Eur. J. Oncol., vol. 14, n. 4, pp. 199-208, 2009

The toxicity of carbon dioxide inhalation

Tossicità da inalazione di biossido di carbonio

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

[1] Service de radiothérapie, Hôpital Pitié-Salpêtrière, Paris, France
[2] Nosco Pharmaceuticals, Paris, France
[3] Biorébus, Paris, France
[4] Radio-Hyperthermia Service, Hospital of Monza, Monza, Italy
[5] Laboratoire des Sciences du Génie Chimique CNRS – ENSIC, Nancy, France
[6] Université Paris-Sud, EA4046, UFR de Bicêtre, Le Kremlin-Bicêtre, France
[7] AP-HP, CHU de Bicêtre, Service de Biophysique et de Médecine nucléaire, Le Kremlin-Bicêtre, France
[8] Epidemiology for Cancer Prevention, Inserm U897, Bordeaux School of Public Health, Victor Segalen Bordeaux 2 University, Bordeaux, France

Summary

Carbon dioxide (CO_2) is naturally present in the atmosphere where its concentration is about 0.038%. It is also a normal constituent of the body arising from cellular respiration or taking part in several metabolic reactions. CO_2 is also one of the main side-products of combustion, and in particular of cigarette smoking. Analysis of primary tobacco smoke demonstrates the presence of a high concentration at 13.5% CO_2. We designed a study to assess if CO_2, either obtained from gas canister or from cigarette combustion, is pro-inflammatory. We analyzed *in vitro* the inflammation response induced by exposure to CO_2 for 48 hours (0 to 20% with a constant O_2 concentration of 21%). In addition, we carried out an *in vivo* experiment where mice were submitted to increasing concentrations of CO_2 (0, 5, 10, 15% with a constant O_2 concentration of 21%) for one hour. The exposure to concentra-

Riassunto

Il biossido di carbonio (CO_2) è naturalmente presente nell'atmosfera ad una concentrazione di circa lo 0,038%. Il CO_2 è un normale costituente del corpo, deriva dalla respirazione cellulare e prende parte a numerose reazioni metaboliche. È inoltre, uno dei principali prodotti finali della combustione, e in particolare del fumo di sigaretta. Analisi del fumo di tabacco dimostrano infatti la presenza di una elevata concentrazione di CO_2, pari al 13,5%. Abbiamo progettato uno studio per valutare se il CO_2, ottenuto sia da bomboletta o da gas di combustione di sigarette, avesse propietà pro-infiammatorie. Per dimostrare questa attività pro-infiammatoria abbiamo analizzato *in vitro* la risposta infiammatoria indotta da esposizione alle emissioni di CO_2 per 48 ore (da 0 a 20%, con una concentrazione costante di O_2 del 21%). Abbiamo, inoltre, condotto un esperimento *in vivo* su topi sottoposti a concentrazioni crescenti

Received/Pervenuto 21.8.2009 - Accepted/Accettato 17.11.2009
Address/Indirizzo: Dr Laurent Schwartz, Service de Radiothérapie, Hôpital Pitié-Salpétrière, boulevard de l'Hôpital, 75013 Paris, France
Tel.+33 6 71 81 33 47 - Fax +33 1 40 70 01 30 - E-mail: laurent.schwartz@biorebus.com

tions above 5% of CO_2 resulted in the dose-dependent secretion of pro-inflammatory cytokines by A549 cells and pulmonary cells retrieved from the exposed mice as demonstrated by ELISA. Lung inflammation was also demonstrated *in vivo* by airway hyperreactivity induction pointed out by plethysmography. This response was mediated by the nuclear translocation of p65 Nuclear Factor kappa-B (NF-κB), itself a consequence of Protein Phosphatase 2A (PP2A) activation. Short inhibiting RNAs (siRNAs) targeted toward catalitic subunit PP2Ac prevented the effect of CO_2, *i.e.* disrupted the NF-κB activation and the pro-inflammatory cytokine secretion. Thus, *in vivo* or *in vitro* exposure to high CO_2 concentrations leads to an inflammation response mediated by the PP2A/NF-κB pathway. These results strongly suggest that exposure to carbon dioxide may be more toxic than previously thought. Because of the described relationship inbetween inflammation and cancer this data may be relevant for carcinogenic effects of combustion products such as those of tobacco. Eur. J. Oncol., 14 (4), 199-208, 2009

