a research ethic board. Mice were exposed for 60 minutes to cigarette smoke generated by a burning cigarette (Marlboro light, Philip Morris) connected to a vacuum pump controlled by a tap. Filtration system consisted of two plexiglas columns placed in series (100 x 0.97 cm Bio-Rad column + 48 x 1.96 cm Pharmacia column), half-filled with 1M potassium hydroxide (KOH) solution or acidified water (pH 5.5 with HCl) at room temperature. Flow of smoke passes over this solution (slightly agitated to increase contact surface) and carbon dioxide is captured. Mouse exposure chamber consisted of 8 conic glass tubes fused to a glass chamber (total volume: 1.6 liter), so that muzzles were in contact with interior of chamber. Cigarette smoke comes out through a tube fitted to upper part of chamber after passing through the filtration system. When stated, the CO2 concentration of the mouse exposure chamber was maintained between 9 and 13% by the injection of a pure CO2 gas capsule (Carboxique). Partial $CO_2$ and $O_2$ pressures in exposure chamber were measured using specific microelectrodes (Lazar Research Laboratories). Partial $H_2O$ pressure was estimated at

about 50 mmHg. The number of cigarette smoke particles present in each size range was counted on a 10 ml sample using a Met One 237A portable airborne particle counter (Hach Ultra).

**Lung histology.** Twenty-eight hours after exposure to cigarette smoke, mice were anesthetized with a urethane injection and lungs were fixed by intratracheal injection of Hydrosafe (LABOnord). Lungs were removed, treated with Hydrosafe solution, and then embedded in paraffin. To evaluate microscopic changes, fixed lungs were cut into 5µm sections and stained with hematoxylin. Evaluation of overall histologic lung inflammation using a semi-quantitative and reproducible blinded scoring system (9,33). Seven animals exposed to cigarette smoke and eight animals exposed to cigarette smoke filtered with potassium hydroxide were analyzed. For each animal, six lung sections stained with either hematoxylin, periodic acid-Shiff 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.

**Pro-inflammatory cytokine secretion measurement.** Mouse pulmonary cells were prepared from mice exposed to cigarette smoke and then cultivated to allow measurement of different cytokines (2). Regulated upon Activation, Normal T-cell Expressed, and presumably, Secreted (RANTES), IL-6, Macrophage Inflammatory Protein 2 (MIP-2) and TNF-α secretion were measured by ELISA (DuoSet ELISA development kit, R&D Systems), as already described (1).

**PP2A Activity Assay.** PP2A activity was measured in fresh cells as already described by Abolhassani (1) using 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 is 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) (1). Five micrograms of proteic extracts were tested for the NF-κB activation by using the NF-κB p65 TransAMTM transcription factor assay kit (Active Motif), an ELISA-based transcription factor detection assay, according to the manufacturer's instructions.

**PP2Ac semi-quantitative RT-PCR.** Total RNA of right lung from mice were 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 M-MLV reverse transcriptase, 5 × RT buffer (Promega). PCR primers for PP2Ac and GAPDH (IBA) were the following: PP2Ac sense (5'-AGACACACTGGATCACATCC-3') and antisense (5'-CCATGATTGCAGCTTGGTTA-3') and GAPDH sense (5'-AATGGTGAAGGTCGGTGTGAAC-3') and antisense (5'- GAAGATGGTGA TGGGCTTCC-3'). The size of amplified PCR products was obtained by subjecting to electrophoresis at 100 V through 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 Mann-Whitney U-test. Statistical difference was accepted at $p < 0.05$. The non-parametric 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 $p < 0.05$, Mann-Whitney U-test with Bonferroni's conservative corrections were applied to compare individual groups

(significant when $p < 0.0083$). Statistical calculations were performed with the statistical packet SPSS 16.0 (SPSS Inc.; Chicago, IL).

**Results**

We designed experiments to assess the proportion of cigarette smoke-induced acute inflammation that is due to carbon dioxide inhalation. We thus designed a specific experimental setup and exposed healthy mice to cigarette smoke for one hour (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 comes out through 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 release carbonic acid ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$). Trapping depends on the $CO_2$ concentration and pH: $pH = pK + \log [HCO_3^- / CO_2]$ (Henderson-Hasselbalch equation). 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 controlled the $CO_2$ and $O_2$ levels. The $O_2$ concentration in the mouse exposure chamber was measured at between 99 and 136 mmHg (Figure 1B) 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 50 mmHg and 106 mmHg (average partial pressure 84.52 mmHg or 12.7%) (Figure 1C). When the smoke is filtered with a potassium hydroxide solution, the $CO_2$ level is reduced to levels between 30 and 52 mmHg (average 38.89 mmHg or 5.8%), which confirms that bicarbonates are

