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Comparative plasma proteomic studies of pulmonary TiO₂ nanoparticle exposure in rats using liquid chromatography tandem mass spectrometry

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Abstract

Mounting evidence suggests that pulmonary exposure to nanoparticles (NPs) has a toxic effect on biological systems. A number of studies have shown that exposure to NPs result in systemic inflammatory response, oxidative stress, and leukocyte adhesion. However, significant knowledge gaps exist for understanding the key molecular mechanisms responsible for altered microvasculature function. Utilizing comprehensive LC-MS/MS and comparative proteomic analysis strategies, important proteins related to TiO₂ NP exposure in rat plasma have been identified. Molecular pathway analysis of these proteins revealed 13 canonical pathways as being significant ($p < 0.05$), but none were found to be significantly up or down regulated ($z > |2|$). This work lays the foundation for future research that will monitor relative changes in protein abundance in plasma and tissue as a function of post-exposure time and TiO₂ NP dosage to further elucidate mechanisms of pathway activation as well as to decipher other affected pathways.

Graphical abstract

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Keywords

Proteomics; biopathway analysis; TiO₂ nanoparticles

Introduction

Over the last several years, the production of engineered nanomaterials (ENM) has increased dramatically. Here nanoparticles (NP) are defined as species exhibiting a single dimension that is less than 100 nm in size. Such species find usage in a wide variety of products ranging from electronic and cosmetic to medical and pharmaceutical[1]. As an indication of their increasing usage, the number of consumer products sold in the United States containing ENM as well as the work force involved in the production of such products are expected to increase by more than an order of magnitude from 2008 to 2020[2]. It has recently been estimated that to support this industry, ~50,000 tons of TiO₂ nanoparticles alone are manufactured per year[3, 4]. With this acceleration in NP production comes a greater need to investigate the impact of increased NP exposure on human health.

At the turn of the 21st century, investigative studies began associating air pollution particulate matter exposure to cardiovascular events [5-9]. Since that time, significant resources[10, 11] have been dedicated to studies aimed at determining the effects of particulate matter exposure on the cardiovascular system[12-14]. Concerned with the limited applicability of macrovascular studies, a number of recent investigations have focused on the effects of particle exposure on microvasculature function [15-25]. With regard to NP exposure, such efforts have led to the development of three primary hypotheses describing processes by which exposure affects microvascular function[26]. These include systemic inflammation and changes in autonomic influence. A third process involves direct tissue-NP contact resulting from particle translocation from the lungs to the systemic microvasculature[26].

These mechanistic hypotheses generate questions regarding the roles of various molecular interactions in altering normal microvascular function. A number of experiments aimed at determining molecules associated with macrovascular dysfunction indicate that particle exposure has been shown to elicit a systemic inflammatory response. For example, circulating levels of tumor necrosis factor alpha (TNF alpha), interleukin 1 (IL-1 beta), interleukin 6 (IL-6), endothelin 1 (ET-1), and c-reactive protein (CRP) are observed to be elevated for human subjects after exposure to particles [27-29]. Analyses of bronchoalveolar lavage fluid (BALF) from animal studies have demonstrated TiO₂ NP exposure increases inflammatory cells, such as neutrophils and lymphocytes; moreover, the observed increase of inflammatory cells was found to be dependent on days after exposure [30-33], dose [32-34], and method of introduction (whole body inhalation vs. intratracheal instillation) [33]. Furthermore, gene expression analyses of lung tissue have shown immune and inflammatory responses as a result of TiO₂ particle exposure [34-36]. Additionally, markers associated with oxidative and nitrosative stresses in the microcirculation increase [20-22, 25].

From such studies a better understanding that particle exposure with microvascular dysfunction is emerging. Briefly, local inflammation in the lungs (resident macrophages and epithelial cells) due to interaction with NPs results in recruitment and activation of polymorphonuclear leukocytes (PMNs)[26]. Additionally, the local response results in increased levels of cytokines and chemokines in the blood. The circulating PMNs and cytokines result in the release of additional molecules associated with inflammation, some of which may enhance leukocyte adhesion in the microvasculature. Attachment of leukocytes to the vascular endothelium is believed to result in myeloperoxidase (MPO) deposition. Production of ROS increases local oxidative stress[25]. This ultimately decreases bioavailable nitric oxide (NO). Additionally, by-products of MPO-associated reactions result in decreased endothelial NO production. The marked decrease in local NO levels results in impaired arteriolar reactivity and further venular leukocyte adhesion.

