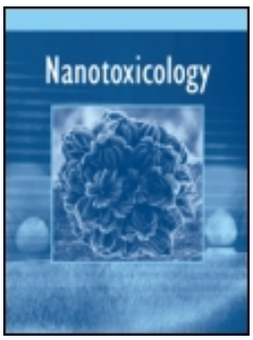

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ORIGINAL ARTICLE

Pulmonary exposure of rats to ultrafine titanium dioxide enhances cardiac protein phosphorylation and substance P synthesis in nodose ganglia

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Abstract

The inhalation of engineered nanoparticles stimulates the development of atherosclerosis and impairs vascular function. However, the cardiac effects of inhaled engineered nanoparticles are unknown. Here, we investigate the effects of ultrafine titanium dioxide ($UFTiO_2$) on the heart, and we define the possible mechanisms underlying the measured effects. Pulmonary exposure of rats to $UFTiO_2$ increased the phosphorylation levels of p38 mitogen-activated protein kinase and cardiac troponin I, but not Akt, in the heart and substance P synthesis in nodose ganglia. Circulatory levels of pro-inflammatory cytokines, and blood cell counts and differentials were not significantly changed after pulmonary exposure. Separately, the incubation of cardiac myocytes isolated from naïve adult rat hearts *in vitro* with $UFTiO_2$ did not alter the phosphorylation status of the same cardiac proteins. In conclusion, the inhalation of $UFTiO_2$ enhanced the phosphorylation levels of cardiac proteins. Such responses are likely independent of systemic inflammation, but may involve a lung-neuron-regulated pathway.

Keywords: Nanoparticles, cardiovascular diseases, titanium dioxide, inhalation study

Introduction

Nanoparticles possess a range of physical and chemical properties depending on their composition and synthetic process (Paull et al. 2003). Animal studies have shown that on an equivalent mass dose basis, nanoparticles are more inflammatory and toxic to the cells and tissues of the lung than larger particles of the same composition (Monteiller et al. 2007); thus, a relationship exists between particle size and biological reactivity. Evidence accumulated from epidemiological studies also confirms a significant pathogenic correlation between the inhalation of small-sized particles, especially nano-sized particles, from ambient air and cardiovascular events, such as angina, arrhythmia, ischemic heart failure and sudden death (Donaldson et al. 2001; Schwartz & Dockery 1992). Engineered nanoparticles are more homogeneous in their physical and chemical properties than naturally existing nanoparticles; therefore, they may cause more consistent and reproducible adverse health effects. A number of studies have shown that pulmonary inhalation of ultrafine particulate matter (diameter <0.1 μm) generated from synthetic nanoparticles, such as single-walled carbon nanotubes (SWCNTs) and ultrafine titanium dioxide ($UFTiO_2$), resulted in the development of vascular abnormalities, including atherosclerosis, increased vascular tone and impaired endothelial-dependent vascular dilation (Cascio et al. 2007; Li et al. 2007; Nurkiewicz et al. 2008). However, there is little information available regarding the biological and pathophysiological effects of inhaled engineered nanoparticles on the heart. Therefore, the goal of the current study was to utilise both *in vivo* and *in vitro* methodologies to determine the effects of engineered $UFTiO_2$ on the heart and elucidate the possible mechanisms underlying the measured effects.

Considerable evidence from epidemiological and animal studies indicates that pulmonary exposure to nanoparticles may not only induce lung injury but also cause cardiovascular dysfunction. Cardiac dysfunction due to nanoparticle exposure could be the result of nanoparticle action on specific cardiac proteins, induction of oxidative stress responses, influence on inflammatory cytokines or influence on neural mechanisms controlling autonomic regulation of the heart.

Cardiac dysfunction may occur through alteration of the phosphorylation level of cardiac proteins, such as p38 mitogen-activated protein kinase (p38 MAPK), cardiac troponin I (cTnI) and Akt. The p38 MAPK and cTnI regulate cardiac muscle contraction, and the Akt protein is involved

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in protecting cardiac cells from oxidative stress and cell death. These three proteins impact myocardial function both *in vivo* and *in vitro* (Bellahcene et al. 2006; Messer et al. 2007). Therefore, the current study examines the biological changes in these proteins in heart tissue and cardiac myocytes after *in vivo* or *in vitro* exposure to UFTiO$_2$.

In humans, there is an association between systemic inflammation and an increased risk of cardiovascular events (Chrysohoou et al. 2009; Mahmud & Feely 2005). Animal studies suggest that inhaled nanoparticles including engineered nanoparticles because of their great surface to mass ratio and surface activity can trigger pulmonary-mediated systemic inflammation as indicated by an increase in the percentage of monocytes and neutrophils in peripheral blood (Liao et al. 2008). Other potential mechanisms are related to the induction of pro-inflammatory cytokines at the target tissue itself. Recently, evidence for engineered nanoparticle-induced production of pro-inflammatory cytokines in the lungs and cardiac myocytes has been reported (Cho et al. 2007; Helfenstein et al. 2008). Pro-inflammatory cytokines have been implicated in the pathogenesis of cardiovascular diseases since the identification of immune and inflammatory mechanisms in heart failure (Mehra et al. 2005); thus, pro-inflammatory cytokines are examined in the current study.

One of the proposed mechanisms underlying the distant effects of nanoparticles is the ability of nanoparticles to translocate to systemic tissue from the lung. Nanoparticles deposited in pulmonary target sites may rapidly pass through the blood–air barrier into the systemic circulatory system and then directly interact with systemic organs. In light of this hypothesis, the oxidative stress response of cardiac myocytes following *in vitro* treatment with UFTiO$_2$ was examined. As mediators of signal transduction pathways, free oxygen radicals induce cytokine production in various cell types (Droge 2002). Nanoparticle-induced oxidative stress has also been reported in different cell types including neonatal cardiac myocytes in culture (Helfenstein et al. 2008). In the current study, we examine the effects of *in vitro* exposure to UFTiO$_2$ on oxidative stress in cardiac myocytes.

