

## The role of nodose ganglia in the regulation of cardiovascular function following pulmonary exposure to ultrafine titanium dioxide

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### Abstract

The inhalation of nanosized air pollutant particles is a recognised risk factor for cardiovascular disease; however, the link between occupational exposure to engineered nanoparticles and adverse cardiovascular events remains unclear. In the present study, the authors demonstrated that pulmonary exposure of rats to ultrafine titanium dioxide (UFTiO<sub>2</sub>) significantly increased heart rate and depressed diastolic function of the heart in response to isoproterenol. Moreover, pulmonary inhalation of UFTiO<sub>2</sub> elevated mean and diastolic blood pressure in response to norepinephrine. Pretreatment of the rats *ip* with the transient receptor potential (TRP) channel blocker ruthenium red inhibited substance P synthesis in nodose ganglia and associated functional and biological changes in the cardiovascular system. In conclusion, the effects of pulmonary inhalation of UFTiO<sub>2</sub> on cardiovascular function are most likely triggered by a lung-nodose ganglia-regulated pathway via the activation of TRP channels in the lung.

**Keywords:** nanoparticles, neural pathways, cardiovascular system, inhalation study

### Introduction

Evidence accumulated from epidemiological studies suggests that there is a significant pathogenic correlation between the inhalation of small-sized or nanosized particles from ambient air and cardiovascular events, such as angina, arrhythmia, ischemic heart failure and sudden death (Dockery et al. 1992; Donaldson et al. 2001; Huang & Ghio 2009; Simkhovich et al. 2008). Recently, a number of studies have shown that pulmonary exposure to engineered nanoparticles (with a diameter <100 nm), such as single-walled carbon nanotubes (SWCNTs), ultrafine titanium dioxide (UFTiO<sub>2</sub>) and silicon dioxide (SiO<sub>2</sub>), can cause biological and pathophysiological effects on the cardiovascular system. For instance, pulmonary inhalation of SWCNTs or UFTiO<sub>2</sub> results 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), while pulmonary inhalation of SiO<sub>2</sub> causes alterations in both blood vessels and the heart (Chen et al. 2008). All of these observations suggest that engineered nanoparticles exhibit the potential to cause adverse effects on the cardiovascular system. In addition, the most interesting finding is that the adverse effects observed on the cardiovascular system induced by engineered nanoparticles are very similar to those reported in epidemiological studies of air pollution (Brook et al. 2009; Niwa et al. 2008; Simeonova & Erdely 2009). This finding indicates that engineered nanoparticles and nanosized particles from ambient air may share a common pathophysiological pathway(s) in the development of adverse cardiovascular effects following pulmonary exposure. Engineered nanoparticles are more homogeneous in their physical and chemical properties than naturally existing nanoparticles; therefore, they may induce more consistent and reproducible adverse health effects. These characteristics of engineered nanoparticles provide researchers with a rationale for using engineered nanoparticles to elucidate the mechanisms underlying adverse cardiovascular effects induced not only by engineered nanoparticles but also by air pollution.

Recently, studies conducted by other investigators have shown that inhalation of ultrafine particles from ambient air can alter cardiovascular autonomic nerve activity and baroreceptor reflex sensitivity (Bartoli et al. 2009; Timonen et al. 2006), providing evidence to support the hypothetical mechanism of an autonomic sensory neuron-regulated pathway. Interestingly, the same observation has also been made in a study conducted by Legramante et al. (2009), who demonstrated that intratracheal instillation of engineered nanoparticles reduces the baroreflex response, thereby altering cardiac autonomic neuron activity. The authors of this study also demonstrated that pulmonary exposure of rats to UFTiO<sub>2</sub> enhanced substance P synthesis in nodose ganglia

of the vagus nerve and was associated with biological changes in the heart, which was evidenced by an increased phosphorylation level of cardiac troponin I (cTnI) (Kan et al. 2012). All of these studies provide strong evidence, suggesting that a neuron-regulated pathway could play an important contributing role in cardiovascular dysfunction caused by air pollution or engineered nanoparticles (Kang et al. 2011).

Nodose and dorsal root ganglia contain pulmonary C-fibre sensory neurons. These sensory neurons detect major irritants in the lung and can be stimulated mechanically, chemically and biologically via the activation of the transient receptor potential (TRP) channels. Increased activity in C-fibre sensory neurons can result in changes to the gene expression of preprotachykinin and neuronal function in nodose and dorsal root ganglia (Curran et al. 2002; Dinh et al. 2004). Moreover, the nerve fibres from the nodose ganglia also project to the brainstem, including the medullar cardiovascular regulatory centre, to regulate autonomic efferent neuron activity (Spyer 1982; Stansfeld & Wallis 1985) or innervate the different chambers of the rat heart and the wall of the aortic arch, where they exhibit a variety of neurochemical phenotypes (Ai et al. 2009; Guic et al. 2010; Kosta et al. 2010). Thus, the sole connection of nodose ganglia could be a critical neuronal pathway that regulates cardiovascular function in response to pulmonary inhalation of nanoparticles. The objective of the present study was to determine cardiovascular reactions following pulmonary exposure to UFTiO<sub>2</sub> and elucidate the underlying mechanism(s). The authors conducted cardiovascular haemodynamic measurements, including heart rate, left ventricular function and systemic blood pressure, and evaluated the role of nodose ganglia in the regulation of cardiovascular function by applying the TRP channel blocker in an *in vivo* animal model.