Despite the success in determining relevant molecular responses (i.e., those potentially affecting the microvasculature) resulting from particle exposure, many factors remain to be elucidated. Currently the connection between pulmonary inflammation and the downstream microvascular effects is not well understood[26]. For example, it is unknown if secondary inflammatory markers released by activated PMNs (occurring at the lungs) affect leukocyte adherence and reactive stress within the microvasculature. Additionally, the role of secondary inflammatory markers in endothelial activation is not well known. Finally, the source of reactive stresses and their relationship to inflammatory markers and vascular activation parameters is unclear.

The current study employs comparative proteomics analyses to begin addressing the potential connections between pulmonary inflammation and microvascular dysfunction from TiO₂ NP exposure by examining relative differential abundances in proteins. Proteomics studies were conducted using LC-MS/MS under positive ion detection. Comparisons of assigned protein abundances between plasma samples obtained from rats exposed to TiO₂ NP's and a control cohort reveal a number of species that may be used to distinguish sample types. These molecules have been used in biopathway analyses to generate a molecular regulation network. The comparative proteomics, as well as the biopathway analyses, are discussed in light of recent findings associating NP exposure and microvascular dysfunction.

Materials and Methods

Samples

Experimental animals—Male Sprague Dawley rats (175 - 250 g) were purchased from Hilltop Laboratories (Scottsdale, PA), and housed in laminar flow cages under controlled temperature and humidity conditions and a 12 hr light/12 hr dark cycle at the West Virginia University Health Sciences Center vivarium. Food and water were provided *ad libitum*. All animals were acclimated for 72-hours before nanomaterial inhalation exposure. To ensure that all methods were performed humanely and with regard to alleviation of suffering, all procedures were approved by the Institutional Animal Care and Use Committee of the West Virginia University.

ENM—Nano-titanium dioxide (TiO₂) powder was obtained from Evonik (Aeroxide TiO₂, Parsippany, NJ). This ENM is a mixture composed of anatase (80%) and rutile (20%) TiO₂, with a primary particle size of 21 nm, and a surface area of 48.08 m²/g[24, 37, 38]. The nano-TiO₂ was prepared for aerosolization by drying, sieving, and storing the powder[19, 24].

Inhalation Exposure—We have previously reported and described our Nanoparticle Aerosol Generator and exposure system used for the current experiments (U.S. Patent #8,881,997)[19, 24, 39, 40]. The system was developed specifically for rodent, whole-body nanoparticle inhalation exposures. Briefly, the apparatus was developed with a vibrating fluidized bed, a Venturi vacuum pump, cyclone separator, impactor and mixing device, an animal housing chamber, and real-time monitoring devices. Aerosols were generated by allowing a high velocity air stream to pass through the vibrating fluidized bed and into the Venturi vacuum pump; drawing air and the TiO₂ nanoparticles as it passes. Aerosols enter the cyclone separator, which is gated to removed agglomerates >400 nm at an input flow rate of 60 L/min of clean dry air before entering the exposure chamber.

Size distribution, mean aerodynamic diameter, and relative mass concentration of the aerosols were monitored in real time (Electrical Low Pressure Impactor (ELPI), Dekati, Tempere, Finland). The particle size distribution was also measured in real-time with a Scanning Mobility Particle Sizer device (SMPS; TSI Inc., St. Paul, MN). The real-time size distribution and mass concentration of the aerosols were monitored between 7-1000 nm with these devices. The count median aerodynamic diameter of the aerosols was ~160 nm. Once the steady-state aerosol concentration was achieved, exposure duration was adjusted to achieve a calculated deposition of 30 µg/animal. Animals were exposed for 4-6 hours per day for at a target mass concentration of 6.0 mg/m³ or to filtered air (0 mg/m³, control).

