Mucin Secretion Induced by Titanium Dioxide Nanoparticles

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Abstract

Nanoparticle (NP) exposure has been closely associated with the exacerbation and pathophysiology of many respiratory diseases such as Chronic Obstructive Pulmonary Disease (COPD) and asthma. Mucus hypersecretion and accumulation in the airway are major clinical manifestations commonly found in these diseases. Among a broad spectrum of NPs, titanium dioxide (TiO2), one of the PM10 components, is widely utilized in the nanotechnology industry due to their vast array of applications that range from mineral ore rutile [10]. It has been reported that TiO2 NP-evoked mucin secretion was a function of increasing intracellular Ca2+ concentration resulting from an extracellular Ca2+ influx via membrane Ca2+ channels and cytosolic ER Ca2+ release. The calcium-induced calcium release (CICR) mechanism played a major role in further amplifying the intracellular Ca2+ signal and in sustaining a cytosolic Ca2+ increase. This study provides a potential mechanistic link between airborne NPs and the pathoetiology of pulmonary diseases involving mucus hypersecretion.

Introduction

Many published reports have demonstrated the association between NP exposure and pulmonary morbidity and mortality [1,2,3]. The adverse effects induced by NPs seem to exacerbate clinical symptoms of pre-existing respiratory illnesses such as asthma, COPD and Cystic Fibrosis (CF) [1,2,3,4,5,6]. During NP exposure, individuals with respiratory diseases showed more incidences of bronchoconstriction, medication use, bronchial hyperreactivity and lung fibrosis [2,7]. TiO2 NPs are widely used in the nanotechnology industry due to their vast array of applications that range from household commodities, such as components of paints and carpets, to personal products that include cosmetics, textiles, sunscreens and foods [8,9]. TiO2 is also one of the PM10 components commonly found in industries or manufacturing plants involved in processing mineral ore rutile [10]. It has been reported that >50% of TiO2 NP exposed workers had respiratory symptoms accompanied by reduction in pulmonary function [10,11]. Other reports have also indicated that inhalation of TiO2 NPs can induce pulmonary inflammatory responses (characterized by neutrophil recruitment), epithelial cell death and increased permeability [2,9]. Furthermore, TiO2 NPs have been shown to play a role in inducing epithelial fibroproliferative changes, stimulating goblet cell hyperplasia and in instigating emphysema-like (such as alveolar enlargement) damages in the lungs [2,10,12]. Overall, nanotoxicity induced by TiO2 NP exposure in both the occupational and ambient environment presents a significant and realistic health concern.

The harmful effects of NPs on the respiratory system not only encompass cellular apoptosis/necrosis, but also mucus hyperproduction which is closely associated with the pathogenesis of pulmonary diseases that include asthma, COPD and CF [2,10,13]. In these chronic pulmonary diseases, mucus hypersecretion and accumulation may lead to recurrent episodes of chronic bacterial infections, limited airflow and chronic inflammatory responses [2,14,15]. However, whether TiO2 NPs can directly induce mucin secretion has not been resolved.

Airway mucus plays a vital role in the constant clearance of inhaled pathogens and particulates. Mucus is a large, highly glycosylated protein consisting of an array of mucin peptides (apomucin) [14]. With their oligosaccharide sidegroups, such as sialic acid, sulfate, and carboxyl (COO−), mucins are usually polyanionic in nature [16]. Mucin secretion is closely regulated by cytosolic Ca2+ concentrations ([Ca2+]c) in various epithelial cells [17]. A rise in [Ca2+]c is crucial for initiating a cascade of downstream events including the mobilization of granule-bound Ca2+, docking of the secretory granules, fusion of the plasma-granule membrane and the formation of secretory pores, therefore leading to the exocytosis of the mucin granules [18].
Agonist-induced opening of various Ca\textsuperscript{2+} channels expressed on the cell membrane allows the influx of extracellular Ca\textsuperscript{2+}, which may serve as the external Ca\textsuperscript{2+} source [19]. The initial upsurge in the [Ca\textsuperscript{2+}]c is usually relayed by triggering a secondary wave of Ca\textsuperscript{2+} propagation from internal stores, such as the ER [19,20,21,22]. Ryanodine receptors (RyRs) on the ER have multiple allosteric Ca\textsuperscript{2+} binding sites responsible for triggering Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) into the cytosol [19,20,21,22]. The resultant increase in [Ca\textsuperscript{2+}]c could activate other cytosolic proteins and modulate secretion of mucin, hormones or various neurotransmitters [17,23,24].