trapped. When stated, the CO2 concentration of the filtered cigarette smoke in the exposure chamber was raised by direct injection of pure gas and measured between 53 and 110 mmHg (average 89.3 mmHg or 13.4%). We analysed particle size distribution profile between cigarette smoke filtered with H2O or KOH and cigarette smoke filtered with KOH with addition of C02. In the experiment, the total particle number varies from 3.17 $10^8$ (± 6.27 $10^7$) to 4.66 $10^8$ (± 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 in the particles size distribution (0.1 to 1.5 μm) of a sample withdrawn at the 30$^{th}$ minute of the experiment.

We first analyzed the consequences of exposure to unfiltered cigarette smoke versus potassium hydroxide-filtered cigarette smoke on mouse lung histology. About twenty-eight hours 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 (Figures 2C and D). Interestingly, in the cases where carbon dioxide is captured, inflammation is strongly hampered (Figure 2B). We established an inflammatory score for each lung sample analyzed using a validated grading system (9, 33) 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 pro-inflammatory cytokine secretion. Normal lung cells were retrieved from the mice exposed to cigarette smoke and cultured for 20 hours in order to perform ELISA pro-inflammatory cytokine (MIP-2, TNF-

α, RANTES and IL-6) measurements (Figure 3) (1, 2). As expected, there is an increase in cytokine secretion in response to cigarette smoke exposure as compared with normal air (Figures 3A to 3D): for RANTES, IL-6, MIP-2 and TNF-α, $p < 0.001$ for the difference between ambient air filtered with $H_2O$ and cigarette smoke filtered with $H_2O$.

To characterize the mechanism of action of cigarette smoke, we measured the PP2A and p65 NF-κB activity (Figure 4). Here, we show that tobacco smoke stimulates the transcription and phosphatase activity of PP2A (Figures 4A and 4B) (Phosphate released: $p < 0.001$ for the difference between ambient air filtered with $H_2O$ and cigarette smoke filtered with $H_2O$). There also is a subsequent translocation of p65 NF-κB from the cytoplasm to the nucleus (Figure 4C) (NF-κB translocation: $p < 0.001$ for the difference between ambient air filtered with $H_2O$ and cigarette smoke filtered with $H_2O$).

In order to show the pro-inflammatory action of the trapped components, we demonstrate that the enhanced cytokine secretion induced by cigarette smoke is clearly reduced by potassium hydroxide filtration (Figures 3A to 3D): for RANTES, IL-6, MIP-2 and TNF-α, $p < 0.001$ for the difference between cigarette smoke filtered with $H_2O$ and cigarette smoke filtered with potassium hydroxide. Similarly, the PP2A and NF-κB inductions caused by cigarette smoke are reduced in the presence of the potassium hydroxide filter (Figures 4A to 4C): phosphate released and NF-κB translocation; $p < 0.001$ for the difference between cigarette smoke filtered with $H_2O$ and cigarette smoke filtered with potassium hydroxide.

Eventually, in order to confirm the specificity of this inflammation response, we injected CO2 in the mouse exposure chamber, after the cigarette smoke had been filtered by potassium hydroxide (Figure 1A and 1B). In this condition, we observed an increase in PP2A activity (phosphate released: $p < 0.001$ for the difference between cigarette smoke

filtered with KOH and cigarette smoke filtered with KOH with addition of C02) (Figure 4B), NF-κB nuclear translocation (NF-κB translocation: $p < 0.001$ for the difference between cigarette smoke filtered with KOH and cigarette smoke filtered with KOH and completed with CO2) (Figure 4C) and pro-inflammatory cytokine secretion (for RANTES, IL-6, MIP-2 and TNF-α, $p < 0.001$ for the difference between cigarette smoke filtered with KOH and cigarette smoke filtered with KOH with addition of C02) (Figure 3). Thus, it appears that carbon dioxide is an major pro-inflammatory component of cigarette smoke.

**Discussion**

Carbon dioxide is known to play a key role in global warming. However, its effect on human health has not been extensively studied. Here, we 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, containing or not carbon dioxide. Several types of carbon dioxide filtration systems have been developed for use in a confined environment such as a submarine or space ship (34) (lithium, monoethanolamine or lime-based), or by the petrol, gas or cement industries (31) (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 containing a potassium hydroxide solution between the cigarette and the mouse chamber. This experimental device has its own limitation, 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 interfere with the particles contained in the smoke.