## Methods and materials

### Animals

Male Sprague-Dawley rats (Hla:(SD) CVF) from Hilltop Lab Animals (Scottsdale, 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 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 rats were allowed to acclimatise to the facilities for 1 week before exposure was performed. 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 and conducted in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

### Pulmonary UFTiO<sub>2</sub> exposure and ruthenium red treatment

Rats (7–8 weeks of age) received UFTiO<sub>2</sub> (primary particle diameter ~ 21 nm) by inhalation. For this study, the UFTiO<sub>2</sub>

aerosol concentration was 6 mg/m<sup>3</sup>, and the exposure duration was 4 h. The aerosol generation system, exposure chamber and physical characterisation of UFTiO<sub>2</sub> aerosol have been described previously (Nurkiewicz et al. 2008). Previous studies have shown that this exposure scheme produced an actual pulmonary deposition of 10 µg UFTiO<sub>2</sub> in rats, which is equivalent to workers exposed to 0.1 mg/m<sup>3</sup> for 27 workdays in a typical occupational environment, and resulted in biological and functional changes in the systemic and cardiac vascular system (Kan et al. 2012; LeBlanc et al. 2009; Nurkiewicz et al. 2008). This equivalency is calculated based on the deposited mass of UFTiO<sub>2</sub> aerosol assuming a slow or no clearance. The rationale for the 27 days was based on the equivalent deposited mass per surface area of alveolar epithelium. Morphometric data for rat and human alveolar surface area are from Stone et al. (1992). Non-selective TRP channel blocker ruthenium red (2.5 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally 1 h prior to the pulmonary exposure of rats to UFTiO<sub>2</sub> or filtered air.

### *In vivo* haemodynamic measurements

At 24 h post-exposure, rats (7–8 per group) were anaesthetised with inhaled 3% isoflurane mixed with oxygen at a flow rate of 2 l/min. A temperature-regulated heating pad was used to maintain the normal body temperature of the rat. Using aseptic technique, a custom catheter made according to the method described by Khanna et al. (2007) was inserted into the left ventricle through the carotid artery. The correct position of the catheter tip in the left ventricle was confirmed by the waveform of left ventricular pressure visualised on a computer monitor. Heart function was assessed by measuring the maximum rate of increase in left ventricular pressure (dP/dt<sub>max</sub>) and minimum rate of decrease in left ventricular pressure (dP/dt<sub>min</sub>). To study vascular function *in vivo*, systemic arterial blood pressure was determined by using a fluid-filled arterial catheter placed in the femoral artery and connected to a pressure transducer coupled to a computerised cardiovascular continuous monitoring system, a PowerLab/4SP analog-to-digital converter (AD Instruments, Colorado Springs, CO, USA). The heart rate was recorded by the same monitoring system at a sampling rate of 1000 Hz. Isoproterenol (ISO) or norepinephrine (NE) (Sigma-Aldrich) was administered through a catheter (polyurethane, 3 French size) preplaced in the jugular vein. Cardiopulmonary responses and spinal reflexes were checked to determine the proper depth of anaesthesia. Each rat was euthanised with a carbon dioxide overdose at the end of the experiment.

### Substance P immunohistochemistry

For tissue preparation, the right and left nodose ganglia and dorsal root ganglia (C1–C2 and T1–T4) were excised, 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. The immunocytochemical procedures for the localisation of substance P immunoreactive neurons were similar to those described previously

(Kan et al. 2012). Briefly, cryostat sections (12  $\mu\text{m}$  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 PBS-Tx containing 1% bovine serum albumin (PBS-Tx + BSA) three times, for 5 min per rinse. The sections were then 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. Then, the coverslips were mounted with fluoromount, and the sections were observed under a fluorescence microscope equipped with a fluorescein (excitation wavelengths from 455 to 500 nm and emission wavelengths >510 nm) filter. The controls consisted of testing the specificity of primary antiserum by absorption with 1  $\mu\text{g}/\text{ml}$  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 performed similarly to the authors' previously described method (Kan et al. 2012).