NPs have been shown to disturb cellular functions by elevating intracellular Ca\textsuperscript{2+} levels [25,26,27,28]. For example, ultrafine carbon black NPs can elicit Ca\textsuperscript{2+}-dependent secretion through the activation of L-type voltage-gated Ca\textsuperscript{2+} channels [25,26,28]. However, little is known regarding the intricate calcium signaling pathway regulating the exocytotic events of secretory products. In this study, we aim to investigate the mechanism through which TiO\textsubscript{2} NPs induce mucin secretion via a Ca\textsuperscript{2+} signaling mediated pathway.

Materials and Methods
1. Culture of ChaGo-K1 cells
The human airway bronchial epithelial cell line ChaGo-K1, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), was used because it expresses MUC proteins and secretes mucin [29]. Cells were cultured in 15 cm cell culture plates (VWR, CA, USA) in RPMI 1640 medium (Invitrogen, CA, USA) supplemented with L-glutamine, 1% penicillin/streptomycin and 10% heat inactivated fetal bovine serum (FBS). Cultures were incubated in a humidified incubator at 37°C/5% CO\textsubscript{2}. Cell counts were performed using trypan blue (Sigma-Aldrich, MO, USA) exclusion and a Bright-Line haemocytometer.

2. Nanoparticles and characterization
A mixture of anatase and rutile forms of ultrafine titanium (IV) dioxide (<75 nm) (Sigma-Aldrich, MO, USA) was used in this study because this form has been shown to result in more severe cellular injuries [30,31]. The TiO\textsubscript{2} NPs have a surface area of 36 m\textsuperscript{2}/g and the dispersion conductivity is 1040 μS/cm (information from Sigma). All NP samples were sonicated before usage. The concentrations used were 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.1 mg/ml, and 0.05 mg/ml. The range of concentrations was consistent with the concentrations of TiO\textsubscript{2} NPs found in previous reports [30]. The TiO\textsubscript{2} NPs were reconstituted with Hank's solution (Invitrogen, CA, USA) before being tested. The size of NPs was independently confirmed using homodyne dynamics laser scattering (DLS) as described in previous studies [32,33].

3. Cell preparation
Cells were seeded at 2 × 10\textsuperscript{5} cells per well in a 24-well plate per well in a 24-well plate and cultured for 24 hrs. TiO\textsubscript{2} NP prepared with calcine fluorescent dye (50 μM) (Invitrogen, CA, USA) in Hank's solution was incubated with the cells for 5 minutes at 37°C. Calcine is a biological inert green-fluorescent molecule of a molecular mass of 623 Da and an estimated molecular radius of 0.6 nm [36]. TiO\textsubscript{2} NP solution containing the calcine dye was then removed and cells were rinsed twice with PBS to remove possible remnants of calcine dye in the extracellular solution. The cells were subsequently stained with a fluorescent nucleus dye, hoechst (10 μM) (Sigma-Aldrich, MO, USA), for 5 minutes at 37°C and thoroughly rinsed again [33]. Fresh Hank's solution was added into each well before taking fluorescent images of calcine and hoechst loaded cells with a Nikon fluorescence microscope. A percentage of calcine loaded cells against total number of cells, as indicated by hoechst fluorescence, was calculated for each of the TiO\textsubscript{2} NP concentrations used in the experiment.

