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Effect of engineered nanoparticles on vasomotor responses in rat intrapulmonary artery

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Abstract

Pulmonary circulation could be one of the primary vascular targets of finest particles that can deeply penetrate into the lungs after inhalation. We investigated the effects of engineered nanoparticles on vasomotor responses of small intrapulmonary arteries using isometric tension measurements. Acute *in vitro* exposure to carbon nanoparticles (CNP) decreased, and in some case abolished, the vasomotor responses induced by several vasoactive agents, whereas acute exposure to titanium dioxide nanoparticles (TiO₂NP) did not. This could be attributed to a decrease in the activity of those vasoactive agents (including PGF_{2α}, serotonin, endothelin-1 and acetylcholine), as suggested when they were exposed to CNP before being applied to arteries. Also, CNP decreased the contraction induced by 30 mM KCl, without decreasing its activity. After endoplasmic reticulum calcium stores depletion (by caffeine and thapsigargin), CaCl₂ addition induced a contraction, dependent on Store-Operated Calcium Channels that was not modified by acute CNP exposure. Further addition of 30 mM KCl elicited a contraction, originating from activation of Voltage-Operated Calcium Channels that was diminished by CNP. Contractile responses to PGF_{2α} or KCl, and relaxation to acetylcholine were modified neither in pulmonary arteries exposed *in vitro* for prolonged time to CNP or TiO₂NP, nor in those removed from rats intratracheally instilled with CNP or TiO₂NP. In conclusion, prolonged *in vitro* or *in vivo* exposure to CNP or TiO₂NP does not affect vasomotor responses of pulmonary arteries. However, acute exposure to CNP decreases contraction mediated by activation of Voltage-Operated, but not Store-Operated, Calcium Channels. Moreover, interaction of some vasoactive agents with CNP decreases their biological activity that might lead to misinterpretation of experimental data.

Keywords

Carbon nanoparticles, titanium dioxide nanoparticles, calcium channels, pulmonary artery, vascular reactivity.

Introduction

Ultrafine particles are defined as particles with a nominal diameter of 100 nm or less. They have been, for a long time, only considered as an important component of ambient particulate pollution, being responsible for the high incidence of cardio-pulmonary morbidity and mortality reported by epidemiological studies (Pope et al., 1991 ; Poloniecki et al., 1997 ; Samet et al., 2000 ; Hoek et al., 2002 ; Peng et al., 2008). Nowadays, with the development of nanotechnology, nanoparticles are recognized as an important class of new-engineered materials, with a wide range of industrial and biomedical applications for the diagnosis and the treatment of diseases (De Jong and Borm, 2008 ; Sandhiya et al., 2009). Those nanoparticles are defined with a nominal diameter smaller than 100 nm. Whatever the origin of those particles, the underlying mechanisms of their potential deleterious effects on health remain poorly understood, and are a matter of intensive research.

Regarding composition, ultrafine particles may be extremely heterogeneous, depending on emission sources. Various constituents, such as transition metals and organic or inorganic compounds, can be adsorbed onto ultrafine particles' cores derived from combustion process. Compared to ultrafine particles from atmospheric pollution, engineered nanoparticles are relatively homogenous in size and composition, and relatively free of adsorbed constituents. Thus, manufactured nanoparticles, especially carbon or titanium dioxide nanoparticles, could be relevant surrogate for airborne particulate matter (Donaldson et al., 2003), and are frequently used in order to discriminate which components (i.e. particulate core, adsorbed constituent or both) are responsible for their deleterious health effects.

Likely because they can deeply penetrate into the lungs after inhalation, such ultrafine fractions produce enhanced pulmonary adverse effects, when compared to larger particles of similar composition (Warheit et al., 2008). Alteration of vasomotor responses by particulate matter has been described in different vascular beds, including the pulmonary circulation, which could be one of the primary vascular targets of inhaled particles (Courtois et al., 2008). It was shown that particles from

urban pollution could affect the NO dependant relaxation pathway, probably by decreasing the activity of cytosolic guanylyl cyclase. In most animal (rat or rabbit) or human studies, *in vivo* or *in vitro* exposure to fine PM_{2.5} (with aerodynamic diameter < 2.5 µm) from urban origin, diesel exhaust or residual-oil fly ash induces vasoconstriction (Brook et al., 2002 ; Huang et al., 2002 ; Li et al., 2005 ; Mills et al., 2005). Such vasoconstrictor effect may contribute to enhance arterial resistances and pressure, which in turn, may have negative impact on cardiac function. However, some other reports demonstrate a relaxant effect of systemic vessels upon exposure to particulate matter (Knaapen et al., 2001). Whether nanoparticles exert a similar effect, i.e. vasoconstriction or vasodilatation, in the pulmonary vasculature is largely unknown.