Pulmonary injuries observed by histology and inflammatory response consecutives to tobacco smoke inhalation confirmed earlier findings (32). Studies assessing the acute inflammatory effects of smoking have demonstrated the development of inflammation and oxidative stress. In the first hours following the stress, inflammation was characterized by the recruitment of alveolar macrophages and neutrophils and by an increased epithelial permeability (32). For example, the release of inflammatory mediators, IL-1β and Monocyte Chemoattractant Protein-1 (MCP-1) was shown one hour after the exposure of alveolar macrophages to cigarette smoke (7). We show that lung inflammation score and pro-inflammatory cytokines secretion were greatly diminished when withdrawing carbon dioxide through a potassium hydroxide filter, thus demonstrating that the lung acute toxicity of cigarette smoke is a consequence of carbon dioxide exposure.

We characterized implicated signalisation proteins and demonstrated the activation of PP2A and NF-κB that control cigarette-smoke induced lung acute inflammation. This is consistent with the literature as, one hour 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 (7). In previous papers (1, 2, 30), we reported that different pro-inflammatory stimuli (hyperosmolarity and hypercapnia) are transduced by PP2A, which controls the translocation of NF-κB from the cytoplasm to the nucleus. This non-canonical role of PP2A, as a positive activator of NF-κB nuclear translocation, has been described by others (5), however, it is still controversial (3, 23).

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 (25, 28). 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 (29), guinea pigs were exposed to up to 15% $CO_2$. The initial phase (6 hours) 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 (2). The exposure of cells and whole mice to $CO_2$ concentrations higher than 5% results in the secretion of multiple pro-inflammatory cytokines mediated by the nuclear p65 NF-κB translocation, itself a consequence of PP2A activation. Short inhibiting RNAs (siRNAs) targeted toward PP2Ac reverse the effect of carbon dioxide both on NF-κB activation and cytokine secretion.

Thus, our study demonstrate that the carbon dioxide contained in cigarette smoke is responsible for a large part of tobacco-related pulmonary pro-inflammatory effects. Furthermore, taking into account the close link between smoking-induced pulmonary inflammation and cancer (12), and more generally the concept of cancer as an inflammation-based disease (8, 17, 22), as well as the old literature describing the potential carcinogenic consequences of the exposure cell cultures to carbon dioxide (4, 24), the implication of carbon dioxide in carcinogenesis should be of major concern in the future.

**Acknowledgments**

We wish to acknowledge the help of Edward Sanders and Jean-Marc Steyaert.

**Grants**

This work was funded by Biorébus.

**References**

1. **Abolhassani M, Wertz X, Pooya M, Chaumet-Riffaud P, Guais A, Schwartz L.** Hyperosmolarity causes inflammation through the methylation of protein phosphatase 2A. *Inflamm Res* 57: 419-429, 2008.

2. **Abolhassani M, Guais A, Chaumet-Riffaud P, Sasco AJ, Schwartz L.** Carbon dioxide inhalation causes pulmonary inflammation. *Am J Physiol Lung Cell Mol Physiol* 296: L657-665, 2009.

3. **Barisic S, Strozyk E, Peters N, Walczak H, Kulms D.** Identification of PP2A as a crucial regulator of the NF-kappaB feedback loop: its inhibition by UVB turns NF-kappaB into a pro-apoptotic factor. *Cell Death Differ* 15: 1681-1690, 2008.

4. **Bauer J.** The effect of carbon dioxide on cells in tissue cultures. *Bull Johns Hopkins Hosp* 37: 420-439, 1925.

5. **Bhattacharyya S, Dudeja PK, Tobacman JK.** Lipopolysaccharide activates NF-kappaB by TLR4-Bcl10-dependent and independent pathways in colonic epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 295: G784-790, 2008.

6. **Burns D.** Epidemiology of smoking-induced cardiovascular disease. *Prog Cardiovasc Dis* 46: 11-29, 2003.

7. **Castro P, Legora-Machado A, Cardilo-Reis L, Valença S, Porto LC, Walker C, Zuany-Amorim C, Koatz VL.** Inhibition of interleukin-1beta reduces mouse lung inflammation induced by exposure to cigarette smoke. *Eur J Pharmacol* 498: 279-286, 2004.

8. **Coussens LM, Werb Z**. Inflammation and cancer. *Nature* 420: 860-867, 2002.