### Western blots

The phosphorylation status of cTnI was examined at 24 h following pulmonary exposure to UFTiO<sub>2</sub>. Cardiac tissue samples were removed from rats, rapidly frozen and crushed with a mortar and pestle at the temperature of liquid nitrogen for protein extraction. Cardiac tissue was prepared in lysis buffer containing 20 mmol/l Tris-HCl, 20 mmol/l NaCl, 0.1 mmol/l ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100 and protease inhibitors and centrifuged at 10,000 *g* for 20 min at 4°C as described previously (Kan et al. 2012). Equal amounts of protein were loaded onto an 8% SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA). Phosphorylation of cTnI was detected by phospho-cardiac troponin-I (Ser 23/24) antibody (Cell Signaling Technology, Danvers, MA, USA). Total cTnI was detected with a rabbit mAb (Cell Signaling Technology).

### Statistical analysis

Data were compared using analysis of variance, followed by pairwise comparisons between the control and treated groups using a Student's *t*-test. All data were analysed using JMP software (version 9.0) and differences were considered statistically significant at  $p < 0.05$ . The values in the figures are expressed as the mean  $\pm$  SD (standard deviation).

## Results

### Effects of UFTiO<sub>2</sub> and TRP channel blocker on heart rate

Pulmonary inhalation of UFTiO<sub>2</sub> did not change the basal heart rate compared with the control group (exposed to filtered air) at 24 h post-exposure. However, ISO-induced increases in heart rate were greater in rats exposed to UFTiO<sub>2</sub> compared with rats exposed to filtered air (Figure 1). Pretreatment of the rats with ruthenium red, a non-selective

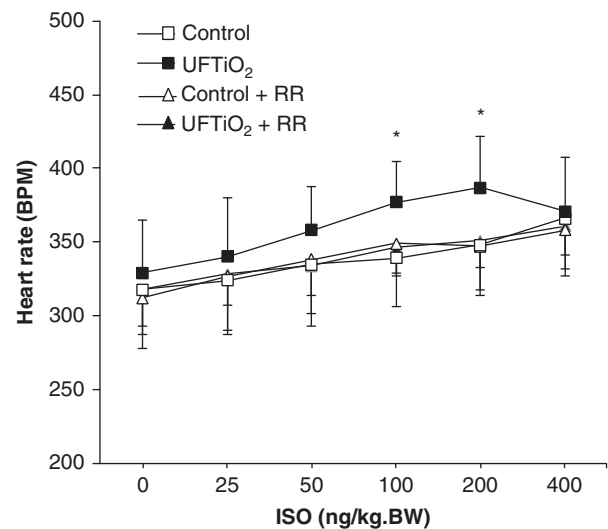


Figure 1. The dose-response curve of heart rate in response to ISO at 24 h after exposure to UFTiO<sub>2</sub> and the effect of ruthenium red (RR) on UFTiO<sub>2</sub>-induced heart rate increase in response to ISO. Each value represents the mean  $\pm$  SD of six rats;  $p < 0.05$  compared with the control group (\*).

TRP channel blocker, did not affect the basal heart rate in rats either exposed to filtered air or UFTiO<sub>2</sub>, but prevented the ISO-induced increase in heart rate in rats exposed to UFTiO<sub>2</sub> (Figure 1). In addition, ruthenium red alone had no effect on heart rate in response to ISO (Figure 1).

### Effects of UFTiO<sub>2</sub> and TRP channel blocker on left ventricular function

Baseline measures of  $dP/dt_{\text{max}}$ , which reflects the systolic function of the left ventricle, were similar in all experimental groups at 24 h post-exposure. Pulmonary inhalation of UFTiO<sub>2</sub> slightly reduced  $dP/dt_{\text{max}}$  in response to higher dosages of ISO, but the difference did not reach statistical significance when compared with the control group ( $p > 0.05$ ) (Figure 2A). The  $dP/dt_{\text{min}}$ , which indicates the diastolic function of the left ventricle, was decreased in response to ISO and reached statistical significance at higher dosages in rats exposed to UFTiO<sub>2</sub> compared with the control group (Figure 2B). Reduced  $dP/dt_{\text{min}}$  in response to ISO was prevented by pretreatment of rats with ruthenium red (Figure 2B).

### Effects of UFTiO<sub>2</sub> and TRP channel blocker on systemic blood pressure

Pulmonary inhalation of UFTiO<sub>2</sub> or pretreatment with ruthenium red did not alter basal levels of mean blood pressure compared with the control group ( $p > 0.05$ ) (Figure 3A). However, UFTiO<sub>2</sub> significantly increased mean blood pressure in response to NE, an  $\alpha$ -adrenergic receptor agonist. Ruthenium red prevented the increase of mean blood pressure in response to NE in rats exposed to UFTiO<sub>2</sub> (Figure 3A). Systolic blood pressure was not affected by pulmonary inhalation of UFTiO<sub>2</sub> either at basal levels or in response to NE (Figure 3B). By contrast, diastolic blood pressure in response to NE was significantly increased at higher NE concentrations in rats exposed to UFTiO<sub>2</sub> at 24 h