More recently, epidemiological and animal studies have suggested that inhalation of ultrafine particles from ambient air or engineered nanoparticles can cause adverse cardiovascular effects by altering the balance of autonomic neuronal activity (Legramante et al. 2009). We have conducted immunohistochemistry experiments to elucidate the effect of pulmonary UFTiO$_2$ exposure on neuronal activity by examining substance P synthesis in the nodose ganglia, which integrates and controls lung and heart function by receiving primary sensory nerve fibres from the lung and transmitting the information to the brainstem, including the medullar cardiovascular regulatory centre (Armour 1999; Kosta et al. 2010).

Materials and methods

Animals

Male Sprague-Dawley (Hla:(SD) CVF) rats from Hilltop Lab Animals (Scottdale, PA, USA), 6–7 weeks of age and free of viral pathogens, parasites, mycoplasmas, *Helicobacter* and cilia-associated respiratory (CAR) bacillus were used for all experiments. The rats were acclimated for 1 week after arrival and housed in cages ventilated with HEPA (high-efficiency particulate air)-filtered air under controlled temperature and humidity conditions and a 12-h light/12-h dark cycle. Food (Teklad 7913) and tap water were provided *ad libitum*. The animal facilities are specific pathogen-free and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All experimental procedures were approved by the Animal Care and Use Committee of the National Institute for Occupational Safety and Health.

Pulmonary UFTiO$_2$ exposure

Male Sprague-Dawley rats (7–8 weeks of age) received UFTiO$_2$ by inhalation exposure. Rats were placed in an exposure chamber and exposed to aerosols of UFTiO$_2$ (primary particle diameter ~ 21 nm). Sham animals (control group) were placed in the chamber but were exposed to filtered air. We used an UFTiO$_2$ aerosol concentration of 6 mg/m^3 and an exposure duration of 4 h. Previous studies had shown that this exposure scheme produced an actual pulmonary deposition of 10 μg UFTiO$_2$ in the rat, which is equivalent to the workers exposed at 0.1 mg/m^3 for 27 workdays in a typical occupational environment, and resulted in biological and functional changes in the systemic and cardiac vascular system (LeBanc et al. 2009; Nurkiewicz et al. 2008).

Isolation of adult rat ventricular myocytes

Naïve rats were euthanized with an overdose of sodium pentobarbital (100 mg/kg, i.p.), and the heart was rapidly removed and perfused with Krebs–Henseleit bicarbonate (KHB) solution to wash out the residual blood in the coronary vasculature. The heart was then immersed in recirculating KHB solution with low Ca^{2+}-containing collagenase (Liberase™ Research Grade; 0.04 mg/ml, Roche Applied Science, Indianapolis, IN, USA) for 40 min until the heart became soft. Heart ventricular tissue was then sliced and placed into a plastic container containing 5 ml of the same enzyme solution. The tissue was aspirated repeatedly by a plastic transfer pipette with a small opening until most of the tissue had 'fallen apart'. The concentration of Ca^{2+} in the KHB solution was increased in four increments (0.05, 0.4, 0.8 and 1.2 mM). Finally, the mixture was passed through 225-μm nylon mesh and centrifuged at 50 $\times g$ for 2 min. The centrifuge procedure was repeated until the preparation contained at least 80% viable adult rat ventricular myocytes (ARVMs). The myocytes exhibited a typical striated and rod-shaped appearance when viewed with light microscope.

Oxidative stress

The solutions containing UFTiO$_2$ were sonicated for 1 min before they were added to the cardiac myocytes. Cardiac myocytes were treated with concentrations of 0, 0.1, 0.5 or 1 μg/ml of UFTiO$_2$ for 4 h. Additional cultures were treated with 100 μM H_2O_2 for 20 min as a positive control. The oxidative stress response of cardiac myocytes following

in vitro treatment with $UFTiO_2$ was measured with the OxiSelect™ ROS Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA). Reactive oxygen species (ROS) activity was determined by fluorescence intensity using a CytoFluor multi-well plate reader (PerSeptive BioSystems, Ramsey, MN, USA).

Western blots

The phosphorylation status of cardiac proteins was examined at 0 and 24 h following pulmonary exposure to $UFTiO_2$. Cultured cardiac myocytes were exposed to $UFTiO_2$ (1 μg/ml) or arsenite (10 μM) for 4 h. Each of cardiac tissue samples removed from the rats was rapidly frozen and crushed in a mortar with pestle at the temperature of liquid nitrogen for protein extraction. Cardiac tissue and ARVM lysates were prepared in lysis buffer containing 20 mmol/l Tris-HCl, 20 mmol/l NaCl, 0.1 mmol/l EDTA (ethylenediaminetetraacetic acid), 0.1% Triton X-100 and protease inhibitors and centrifuged at 10,000 $\times g$ for 20 min at 4°C as described previously (Kan et al. 2005). Equal amounts of protein were loaded onto an 8% SDS-PAGE gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA). Phosphorylation of p38 MAPK, cTnI and Akt were detected by phospho-p38 MAP kinase (Thr180/Tyr182), phospho-cTnI (Ser23/24) and phospho-Akt (Ser473) antibodies, respectively (Cell Signaling Technology, Danvers, MA, USA). Total p38 MAPK, cTnI and Akt were detected with rabbit monoclonal antibodies (mAbs) (Cell Signaling Technology).