9. **Curtis JL, Byrd PK, Warnock ML, Kaltreider HB.** Requirement of CD4-positive T cells for cellular recruitment to the lungs of mice in response to a particulate intratracheal antigen. *J Clin Invest* 88: 1244-1254, 1991.

10. **Doll R, Hill AB.** Smoking and carcinoma of the lung; preliminary report. *Br Med J* 2: 739-748,1950.

11. **Dube MF, Green CR.** Methods of collection of smoke for analytical purposes. *Rec Adv Tob Sci* 8: 42–102, 1982.

12. **Engels EA.** Inflammation in the development of lung cancer: epidemiological evidence. *Expert Rev Anticancer Ther* 4: 605-615, 2008.

13. **Ezzati M, Henley SJ, Lopez AD, Thun MJ.** Role of smoking in global and regional cancer epidemiology: current patterns and data needs. *Int J Cancer* 116: 963-971, 2005.

14. **Hammond D, O'Connor RJ.** Constituents in tobacco and smoke emissions from Canadian cigarettes. *Tob Control* 17: i24-31, 2008.

15. **Hecht SS.** Cigarette smoking, cancer risks, carcinogens, and mechanisms. *Langenbecks Arch Surg* 391: 603-613, 2006.

16. **Hoffmann D, Hoffmann I.** The changing cigarette, 1950-1995. *J Toxicol Environ Health* 50: 307-364, 1997.

17. **Israël M, Schwartz L.** *Cancer as a dysmethylation syndrome.* Paris, France: John Libbey Eurotext, 2006.

18. **Jauniaux E, Burton GJ.** Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev* 83: 699-706, 2007.

19. **Kabat G.** Fifty years' experience of reduced-tar cigarettes: what do we know about their health effects? *Inhal Toxicol* 15: 1059-1102, 2003.

20. **Keeling C, Whorf T.** Atmospheric $CO_2$ concentrations derived from flask air samples at sites in the SIO network. *In Trends: A Compendium of Data on Global Change*. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee, U.S.A, 2004. Available at: http://cdiac.ornl.gov/trends/co2/sio-keel-flask/sio-keel-flaskmlo_c.html [accessed 16 January 2009].

21. **Lee PN, Sanders E.** Does increased cigarette consumption nullify any reduction in lung cancer risk associated with low-tar filter cigarettes? *Inhal Toxicol* 16: 817-33, 2004.

22. **Mantovani A, Allavena P, Sica A, Balkwill F**. Cancer-related inflammation. *Nature* 454: 436-444, 2008.

23. **Marasa BS, Xiao L, Rao JN, Zou T, Liu L, Wang J, Bellavance E, Turner DJ, Wang JY.** Induced TRPC1 expression increases protein phosphatase 2A sensitizing intestinal epithelial cells to apoptosis through inhibition of NF-kappaB activation. *Am J Physiol Cell Physiol* 294: C1277-1287, 2008.

24. **Mottram J.** On the division of cells under varying tensions of carbon dioxide. *Br J Exp Path* 9: 240-244, 1928.

25. **Niemoeller H, Schaeffer KE.** Development of hyaline membrane and atelectases in experimental chronic respiratory acidosis. *Proc Soc Exp Biol Med* 110: 804-808, 1962.

26. **Norman V.** An overview of the vapor phase, semivolatile and nonvolatile components of cigarette smoke. *Rec Adv in Tob Sci* 3: 28-58, 1977.

27. **Parkin DM, Bray F, Ferlay J, Pisani P.** Global cancer statistics, 2002. *CA Cancer J Clin* 55: 74-108, 2005.

28. **Schaefer KE, Hastings BJ, Carey CR, Nichols G, Jr**. Respiratory acclimatization to carbon dioxide. *J Appl Physiol* 18: 1071-1078, 1963.

29. **Schaefer KE, Avery ME, Bensch K**. Time course of changes in surface tension and morphology of alveolar epithelial cells in $CO_2$-induced hyaline membrane disease. *J Clin Invest* 43: 2080-2093, 1964.

30. **Schwartz L, Abolhassani M, Pooya M, Steyaert J-M, Wertz X, Israël M, Guais A, Chaumet-Riffaud P.** Hyperosmotic stress contributes to mouse colonic inflammation through the methylation of Protein Phosphatase 2A. *Am J Physiol Gastrointest Liver Physiol* 295: G934-941, 2008.