4. Measurements of cytosolic Ca\textsuperscript{2+} concentrations induced by TiO\textsubscript{2} exposure
The cells were then loaded with a Rhod-2 AM dye (1 μM) (K\textsubscript{d} = 570 nM, λ\textsubscript{Em} = 532 nm and λ\textsubscript{Em} = 581 nm (Invitrogen, CA, USA) for 45 minutes. After the dye loading, the cells were rinsed, incubated with either normal Hanks' or Ca\textsuperscript{2+}-free Hanks' solution, and treated with the appropriate TiO\textsubscript{2} concentrations. All calcium signaling experiments were carried out on a thermostated stage at 37°C mounted on a Nikon microscope (Nikon Eclipse TE2000-U, Tokyo, Japan). ChaGo-K1 cells were incubated with cadmium chloride (200 μM; Sigma-Aldrich, MO, USA) to block the membrane Ca\textsuperscript{2+} channels [34], followed by TiO\textsubscript{2} NP stimulation. To investigate the interaction between TiO\textsubscript{2} and membrane Ca\textsuperscript{2+} channels, nifedipine (10 μM; Sigma-Aldrich, MO, USA), an L-type Ca\textsuperscript{2+} channel blocker [27], was added to ChaGo-K1 cells prior to the exposure of TiO\textsubscript{2}. Antioxidant N-acetylcycteine (NAC, 250 μM; Sigma-Aldrich, MO, USA) was also added to ChaGo-K1 cells to study the involvement of reactive oxygen species (ROS) [27-35], possibly generated as a result of TiO\textsubscript{2} stimulation, and the activation of Ca\textsuperscript{2+} channels. Thapsigargin (100 nM; Sigma-Aldrich, MO, USA) [19] and ryanodine (100 μM; Sigma-Aldrich, MO, USA) were added separately to deplete the ER Ca\textsuperscript{2+} content and to inhibit the CICR mechanism [20,21], correspondingly. These two blockers were utilized to investigate the contribution from the internal ER Ca\textsuperscript{2+} pool.

5. Calcein dye leakage measurements
ChaGo-K1 cells were seeded at the density of 2 × 10\textsuperscript{5} cells per well in a 24-well plate and cultured for 24 hrs. TiO\textsubscript{2} NP prepared with calcine fluorescent dye (50 μM) (Invitrogen, CA, USA) in Hank's solution was incubated with the cells for 5 minutes at 37°C. The cells were then loaded with calcine fluorescent dye (50 μM) (Invitrogen, CA, USA) in Hank's solution for 5 minutes at 37°C, and thoroughly rinsed again [33]. Fresh Hank's solution was added into each well before taking fluorescent images of calcine and hoechst loaded cells with a Nikon fluorescence microscope. A percentage of calcein loaded cells against total number of cells, as indicated by hoechst fluorescence, was calculated for each of the TiO\textsubscript{2} NP concentrations used in the experiment.

6. Mucin secretion and ELLA Preparation
The cells were seeded at 2 × 10\textsuperscript{5} cells per well in a 24-well plate and cultured for 24 hrs. ChaGo-K1 cells were then rinsed with PBS and treated with BAPTA-AM (Invitrogen, CA, USA), thapsigargin (Sigma-Aldrich, MO, USA) or ryanodine (Sigma-Aldrich, MO, USA) for at least 30 minutes. Afterward the cells were stimulated for 15 minutes with the corresponding TiO\textsubscript{2} NP concentrations (0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.1 mg/ml) or ionomycin (1 μM) (positive control) (Sigma-Aldrich, MO, USA), both prepared in PBS. The supernatant containing secreted mucin was collected and briefly centrifuged at 8,000 rpm to remove the residual TiO\textsubscript{2} NPs. The supernatant was then incubated in a 96 well (Nunc MaxiSorp, VWR, CA, USA) plate overnight at 4°C. Afterward the 96-well plate was washed with PBST (PBS + 0.05% Tween-20) and then blocked with 1% BSA. The 96 well plate was washed again with PBST and incubated with lectin (Wheat germ agglutinin, WGA) (Sigma-Aldrich, MO, USA), conjugated to horseradish peroxidase (HRP; 3 μg/ml (Sigma-Aldrich, MO, USA), at 37°C for 1 hr. The substrate, 3,3',5,5'-Tetramethylbenzidine (TMB; Sigma-Aldrich, MO, USA), was added to each well at room temperature followed by H\textsubscript{2}SO\textsubscript{4} (Sigma-Aldrich, MO, USA) in order to terminate the reaction. The optical density was measured at 450 nm [37].
7. Image Analysis

After staining the treated cells, image analysis was performed with an inverted Nikon Eclipse TE2000-U fluorescent microscope. Each photo was taken at a magnification of 200× and analyzed using SimplePCI (Compix Inc., Imaging Systems, Sewickley, PA, USA). The data shown is a representative of Ca²⁺ signals of more than 200 cells.