In this study, we have therefore investigated the effect of carbon nanoparticles (CNP) or titanium dioxide nanoparticles (TiO₂NP) on the vasomotor response induced by a variety of agents in small rat intrapulmonary arteries using isometric tension measurements. The influence of acute or long-term *in vitro* treatment with CNP or TiO₂NP, as well as that of *in vivo* instillation, has been examined. Responses induced by both contractile agents acting on vascular smooth muscle (following activation of plasma membrane receptors, or direct activation of calcium entry through opening of Voltage-Operated or Store-Operated Calcium Channels, i.e. VOCC and SOCC respectively), and relaxing agents acting either on vascular smooth muscle (via the intracellular messengers cyclic AMP or cyclic GMP) or endothelium (via the release of endothelial NO, which in turn activates cyclic GMP pathway) were analysed.

Methods

Chemicals.

Each salts for physiological salt solution (PSS) preparation, 3-[(3-cholamido-propyl)dimethylammonio]-1-propanasulfonate (CHAPS), acetylcholine chloride, phenylephrine, sodium nitroprusside, forskoline, endothelin-1, serotonin, U-46619, thapsigargin, 2-aminoethyl diphenylborinate, nicardipine and caffeine were purchased from Sigma Chemical Co. (St Quentin-Fallavier, France). Prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$, Dinolytic®) was purchased from Centravet (Libourne, France). Carbon nanoparticles, i.e. CNP (primary particle size of 13-14 nm), and titanium dioxide nanoparticles, i.e. TiO $_2$ NP (primary particle size of 15 nm), were obtained from Degussa (Germany) and Sigma Chemical Co. (St Quentin-Fallavier, France), respectively. Those nanomaterials were already described in more details according to their morphology and surface reactivity (Rouzaud et al., 2004 ; Xia et al., 2006 ; Setyan et al., 2009). Nanoparticles (CNP and TiO $_2$ NP) were suspended in deionised water (final concentration of 10 mg/ml) and were subjected to sonication before use.

Treatment of vasoactive agents with CNP.

Vasoactive agents were incubated in PSS (containing in mM: 119 NaCl, 4.7 KCl, 1.5 CaCl $_2$, 1.17 MgSO $_4$, 1.18 KH $_2$ PO $_4$, 25 NaHCO $_3$ and 5.5 glucose) in the presence of 200 µg/ml CNP at 37°C. After 15 minutes incubation period, nanoparticles were pelleted by centrifugation (11 000 g, 15 min). The supernatant was collected for evaluation of biological activity (see below). In addition, phenylephrine water concentrations was assayed before addition of CNP and in the supernatant collected after incubation with CNP by LC-MS/MS (see below).

Measurement of phenylephrine concentrations after exposure to CNP by LC-MS/MS.

Phenylephrine water solutions (2 µl), collected before and after nanoparticles exposure, were injected into a liquid chromatography-tandem mass spectrometry system. The LC-MS/MS method consisted of an Acquity UPLC® separation module coupled with an Acquity TQD® detector with electrospray ionisation in positive ion mode. The chromatographic separation was performed at 25°C on an XTerra RP C₁₈ column (100x2.1mm; 5µm, Waters). The mobile phase consisted of a gradient of acetonitrile (A) and ammonium formate buffer (B) (4 mmol/l; pH 3.4) at 0.2ml/min flow rate. The mobile phase started at 2% (A); linearly increased to 35% in 4min and kept for 1min then decreased to 2%, with total separation time of 7min.

The MS system was operated with a capillary voltage of 1kV, a source temperature of 115°C and a drying gas temperature of 300°C. Argon was used as collision gas (0.2 ml/min flow rate). The multiple reaction monitoring was performed by monitoring the transitions between *m/z* parent and daughter ion: 168.2>150 (phenylephrine). The cone voltage was set at 22V and the collision energy at 10eV. The method is validated with a good linearity between $5 \cdot 10^{-7}$ to $5 \cdot 10^{-5}$ M (i.e. 100-10000ng/ml; $r^2 > 0.995$) for phenylephrine.