White blood cell differential count

Rats were euthanized with an overdose of sodium pentobarbital, as described above. The blood was quickly drawn from the posterior vena cava using a vacutainer blood collection tube containing sodium ethylenediamine tetraacetate as an anticoagulant. The white blood cell differential count was conducted by flow cytometry. Monocytes and neutrophils were gated by side-scattering and forward-scattering and were quantified. The blood levels of cytokines were determined using a commercial ELISA (enzyme-linked immunosorbent assay) kit (BD Biosciences, San Jose, CA, USA).

Substance P immunohistochemistry

For tissue preparation, the right and left nodose ganglia were dissected out, fixed in picric acid-formaldehyde for 3 h and rinsed three times with 0.1 M phosphate-buffered saline containing 0.3% Triton X-100 (PBS-Tx) (pH 7.8). The nodose ganglia were then frozen in isopentane, cooled with liquid nitrogen, and stored in airtight bags at -80°C. Immunocytochemical procedures for localisation of substance P immunoreactive neurons were similar to those described previously (Wu et al. 2008). Briefly, cryostat sections (12 μm thickness) of nodose ganglia were mounted on gelatin-coated coverslips, dried briefly at room temperature and then incubated with rabbit anti-substance P antiserum (Peninsula, Belmont, CA, USA) at a dilution of 1:100 for 60 min in a humidified chamber at 37°C. The coverslips were rinsed with a PBS-Tx containing 1% bovine serum albumin (PBS-Tx + BSA) three times, for 5 min per rinse. Then, the sections were incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (ICN Immunobiologicals, Inc., Costa Mesa, CA, USA) at a dilution of 1:100 for 60 min at 37°C. The coverslips were rinsed again in PBS-Tx + BSA three times. Then the sections were processed for protein gene product (PGP) 9.5 immunoreactivity using mouse anti-PGP 9.5 antiserum (1:100) and goat anti-mouse IgG labelled with rhodamine (1:100). PGP 9.5 labelling allowed efficient identification of neurons. After all immunocytochemical procedures were conducted, the coverslips were mounted with fluoromount and observed under a fluorescence microscope equipped with fluorescein (excitation wavelengths from 455 to 500 nm and emission wavelengths >510 nm) and rhodamine (excitation 540–504 nm, emission >580 nm) filters. Controls consisted of testing the specificity of primary antiserum by absorption with 1 μg/ml of the specific antigen. Non-specific background labelling was determined by omission of primary antiserum.

The quantitative measurement of fluorescence intensity in nodose ganglia was similar to as described previously (Wu et al. 2008). Briefly, we recorded images using an AX70 microscope (Olympus America, Melville, NY, USA) with the SPOT 2 digital camera (Diagnostics Instruments, Sterling Heights, MI, USA). Fluorescence intensity of substance P was measured using commercial image processing software (Optimas 6.5; Media Cybernetics, Silver Spring, MD, USA). The intensity recordings were calibrated with the InSpeck Green (505/515) microscope image intensity calibration kit (Molecular Probes, Eugene, OR, USA). The fluorescence intensity in nodose ganglia was reported as gray level on a scale of 255 for each neuron. Neurons with a gray level <50 were considered negative because they were at or below the general background. Fluorescence intensities of ≥50 were counted as labelled neurons. All identifiable nodose ganglia were evaluated in every fifth section collected from serial sections, usually amounting to a total of 10–15 sections analysed.

Statistical analysis

Data were compared using analysis of variance followed by pairwise comparisons between control and treated groups using Student's t-test. All data were analysed using JMP software (Version 9.0) and differences were considered statistically significant at the level of $p < 0.05$. The values in the figures are expressed as mean ± SE.

Results

Cardiac protein phosphorylation

Western blots indicated that elevated phosphorylation levels of p38 MAPK and cTnI can be detected as early as 0 h post-exposure and remain detectable at 24 h after pulmonary $UFTiO_2$ exposure in rats (Figure 1). No change in the phosphorylation level of Akt in cardiac tissue was noted in response to $UFTiO_2$ inhalation. Protein expression levels of p38 MAPK, cTnI and Akt were not changed in rats exposed to $UFTiO_2$ compared with the control group at either 0 or 24 h post-exposure as determined by their respective total antibodies (Figure 1).