8. Statistical Analysis

The data was presented as means±SD. Each experiment was performed independently at least three times. Statistical significance was determined using a Student’s t-test analysis with p values <0.05 (GraphPad Prism 4.0, GraphPad Software, Inc., San Diego, CA, USA).

Results

TiO₂ NP characterization

Dynamic laser scattering (DLS) was used to characterize the TiO₂ NPs. The particle size distribution ranged from ~9 to 80 nm due to minor aggregation or agglomeration while the predominant size is ~50 nm (Fig. 1A).

TiO₂ NPs induce cytosolic Ca²⁺ concentration increase

To investigate whether TiO₂ NPs could generate an increase in \([\text{Ca}^2+]_\text{C}\), ChaGo-K1 cells were loaded with Rhod-2 AM dye and exposed to 0.05–1 mg/ml of TiO₂ NPs. The change in \([\text{Ca}^2+]_\text{C}\) as represented by the fluorescence intensity within ChaGo-K1 cells, was monitored for 60 seconds. Figure 1B shows that 1 mg/ml of TiO₂ NPs induced an approximate 150% increase, while lower TiO₂ concentrations (~0.1 mg/ml) caused a minor elevation (~10%) in \([\text{Ca}^2+]_\text{C}\) when compared with untreated cells. The effect of TiO₂ treatment on the \([\text{Ca}^2+]_\text{C}\) of ChaGo-K1 cells followed a concentration-dependent manner (Fig. 1B).

Extracellular source for Ca²⁺ increase

To determine the main source of elevated \([\text{Ca}^2+]_\text{C}\) upon stimulation, ChaGo-K1 cells were exposed to TiO₂ NPs in Ca²⁺-free Hanks’ solution. EGTA (2 mM) was added in Hanks’ solution to chelate possible traces of Ca²⁺. TiO₂ (0.05 mg/ml–1 mg/ml) treatment under Ca²⁺-free conditions failed to instigate a significant increase in \([\text{Ca}^2+]_\text{C}\) (Fig. 2A). Our data suggests that the extracellular Ca²⁺ pool is the primary source of the observed cytosolic Ca²⁺ increase. We then tested whether TiO₂ NPs can induce a Ca²⁺ influx via membrane channels. Blocking the channels with CdCl₂ (200 μM) significantly inhibited an increase in \([\text{Ca}^2+]_\text{C}\) (Fig. 2B). Co-treatment of cells with TiO₂ NPs and nifedipine greatly blocked the NP-induced \([\text{Ca}^2+]_\text{C}\) increase (Fig. 2C). However, the incomplete blockage of extracellular Ca²⁺ influx via channels postulates additional Ca²⁺ leakage through perturbed cell membranes. To confirm whether TiO₂ can instigate membrane disruption, thereby permitting unspecific extracellular Ca²⁺ entry, cytosolic leakage was assessed using the fluorescent calcine dye. It was found that the dye permeation ratio increased from approximately 4 to 13% with elevated TiO₂ concentrations ranging from 0.1 to 1 mg/ml (Fig. 2D).

Oxidative stress induced Ca²⁺ influx

To demonstrate that TiO₂-evoked \([\text{Ca}^2+]_\text{C}\) increase can be associated with oxidative stress, cells were pretreated with an antioxidant, N-acetylcysteine (NAC) [27]. Pre-treatment with NAC was able to partially attenuate the increase in cytosolic Ca²⁺ level triggered by 1 mg/ml and 0.75 mg/ml TiO₂ exposure (Fig. 2E). These results support the idea that oxidative stress, induced by TiO₂ NPs, contributes to the observed \([\text{Ca}^2+]_\text{C}\) increase and promote Ca²⁺-dependent mucin secretion.

The ER as an intracellular source of Ca²⁺

In order to determine the involvement of ER Ca²⁺ pool, it was depleted by pre-incubating the cells with thapsigargin. Pre-treatment with thapsigargin impeded TiO₂ NPs from triggering a sustained increase in the cytosolic Ca²⁺ level (Fig. 3A). We then investigated the role of the CICR mechanism by blocking RYRs (ryanodine receptors) [20]. Our results revealed that CICR was largely inhibited by ryanodine (a blocker for RYR associated with the CICR response) resulting in a significantly diminished \([\text{Ca}^2+]_\text{C}\) increase induced by NPs (Fig. 3B).