Blood collection and plasma isolation—24 hours after ENM inhalation exposure, rats were anesthetized by an intraperitoneal injection of either sodium thiopental or thiobutabarbital sodium (100 mg/kg) and placed on a heating pad to maintain a rectal temperature of 37 °C. The trachea was intubated to ensure a patent airway, and the right carotid artery was cannulated to measure arterial blood pressure and sample whole blood. Whole blood was collected via the carotid artery cannula from live animals directly into Vacutainers containing K₂ EDTA (BD Biosciences, San Jose, CA). Blood samples were immediately centrifuged for 10 minutes at 2500 g. The plasma layer (supernatant) was then transferred into 1.5 mL microcentrifuge tubes. These tubes were snap frozen in liquid nitrogen before being placed at -80 °C for long term storage.

Proteomics Extraction Procedures

Immunodepletion—A Seppro IgY14 immunodepletion kit was utilized (Sigma-Aldrich, St. Louis, MO) for immunodepletion of the 14 most abundant proteins in plasma. Prior to depletion, 20 µL of plasma was diluted with 500 µL of 1× Dilution buffer, added to a Corning Costar Spin X 0.45 µm column (Sigma-Aldrich, St. Louis, MO), and centrifuged at 10000 g for 1 minute. 500 µL of the diluted plasma was immunodepleted using Seppro IgY14 spin columns according to the manufacturer's protocol. Immunodepleted samples

were frozen at -80°C overnight and then dried down in a vacuum concentrator at 35°C for 8 hours. Samples were reconstituted in 1 mL of 200 mM ammonium bicarbonate with 6 M urea in water for further sample preparation.

Bradford Analysis—Total protein concentration of the immunodepleted samples was determined using a Bradford Assay. Standards of BSA (0, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 $\mu\text{g}/\mu\text{L}$) were prepared and all samples and standards were analyzed in duplicate. Protein concentrations were determined at 595 nm on an Epoch Biotek UV-vis spectrophotometer (BioTek, U.S., Winooski, VT). The average sample concentration was calculated and the amount of sample needed to react with 2 μL of 0.01 M DTT at a concentration of 1:40 (protein:DTT) was determined.

Protein reduction, alkylation, and digestion—Plasma protein extractions were prepared as outlined by Valentine, *et al* with modifications[41]. The calculated aliquot of sample was diluted to a volume of 1 mL with 200 mM ammonium bicarbonate with 6 M urea. 2 μL 0.01M of DTT was added and the samples were incubated at 37°C for 2 hours. Samples were placed on ice and 4 μL of 0.01 M iodoacetamide (IAM) was added. Samples were incubated at 4°C for 2 hours in the dark. 2 μL of 0.01 M cysteine was added and samples were incubated at room temp for 30 minutes. 1 mL of 25 $\mu\text{g}/\text{mL}$ trypsin was added and samples were incubated at 37°C for 18 hours. After trypsin digestion, samples were stored at -80°C overnight and then dried down in a vacuum concentrator for 8 hours at 35°C . Samples were reconstituted in 150 μL sample buffer (95/5 $\text{H}_2\text{O}/\text{ACN}$ + 0.5% TFA) and desalted using Pierce C18 Spin columns and manufacturer's protocol. After desalting, samples were dried down at 35°C and reconstituted in 50/50 $\text{H}_2\text{O}/\text{ACN}$ with 0.1% formic acid for LC-MS/MS analysis.

Proteomic analysis by LC-MS/MS—A Thermo Scientific Q Exactive with an Easy-nLC 1000 nano-LC was utilized (Thermo Fisher, San Jose, CA). 18 μL of sample was injected onto a Thermo Scientific EASY-spray PepMap C18, 3 μm , 75 $\mu\text{m} \times 150$ mm column (Thermo Fisher, San Jose, CA) with the column temperature set to 35°C . Mobile phases were A – H_2O with 0.1% formic acid, and B – ACN with 0.1% formic acid. At a flow rate of 20 $\mu\text{L}/\text{min}$, the sample gradient was as follows: 40 minute linear gradient from 2% to 60% B; 20 minute linear gradient to 90% B and hold for 10 minutes; 5 minute linear gradient back to initial conditions and hold for 5 minutes. MS settings were: positive mode; m/z range 150-2000; MS resolution 70,000 and automatic gain control (AGC) 1E6; data dependent MS/MS top 5 with S/N 5, resolution of 140,000, AGC 1E5, normalized collision energy (NCE) 30, and isolation window 4.0 m/z ; spray voltage 1.80 kV; and S lens 60 V. Samples were analyzed in a random order.