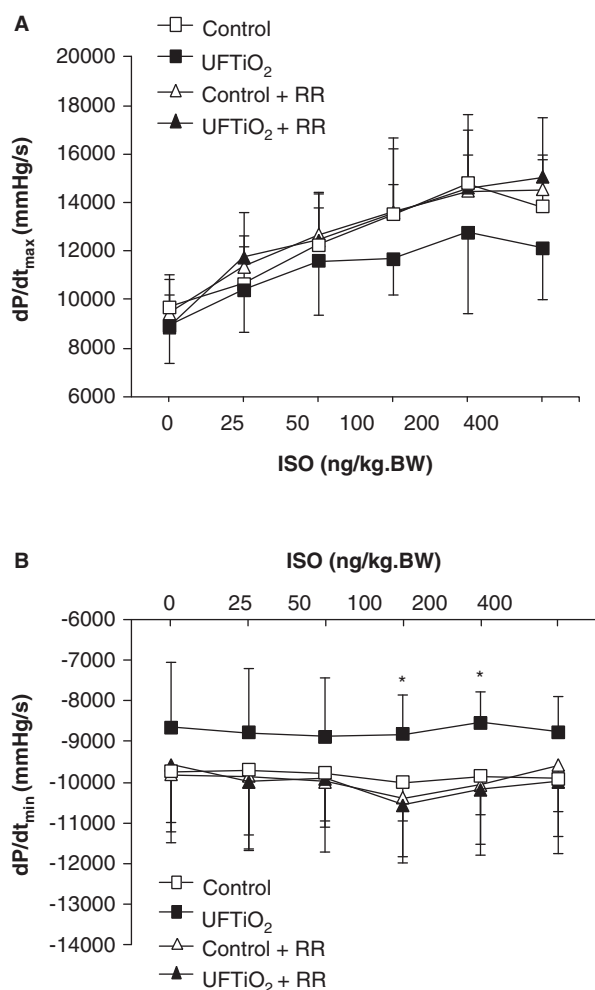


Figure 2. Effects of UFTiO<sub>2</sub> and ruthenium red (RR) on cardiac function. (A) The contractile ability of the heart at 24 h after exposure to UFTiO<sub>2</sub> was assessed by measuring the maximum rate of increase in left ventricular pressure, dP/dt<sub>max</sub>. (B) The diastolic function of the heart at 24 h after exposure to UFTiO<sub>2</sub> was assessed by measuring the minimum rate of decrease in left ventricular pressure, dP/dt<sub>min</sub>. Each value represents the mean  $\pm$  SD of six rats;  $p < 0.05$  compared with the control group (\*).

post-exposure compared with the control group (Figure 3C). Pretreatment of rats with ruthenium red prevented increased diastolic blood pressure in response to NE in rats exposed to UFTiO<sub>2</sub> (Figure 3C).

#### Effect of TRP channel blocker on SP synthesis in nodose and dorsal root ganglia and cTnI phosphorylation

Pulmonary inhalation of UFTiO<sub>2</sub> significantly increased neuronal substance P immunoreactivity in the nodose ganglia, but not in the dorsal root ganglia at 24 h post-exposure (Figure 4A). Immunohistochemistry revealed that only  $3.42 \pm 0.65\%$  of neurons displayed substance P immunoreactivity in control rats, whereas  $10.23 \pm 1.67\%$  ( $p \leq 0.05$  compared with the controls) of neurons were substance P positive in rats exposed to UFTiO<sub>2</sub>. Substance P immunoreactivity in nodose ganglia by UFTiO<sub>2</sub> was reduced to  $4.98 \pm 0.59\%$  in rats pretreated with ruthenium red (Figure 4B). Western blots confirmed that pulmonary inhalation of UFTiO<sub>2</sub> increased the phosphorylation level of cTnI in the heart at 24 h post-exposure (Figure 5A and B). However, pretreatment with

ruthenium red prevented UFTiO<sub>2</sub>-elevated phosphorylation levels of cTnI (Figure 5A and B).