Animals, exposition to CNP and TiO₂NP and tissue preparation.

Healthy male Wistar or Sprague-Dawley rats, weighing 400-550 g (10-14 weeks old), were obtained from Elevage Janvier (Le Genest Saint Isle, France). Animals were housed in a regulated animal facility where 12 h light/dark cycle was maintained. The rats were fed with standard animal food and water received ad libitum. The investigation conforms to the Guiding Principles for the Use of Animals in Toxicology, which were adopted by the Society of Toxicology in 1989. Agreement (number A 33409) was obtained by French authorities.

Intralobar pulmonary arteries (2nd order branch, internal diameter 500 - 800 µm) were dissected from male Wistar rats and mounted in wire myograph, as previously described (Courtois et al., 2008). In some cases, endothelium was chemically removed before mounting arterial segments in myograph, by perfusion of a non-denaturing zwitterionic detergent CHAPS (0.3 %), as previously described (Pourageaud et al., 2005). In another set of experiments, intrapulmonary arteries were first incubated in DMEM for 24 h at 37°C in a humidified atmosphere of 95% air

/ 5% CO₂, in absence or presence of CNP or TiO₂NP (200 µg/ml) and then carefully washed with PSS before mounting in myograph.

Finally, some experiments were also performed in intralobar pulmonary arteries removed from Sprague-Dawley rats, which were previously instilled with CNP or TiO₂NP. For these experiments, rats were anesthetised with ketamine (50 mg/kg) and xylazine (4 mg/kg), and intratracheally instilled with 100 µg of carbon or titanium dioxide nanoparticles in 0.5 ml of saline (NaCl 0.9 %) or saline alone. After a 21 days recovery period, intrapulmonary arteries were prepared and mounted in myograph as previously described (Courtois et al., 2008).

After 60 min of equilibration period under resting tone, a PSS containing 80 mM KCl (equimolar substitution with NaCl) was added to organ bath in order to obtain a maximal contraction and ascertain the viability of our preparation. Arteries developing a wall tension below 1 mN.mm⁻¹ were discarded. Washout with normal PSS allowed recovery of resting tone. Arteries were then submaximally pre-contracted with PGF_{2α}, and once contraction reached a steady-state level, acetylcholine (an agent which produces endothelium-dependent relaxation) was applied. While 30 µM acetylcholine produced 68.23 ± 3.51 % relaxation (n = 10) in control arteries, it failed to produce significant relaxation in CHAPS-treated ones (-0.09 ± 1.65 % relaxation, n = 6).

Experimental protocols.

In order to assess the direct effect of nanoparticles on vascular tone, intrapulmonary arteries were first submaximally contracted with 10 µM PGF_{2α} or 30 mM KCl. Once stable contraction was obtained, cumulative concentrations of nanoparticles, i.e. CNP or TiO₂NP (0 - 200 µg/ml) were added and wall tension was recorded.

In experiments in which interaction between contractile agents and CNP was investigated, solutions of nanoparticle-treated contracting agents (PGF_{2α}, 10 µM; U-46619, 0.3 µM; phenylephrine, 0.3 µM; serotonin, 30 µM; endothelin-1, 30 nM; and KCl, 30 mM) were added to the organ bath. In experiments in which relaxant agents were studied, intrapulmonary arteries were submaximally contracted with PGF_{2α} (10 µM). Once stable contraction was obtained, nanoparticle-treated solution of acetylcholine (10 µM), forskoline (0.3 µM) or sodium nitroprusside (1 µM) was added.

The concentrations of these agents were chosen from preliminary experiments to produce between 40 - 80 % of their maximal effect. The effect of nanoparticle-treated vasoactive agents was compared to that of untreated-ones, which was determined either in the same or in a different arterial preparation, depending on the reproducibility of the response.