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Figure 1. TiO₂ NP characterization and resultant \([\text{Ca}^2+]_\text{C}\) changes after NP treatment. A) DLS assessment of TiO₂ NPs in Hanks’ solution showed a size distribution of ~9 to 80 nm. B) Cells were treated with TiO₂ NPs with concentrations of 0.05 mg/ml (yellow), 0.1 mg/ml (Light Blue), 0.25 mg/ml (Purple), 0.5 mg/ml (Green), 0.75 mg/ml (Red), and 1 mg/ml (Blue) in normal Hanks’ solution. Each line represents the average fluorescence intensity of approximately 200 cells per well. doi:10.1371/journal.pone.0016198.g001
Ca²⁺-dependency of TiO₂-induced mucin secretion

Enzyme-linked lectin assay (ELLA) was used to assess the amount of mucin secreted from ChaGo-K1 cells when stimulated with TiO₂ NPs. When compared to the control, TiO₂ NPs increased mucin secretion by 113%, 125%, 133%, 137% and 150% at 0.05, 0.1, 0.25, 0.5 and 0.75 mg/ml, respectively (Fig. 4A). Chelating the intracellular Ca²⁺ with BAPTA-AM yielded a significant reduction in mucin secretion (Fig 4B). Addition of thapsigargin (Fig. 4C) or ryanodine (Fig. 4D) also resulted in diminished mucin secretion induced by TiO₂ NPs. Our data indicates that TiO₂-induced mucin secretion is dependent on the [Ca²⁺]ᵣ, attributed to both external and internal Ca²⁺ pools (Fig. 4A–D). Ionomycin (a Ca²⁺ ionophore) was used to elicit mucin secretion as a positive control (Fig. 5).

Figure 2. Measurement of the [Ca²⁺]ᵣ and calcein dye leakage after TiO₂ NP treatment. Cells were treated with TiO₂ NPs with concentrations ranging from 0.05 mg/ml–1 mg/ml, in A) Ca²⁺-free Hanks' solution, B) in the presence of CdCl₂ (200 µM), C) nifedipine (10 µM), D) calcein (50 µM) (n = 12, **P<0.005), and E) NAC (250 µM) (colors are as depicted in Figure 1B). doi:10.1371/journal.pone.0016198.g002
Figure 3. Measurement of $[\text{Ca}^{2+}]_c$ after stimulation by TiO$_2$ NPs. Cells were treated with TiO$_2$ NPs with concentrations ranging from 0.1 mg/ml – 1 mg/ml, in the presence of A) thapsigargin (100 nM), and B) ryanodine (100 µM) (colors used are consistent with Figure 1).

doi:10.1371/journal.pone.0016198.g003

Figure 4. Measurement of mucin secretion triggered by TiO$_2$ NPs. Cells were treated with TiO$_2$ NP concentrations ranging from 0.05 mg/ml – 0.75 mg/ml. Figure 4A) shows the relative quantification of mucin secreted after TiO$_2$ stimulation under normal conditions (n=7, **P<0.005), 4B) in the presence of BAPTA-AM (50 µM) (n=9), 4C) with pre-treatment of thapsigargin (100 nM) (n=8), 4D), and with ryanodine (100 µM) (n=5).

doi:10.1371/journal.pone.0016198.g004
Our data demonstrated that TiO2 NPs induced a concentration dependent increase in \([\text{Ca}^{2+}]_{\text{C}}\) when challenged with ZnO NPs [27]. Our results support the idea that NAC and other antioxidants may be effective in reducing NP-instigated mucin hypersecretion.

TiO2 NPs can damage cell membrane integrity by possible lipid peroxidation [27,31], thereby creating pores on the lipid bilayer [49] that may allow the transient influx of extracellular Ca2+. Our data further demonstrated that co-administration of TiO2 NPs and fluorescent calcein dye lead to intracellular leakage and the permeation efficiency increased in a TiO2 concentration dependent manner (Fig. 2D).