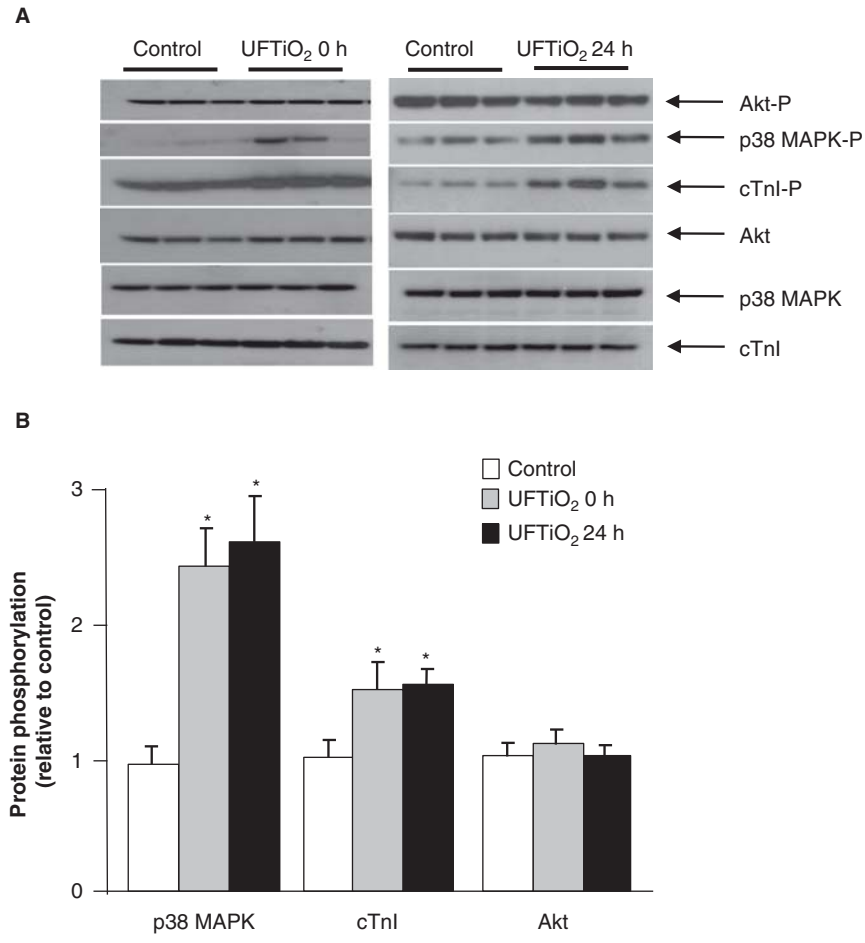

Figure 1. Phosphorylation of cardiac proteins at 0 and 24 h post-inhalation exposure to $UFTiO_2$. A representative western blot for cardiac p38 MAPK-P, cTnI-P and Akt-P is shown. The antibodies of total p38 MAPK, cTnI and Akt were used to indicate the same expression levels of each protein (A). Densitometry values of specific bands in the control and $UFTiO_2$ groups were compared at different time points (B). Each value represents the mean ± SE of six rats; $p < 0.05$ compared with the control group (*). cTnI: cardiac troponin I; MAPK: mitogen-activated protein kinase; $UFTiO_2$: ultrafine titanium dioxide.

Cytokine gene expression of TNF-α, IL-1 and IL-6 in cardiac tissue was examined at 0 and 24 h following exposure of rats to $UFTiO_2$ via inhalation. Except for barely detectable gene expression of TNF-α at 24 h after exposure, pulmonary exposure to $UFTiO_2$ did not induce cytokine gene expression in cardiac tissue (data not shown). Similarly, protein synthesis of TNF-α and IL-1 in the peripheral blood was not elevated in rats exposed to $UFTiO_2$ compared with the control group at either 0 or 24 h post-exposure (Figure 2).

White blood cell differential count

Pulmonary inhalation of $UFTiO_2$ resulted in an initial increase in the percentage of monocytes detected at 0 h after exposure which returned to control levels at 24 h, however, there was no difference in the percentage of neutrophils at 0 or 24 h following exposure compared with the control group (Figure 3).

Direct effects of $UFTiO_2$ on cardiac myocytes

Unlike arsenite, a chemical compound that increases the phosphorylation level of p38 MAPK in isolated cardiac myocytes (Chen et al. 2003), $UFTiO_2$ (1 μg/ml) did not alter the phosphorylation status of p38 MAPK. Neither arsenite nor $UFTiO_2$ affected Akt phosphorylation in cardiac myocytes (Figure 4). Arsenite (10 μM), but not $UFTiO_2$, increased cTnI phosphorylation in cardiac myocytes (Figure 4). Both $UFTiO_2$ and arsenite did not alter protein expression levels of p38 MAPK, cTnI or Akt in cardiac myocytes (Figure 4).

Oxidative stress

In vitro exposure of cardiac myocytes to $UFTiO_2$ at concentrations of 0.1, 0.5 and 1 μg/ml for 4 h did not increase fluorescence intensity compared with the control group. By contrast, the positive control group, receiving 100 μM of hydrogen peroxide (H_2O_2), increased in fluorescence intensity by almost twofold in cardiac myocytes compared with the untreated control group (Figure 5).

Neuronal effect of pulmonary $UFTiO_2$ exposure

Pulmonary exposure to $UFTiO_2$ for 4 h increased neuronal substance P immunoreactivity in the nodose ganglia at 0 and 24 h after exposure (Figure 6). Double-labelling immunohistochemistry revealed that only 4.13 ± 0.49% of PGP 9.5-positive neurons displayed substance P immunoreactivity in control rats, whereas 9.62 ± 0.83% and 11.22 ± 1.04% ($p \leq 0.05$ compared with control) of PGP 9.5-positive neurons

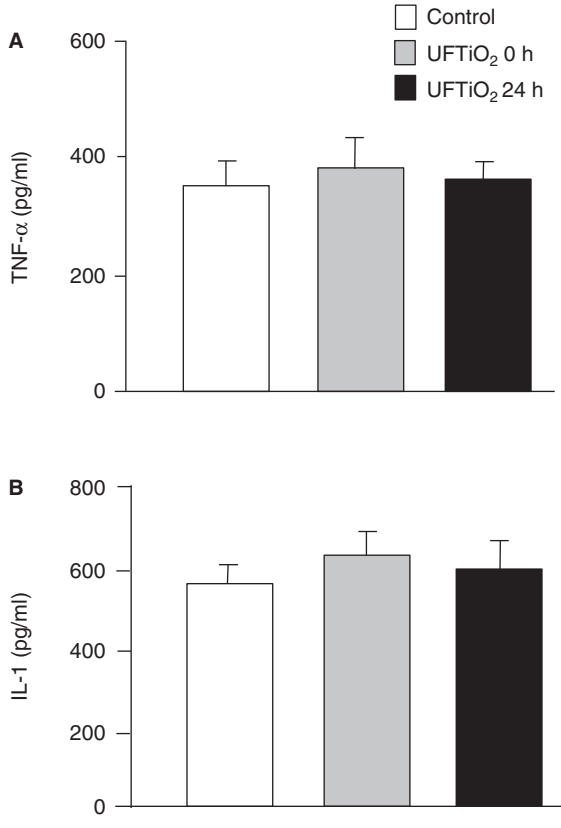

Figure 2. Cytokine levels in peripheral blood measured at 0 and 24 h post-inhalation exposure to $UFTiO_2$. Bar graphs represent the concentrations (pg/ml) of TNF-α (A) and IL-1 (B) detected in the control and $UFTiO_2$ groups at different time points. Each value represents the mean ± SE of five rats. $UFTiO_2$: ultrafine titanium dioxide.