The effect of CNP on the Store-Operated Calcium Channels (SOCC) component of contraction was determined according to the following protocol. To ensure calcium store depletion, endothelium-denuded intrapulmonary arteries were bathed for 30 min in calcium-free PSS (119 mM NaCl, 4.7 mM KCl, 1.17 mM MgSO₄, 1.18 mM KH₂PO₄, 25 mM NaHCO₃ and 5.5 mM glucose), in the presence of thapsigargin (1 µM) and caffeine (5 mM). After washout in calcium-free PSS, CaCl₂ (1.5 mM) was added to organ bath. Exposure to calcium induced a contractile response, which is inhibited by 4.10⁻⁵ M of 2-aminoethyl diphenylborinate (a SOCC inhibitor, Park et al., 2008), and therefore attributed to calcium influx from SOCC (Marthan, 2004). Once stable level of contraction was obtained, CNP (100 µg/ml) were added. For evaluation of the effect of CNP on the Voltage-Operated Calcium Channels (VOCC) component of contraction, endothelium-denuded intrapulmonary arteries were exposed to calcium-free PSS, thapsigargin, caffeine and CaCl₂. Such protocol induced a contractile response attributed to calcium influx from SOCC as described above. Then KCl (30 mM) was added to organ bath. Under these conditions, KCl-induced second contraction, which is inhibited by 10⁻⁵ M of nifedipine (a VOCC inhibitor), was attributed to calcium influx through VOCC (Marthan, 2004). Once stable contraction to KCl was achieved, CNP (100 µg/ml) were added.

In another set of experiments, intrapulmonary arteries which were previously incubated for 24 h in the absence or presence of CNP or TiO₂NP (200 µg/ml) or which were removed from saline or CNP- or TiO₂NP-instilled rats, were exposed to cumulative concentration of PGF_{2α}. After washout, preparations were submaximally contracted with PGF_{2α}, and once stable level of contraction was obtained, acetylcholine was applied in a cumulative manner.

Data Expression and Statistical Analysis.

Contractile responses are expressed in wall tension ($\text{mN}\cdot\text{mm}^{-1}$). Relaxant responses are expressed as the percentage of decrease of the initial tone induced by either $\text{PGF}_{2\alpha}$ or KCl. Data are given as means $\pm$ s.e.m from n experiments (with n : number of rats). Concentration-response curves were compared using two-way analysis of variance (ANOVA). Student t test (paired or unpaired, as appropriate) was used for other statistical comparisons. Differences were considered statistically significant when $P < 0.05$.

Results

Effect of acute *in vitro* treatment with CNP or TiO₂NP on vasomotor responses induced by various agents.

Effect of increasing concentration of CNP or TiO₂NP was evaluated in rat intrapulmonary arteries precontracted with 10 μ M PGF_{2 α} . CNP decreased tone concentration-dependently in both endothelium intact and denuded arterial preparations (**Figure 1**). This effect started for a particles concentration of approximately 30 μ g/ml and was maximal for 200 μ g/ml (90.20 ± 2.16 and 85.27 ± 3.82 percent decrease of tone, in endothelium intact and endothelium denuded arteries, respectively). By contrast, TiO₂NP failed to alter vascular tone in endothelium intact arteries (**Figure 1**) whatever the particles concentration used.

To assess whether this relaxing effect could be attributed to an effect of CNP on arterial tissue or to a direct interaction of these nanoparticles with the contractile agonist, PGF_{2 α} was exposed to CNP, before being applied to intrapulmonary arteries (see methods). As shown in **Figure 2b**, previous exposure of PGF_{2 α} to CNP resulted in a strong diminution of PGF_{2 α} -induced contraction, compared to the effect of untreated PGF_{2 α} (**Figure 2a**).

In order to assess whether this effect of CNP was restricted to PGF_{2 α} or could also occur with alternative vasoactive agents with different physico-chemical properties, the biological activity of several contracting or relaxant agents was assessed, after exposure or not to CNP. As summarized in **Table 1**, not only contractile responses to PGF_{2 α} and U46619 (fatty-acid derivatives) but also those of other agonists of G-protein coupled receptors such as the hydrophilic amine derivatives phenylephrine and 5-HT, and those of the 21 amino acid peptide ET-1 were markedly decreased after exposure to CNP. Vasorelaxant responses to acetylcholine (a hydrophilic quaternary ammonium compound, which induces endothelium and NO-dependent relaxant effect, **Figure 2c** and **2d**, **Table 1**) and to forskoline (a diterpene from *Coleus forskohlii*, which induces cyclic AMP-dependent relaxation, **Table 1**) were

also markedly diminished after exposure to CNP. By contrast, vasomotor responses elicited by the inorganic compounds KCl (which triggers the opening of voltage-dependent calcium channels and induces contraction) or sodium nitroprusside (which induces NO-dependent but endothelium-independent relaxation) were not affected by CNP (**Table 1**).