#### Effect of UFTiO<sub>2</sub> on reactive oxygen species in the heart and aortic artery

Pulmonary exposure to nanoparticles or polluted air can increase reactive oxygen species (ROS) generation in the lung and the secondary organs, which causes tissue damage (Lodovici & Bigagli 2011). As reported previously, inhalation exposure to UFTiO<sub>2</sub> at levels and duration used in the present study did not alter bronchoalveolar lavage markers of inflammation and lung damage. However, histological evaluation demonstrated alveolar macrophages associated with deposition sites of particles in scattered alveoli (Nurkiewicz et al. 2008). In the present study, the authors

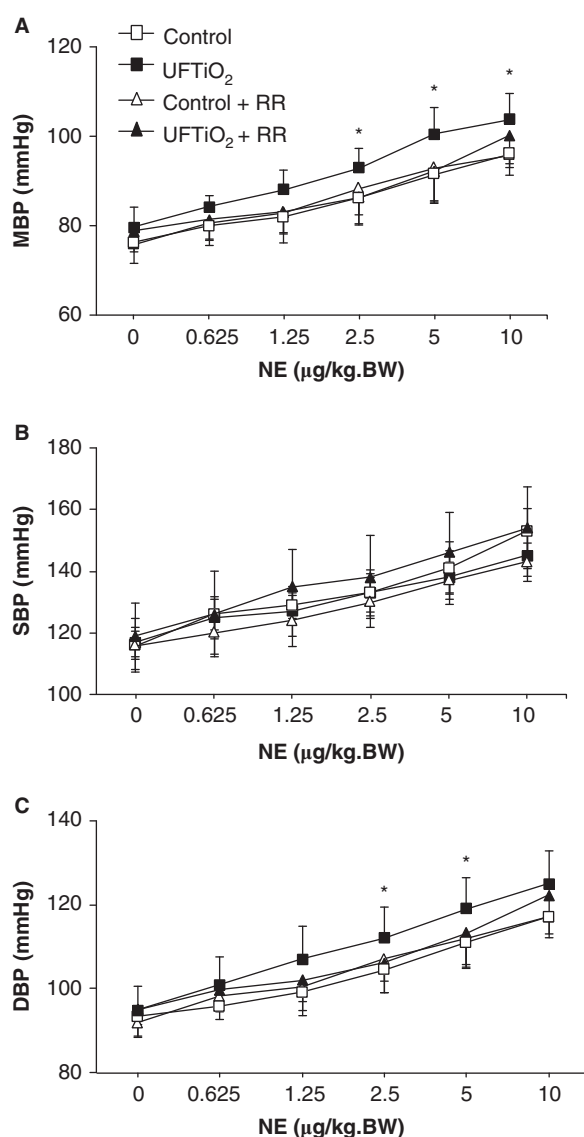


Figure 3. Effects of UFTiO<sub>2</sub> and ruthenium red (RR) on blood pressure. (A) The dose-response curve of mean blood pressure (MBP) in response to NE at 24 h after exposure to UFTiO<sub>2</sub>. (B) The dose-response curve of systolic blood pressure (SBP) in response to NE at 24 h after exposure to UFTiO<sub>2</sub>. (C) The dose-response curve of diastolic blood pressure (DBP) in response to NE at 24 h after exposure to UFTiO<sub>2</sub>. Each value represents the mean  $\pm$  SD of six rats;  $p < 0.05$  compared with the control group (\*).



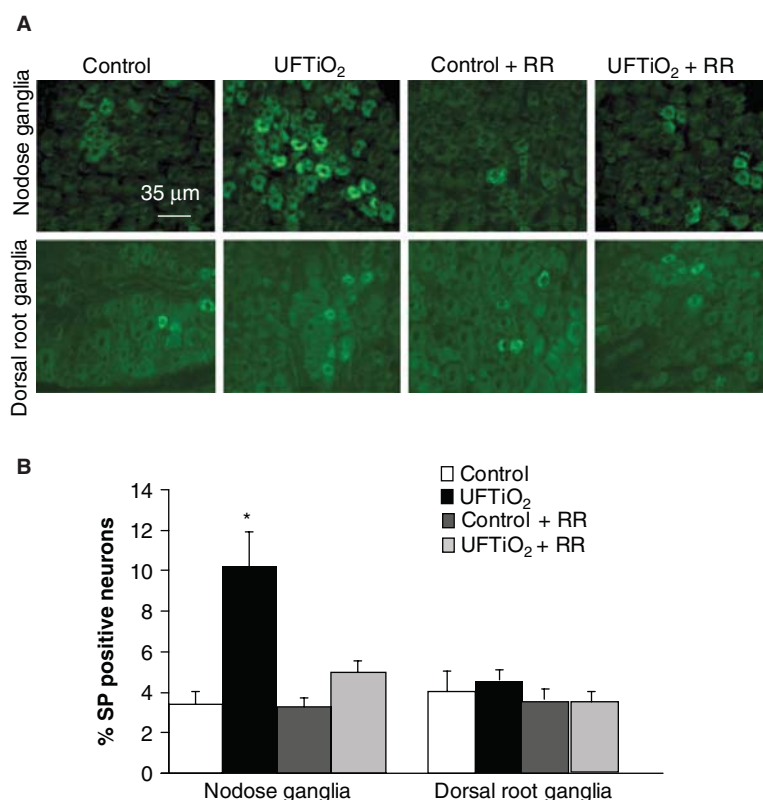


Figure 4. Fluorescence photomicrographs of substance P (SP) immunoreactivity in nodose and dorsal root ganglia. (A) SP immunoreactivity was detected in different groups at 24 h post-exposure. (B) The SP immunoreactivity in nodose and dorsal root ganglia was quantified (B). Each value represents the mean  $\pm$  SD of 5 rats;  $p < 0.05$  compared with the control group (\*).

also examined the formation of ROS in the heart and aortic artery after UFTiO<sub>2</sub> exposure. Hydroxyl radicals were indirectly measured in heart and aorta tissue slices according to methods previously described (Ide et al. 2000). The results indicated that UFTiO<sub>2</sub> exposure did not increase the amount of ROS production in the heart and aortic artery (data not shown).