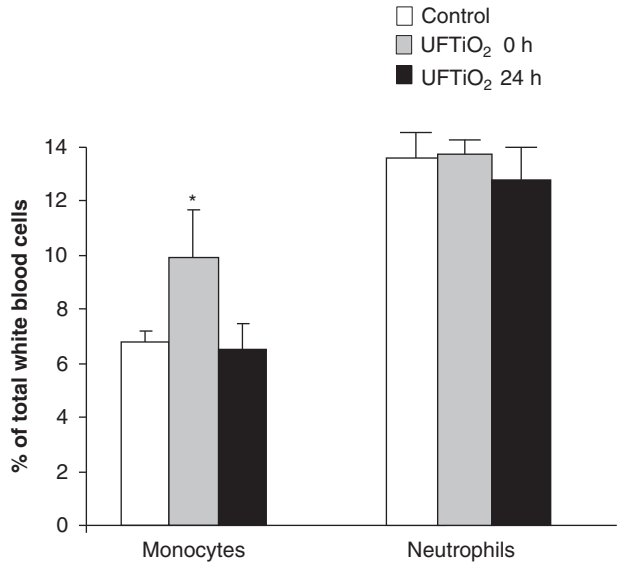

Figure 3. Percent changes in monocyte and neutrophil counts after inhalation exposure of rats to $UFTiO_2$. Blood was drawn and prepared for cell counts at 0 and 24 h post-exposure to $UFTiO_2$. Each value represents the mean ± SE of five rats; $p < 0.05$ compared with the control group (*). $UFTiO_2$: ultrafine titanium dioxide.

were substance P positive in rats at 0 or 24 h post-exposure, respectively.

Discussion and conclusion

The present study provides evidence that the effects of pulmonary exposure to $UFTiO_2$ on cardiac function are more likely mediated by indirect mechanisms than through a direct interaction between the nanoparticles and cardiac tissue. The rapid increase in the phosphorylation levels of p38 MAPK and cTnI along with the increased substance P immunoreactivity detected in the nodose ganglia at 0 and 24 h post-exposure are consistent with this hypothesis. Further, *in vitro* exposure of ARVM to $UFTiO_2$ had no direct effect on the phosphorylation of p38 MAPK or cTnI and did not induce ROS generation.

In the present study, we demonstrated that pulmonary exposure to $UFTiO_2$ rapidly increased the phosphorylation levels of p38 MAPK and cTnI, but not Akt in cardiac tissue. Both p38 MAPK and cTnI are key mediators in the regulation of cardiac function (Chen et al. 2003; Kan et al. 2005; Messer et al. 2007). These changes may partially explain the pathogenesis of nanoparticle inhalation-induced cardiovascular incidents, such as acute heart failure and irregular heartbeat, observed in the epidemiologic and animal studies. Under basal conditions, cardiac p38 MAPK activity is tightly regulated and maintained at a relatively low level. Increased p38 MAPK activity resulting from phosphorylation can depress cardiac contractility through a pro-inflammatory cytokine-dependent or a pro-inflammatory cytokine-independent pathway (Kan et al. 2005). In our study, lack of gene expressions of TNF-α, IL-1 or IL-6 in cardiac tissue and the protein synthesis of these cytokines in the peripheral bloodstream exclude the role of a p38 MAPK-mediated pro-inflammatory cytokine-dependent pathway in the regulation of cardiac function. Recently, increased p38 MAPK activity has also been related to arrhythmogenesis in cardiomyocytes by up-regulating T-type Ca^{2+} channel expression (Morishima et al. 2009). This up-regulation of T-type Ca^{2+} channel may be one of the possible mechanisms underlying cardiac arrhythmia in response to exposure to ambient particulate matter or engineered nanoparticles (Emilsson 2008). On the other hand, cTnI is a cardiac regulatory protein involved in the coupling of excitation to contraction (E-C coupling), which is vital for maintaining the normal contractile function of the heart. The phosphorylation level of cTnI is mainly regulated via protein kinase A activation, secondary to β-adrenoceptor stimulation. A prolonged increase or decrease of cTnI phosphorylation at Ser23/24 usually suggests an impairment of the cardiac β-adrenoceptor-mediated signalling pathway and can result in reducing cardiac contractility by altering the sensitivity of myofilaments to intracellular Ca^{2+} (Messer et al. 2007; Tavernier et al. 2001).