In addition the concentrations of phenylephrine, either unexposed or exposed to carbon nanoparticles, were evaluated by liquid chromatography-tandem mass spectrometry. In unexposed phenylephrine, we measured a concentration of $31.57 \pm 0.6 \mu\text{M}$, whereas in these same nanoparticles-exposed solutions phenylephrine concentration was $27.61 \pm 0.16 \mu\text{M}$ ($p < 0.01$). It is important to notice that these concentrations used are in the same range of the concentrations used to detect an adsorption of vasoactive agent onto particles surfaces by functional experiments.

Effect of acute *in vitro* treatment with CNP on vasomotor response induced by KCl.

Since the aboved-mentioned experiments demonstrated the absence of CNP direct interaction with KCl; we have tempted to evaluate CNP direct effect on smooth muscle contraction, the effect of these nanoparticles was studied in endothelium-denuded KCl-precontracted intrapulmonary arteries. After precontraction with 30 mM KCl, CNP produced a significant and concentration-dependent decrease in tone (25 % for 200 $\mu\text{g/ml}$ CNP), as compared to time-matched control vessels (**Figure 3**).

Vascular smooth muscle contraction to KCl is mainly initiated by a rise in cytoplasmic calcium concentration following extracellular calcium entry. The following experiments were designed to evaluate the effect of CNP on two types of channels involved in such entry, namely Store-Operated Calcium Channels (SOCC) and Voltage-Operated Calcium Channels (VOCC). To study SOCC component, endoplasmic reticulum calcium stores were first depleted by concomitant addition of thapsigargin (SERCA inhibitor) and caffeine (which induces calcium release by activation of ryanodine receptors) in arterial preparations bathed in calcium-free medium (see methods and **Figure 4** for experimental protocol). After washout with calcium-free medium (it should be notice that such washout protocol was ineffective

to remove thapsigargin), addition of CaCl_2 (1.5 mM) induced a contractile response (**Figure 4a**), which is significantly inhibited by $4 \cdot 10^{-5}$ M of 2-aminoethyl diphenylborinate (a SOCC inhibitor, Park et al., 2008), and therefore attributed to calcium influx from SOCC (data not shown). Further addition of CNP failed to modify CaCl_2 -induced contraction (**Figure 4a** for a representative trace of arterial tone and **Figure 4c** for statistical analysis from $n = 4$ experiments). The VOCC component was studied after depletion of endoplasmic reticulum calcium store (with thapsigargin and caffeine in calcium-free medium). After medium withdrawal and washout with calcium-free medium, SOCC was activated by addition of 1.5 mM CaCl_2 (see methods and **Figure 4** for experimental protocol). Subsequent addition of 30 mM KCl induced a further contractile response (**Figure 4b**), which is significantly inhibited by 10^{-5} M of nicardipine (a VOCC inhibitor), and therefore attributed to calcium influx through VOCC (data not shown). Once stable contraction to KCl was achieved, CNP (100 $\mu\text{g/ml}$) were added. Under these conditions, addition of CNP elicited a significant decrease of contraction of about 25 % (**Figure 4b** for a representative trace of arterial tone and **Figure 4d** for statistical analysis from $n = 4$ experiments), similar to what is shown in figure 3.

Effect of long-term *in vitro* treatment with CNP or TiO_2NP on vasomotor responses induced by various agents.

Since acute direct interaction between CNP and some vasoactive agents may mask effects of these nanoparticles on arterial tissue, the following series of experiments were designed to evaluate whether prolonged exposure of pulmonary arteries to CNP could affect their contractile responses. In this set of experiments, intrapulmonary arteries were first incubated in culture medium (see methods) in absence or presence of 200 $\mu\text{g/ml}$ CNP. After 24 h exposure, arteries were carefully washed with PSS and mounted in myograph to evaluate their contractile responses to either $\text{PGF}_{2\alpha}$ or KCl, or their relaxant responses to acetylcholine. As shown in **Figure 5**, 24 h exposure of intrapulmonary arteries to CNP resulted neither in significant impairment of KCl or $\text{PGF}_{2\alpha}$ -induced contraction nor of acetylcholine-induced relaxation. Similarly, as shown in **Figure 6**, prolonged exposure to TiO_2NP did not modify responses to those vasoactive agents.

Effect of *in vivo* treatment with CNP or TiO₂NP on vasomotor responses induced by various agents.