Titanium Dioxide Stimulates Mucin Secretion

Discussion

Recently, an increasing number of reports have shown that airborne particulate pollution found in both the ambient and working environments, particularly TiO2 NPs, can exacerbate airway diseases [1,2,3,4,5,6,10,11,38]. Aggrivated clinical manifestations of COPD, CF and asthma may include intensified symptoms of mucociliary transport impairment and mucus hypersecretion [15,39]. The resultant accumulation of thick obstructive mucus usually occupies airway lumen, thereby limiting airflow and leading to morbidity [15,39]. Despite documentations of TiO2-induced cellular nanotoxicity effects, pulmonary inflammatory responses and emphysema-like pathology [12], whether TiO2 NPs can directly trigger mucin secretion has not been resolved. In this study, we demonstrate that TiO2 NPs can stimulate mucin secretion from bronchial epithelial ChaGo-K1 cells via a Ca2+-dependent pathway.

Our study showed that TiO2 NPs can induce mucin secretion that increases as a function of TiO2 NP concentration (Fig. 4A). The TiO2 concentration range used in our study is consistent with previous reports representing the concentration found in ambient and nanotechnology industries [30,40,41,42]. While NP exposure has been long associated with increasing mucin synthesis due to goblet cell hyperplasia [13], our study indicates that TiO2 NPs can directly trigger mucin secretion in the airway.

It has been well established that intracellular Ca2+ plays a vital role in stimulus-secretion coupling [43]. Previous reports have documented that an elevated \([\text{Ca}^{2+}]_{\text{C}}\) precedes mucin granule exocytosis [17]. NP exposure has been shown to trigger an intracellular Ca2+ increase in various cells; therefore, we examined the cellular Ca2+ signaling pathway involved during TiO2 stimulation [25,28,44]. At TiO2 concentrations of 0.5, 0.75, and 1 mg/ml, there was a sustained elevation in \([\text{Ca}^{2+}]_{\text{C}}\). At lower doses (0.05, 0.1 and 0.25 mg/ml), the \([\text{Ca}^{2+}]_{\text{C}}\) increased gradually within the 1st minute (Fig. 1B). Our data demonstrated that TiO2 NPs induced a concentration dependent increase in \([\text{Ca}^{2+}]_{\text{C}}\), which is consistent with results from the mucin secretion measurements (Fig. 4A).

The stimulus-induced intracellular Ca2+ signal can be evoked by the entry of Ca2+ through voltage-gated Ca2+ channels, or by the release of Ca2+ from intracellular Ca2+ stores [43,43,46]. Previous researches have suggested that extracellular Ca2+ influx plays an important role in the elevated \([\text{Ca}^{2+}]_{\text{C}}\) during NP stimulation [25,27,28,47]. Data from experiments performed in Ca2+-free Hanks’ solution confirmed that \([\text{Ca}^{2+}]_{\text{C}}\) failed to increase when treated with TiO2 NPs (Fig. 2A). To characterize the nature of the Ca2+ influx induced by TiO2 NPs, we first evaluated the effect of cadmium chloride (CdCl2), a general Ca2+ channel blocker [34,48]. Figure 2B shows that the \([\text{Ca}^{2+}]_{\text{C}}\) remained low and relatively unchanged with CdCl2. Secondly, nifedipine, a widely used L-type Ca2+ channel blocker, markedly diminished the increase in \([\text{Ca}^{2+}]_{\text{C}}\) (Fig. 2C). The effect of nifedipine implies that TiO2 NPs can activate L-type voltage gated Ca2+ channels, allowing extracellular Ca2+ influx into the cytosol. This observation is consistent with previous reports showing that ultrafine carbon black and ZnO NP-induced \([\text{Ca}^{2+}]_{\text{C}}\) elevation can also be attenuated by nifedipine [27,28]. In addition, several reports have suggested that oxidative stress induced by NPs can exert an impact on the intracellular Ca2+ signaling pathway and that the activity of Ca2+ channels may be altered by ROS [27,28,44]. Results from Figure 2E showed that NAC significantly reduced the rising \([\text{Ca}^{2+}]_{\text{C}}\) generated by TiO2 NPs. Huang et al, has also demonstrated that NAC can attenuate the intracellular Ca2+ level when challenged with ZnO NPs [27]. Our results support the idea that NAC and other antioxidants may be effective in reducing NP-instigated mucin hypersecretion.