## Discussion and conclusion

The present study reports that pulmonary exposure of rats to UFTiO<sub>2</sub> did not increase ROS production in the heart and aortic artery, but significantly increased heart rate, depressed diastolic function of the heart and elevated mean and diastolic blood pressure in response to adrenergic stimuli. Pretreatment of the rats with ruthenium red, a non-selective TRP channel blocker, not only inhibited substance P synthesis in nodose ganglia but also inhibited the phosphorylation level of cTnI and prevented the changes in cardiovascular function associated with substance P synthesis in nodose ganglia induced by UFTiO<sub>2</sub>.

An increase in heart rate (Figure 1) associated with altered diastolic function in response to ISO at 24 h after exposure to UFTiO<sub>2</sub> suggests an acute effect of UFTiO<sub>2</sub> on cardiac function (Figures 1 and 2). A change in heart rate usually results from an alteration in autonomic nervous system activity. Alterations in autonomic nervous system activity have been observed in epidemiological studies and are positively correlated with the level of small-sized particles in ambient air

(Pope et al. 1999). Increased heart rate resulting from exposure to engineered nanoparticles was first reported in a study using an isolated rodent heart. The increase in heart rate in this experimental model was primarily due to an increase of catecholamine released from adrenergic nerve endings within the heart (Stampfl et al. 2011). In the present study, it is likely that pulmonary inhalation of engineered nanoparticles is able to influence the activity of the autonomic nervous system, which in turn modulates neurotransmitter synthesis or release from neuronal terminals that innervate the heart. This proposed mechanism is based on the finding that pulmonary inhalation of UFTiO<sub>2</sub> increased substance P synthesis in nodose ganglia, which may alter the activity of nodose ganglia neurons that project to the medulla and different parts of the heart. Such a mechanism may also partially explain why increases in heart rate are observed during air pollution episodes (Peters et al. 1999).

This study also demonstrated that pulmonary inhalation of UFTiO<sub>2</sub> had a significant effect on cardiac function, with diastolic function of the heart affected more than systolic function. This observation, together with the previous finding that inhalation of UFTiO<sub>2</sub> resulted in an increase in phosphorylated cTnI, suggests that UFTiO<sub>2</sub>-induced cTnI phosphorylation may serve as one of the factors underlying the cardiac dysfunction observed in the present study and epidemiological studies of air pollution. *In vivo* and *in vitro* studies have confirmed that a sustained increase in cTnI phosphorylation regulated by protein kinase A (PKA) changes myofilament calcium binding affinity and reduces

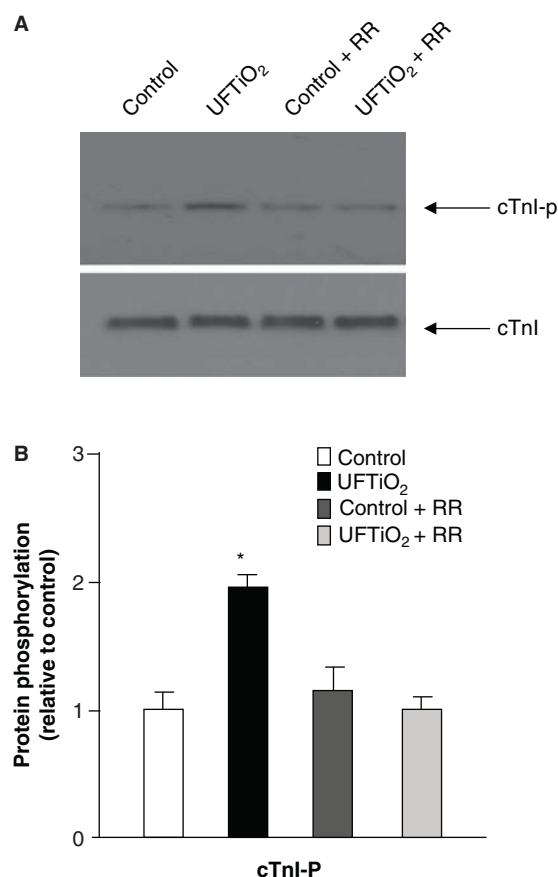


Figure 5. The Western blot represents cardiac TnI phosphorylation and expression in different groups at 24 h post-exposure. (A) Each lane of the Western blot shows cardiac TnI phosphorylation and expression in a myocyte preparation from an individual rat. (B) Densitometry values of specific bands were compared between each treatment and the control group. Each value represents the mean  $\pm$  SD of three different experiments;  $p < 0.05$  compared with the control group (\*).