31. **Siriwardane R, Shen M, Fisher E, Poston J, Shamsi A**. Adsorption and desorption of $CO_2$ on solid sorbents. Published in the *Proceedings of the First National Conference on Carbon Sequestration,* 2001.

32. **Van der Vaart H, Postma DS, Timens W, Ten Hacken NH**. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* 59: 713-721, 2004.

33. **Van Hove CL, Moerloose K, Maes T, Joos GF, Tournoy KG**. Cigarette smoke enhances Th-2 driven airway inflammation and delays inhalational tolerance. *Respir Res* 9: 42, 2008.

34. **Warkander D, Lillo R, Navy experimental diving unit Panama City Fl.** Review of Two Methods to Remove $CO_2$ Using Seawater From Submarines during Emergency Conditions. US Navy report A352983, 2000. Defense Technical Information Center, Fort Belvoir, Virginia, USA.

**Figure legends**

**Fig. 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. Filtration system consisted of two plexiglas columns placed in series, half-filled with 1M potassium hydroxide (KOH) solution or acidified water (pH 5.5 with HCl). Flow of smoke passes (dark arrow) over this solution and carbon dioxide is captured. Mouse exposure chamber consisted of 8 conic glass tubes fused to a glass chamber, so that muzzles were in contact with interior of chamber. Cigarette smoke comes out through a tube fitted to upper part of chamber after passing through the filtration system. When stated, the CO2 was directly injected in the mouse exposure chamber.(B-C) Partial $CO_2$ (B) and $O_2$ (C) pressures (mmHg) in exposure chamber were measured (dark lines: cigarette smoke, plain lines: KOH-filtered cigarette smoke, dashed lines: KOH-filtered cigarette smoke with addition of C02). The graphs present means ± SEM from two experiments. (D) Cigarette smoke particles number (expressed in percentage of total particles number) present in each size range (from 100 to 1500 nm) was counted in a sample withdrawn at the $30^{th}$ minute (white columns: cigarette smoke, dark columns: KOH-filtered cigarette smoke, grey columns: KOH-filtered cigarette smoke with addition of C02). The graphs shown are representative of two experiments with SD.

**Fig. 2: Consequences on mouse lung histology of exposure to cigarette smoke with/without potassium hydroxide 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 semi-quantitative and reproducible scoring system. Data are expressed as mean ± SEM.

**Fig. 3: Cigarette smoke exposure induces the secretion of pro-inflammatory cytokines, strongly reduced in the presence of a potassium hydroxide filter and recovered with CO2 addition.**