Recently, a study conducted in humans suggested that inhalation of ultrafine particles from ambient air can alter cardiovascular autonomic nerve activity (Timonen et al. 2006). Similar results have also been reported by several investigators in animal studies. For instance, a study conducted by Legramante et al. (2009) demonstrated that intratracheal instillation of SWCNTs alters cardiac autonomic neuron activity resulting from a reduced baroreflex (BRF)

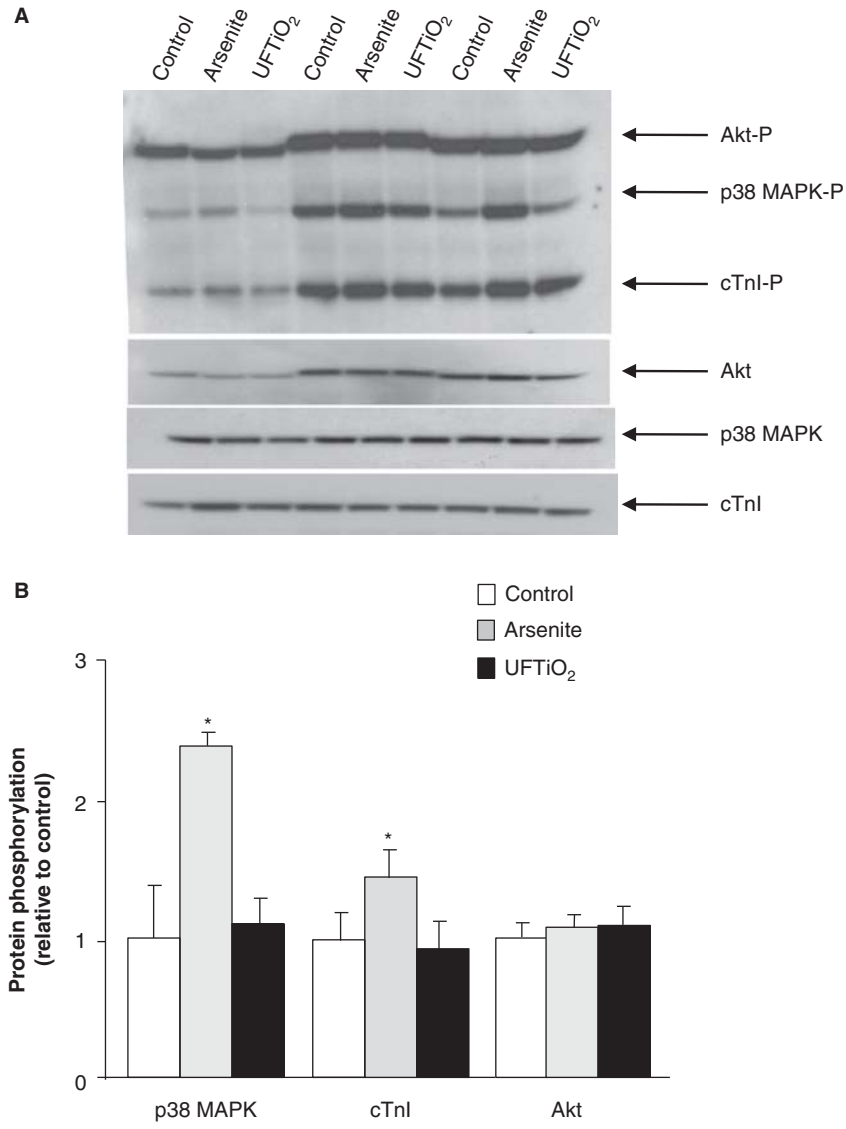

Figure 4. Phosphorylation of cardiac proteins in ARVM isolated from naïve rats. Myocytes were exposed in vitro to $UFTiO_2$ (1 μg/ml) or arsenite (10 μM) for 4 h. The western blot represents cardiac protein phosphorylation and expression. Each lane of the western blot shows cardiac protein phosphorylation and expression in a myocyte preparation from an individual rat. Samples were prepared from three different rats (A). Densitometry values of specific bands were compared between the different treatment and the control (B). Each value represents the mean ± SE of three different experiments; $p < 0.05$ compared with the control (*).ARVM: adult rat ventricular myocytes; cTnI: cardiac troponin I; MAPK: mitogen-activated protein kinase; $UFTiO_2$: ultrafine titanium dioxide.

response. Another study conducted by Nurkiewicz and his group reported that pulmonary exposure to $UFTiO_2$ inhibits endothelium-dependent dilation in systemic and cardiac arterioles in the absence of significant lung damage or systemic inflammation in rats (LeBanc et al. 2009; Nurkiewicz et al. 2008). This defect in dilatory function can be partially reversed by local application of tetrodotoxin, a potent neurotoxin, suggesting the involvement of autonomic nerve regulation (Nurkiewicz et al. 2009). More recently, Hazari et al. (2011) reported that rats exposed to diesel exhaust, which has average size of particles less than 100 nm, increased the sensitivity of the heart to triggered arrhythmias. This adverse cardiac effect of diesel exhaust exposure can be prevented by pretreatment of the rats with sympathetic blockade. These findings provide strong evidence to support the hypothetical mechanism of an autonomic sensory neuron-regulated pathway in cardiovascular events in response to pulmonary nanoparticle exposure.

Disruption of normal autonomic neuronal regulation in the cardiovascular system is one of the mechanisms that contributes to the alteration of heart rate variability, development of arrhythmia and heart muscle ischaemia/injury through a p38 MAPK-dependent or a p38 MAPK-independent pathway (Armour 1999; Ballard-Croft & Horton 2002; Curtis & O'Keefe 2002). In addition, the phosphorylation level of cTnI is regulated by sympathetic nerve activity through a β-adrenergic signalling pathway. A sympathetic overdrive-induced β-adrenergic signalling pathway defect, which consequently results in an abnormality in E-C coupling due to altered phosphorylation levels of cTnI, is one mechanism that leads to heart failure (Messer et al. 2007). However, the mechanism underlying pulmonary exposure to nanoparticle-induced alteration of autonomic nerve activity