Key words: **cancer, carbon dioxide, inflammation, Protein Phosphatase 2A, NF-κB, tobacco smoke**

di CO_2 (0, 5, 10, 15%, con una concentrazione costante di O_2 del 21%) per un'ora. L'esposizione a concentrazioni superiori al 5% di CO_2 ha portato alla secrezione di citochine pro-infiammatorie da cellule A549 in modo dose-dipendente e le cellule polmonari ottenute dai topi esposti, hanno dimostrato infiammazione polmonare tramite test ELISA. In più è stata dimostrata *in vivo* tramite pletismografia anche una iperattività delle vie aeree. Questa ultima risposta è stata mediata dalla traslocazione nucleare di p65 fattore nucleare kappa-B (NF-κB), a sua volta una conseguenza della attivazione della proteina fosfatasi 2A (PP2A). Inibitori dell'RNA (siRNA) specifici verso il dominio PP2Ac hanno impedito l'effetto pro infiammatorio del CO_2, vale a dire hanno inibito l'attivazione del NF-κB e la secrezione di citochine pro-infiammatorie. Sia *in vivo* che *in vitro* l'esposizione a concentrazioni di CO_2 alta porta a una riposta infiammatoria mediata dalla via PP2A/NF-κB. Questi risultati suggeriscono che l'esposizione al biossido di carbonio possa essere più tossica di quanto si pensasse. A causa del rapporto descritto fra il processo infiammatorio e il cancro, questi dati potrebbero essere rilevanti per gli effetti cancerogeni dei prodotti di combustione come quelli del tabacco. Eur. J. Oncol., 14 (4), 199-208, 2009

Parole chiave: **cancro, biossido di carbonio, infiammazione, proteina fosfatasi 2A, NF-κB, fumo di tabacco**

Abbreviations

MCP-1: Monocyte Chemoattractant Protein-1
IL-8: Interleukin-8
PP2A: Protein Phosphatase 2A
NF-κB: Nuclear Factor kappa B
ELISA: Enzyme-Linked ImmunoSorbent Assay
CO_2: carbon dioxide
siRNA: short inhibiting RNA
AHR: airway hyperreactivity
ELISPOT: enzyme-linked immunospot
RANTES: Regulated upon Activation, Normal T-cell Expressed, and Secreted or CCL5
SFCs: Spot forming cells
Penh: Enhanced pause
LPS: lipopolysaccharide

Introduction

Carbon dioxide (CO_2) is naturally present in the atmosphere where its concentration is about 0.038% (0.29 mmHg or 380 ppm). It is a colorless gas that is heavier than air and which has a faintly pungent odor.

Carbon dioxide is a normal constituent of the body arising from cellular respiration. It diffuses from the tissues into the surrounding capillaries and is carried by blood in chemical combination with hemoglobin, in physical solution as dissolved carbon dioxide, carbonic acid, or bicarbonate ions, and as minor

amounts of other carbamino compounds (carbon dioxide in combination with plasma proteins). The partial pressure of carbon dioxide in pulmonary capillary blood is about 7% or 46 mmHg, which is greater than that in alveolar air (6% or 40 mmHg). The gas is freely exchanged through the alveolar membrane by diffusion, because of the concentration gradient existing between the blood and the air in the alveoli, and is thus released from the lungs by convection.

Carbon dioxide is not an inert molecule. It reacts with water to undergo hydration and release carbonic acid ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$). As with other buffers, pH is related to concentrations of CO_2 and bicarbonates according to the Henderson-Hasselbalch equation. There are several reports in the literature suggesting that exposure to high concentrations of CO_2 can be deleterious. These studies were essentially carried out to assess the effects of breathing in a confined environment such as a submarine or space ship (1, 2).

There is little, if any, data on the toxicity of carbon dioxide released during combustion. Analysis of primary tobacco smoke demonstrates the presence of a very high concentration of 12.5% carbon dioxide (3, 4). Similar concentrations are detected in car exhausts or coal power plant emissions (5).

In this work, we performed a feasibility study to validate the inflammatory effect of carbon dioxide, decipher its mechanism of action and suggest that the toxicity of smoke may be, at least partially, mediated by carbon dioxide.

Materials and Methods

Animals

BALB/c mice were obtained from the Centre d'Elevage Janvier (Le Genest, St Isle, France) and maintained in accordance with the European Community's guidelines (1987 regulation) concerning the care and use of laboratory animals.

Cell Cultures

The human pulmonary cancer cell line, A549 (ATCC number CCL-185; LGC Promochem, Molsheim, France) were cultured in Dulbecco's minimal essential medium (DMEM, Gibco) supplemented with 10% decomplemented fetal bovine serum (FBS, Eurobio, Les Ulis, France) and 1% non-essential amino acids, in a humid atmosphere with various concentrations of CO_2 at 37°C up to 48 h with a constant O_2 concentration of 21%.

ELISA

Cytokine secretions into the supernatants of A549 human cells into the culture medium after a 48 h of exposure to CO_2 were quantified using the DuoSet Enzyme-Linked ImmunoSorbent Assay (ELISA) development kit (R&D Systems, Minneapolis, MN) for human IL-8 and MCP-1, as previously described (6).

PP2A Activity Assay

PP2A activity was measured in fresh cells as already described by Abolhassani (6) using R&D systems (Minneapolis, MN) 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) (6). 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 siRNA

Combinations of three short inhibiting RNAs (siRNAs) targeting different positions within the β

isoform of human *PP2Ac* mRNA (PP2Ac-siRNA) were used (Qiagen) and a non-silencing siRNA (Non-PP2A siRNA) was included as control (7). Transfection of cells was carried out by electroporation using the Lipofectamin 2000 (Invitrogen). Analyses (NF-κB nuclear translocation and cytokine secretion measurements) were performed 14 hours after transfection.