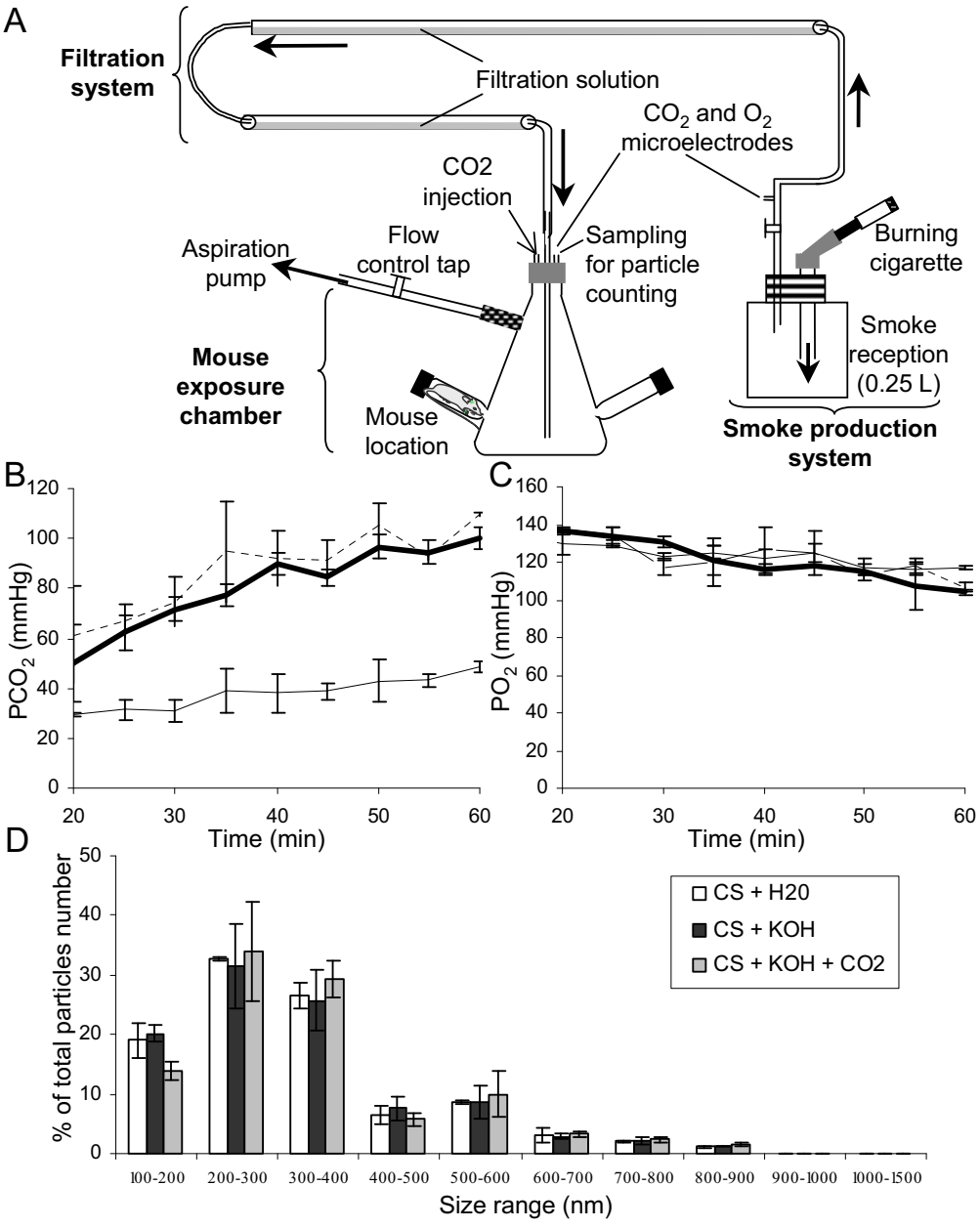
Mouse pulmonary cells were prepared from mice exposed to cigarette smoke filtered with water (CS + $H_2O$) or potassium hydroxide (CS + KOH) or with addition of C02 (CS + KOH + CO2), as compared with ambient air respiration (Air + $H_2O$ or Air + KOH). Retrieved cells were then cultivated to allow measurement 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=16). Groups were compared using the Kruskall-Wallis test to screen significant differences between the groups (upper values). When $p < 0.05$, Mann-Whitney U-test with

Bonferroni's conservative corrections were applied to compare individual groups (values above a pair of strip charts, significant when $p < 0.0083$). NS: non significant.

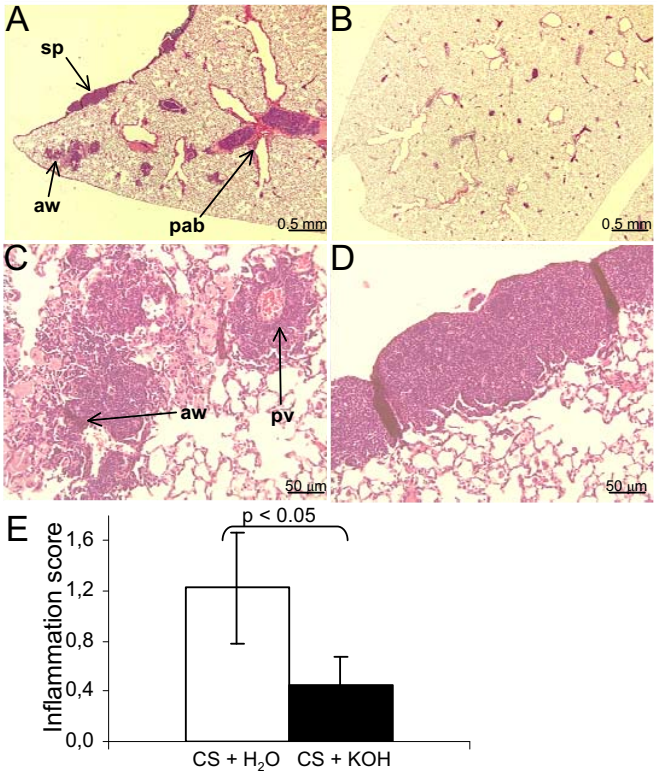
**Fig. 4: Cigarette smoke exposure causes PP2A and NF-κB activation, strongly reduced in the presence of potassium hydroxide and recovered with CO2 addition.**

Mouse pulmonary cells were prepared from mice exposed to cigarette smoke filtered with water (CS + $H_2O$) or potassium hydroxide (CS + KOH) or with addition of C02 (CS + KOH + CO2), as compared with ambient air respiration (Air + $H_2O$ or Air + KOH). (A) PP2Ac transcription was measured by semi-quantitative 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=16). Statistical analysis were performed as described in Figure 3.