Increasing the \([\text{Ca}^{2+}]_{\text{C}}\) of human goblet cells has been shown to trigger degranulation [17]. We used BAPTA (cytosolic Ca2+ chelator) to test whether the increase in Ca2+ induced by TiO2 NPs could stimulate mucin exocytosis. It is evident that BAPTA significantly inhibited mucin exocytosis (Fig. 4B), indicating that TiO2 NPs can elicit a \([\text{Ca}^{2+}]_{\text{C}}\) increase, thereby leading to mucin secretion.

Besides the external Ca2+ source (Hanks’ solution), the ER is one of the major internal Ca2+ stores. Figures 3A and 4C revealed that when the ER Ca2+ had been depleted by pretreatment with thapsigargin, the TiO2 NP-induced \([\text{Ca}^{2+}]_{\text{C}}\) failed to increase significantly, and the subsequent mucin secretion was abolished. Our data indicates that the ER plays a critical role in relaying TiO2-induced Ca2+ signaling. CICR is a positive feedback mechanism where the ER amplifies a small increase in \([\text{Ca}^{2+}]_{\text{C}}\), (e.g. due to voltage-gated Ca2+ influx [22]), with the activation of RYRs that will lead to the release of more Ca2+ from the ER [19,20]. Previous studies have shown that through activation of RYRs with Ca2+, CICR can generate an overall increase in \([\text{Ca}^{2+}]_{\text{C}}\) [20,21,22]. Our data showed that ryanodine inhibited a continual rise in \([\text{Ca}^{2+}]_{\text{C}}\) when applying TiO2 NPs (Fig. 3B). Therefore, it is indicative that the TiO2-instigated increase in \([\text{Ca}^{2+}]_{\text{C}}\) was also CICR dependent. The effect of ryanodine was further demonstrated by the lack of mucin secretion under TiO2 NP stimulation (Fig. 4D).

Figure 5. Mucin secretion in response to ionomycin application (positive control, n=3). Concentration of ionomycin used was 1 μM. doi:10.1371/journal.pone.0016198.g005
In summary, our study indicates that cellular exposure to TiO$_2$ NPs can activate membrane L-type Ca$^{2+}$ channels, induce ROS production and possibly disrupt the cellular membrane. Influx of extracellular Ca$^{2+}$ into the cytoplasm raises [Ca$^{2+}]_{c}$, which in turn can trigger ryanodine receptors on the ER to release ER resident Ca$^{2+}$. The CICR mechanism of the cytosolic Ca$^{2+}$ level results in subsequent mucin secretion. More importantly, our results provide a direct link between airborne particulate matters and the pathogenesis of chronic obstructive pulmonary diseases involving mucus hypersecretion and airway obstruction. In addition, we demonstrate that once thought inert and harmless TiO$_2$ NPs can indeed interfere with intracellular Ca$^{2+}$ signaling, possibly leading to pathological states.