For these experiments, anaesthetised rats were intratracheally instilled with 100 µg CNP or TiO₂NP particles in 0.5 ml of saline (NaCl 0.9 %) or saline alone as control. After a 21 days recovery period, intrapulmonary arteries were prepared and mounted in myograph to evaluate *ex vivo* their vasomotor responses to PGF_{2α}, KCl and acetylcholine. As illustrated in **Figure 7** and **Figure 8**, *in vivo* instillation of CNP or TiO₂NP altered neither the contractile response to KCl and PGF_{2α} nor the relaxant response to acetylcholine.

Discussion

The present results indicate that carbon-manufactured nanoparticles, but not titanium dioxide nanoparticles, alter rat intralobar pulmonary arteries reactivity. However, we also suggest a binding of organic (but not inorganic) pharmacological agents to carbon-manufactured nanoparticles. Such binding reduces the biological activity of these pharmacological agents. This nanoparticle-induced alteration in agonist biological activity should be systematically investigated to avoid misinterpretation of experimental results.

Depending of emission sources, airborne particulate matter is extremely heterogeneous in terms of core size and composition. Regarding particulate matter, the ultrafine fraction is considered to produce the most hazardous health effect (Donaldson et al., 2001). Additionally, various constituents from mineral, organic or biologic origin could be adsorbed onto particle surface and, hence, exert their own toxicity. The precise underlying mechanisms leading to particulate matter-induced adverse health effects remain poorly understood. Moreover, determining which components, i.e. particulate core, adsorbed constituent or both, are involved is often difficult. To circumvent this complex issue, several studies have used coarse, fine or ultrafine carbon or TiO₂ particles as a surrogate for airborne particulate matter (Donaldson et al., 2003). Those surrogate particles are homogenous in size and relatively free of adsorbed constituents. In line with the aforementioned observations, the present study was designed to investigate the influence of different nanoparticles' cores on vasomotor responses in rat intrapulmonary arteries, since the pulmonary circulation could be a privileged target of inhaled particles, and any dysfunction in pulmonary vascular homeostasis may have negative impact on cardiopulmonary function.

A direct effect of CNP on intrapulmonary vascular tone was clearly evidenced in rat intrapulmonary arteries. By contrast TiO₂NP failed to alter vascular tone whatever the concentration used. These results indicate that chemical composition of nanoparticles determines their biological effects, especially on intrapulmonary vascular tone. Furthermore, when using manufactured nanoparticles as surrogate

model of ultrafine particulate matter pollution in order to discriminate the biological effects of particles core from constituents adsorbed onto particles, the interpretation of such observed results should be cautious.

CNP induced a large, sustained and concentration-dependent relaxation in $\text{PGF}_{2\alpha}$ precontracted pulmonary artery. This relaxation was endothelium independent since it was induced in both endothelium intact and endothelium free arteries, and reached the same amplitude. In other vascular beds, i.e., rat aorta or mesenteric arteries, relaxation elicited by particulate matter has already been observed (Knaapen et al., 2001 ; Bagaté et al., 2004). A possible mechanism could be the generation of reactive oxygen species upon particles exposure that was evidenced in several acellular and cellular models (Li et al., 1996; Dick et al., 2003 ; Foucaud et al., 2007), including vascular one (Li et al., 2006). Indeed, CNP enhances the production of oxidant like H_2O_2 which has been already reported or suggested to induce vasorelaxation of precontracted vessels rings *in vitro* (Burke and Wolin, 1987 ; Fraile et al., 1994 ; Fresquet et al., 2006). Moreover, some studies have demonstrated that particulate matter-induced relaxation was reduced using the non-specific antioxidant Trolox (Knaapen et al., 2001). Therefore, a relaxation induced by H_2O_2 derived from CNP exposure, or H_2O_2 generated upon the interaction between CNP and vasoactive agents, could be hypothesized in our *in vitro* pulmonary artery model.

An alternative, and more plausible, explanation is provided by the present observation that the biological activity of almost all of the contracting or relaxing agents used was strongly diminished after pre-treatment with CNP. Indeed, it can be suggested that these agents are adsorbed onto CNP surface reducing their availability to their cellular targets and thus their biological activity. Consistently, for a particles concentration similar to those used in the present study, a strong depletion of Bovine Serum Albumin (BSA) or cytokines has already been reported after exposure to either fine or ultrafine carbon particles (Brown et al., 2000; Kocbach et al., 2008), thus suggesting that proteins might be adsorbed onto the CNP surface. Cytokines binding was the strongest in the absence of serum (superior to 85% binding), and increasing serum or BSA concentration resulted in a significant reduction in cytokines binding. In our model, all experiments were performed in the absence of serum or BSA. Consistently, we observed a decrease in phenylephrine

concentrations after CNP exposure, which likely contributes to decreased biological effect of the agonist.