Measurement of Airway Hyperreactivity

Airway hyperreactivity (AHR) was measured in conscious, unrestrained mice in a preconditioned whole-body plethysmograph (Buxco Electronics Inc., Troy, NY). BALB/c mice (n = 8/group) were exposed to high CO_2 concentrations (0, 5, 10 or 15% with a constant O_2 concentration of 21%) directly injected in the plethysmograph chambers during 60 minutes. Respiratory parameters were recorded and enhanced pause (Penh), which reflects pulmonary resistance (8, 9), was calculated using IOX software (EMKA Technologies, Paris, France).

Mouse Hypercapnia One Hour-long Exposure

Thirty-two male BALB/c mice 6-7 weeks old (8 mice/group) were administered non-restrictively with high concentrations of carbon dioxide (continuous injection with manual control of the tap) in a large plethysmography chamber (EMKA Technologies PLT-UN2-GT) for 60 minutes at room temperature. The partial CO_2 and O_2 pressures in the chamber were measured every two minutes and every minute respectively using specific microelectrodes (Lazar Research Laboratories, CA). In all cases, the O_2 concentration was maintained at 149 mmHg by the injection of a pure dry O_2 gas capsule (Carboxique). The CO_2 concentration was maintained by the injection of a pure dry CO_2 gas capsule (Carboxique) at 35.5 mmHg (5%), 71 mmHg (10%) and 106.5 mmHg (15%). The partial H_2O pressure was estimated at about 50 mmHg.

Lung Cell Purification

Mice were sacrificed 4 hours after exposure to CO_2 (intraperitoneal injection of 800 µl of a 5% aqueous urethane solution). Lungs were deblooded by severing of the inferior vena cava, washed *in situ* with PBS, and removed. Lungs were finely chopped and homogenized in RPMI culture medium containing 30 mg/mL of DNase 1 (SIGMA) and 150 units/mL of collagenase (Worthington, Lakewood, NJ) in a Heidolph homogenizer (RZR 2102). Digestion took place for 1 h at 37°C to eliminate all the extracellular matrix elements for culture. The homogenate was passed over a cell strainer and the single cell suspension was washed in HBSS. This mixture of primary lung cells were resuspended in culture medium and counted.

Cytokine ELISPOT Assay

For enzyme-linked immunospot (ELISPOT) assay of RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted or CCL5), we used anti-mouse RANTES ELISPOT Kit from R&D Systems. Cells were briefly seeded onto polyvinylidene difluouride (PVDF)-backed microplates (Multiscreen, Millipore) coated with cytokine-specific antibodies at a concentration of $2x10^5$ cells/well. Cells were cultivated in duplicate for 20 h. Unbound cells were washed away and a biotinylated antibody specific for the given cytokine was added. Spots (corresponding to cytokine-secreting cells) were developed as described and the number of spot forming cells (SFC) was determined using a dissecting microscope (10).

Lung Histology

Four hours after exposure to CO_2, mice were anesthetized with urethane injection and mice lungs were fixed by intratracheal injection of Hydrosafe (80% acetic acid, methanol and ethanol) (LABOnord, France). Lungs were removed, treated with Hydrosafe solution, and then embedded in paraffin. To evaluate microscopic changes, fixed lungs were cut into 5 µm sections and stained with Hematoxylin-Eosin.

Statistical Analysis

The non-parametric distribution-free Kruskal-Wallis test was used to compare three or more independent groups of sampled data. When significant

differences were found, multiple comparison tests (Tukey test) were carried out to identify which groups are different.

Dose dependence of cytokine production was tested by means of ANOVA and regression analysis.

All statistical analyses were performed using R software (11). Values were considered statistically significant when p was less than 0.05.

Results

A549 cells were exposed for 48 h to various concentrations of carbon dioxide with a constant O_2 concentration of 21% and pro-inflammatory cytokines secretions was measured by ELISA analysis (fig. 1). Exposure to five per cent CO_2 does not appear to be toxic. Between 10 and 20% CO_2, there is an induction of the secretion of MCP-1 and IL-8. Statistical analyses revealed a highly significant dose dependence between carbon dioxide levels and the pro-inflammatory cytokines produced (for instance MCP-1: $p < 0.0001$).