relaxation rate, thereby impairing the diastolic function of the heart (Sakthivel et al. 2005). Other studies have shown that increases of sympathetic nerve activity or catecholamine concentrations in the blood can result in an elevation of cTnI phosphorylation, which indicates a neuron-regulated effect on the phosphorylation status of cTnI (van Dijk et al. 2008). Altered diastolic function of the heart is considered an early pathophysiological indicator of many forms of human heart failure (Krajnak et al. 2011; Satpathy et al. 2006). However, the impact of altered diastolic function on the heart usually produces no symptoms in its early stage, unless it has progressed to the point of diminishing heart function. The alterations in the diastolic function of the heart resulting from pulmonary inhalation of UFTiO<sub>2</sub> in this study suggest that pulmonary exposure to nanoparticles may increase the risk of a cardiac event in workers exposed daily to nanoparticles.

Pulmonary inhalation of UFTiO<sub>2</sub> also altered vasculature function, as evidenced by an increase in mean blood pressure in response to NE (Figure 3A). This increased mean blood pressure was primarily due to elevated diastolic blood pressure (Figure 3C) because while the increases in systolic blood pressure in response to NE were greater in rats exposed to UFTiO<sub>2</sub> (Figure 3B), they did not reach significant

levels. The authors and other investigators have reported that inhalation of pulmonary irritants, including UFTiO<sub>2</sub>, can result in changes in the vascular function of small vessels primarily by increasing vascular tone through a sympathetic nerve-regulated mechanism and/or impairing acetylcholine-induced vasodilation through an endothelium-regulated pathway (Krajnak et al. 2011; LeBlanc et al. 2009; Nurkiewicz et al. 2008). The impact of endothelial dysfunction on blood pressure could be different, as it affects diastolic more than systolic blood pressure in rats (de Belchior et al. 2012). This could be the mechanism of isolated diastolic hypertension, one subtype of hypertension diagnosed in the clinic (Arima et al. 2012).

The alterations in cardiovascular reactions in response to adrenergic stimuli in this study indicate that pulmonary inhalation of UFTiO<sub>2</sub> may have an influence on the activity of the autonomic nerves that regulate cardiovascular function. Recently, other investigators have reported that local treatment with an  $\alpha$ -adrenergic receptor antagonist partially blocked vascular dilatory dysfunction induced by pulmonary exposure to nanoparticles (Brook et al. 2009; Nurkiewicz et al. 2008), which also suggested an involvement of the autonomic nervous system in response to nanoparticle exposure. The present study showed that UFTiO<sub>2</sub> exposure-increased neurotransmitter substance P synthesis occurred predominantly in the nodose ganglia, but not in the dorsal root ganglia (Figure 4A), indicating that the sensory neurons in the nodose ganglia are likely associated with cardiovascular functional and biological changes after exposure to UFTiO<sub>2</sub>. This was supported by the interesting finding that pretreatment of rats with a non-selective TRP channel blocker not only inhibited substance P synthesis in the nodose ganglia but also prevented the cardiovascular functional changes and increases in phosphorylated cTnI (Figures 4 and 5). Both nodose and dorsal root ganglia contain cell bodies of pulmonary sensory neurons, however, the neuronal pathways that functionally transfer signals from the lung to the cardiovascular regulatory centre in the medulla, the heart and the peripheral vasculatures are different. It has been reported that the cardiovascular responses to the inhalation of certain irritants can be blocked by bilateral vagotomy, suggesting that cardiovascular responses induced by certain irritants in the lung can be mediated mainly through nodose ganglia (Wang et al. 1996). In addition, nodose ganglia also contain the cell bodies of the aortic baroreceptor neurons involved in the baroreflex, one of the body's most important homeostatic mechanisms for maintaining normal blood pressure (Li et al. 2008). Therefore, considering the unique neuronal connections of the nodose ganglia with the cardiovascular system, the observations from the current study indicate that the nodose ganglia play a significant role in the regulation of UFTiO<sub>2</sub> exposure-induced changes in the cardiovascular system by acting as an intermediary centre for integrating signalling from the lung and then further transmitting such signals to either the central nervous system or peripheral nerves, which regulate and control cardiovascular function (Figure 6). Nevertheless, these findings are sufficiently compelling enough to warrant more definitive research.