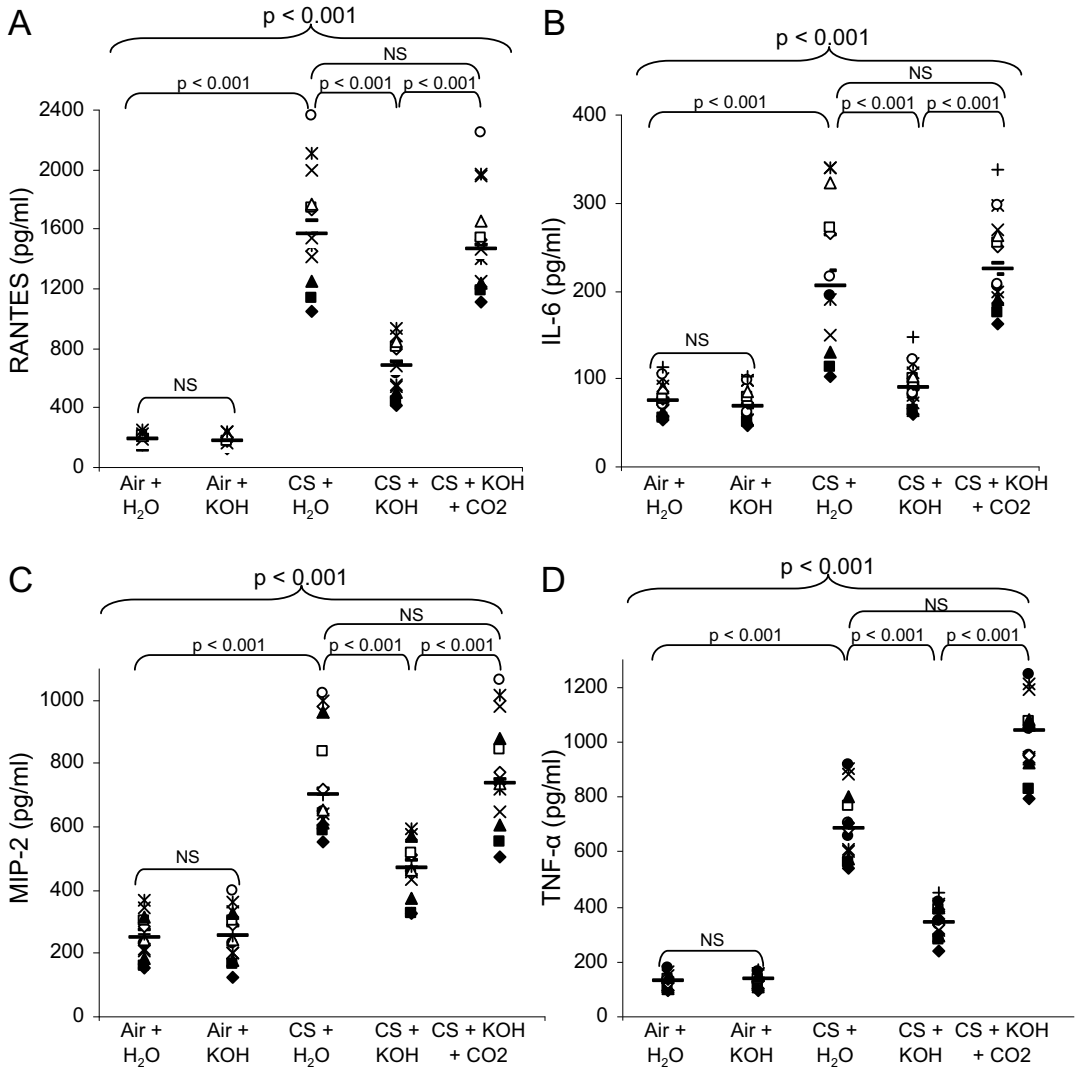
# Figure 1


A
Filtration system
Filtration solution
CO2 and O2 microelectrodes
CO2 injection
Aspiration pump
Flow control tap
Sampling for particle counting
Burning cigarette
Mouse exposure chamber
Mouse location
Smoke reception (0.25 L)
Smoke production system
B
PCO2 (mmHg)
Time (min)
C
PO2 (mmHg)
Time (min)
D
% of total particles number
CS + H20
CS + KOH
CS + KOH + CO2
100-200
200-300
300-400
400-500
500-600
600-700
700-800
800-900
900-1000
1000-1500
Size range (nm)