References

1. Alfaro-Moreno E, Navoret TS, Nemmar A, Nemery B (2007) Particulate matter in the environment: pulmonary and cardiovascular effects. Curr Opin Pulm Med 13: 86–96.
2. Gwin MR, Valkoath V (2006) Nanoparticles: health effects—pros and cons. Environ Health Perspect 114: 1818–1825.
3. Sethi S (2004) New developments in the pathogenesis of acute exacerbations of chronic obstructive pulmonary disease. Curr Opin Infect Dis 17: 113–119.
4. Aitken RW, Anderson HR, Sunyer J, Ayres JG (2001) Calcium and ROS-mediated activation of transcription factors and TNF-alpha cytokine gene expression in macrophages exposed to ultraparticles. Am J Physiol Lung Cell Mol Physiol 280: L545–L549.
5. Brown DM, Huchton L, Donaldson K, Stone V (2007) The effects of PM$_{10}$ particles and oxidative stress on macrophages and lung epithelial cells: modulating effects of calcium-signaling antagonists. Am J Physiol Lung Cell Mol Physiol 292: L1444–L1451.
6. Huang CC, Aronstam RS, Chen DR, Huang YW (2009) Oxidative stress, calcium homeostasis, and altered gene expression in human lung epithelial cells exposed to ZnO nanoparticles. Toxicol In Vitro 24: 45–55.
7. Stone V, Tumman M, Vamvakopoulos JE, Shaw J, Brown D, et al. (2000) Increased calcium influx in a monocyte cell line on exposure to ultrafine carbon black. Eur Respir J 15: 297–303.
8. Daihya R, Kwak KS, Byrd JC, Ho S, Yoon WH, et al. (1995) Mucin synthesis and secretion in various human epithelial cancer cell lines that express the major activity of diesel exhaust particles (PM$_{10}$) exposure. Toxicol Appl Pharmacol 125: 331–340.
9. Chen EY, Yang N, Quinton PM, Chin WC (2010) Functionalized Positive Nanoparticles Reduce Mucin Swelling and Dispersion. PLoS One 5: e15454.
10. Chen EY, Yang N, Quinton PM, Chin WC (2010) A New Role for Bicarbonate in Mucus Formation. Am J Physiol Lung Cell Mol Physiol.
11. Nguyen T, Chin WC, O’Brien JA, Verduzco P, Berger AJ (2004) Intracellular pathways regulating ciliary beating of rat brain ependymal cells. J Physiol 545: 131–140.
12. Kawasaki S, Takizawa H, Takami K, Desaki M, Okazaki H, et al. (2001) Benzeno-extracted components are important for the major activity of diesel exhaust particles: effect on interleukin-8 gene expression in human bronchial epithelial cells. Am J Respir Cell Mol Biol 24: 414–426.
13. Edwards DA, Pauvinet MR, Langer R, Weaver JC (1995) Analysis of Enhanced Transdermal Transport by Skin Electroperoration. Journal of Controlled Release 34: 211–221.
14. Krum PA, Sugar RA, Jackson AD (2004) Nucleotide-mediated mucin secretion from differentiated human bronchial epithelial cells. Am J Respir Cell Mol Biol 31: 446–455.
15. Stone V, Johnston H, Cliff MJ (2007) Air pollution, ultrafine and nanoparticle toxicology: cellular and molecular interactions. IEEE Trans Nanobioscience 6: 331–340.
16. Randell SH, Boucher RC, Grop UNCVL (2006) Effective mucus clearance is essential for respiratory health. Am J Respir Cell Mol Biol 35: 20–28.
17. Benzonex-L black. Eur Respir J 15: 297–303.
18. Sayes CM, Wahi R, Kurlan PA, Liu Y, West JL, et al. (2006) Correlating nanoscale titanium structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. Toxicol Sci 92: 174–185.
19. Zhang AP, Sun YP (2003) Photocatalytic killing effect of TiO$_2$ nanoparticles on L201–210. Mol Physiol 292: L1444–L1451.
20. Petersen CC, Toescu EC, Petersen OH (1991) Different patterns of receptor-activated cytoplasmic Ca$^{2+}$ oscillations in single pancreatic acinar cells: dependence on receptor type, agonist concentration and intracellular Ca$^{2+}$ buffering. J Biol Chem 278: 9896–9904.

Acknowledgments

The authors thank Prof. Pedro Verdugo and Paul Quinton for their support and encouragement during the preparation of this manuscript.

Author Contributions

Conceived and designed the experiments: EYTC MG YCW CSC WCC. Performed the experiments: EYTC MG YCW. Analyzed the data: EYTC MG YCW. Contributed reagents/materials/analysis tools: EYTC MG YCW CSC. Wrote the paper: EYTC WCC.
45. Berridge MJ, Irvine RF (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312: 315–321.
46. Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signalling. Nature 341: 197–205.
47. Brown DM, Hutchison L, Donaldson K, MacKenzie SJ, Dick CA, et al. (2007) The effect of oxidative stress on macrophages and lung epithelial cells: the role of phosphodiesterases 1 and 4. Toxicol Lett 168: 1–6.
48. Boulton CL, O'Shaughnessy CT (1991) The Effect of Calcium Channel Antagonists on Spontaneous and Evoked Epileptiform Activity in the Rat Neocortex In Vitro. Eur J Neurosci 3: 992–1000.
49. Kelly CV, Leroueil PR, Orr BG, Banazak Holl MM, Andricioaei I (2008) Poly(amidoamine) dendrimers on lipid bilayers II: Effects of bilayer phase and dendrimer termination. J Phys Chem B 112: 9346–9353.
50. Chou HT, Wen HW, Kuo TY, Lin CC, Chen WJ (2010) Interaction of cationic antimicrobial peptides with phospholipid vesicles and their antibacterial activity. Peptides 31: 1011–1020.