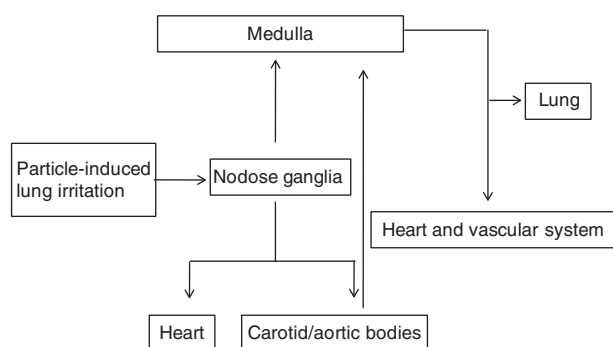


Figure 6. Schematic diagram describes a nodose ganglia-involved neuronal pathway in the regulation of cardiovascular function after pulmonary exposure to nanoparticles. Activation of TRP channels in the lung by nanoparticles results in transmission of stimulatory signals to nodose ganglia, where signals are integrated and processed. These signals are then transmitted to the cardiovascular centre in the medulla or the peripheral organs through the afferent neurons of nodose ganglia, resulting in modification of cardiovascular function.

Recently, Hazari et al. (2011) demonstrated that TRP channels in the lung play a crucial role in the regulation of cardiac rhythm after exposure to diesel particles, and blockage of TRP channels by ruthenium red abolished diesel exposure-induced cardiac arrhythmias. TRP channels are a group of ion channels ( $\text{Ca}^{2+}$  and  $\text{Na}^{+}$ ) that function as cellular sensors. They are expressed in a variety of tissues, including the endings of nodose C-fibres in the lung and cardiomyocytes in the heart (Vennekens 2011), and respond to diverse signals, including intracellular and extracellular messengers, exogenous chemicals, temperature and mechanical stress (Glazebrook et al. 2005). In this study, pretreatment of rats with a non-selective TRP channel blocker prevented UFTiO<sub>2</sub> exposure-induced biological and functional changes in the cardiovascular system, which indicates an activation of TRP channels by pulmonary inhalation of UFTiO<sub>2</sub>. The mechanism by which UFTiO<sub>2</sub> exposure activates TRP channels in the lung is unclear and requires further investigation. However, it has been shown that nanoparticles, including engineered nanoparticles like UFTiO<sub>2</sub>, have high surface activity and can interact with macrophages, epithelial cells or leukocytes that can evoke the production of ROS and pro-inflammatory factors in the lung (Rahman et al. 2002; Scherbart et al. 2011). Therefore, the possible mechanisms responsible for the activation of TRP channels following UFTiO<sub>2</sub> exposure include the direct stimulation of TRP channels by UFTiO<sub>2</sub> or locally generated ROS and/or inflammatory factors in the alveoli.

It has been reported that TRP channels are also expressed in the heart and vasculature and involved in several fundamental cell functions, such as contraction, proliferation and cell death (Watanabe et al. 2008). Ruthenium red is a non-selective TRP channel blocker, it can block ryanodine receptors in the heart and reduce cardiac muscle contractility *in vitro* (Aschar-Sobbi et al. 2012). However, the effects of ruthenium red on the cardiovascular function *in vivo* are uncertain. In the present study, pretreatment of rats with ruthenium red did not affect cardiovascular function either at basal levels or in response to adrenergic stimuli, which

suggests that the effects of ruthenium red on preventing UFTiO<sub>2</sub> exposure-induced biological and functional changes observed in this study are not likely via the blocking of TRP channels in the heart and vasculature.

In conclusion, the *in vivo* observations together with the previous findings that pulmonary inhalation of UFTiO<sub>2</sub> did not cause significant systemic inflammation and that direct exposure of cardiomyocytes to UFTiO<sub>2</sub> did not induce biological changes provide compelling evidence to support a role for nodose ganglia in the neuronal-mediated pathway that regulates cardiovascular function following pulmonary inhalation of UFTiO<sub>2</sub> (Kan et al. 2012). Activation of TRP channels appears to be an initial step to trigger this entire event.

The significance of the findings is that this proposed mechanism may not only be critical for engineered nanoparticle exposure-regulated alterations in cardiovascular function but may also be an important mechanism underlying the increased incidence of cardiovascular diseases associated with air pollution. Unchanged basal cardiovascular function, but altered cardiovascular reactions in response to adrenergic stimuli after pulmonary inhalation of UFTiO<sub>2</sub> indicate that pulmonary inhalation of UFTiO<sub>2</sub> or ambient nanoparticles may not pose an immediate health risk to healthy people; however, in people predisposed to cardiovascular diseases, such as coronary artery disease, congestive heart failure or essential hypertension associated with high circulating plasma catecholamine, pulmonary inhalation of nanoparticles may worsen the disease state and trigger a cardiovascular event.

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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 National Institute for Occupational Safety and Health, Center for Disease Control and Prevention of the USA.

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