By contrast, the biological activity of the contracting agent KCl and the relaxing agent sodium nitroprusside was not affected by CNP treatment suggesting that neither of these two agents binds onto nanoparticles surface. Interestingly it should be noticed that, among all agents tested in our study, only these two later are inorganic chemicals whereas all the others are organic. In line with this, as above mentioned, cytokines (i.e. organic compounds) were found not to interact with mineral particles (Kocbach et al., 2008).

Although carbon nanoparticles did not interact with KCl, they induced a slight but significant decrease in vascular tone in endothelium free vessels precontracted by this agent. The relaxant effect of CNP could not be attributed to a general deterioration of arterial tone with time since the time-matched control was not affected. In arteries, KCl-induced contraction occurred mainly through a rise in cytoplasmic calcium concentration after membrane depolarization and subsequent opening of VOCC generating an intracellular calcium influx. Additionally after a calcium release from intracellular stores, a calcium entry is triggered through the activation of SOCC in order to refill intracellular calcium stores (Guibert et al., 2008). Activation of VOCC by KCl could also subsequently induce a calcium-induced calcium release, which depletes intracellular calcium stores, and stimulates SOCC opening. These voltage-gated calcium channels are important pathways for calcium influx that trigger excitation-contraction coupling in vessels. They are involved in pressure-induced constriction in small arteries and contribute to the dynamic regulation of blood pressure in several vascular beds (Sonkusare et al., 2006). Our results clearly demonstrate that, unlike the SOCC component, CNP affect the VOCC component of KCl-induced contraction. Thus, VOCC alteration in small arteries may disrupt the control of vascular tone and alter blood flow repartition in target organs. Nevertheless, we observed that acute exposure to CNP did not modify intracellular calcium store, assessed by using the $[Ca^{2+}]_i$ -sensitive fluorophore indo-1, in isolated rat pulmonary vascular smooth muscle cells (data not shown). Therefore it cannot be excluded that CNP also modified contractile apparatus sensitization to calcium that produced a decrease in vessel tone induced after KCl addition (Ratz et al., 2005).

In conclusion, we report that carbon manufactured nanoparticles could alter pulmonary vascular tone, whereas another class of nanomaterials, i. e., titanium dioxide nanoparticles, did not. The present results demonstrate that carbon manufactured nanoparticles affect KCl-induced contraction in rat intralobar pulmonary arteries. Additionally, we suggest a binding of organic but not inorganic pharmacological agent to manufactured carbon nanoparticles. Such binding that can occur with several agents may potentially lead to misinterpretation of results and conclusions. Therefore, from a practical point of view, when studying the effects of nanoparticles, such interactions should be systematically evaluated prior experiments. In addition, the conclusions made from the interpretation of results obtained with manufactured nanoparticles used as surrogate of particulate matter pollution should also be performed cautiously.

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Figure legends

Figure 1: Effect of addition of increasing concentrations of nanoparticles on the contractile tone induced by 10 μM $\text{PGF}_{2\alpha}$ in rat intrapulmonary arteries with (open symbol) or without (closed symbol) functional endothelium. Open circles correspond to increasing concentrations of titanium dioxide nanoparticles, whereas open and closed squares correspond to increasing concentrations of carbon nanoparticles. Results are expressed as mean $\pm$ s.e.m of 3 - 6 experiments. Not significant between endothelium intact and endothelium free arteries for CNP.

Figure 2. (a, b): Representative traces of the contractile effect of 10 μM $\text{PGF}_{2\alpha}$ **(a)** and CNP-pretreated $\text{PGF}_{2\alpha}$ **(b)** in rat intrapulmonary arteries. **(c, d):** Representative traces of the relaxant effect of 10 μM acetylcholine **(c)** and CNP-pretreated acetylcholine **(d)** in rat intrapulmonary arteries. The addition of native vasoactive agent is represented by solid arrows, while that of CNP-pretreated agents is represented by dashed arrows.

Figure 3. Effect of addition of increasing concentrations of CNP on the contractile tone induced by 30 mM KCl in rat intrapulmonary arteries (closed square). Tone of time matched control arteries is included for comparison (open square). Results are expressed as mean $\pm$ s.e.m of 6 experiments. **: $P < 0.01$.