In order to decipher the mechanism of action of carbon dioxide, we measured the activity of PP2A and of NF-κB. In previous papers (6, 12), we reported that PP2A controls the translocation from the cytoplasm to the nucleus of p65 NF-κB. Increased concentration of carbon dioxide stimulates the activity of PP2A such as the release of phosphate (fig. 1C). According to our previous work, a translocation from the cytoplasm to the nucleus of p65

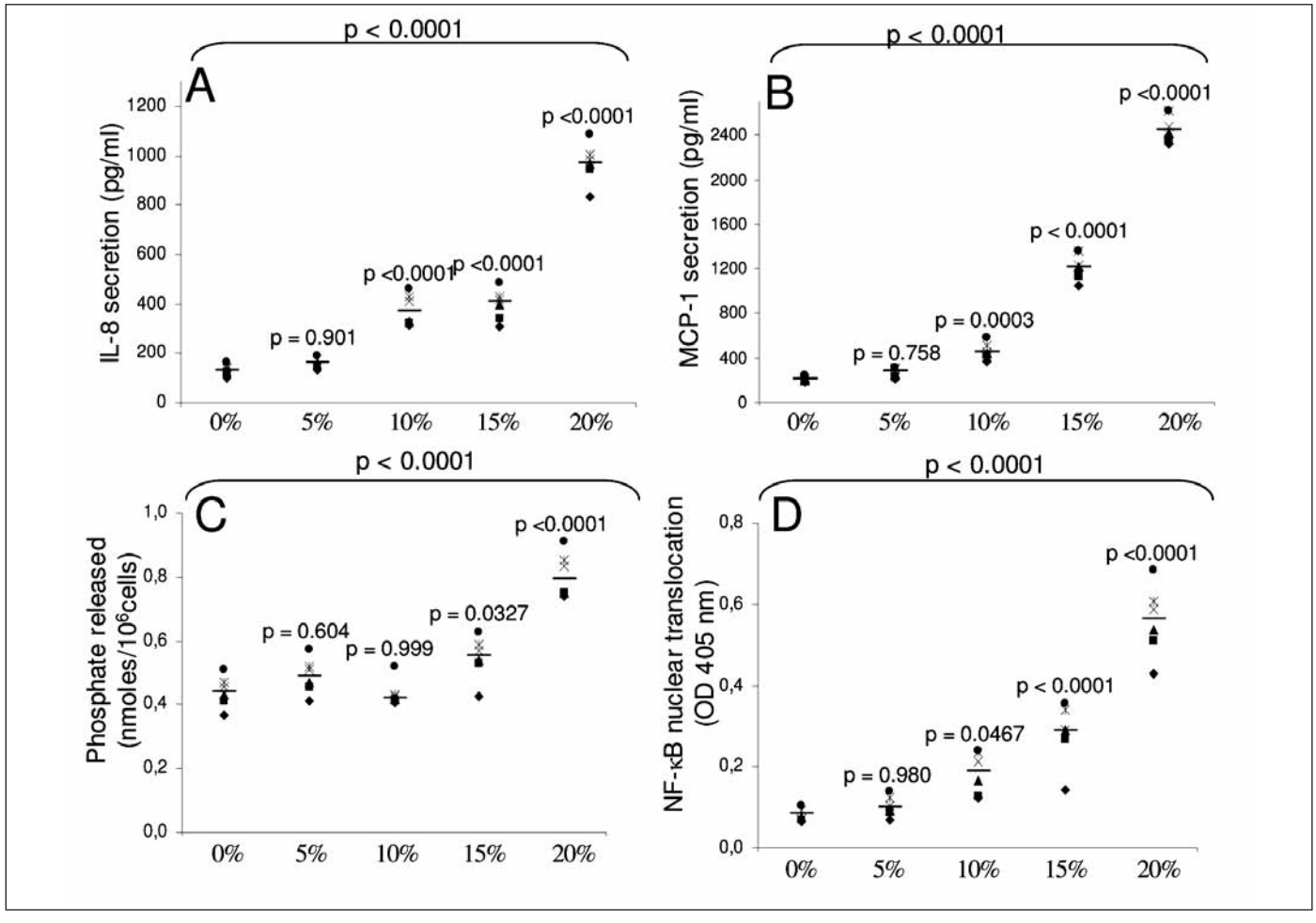

Fig. 1. Increased cytokine secretion associated with PP2A and NF-κB activation following exposure to CO_2. Human A549 cells were exposed to increasing CO_2 concentrations (+: 0, ●: 5, ■: 10, ◆: 15%) during 48 hours with a constant O_2 concentration of 21% (n = 6) and IL-8 (A) and MCP-1 (B) cytokines secretion were measured by ELISA. The amount of phosphate released by PP2A (C) and the p65 NF-κB (D) nuclear translocation were also quantified. For each group, the horizontal bar is the median. Upper values represent the global p-values (Kruskal-Wallis test). The p-value given by the Tukey test above a stripchart indicates the result of the comparison of this group *versus* control.

NF-κB takes place (fig. 1D). However, as NF-κB nuclear translocation appears to occur at a lower concentration of CO_2 than that of PP2A activation, it seems that at least in this model, NF-κB nuclear translocation is controlled by other factors.