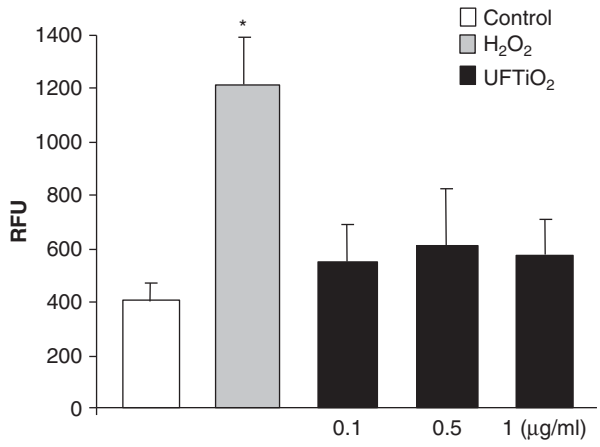

Figure 5. ROS activity in ARVM. Myocytes were exposed to $UFTiO_2$ at various concentrations for 4 h and H_2O_2 (100 μM) for 20 min. The bar graph represents three different experiments; $p < 0.05$ compared with the control (*). RFU: relative fluorescence units; UFTiO2: ultrafine titanium dioxide.

remains unknown. In the present study, we found that pulmonary exposure to $UFTiO_2$ increased neurotransmitter substance P immunoreactivity in the nodose ganglia. The nodose ganglia have been previously reported to be involved in the integration and control of lung and heart function by receiving primary sensory nerve fibres, A and C type, from the lung and transmitting this information to the brainstem, including the medullar cardiovascular regulatory centre, to regulate autonomic efferent neuron activity (Spyer 1982; Stansfeld & Wallis 1985). C type nerve fibres represent a major irritant sensor in the lung and can be stimulated mechanically, chemically and biologically (Gu et al. 2003; Kajekar et al. 1999). More recently, the nodose ganglia's afferent neurons were also shown to project directly to different chambers of the rat heart and exhibit a variety of neurochemical phenotypes (Guic et al. 2010; Kosta et al. 2010). On the other hand, substance P, as a co-neurotransmitter, has a modulatory effect on the synthesis and release of other neurotransmitters, such as acetylcholine, neuropeptide Y and norepinephrine, which results in altered local autonomic neuron-regulated organ function (Chiao & Caldwell 1995; Mukda et al. 2009). Therefore, it is possible that the increase in substance P synthesis in the nodose ganglia in response to pulmonary exposure to $UFTiO_2$ alters cardiac autonomic neuron activity either through the medullar cardiovascular regulatory centre or through the nodose ganglia's afferent neurons, which directly project to the cardiac chambers and modulate the synthesis and release of other co-existing neurotransmitters.

There is a close correlation between pro-inflammatory cytokines, systemic inflammation and cardiac dysfunction (Chrysohoou et al. 2009). Epidemiological and animal studies indicate that exposure to concentrated ambient air or ultrafine particles (size <0.1 μm) up-regulates the mRNA and protein expression of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6 (Totlandsdal et al. 2010; Wilson et al. 2010); alters the white blood cell profile; and results in systemic inflammation (Liao et al. 2008). In human and animal studies, elevation of circulatory inflammatory mediators, particularly TNF-α, IL-1 and IL-6 have reduced the left ventricular ejection fraction (LVEF), a typical clinical sign that occurs in patients with heart failure (Bregagnollo et al. 1999), through p38 MAPK-dependent or non-dependent pathways. An *in vitro* study confirmed that TNF-α and IL-1 can induce cardiac dysfunction by activation of p38 MAPK via the p38 MAPK upstream protein MKK3/6. Numerous epidemiologic and clinical studies have shown that alteration of the white blood cell profile in peripheral blood, in particular an increase of monocyte and/or neutrophil count, is associated with an increased risk of cardiovascular events in humans (Gillum et al. 2005). However, in our study, inhalation of $UFTiO_2$ at a low lung burden of 10 μg did not up-regulate mRNA and protein expression of cytokines in cardiac tissue (data not shown) or the circulatory system and did not induce significant systemic inflammation. Therefore, the biological effects we observed on the heart most likely occur through a systemic inflammation-independent mechanism.

Nanoparticles pass through the air–blood barrier between the alveoli and pulmonary capillaries and then into the bloodstream, which allows nanoparticles to directly interact with remote organs. The direct effect of nanoparticles generated from diesel engines or synthetic sources on different cell types, including cardiac myocytes, has been explored recently (Gojova et al. 2007,2009; Helfenstein et al. 2008; Totlandsdal et al. 2008a,2008b). For example, direct exposure of neonatal rat ventricular cardiomyocytes (NRVCM) to combustion-derived ultrafine particles or ultrafine carbon black can induce ROS production, change cardiomyocyte function, deteriorate myofibrillar structure in a dose-dependent manner (Helfenstein et al. 2008) and elicit a notable cytokine response (Totlandsdal et al. 2008a,2008b). By contrast, in the present study, *in vitro* exposure of ARVM to $UFTiO_2$ at concentrations from 0.1 to 1 μg/ml for 4 h did not induce ROS generation or mRNA expression of TNF-α, IL-1 or IL-6 (data are not shown). ROS is a mediator that increases the activity of p38 MAPK in cardiac tissue (Fan et al. 2009). The absence of an ROS response in ARVM could be partially due to differences of molecular biological profiles of NRVCM and ARVM. For example, changes in expression of ion channels, in contractile protein isoforms and in metabolic pathways are taking place during myocardial development (Mitcheson et al. 1998). A second explanation is the lower dose of $UFTiO_2$ used in the current experiment. A low *in vitro* exposure dose seems most relevant because one would not expect the cardiac tissue concentration of $UFTiO_2$ to be very high after pulmonary exposure. In our *in vitro* study, lack of biological responses, such as the absence of increased phosphorylation levels of cardiac proteins or ROS generation suggests that translocation of $UFTiO_2$ from pulmonary deposition sites into the circulatory system and direct interaction with cardiac myocytes does not appear to be the mechanism to explain the pulmonary $UFTiO_2$ exposure-induced biological changes in the heart. However, we cannot exclude the possibility that $UFTiO_2$ may interact with other cell types, such as blood cells or endothelial cells, which may release mediators not evaluated in our study and induce the pronounced biological effects we have observed *in vivo*. This possibility is based on