Figure 4. Schematic representation of the experimental protocols, which were used to study the SOCC **(a)** and the VOCC **(b)** -dependent contraction in rat intrapulmonary arteries. **(a, b):** Representative traces depicting the effect of addition of CNP (100 $\mu\text{g/ml}$) on either SOCC **(a)** or VOCC **(b)** -dependent contraction, the addition of CNP is indicated by a black arrow. **(c, d):** Wall tension before (open column) and after (closed column) the addition of CNP (100 $\mu\text{g/ml}$). Results in **(c)** and **(d)** are expressed as mean $\pm$ s.e.m of 4 experiments. **: $P < 0.01$.

Figure 5: Contractile responses to 80 mM KCl **(a)** or $\text{PGF}_{2\alpha}$ **(b)**, and relaxant responses to acetylcholine **(c)** in rat intrapulmonary arteries incubated for 24 h in the absence (open square and open column) or in the presence of 200 $\mu\text{g/ml}$ CNP

(closed square and closed column). Results are expressed as mean $\pm$ s.e.m of 8 - 10 experiments. Not significant between the two conditions.

Figure 6: Contractile responses to 80 mM KCl **(a)** or $\text{PGF}_{2\alpha}$ **(b)** and relaxant responses to acetylcholine **(c)** in rat intrapulmonary arteries incubated for 24 h in the absence (open circle and open column) or in the presence of 200 $\mu\text{g/ml}$ TiO_2NP (closed circle and closed column). Results are expressed as mean $\pm$ s.e.m of 5 - 8 experiments. Not significant between the two conditions.

Figure 7: Contractile responses to 80 mM KCl **(a)** or $\text{PGF}_{2\alpha}$ **(b)**, and relaxant responses to acetylcholine **(c)** in intrapulmonary arteries removed from rats intratracheally instilled with 0.9% saline (open square and open column) or 100 μg CNP in 0.5 ml saline (closed square and closed column). Results are expressed as mean $\pm$ s.e.m of 7 experiments. Not significant between the two conditions.

Figure 8: Contractile responses to 80 mM KCl **(a)** or $\text{PGF}_{2\alpha}$ **(b)**, and relaxant responses to acetylcholine **(c)** in intrapulmonary arteries removed from rats intratracheally instilled with 0.9% saline (open circle and open column) or 100 μg TiO_2NP in 0.5 ml saline (closed circle and closed column). Results are expressed as mean $\pm$ s.e.m of 3 - 6 experiments. Not significant between the two conditions.

Table 1. Vasomotor responses induced by various contractile or relaxing agents in rat intrapulmonary arteries. Vasoactive agents were pre-treated or not with 200 $\mu\text{g/ml}$ CNP. The effect of contractile agents is expressed as a percentage of a reference contraction induced by 80 mM KCl for each vessel. The effect of relaxing agents is expressed in percentage of decrease of the initial tone induced by 10 μM $\text{PGF}_{2\alpha}$.

Conflict of interest statement

All authors declare they have no competing financial interest.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Table 1

	Control	Treated agonist	<i>P</i>
Prostaglandin F _{2α} (10 ⁻⁶ M)	61.80 ± 8.58 (8)	10.67 ± 1.55 (3)	< 0.01
Phenylephrine (3.10 ⁻⁷ M)	54.25 ± 3.42 (4)	24.38 ± 8.13 (4)	< 0.01
5-HT (3.10 ⁻⁵ M)	41.44 ± 0.87 (4)	26.36 ± 5.38 (4)	< 0.05
ET-1 (3.10 ⁻⁸ M)	80.80 ± 8.02 (3)	2.83 ± 0.27 (2)	< 0.01
U-46619 (3.10 ⁻⁷ M)	38.68 ± 4.18 (2)	3.14 ± 0.71 (2)	< 0.01
KCl (3.10 ⁻² M)	38.97 ± 2.30 (2)	40.78 ± 5.78 (2)	> 0.05
Acetylcholine (10 ⁻⁶ M)	58.63 ± 5.05 (5)	37.76 ± 9.91 (5)	< 0.05
Sodium Nitroprusside (10 ⁻⁶ M)	81.72 ± 7.06 (4)	71.56 ± 6.35 (4)	> 0.05
Forskolin (3.10 ⁻⁷ M)	67.74 ± 7.70 (7)	1.96 ± 1.96 (3)	< 0.01