In order to confirm the key rôle of PP2A in CO_2 induced inflammation, we transfected A549 cancer cells with silencing RNA. The cells were either treated with non-specific siRNAs or with siRNAs targeted toward the catalytic subunit of PP2A (7). Fig. 2 shows that transfection with siRNAs targeted toward the catalytic subunit of PP2A significantly diminishes the effect of 15% carbon dioxide. After transfection, treatment with carbon dioxide hardly increase the level of IL-8 and MCP-1 (fig. 2A and B). The nuclear translocation of NF-κB is strongly reduced (fig. 2C). PP2Ac siRNA does not completely abolishes NF-κB activation and cytokines secretion. This could be attribute to the implication of another transduction pathway in few proportion of inflammation, otherwise the siRNA inhibition efficacy is not complete. As expected, non-specific siRNA has no effect on the secretion of IL-8, MCP-1, or on the nuclear translocation of NF-kB.

We followed the results obtained on human cancer cell lines by *in vivo* experiments.

Normal pulmonary cells were retrieved from mice exposed to carbon dioxide for one hour (with a constant O_2 concentration of 21%). These cells were then cultured for 20 hours in order to perform ELISPOT pro-inflammatory cytokine measurements. Like human cancer cells, these murine normal cells respond to carbon dioxide. Fig. 3A shows that there is an increase in RANTES secretion by pulmonary cells isolated from mice exposed to one-hour long hypercapnia. As it is shown *in vitro*, PP2A (fig. 3C) and NF-κB (fig. 3D) activations are induced in lung cells exposed to carbon dioxide.

Exposure of mice to carbon dioxide causes an inflammatory syndrome. Mice were exposed to either normal air or to increased concentrations of carbon dioxide (with a constant O_2 concentration of 21%). The lungs were removed four hours later, embedded in paraffin, cut into 5 μm sections and stained with Hematoxylin-Eosin. The photographs confirm that increased CO_2 in the inhaled air results in an inflammation of the lung (fig. 4A and B). There is a recruitment of leukocytes in close proximity to