# Figure 2


A
sp
aw
pab
0.5 mm
B
0.5 mm
C
aw
pv
50 µm
D
50 µm
E
p < 0.05
Inflammation score
1,6
1,2
0,8
0,4
0,0
CS + H2O
CS + KOH

# Figure 3


A
p < 0.001
NS
p < 0.001
p < 0.001
p < 0.001
RANTES (pg/ml)
2400
2000
1600
1200
800
400
0
NS
Air + H2O
Air + KOH
CS + H2O
CS + KOH
CS + KOH + CO2
B
p < 0.001
NS
p < 0.001
p < 0.001
p < 0.001
IL-6 (pg/ml)
400
300
200
100
0
NS
Air + H2O
Air + KOH
CS + H2O
CS + KOH
CS + KOH + CO2
C
p < 0.001
NS
p < 0.001
p < 0.001
p < 0.001
MIP-2 (pg/ml)
1000
800
600
400
200
0
NS
Air + H2O
Air + KOH
CS + H2O
CS + KOH
CS + KOH + CO2
D
p < 0.001
NS
p < 0.001
p < 0.001
p < 0.001
TNF-α (pg/ml)
1200
1000
800
600
400
200
0
NS
Air + H2O
Air + KOH
CS + H2O
CS + KOH
CS + KOH + CO2

# Figure 4

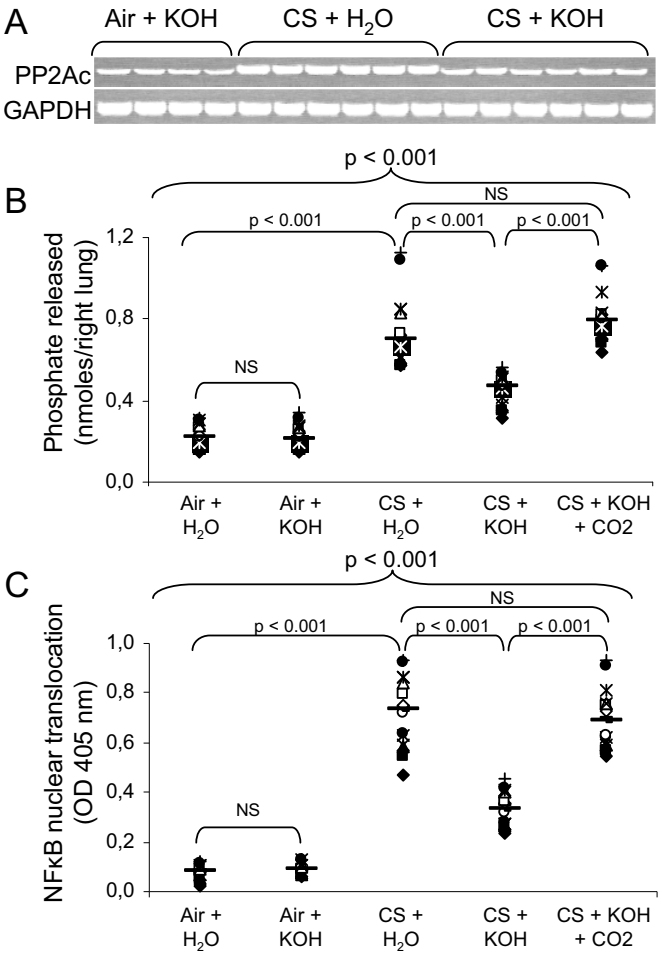

A
Air + KOH
CS + H2O
CS + KOH
PP2Ac
GAPDH
B
p < 0.001
NS
p < 0.001
p < 0.001
p < 0.001
Phosphate released (nmoles/right lung)
1,2
0,8
0,4
0,0
NS
Air + H2O
Air + KOH
CS + H2O
CS + KOH
CS + KOH + CO2
C
p < 0.001
NS
p < 0.001
p < 0.001
p < 0.001
NFκB nuclear translocation (OD 405 nm)
1,0
0,8
0,6
0,4
0,2
0,0
NS
Air + H2O
Air + KOH
CS + H2O
CS + KOH
CS + KOH + CO2

**Table 1. Histologic grading criteria.**

Following the method described by Curtis with a few adaptations (16,17), a value between 0 and 3 was attributed to the tissue sections for each grading criterion.

| | |
|---|---|
| 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. |