Informatics

Proteomics—Raw data files were analyzed with Proteome Discoverer (v. 1.4.0.288; Thermo Fisher, San Jose, CA) using the Sequest algorithm to search against the rat.fasta database. Precursors were selected in a retention time window from 0 to 60 minutes with a mass range from 150 to 5000 Da. Charge states examined were +1 to +4. The mass analyzer was set to FTMS, activation type to HCD, ionization source to nanospray, and polarity to

positive mode. The Sequest algorithm searched for peptides ranging in length from 6 to 144 residues, allowing for 2 missed cleavages. A single dynamic modification of carbamidomethyl was selected and the maximum modifications per peptide were set at 3. Percolator confidence intervals were set at $p = 0.05$. The decoy database search, which attempts to match the peptide spectra against a reversed protein database, was set at $p = 0.1$ for relaxed target and $p = 0.05$ for strict target. All other default settings were used. Protein identifications within this publication and supplementary material are noted by their Uniprot Accession identifier.

Protein comparisons based on the total number of high-confidence peptide ion assignments were carried out using the Access Database software (Microsoft Office 2007, Microsoft Corporation, Redmond, WA). Additional abundance comparisons were performed by first determining an abundance score based on the exponentially modified protein abundance index (emPAI) technique described previously[42, 43]. The emPAI score (S) is computed as[43]

$$S = 10^{\frac{N_{obs}}{N_{exp}}} - 1, \quad (1)$$

where N_{obs} is the total number of peptide ion assignments that are observed for a specified protein and N_{exp} is the expected number of digest peptides from the protein sequence. For these comparisons, N_{exp} is determined using the protein sequence length and the frequency of occurrence of Lysine (~7.2%) and Arginine (~4.2%) amino acid residues[44]. The number of expected tryptic digest peptides for a given protein was estimated to be the product of the sequence length and the sum of occurrence frequencies (expressed as a decimal) of the two amino acid residues. This emPAI score represents protein abundances in principal component analysis (PCA), t-tests, and Ingenuity Pathway Analysis (IPA).

The top 58 proteins in the highest abundance (at least 3 sample hits) across all samples were chosen to supply the y -values (protein emPAI scores) for unsupervised PCA using the JMP Statistical software package (v. 11.2.0, SAS Institute Inc., Cary, NC)[45]. The PCA report includes a loadings matrix value (LM), which demonstrates the degree of effect that a y -variable has on the principal component. LMs range from 0 to 1, where values closer to 1 signify a greater influence on the separation associated with a component. Our data showed 19.4% of the difference between the two groups was in principal component 1 (PC1) and 16.6% in principal component 2 (PC2). The center of mass for each group was found by averaging the PC1 and PC2 coordinates of the group members. The x and y distances between the center of masses in PC1 and PC2 were used as weighting factors to loadings matrix values. A loadings matrix vector value (LMV) for each protein was then calculated by equation 2:

$$LMV = \sqrt{(w_1)(v_1)^2 + (w_2)(v_2)^2} \quad (2)$$

where w_1 and w_2 are the weighting factors for PC1 and PC2, respectively, and v_1 and v_2 are the respective PC1 and PC2 loadings matrix values for each protein. The sample coordinates, centers of mass, and weighting factors can be found in the supplementary material on the “JMP loadings results” tab. The LMVs were then used to determine proteins

exhibiting the greatest degree of variation between the control and exposed samples. The proteins (38 in total) that contributed the most ($LMV > 0.8$) to the separation PC1 and PC2 were input into QIAGEN's IPA (QIAGEN Redwood City, www.qiagen.com/ingenuity) to determine molecular pathways influenced by pulmonary nanoparticle exposure.

Biopathway Analysis—Biological pathways associated with the proteins with the greatest degree of variation between the two sample sets were generated using IPA. The biological pathways were obtained from a molecular input list comprised of the 38 proteins and the fold difference between the average normalized emPAI score of the exposed samples to the average normalized emPAI score of the control samples. IPA uses the fold differences in order to determine up or down regulation of pathways. The 20 most abundant proteins by LMV value and their fold differences are listed in Table 1. For the full complement of data, see supplementary data “IPA input” tab. To generate the biological pathways, the core analysis function in the Ingenuity software was performed on the input molecules. The Ingenuity Knowledge Base was used as the reference set for analysis considering direct and indirect relationships. Molecules and relationships were restricted to rat or undefined species. The number of molecules in networks was restricted to 35 with 25 maximum networks. The top 3 canonical pathways that were found to be most significant based on a calculated p score have been selected for discussion (see Biopathway analysis).