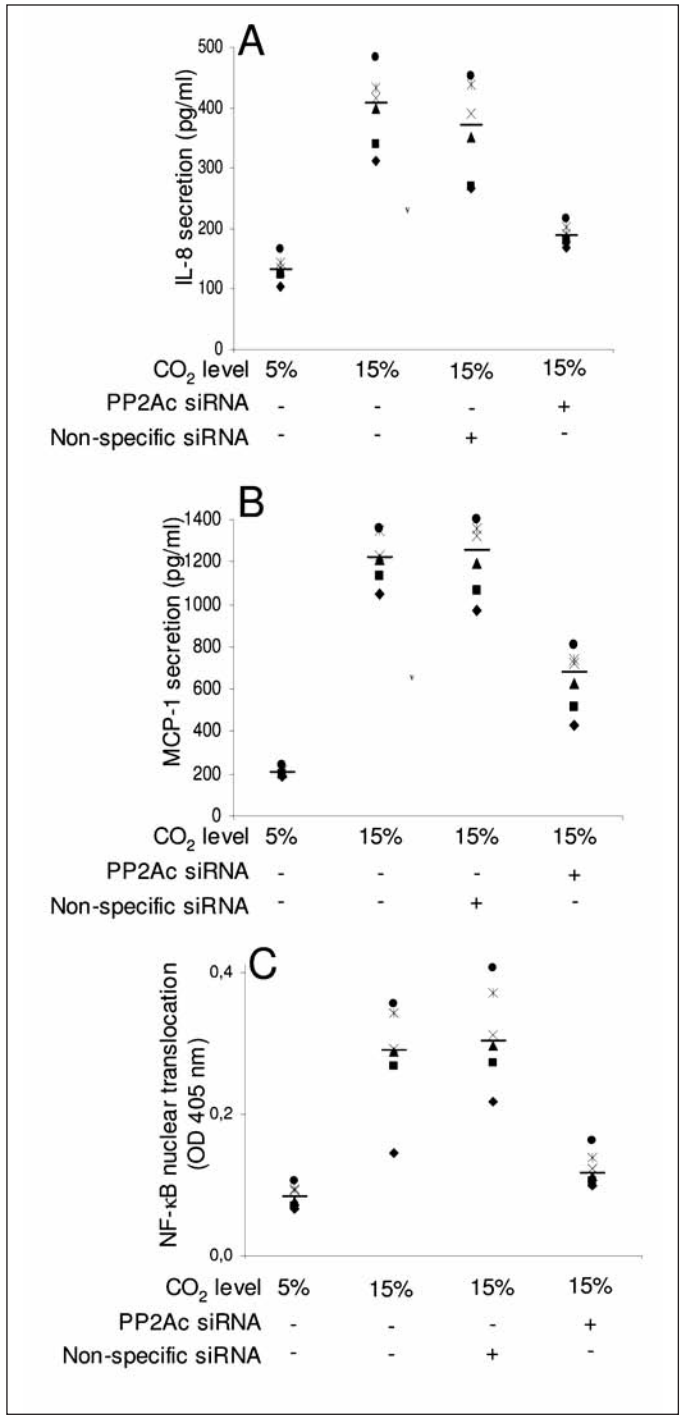

Fig. 2. Effect of PP2Ac siRNAs on the inflammatory response caused by CO_2 exposure A549 cells were transfected with PP2Ac specific and non-specific siRNAs and then incubated with increasing CO_2 levels (+: 0, ●: 5, ■: 10, ◆: 15%) (with a constant O_2 concentration of 21%) for 14 hours (n = 6). IL-8 (A) and MCP-1 (B) pro-inflammatory cytokine production (ELISA), as well as NF-κB nuclear translocation (C) are specifically reduced in the presence of PP2Ac siRNAs. For each group, the horizontal bar is the median. Upper values represent the global p-values (Kruskal-Wallis test). The p-value given by the Tukey test above a stripchart indicates the result of the comparison of this group *versus* control.

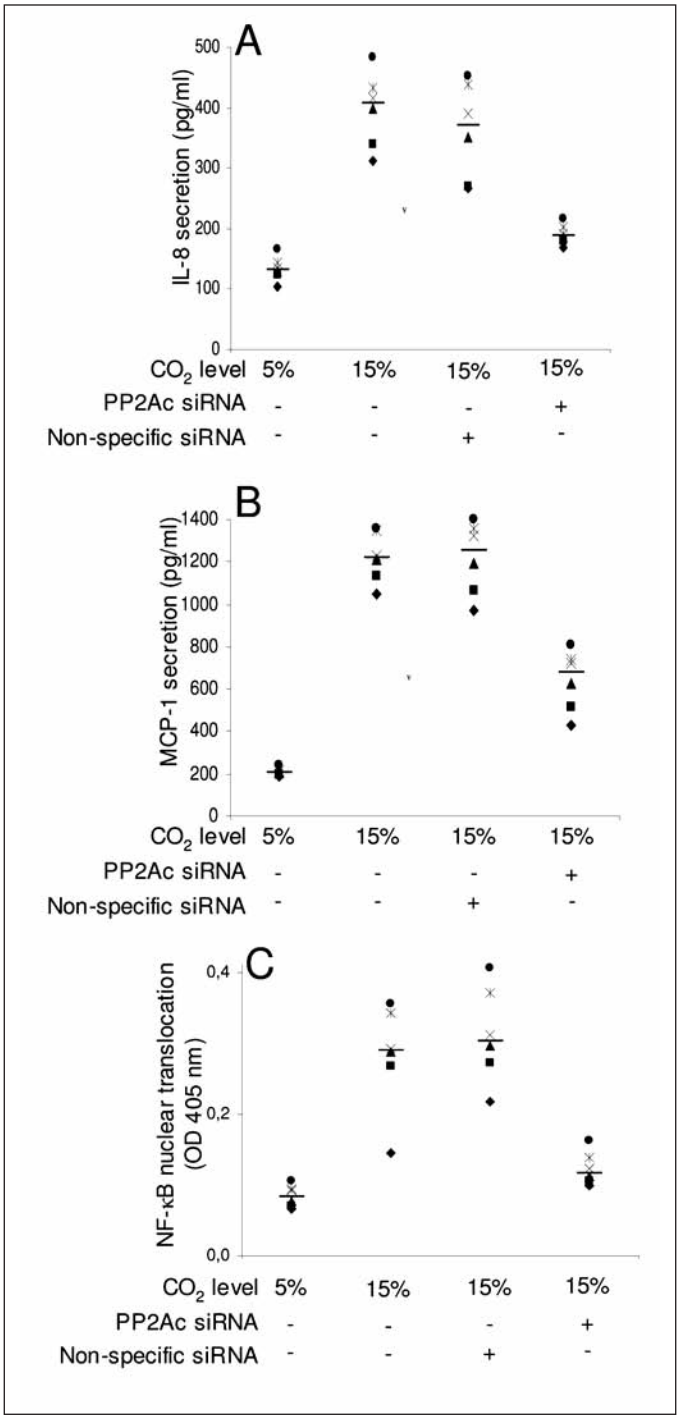

Fig. 3. Pro-inflammatory cytokine production, PP2A and NF-κB activation in response to *in vivo* hypercapnia. (A) RANTES Cytokine secretion was measured using ELISPOT after a 20 hours culture (2.10^5 cells/well). Pulmonary cells had been isolated after sacrifice of BALB/c mice 4 hours after the end of one hour long exposure to CO_2 (+: 0, ●: 5, ■: 10, ◆: 15%). SFC: Spot Forming Cells. The amount of phosphate released by PP2A (B) and the p65 NF-κB nuclear translocation (C) were also measured (n = 6). For each group, the horizontal bar is the median. Upper values represent the global p-values (Kruskal-Wallis test). The p-value given by the Tukey test above a stripchart indicates the result of the comparison of this group *versus* control.

the alveolar lumen. The alveolar membranes of CO_2-exposed mice are thicker than the normal one.