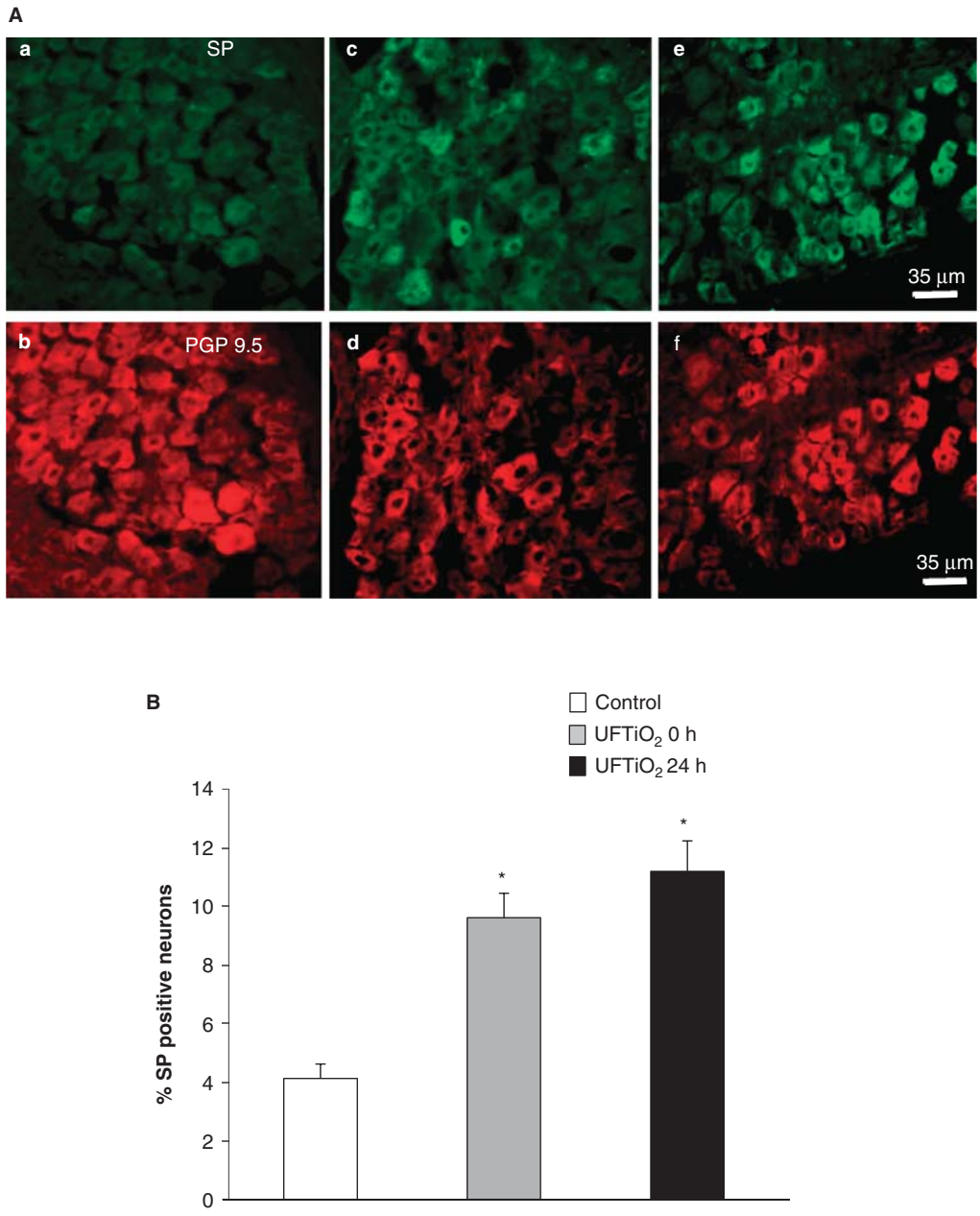

Figure 6. Fluorescence photomicrographs of substance P (green) and PGP 9.5 (red) immunoreactivity in the nodose ganglia. Control (a and b), 0 h (c and d) and 24 h (e and f) post-$UFTiO_2$ exposure. Neuronal cells were identified by PGP 9.5, a neuron-specific protein marker (b, d and f). The increased substance P immunoreactivity was detected at 0 and 24 h post-$UFTiO_2$ exposure (c and e, respectively) compared with the control (a) (A). The substance P immunoreactivity in the nodose ganglia was quantified (B). Each value represents the mean ± SE of five rats; $p < 0.05$ compared with the control (*). PGP: protein gene product; $UFTiO_2$: ultrafine titanium dioxide.

the recent finding that engineered nanoparticles can cause DNA damage across a cellular barrier *in vitro* (Bhabra et al. 2009), which warrants further study.

In conclusion, we have provided compelling evidence that pulmonary exposure to $UFTiO_2$ may cause adverse cardiac effects by increasing the phosphorylation levels of p38 MAPK and cTnI in the heart. Increased substance P synthesis in nodose ganglia in response to pulmonary $UFTiO_2$ exposure may partially account for the biological responses in the heart through a mechanism that involves a lung-neuron-regulated pathway. The current experiments focused on changes in signalling molecules in response to pulmonary $UFTiO_2$ exposure. Additional studies are ongoing in the laboratory, utilising physiological and pharmacological tools in *in vivo* and *in vitro* experiments to study the linkage between the described biological changes and cardiac function.

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Declaration of interest

The opinions expressed in this manuscript are those of the authors and do not necessarily represent the views of the

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