Enhanced pause (Penh) is a well-accepted marker of airway reactivity (8). Mice were exposed in a barometric plethysmography chamber for 60 minutes to various concentrations of carbon dioxide (with a constant O_2 concentration of 21%). Penh, which reflects airway obstruction, was measured every minute during the experiment and average values are presented (fig. 4C). Inhalation of CO_2 increases the reactivity of the airway as shown by Penh increase in hypercapnic conditions. The airway hyperreactivity is only slightly dependent on the duration of exposure. However, the very early increase of Penh (first 30 minutes) can not be related to PP2A/NF-κB pathway activation. In fact, it probably reflects the hypothalamic response (presumably activated by CO_2 receptors located on the neurons of the lungs and brain (13) that accelerates the breathing system.

These studies demostrate the inflammatory effect of carbon dioxide. Nevertheless, there appears to be a threshold. Our work suggests the weak toxicity of 5% carbon dioxide, a concentration that is similar to the carbon dioxide concentration in the alveolar sac (14).

Discussion

In a first attempt to decipher the potential inflammatory rôle of carbon dioxide, we confirm the deleterious effect of short-term exposure to CO_2 with a constant O_2 concentration of 21%. *In vitro*, exposure to 5% CO_2 has only a limited effect, if any. This may be due in part to the fact that cells are usually grown in 5% CO_2. Above this threshold, there clearly is a dose-dependent response in the secretion of multiple pro-inflammatory cytokines. This secretion is mediated by the nuclear translocation of p65 NF-κB, itself a consequence of PP2A activation. Short inhibiting RNAs (siRNAs) targeted toward PP2Ac prevent the effect of carbon dioxide.

PP2A comprises a family of serine-threonine phosphatases implicated in the regulation of many signaling pathways, and in particular inflammation. PP2A holoenzyme is composed of an association of three subunits: regulatory (A), structural (B) and

catalytic (C). One of the functions of the methylated and active PP2Ac is the activation of NF-κB pathway (15). This pathway has been deciphered in other instances, most notably hyperosmotic stress (6) or during immune response (12).

Our data are consistent with the literature. Niemoeller exposed guinea pigs and rats to different CO_2 concentrations (for 2 to 42 days at 1.5, 3, 15% CO_2 in 21% O_2). He noticed extensive lung inflammation with loss of surfactant, hyaline membrane formation (present at 3% CO_2 for 4 days and in all animals at 15% CO_2 for 1-2 days) and atelectasis (from 1.5% CO_2). Hyaline membrane formation was associated with respiratory distress syndrome (1).

In a follow-up study (16), guinea pigs were exposed to up to 15% CO_2. The authors identified four phases of pulmonary changes caused by 15% carbon dioxide. 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 (type II) pneumocytes. The second phase (6-24 hours) was associated with hyaline membrane formation. During the third phase (days 2-7), the surface tension returned to normal, the pulmonary edema diminished and hyaline membranes disappeared. The final phase was one of recovery, although the CO_2 concentration remained high.

The rôle of CO_2 in inflammation has long been controversial. Very high concentrations (>90%) have been reported to decrease the risk of peritonitis after coelioscopy (17). This type of insufflation causes transient and local acidification that inhibits LPS-

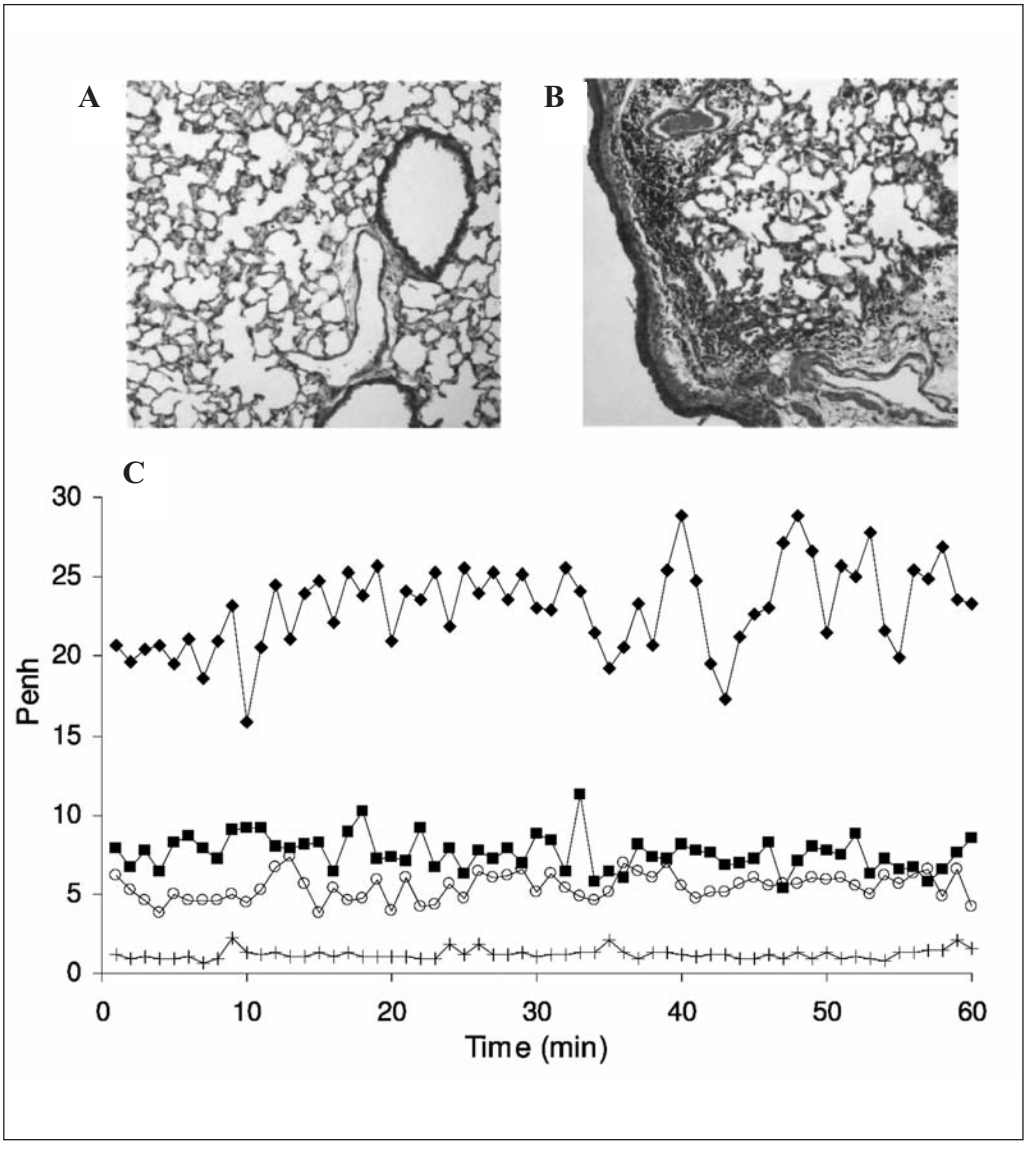

Fig. 4. Lung inflammation in response to *in vivo* hypercapnia (A-B). Sections of lung exposed to one hour long normal CO_2 rate (A) or 15% CO_2 (B). Four hours after exposure, lungs were fixed, embedded in paraffin, cut into 5 μm sections and stained with Hematoxylin-Eosin (magnification X800). As compared to control (A), there is a recruitment of leukocytes in close proximity to the alveolar lumen and a thickening of the alveolar membranes in hypercapnic conditions (B). (C) Induction of mouse airway hyperreactivity by hypercapnia was measured in BALB/c mice (n = 8/group) exposed to increasing CO_2 concentrations (+: 0, ○: 5, ■: 10, ◆: 15%, with a constant O_2 concentration of 21%) for 60 min. Enhanced pause (Penh), which reflects airway obstruction, was measured by barometric plethysmography every minute during the experiment and mean values are presented.

stimulated release of pro-inflammatory cytokines (IL-1 and TNF) by macrophages (18). However, in isolated alveolar type II epithelial cells, cell injury, mediated by nitric oxide activity, was enhanced in cells exposed to high concentrations of CO_2 (19).

Smoking is one of the leading causes of death worldwide. For most authors, the toxicity is mostly linked to tar, a complex mixture of thousands of chemicals, such as polycyclic aromatic hydrocarbons and nitrosamines (20). However, the exact rôle of each cigarette smoke component in lung carcinogenesis is not really defined. In addition to cytotoxic compounds found in the particulate phase, the vapor phase of mainstream cigarette smoke contains a number of cytotoxic constituents capable of damaging cells and inducing pulmonary inflammation (21). A large body of evidence suggests that smoking-induced pulmonary inflammation may play an important rôle in increasing lung cancer risk in smokers (22-25).

The combustion of tobacco like any carbon-rich compound releases CO_2 (12.5% by weight in whole mainstream smoke). Our data suggest that concentrations of CO_2 such as the one released during combustion causes inflammation of the respiratory tract. The toxicity of carbon dioxide is mediated by the activation of PP2A, which results in the translocation of NF-κB (6, 12). It is possible that the effect of CO_2 is due to the increased osmolarity of the extra cellular medium. It is also possible that acidosis, inhibition of the Krebs cycle for stoechiometric reasons, and hyperosmolarity activate a similar pathway.

Smokers are prone to multiple diseases and have a shortened life expectancy. Smokers age faster than the non-exposed population. The link between inflammation and ageing has been well established (26). The rôle of CO_2 in ageing has not been explored. It is our hypothesis that chronic intermittent exposure to carbon dioxide will be a powerful tool for the investigation of the intertwined links between inflammation, ageing and cancer.

Acknowledgements

We wish to acknowledge the help of Jean-Marc Steyaert. This work was funded by Biorébus.

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