It should be noted that there are limitations associated with using biopathway analysis software to determine relevant biological pathways based on differentially expressed genes, and protein and small molecule abundance differences [46]. For example, in using pathway topology software such as IPA, biopathways are dependent on the type of cell and condition or disease state analyzed. The results are a snapshot of the biological system at a given time point and are not able to fully characterize the dynamic states of the system. Furthermore, the interdependence of pathways may not be realized due to weak links between pathways [46]. Despite the limitations, the information gained from biopathway analysis is useful as a starting point in understanding cause and effect relationships resulting from biological stressors.

Results and Discussion

Identified proteins and peptides

All identified proteins and peptides were analyzed at the 95% confidence interval. The LC-MS/MS analysis employed here resulted in identification of 8275 total peptides with 1361 unique peptides identified; 1114 and 881 unique peptides were from control and exposed samples, respectively. Lengths of peptides observed ranged from 6 to 47 for both the control and exposed samples. Peptide charge states for the control samples have been identified as $[M+4H]^{4+}$, $[M+3H]^{3+}$, $[M+2H]^{2+}$, and $[M+H]^+$, and comprise ~7.9%, ~36.9%, ~44.9%, and ~10.2%, respectively, of the total peptide compliment. Similarly, the same peptide charge states for the exposed samples were found to be ~7.6%, ~39.2%, ~47.7%, and ~5.4%, respectively of the total peptide compliment. Using the ratio of the total number of high confidence peptides to the total number of strict target decoy database peptide hits yielded an actual false discovery rate (FDR) of 3.8%[47].

367 total proteins were identified from all the samples; 78 proteins were identified by at least 2 unique peptides. Control samples (n=7) had 260 total proteins and 72 proteins with at least 2 unique peptide hits, and exposed samples (n=7) had 207 total proteins and 61 proteins with at least 2 unique peptide hits. In order to determine the FDR of proteins, peptide hits were randomly removed equal to the number of false positives (3.8% of 8275) [48]. The number of proteins identified by the number of remaining peptides was divided by the number of total proteins identified and subtracted from 100%. This resulted in an FDR of 2.3%. For a full description of the protein complement, see the supplementary information “Appended emPAI scores” and “emPAI scores from Access” tabs.

The protein locations and functions were found using the QuickGO browser [49]. The 367 identified proteins represent 195 unique locations within the cell of 833 total locations identified. The top 19 protein locations totaling 500 hits and their location counts are shown in Figure 1A. 319 unique protein functions were identified from a total of 833 functions. The top 18 functions totaling 308 hits and their individual function counts are shown in Figure 1B. For a complete list of the locations and functions see supplemental material “Protein locations” and “Protein biofunctions” tabs.

The dynamic range of proteins in rat plasma is similar to that of humans, approximately 9 orders of magnitude [50]. Immunodepletion columns are commonly used to remove a large portion of the most abundant proteins within plasma; for example, albumin and IgG are reported to comprise 80-85% of the total plasma protein. The immunodepletion process does not completely deplete the targeted compounds; in our analysis albumin was still found to comprise a significant fraction of the protein content. Even after immunodepletion the dynamic range of the serum proteins is still several orders of magnitude; however, the detection of mid and low abundant compounds is improved [50].

Protein comparison

emPAI scores were calculated (see equation 1 in Methods: Informatics above) for each identified protein in each sample. The resulting scores and associated proteins were input into a database (Microsoft Access) for query analysis. The compiled emPAI scores for each protein were normalized to the total emPAI value for each sample. Proteins that had 3 or more sample hits were selected to be used for inputs for PCA. Proteins with 2 or fewer samples hits were considered to be indeterminate because they were found in only less than 15% of the samples, whereas proteins with 3 or more sample hits are found in approximately 20% of the samples. See supplementary information for emPAI scores used for PCA inputs on the “Selection of proteins” tab. As a result of this filtering, a total of 58 proteins were selected to be used as inputs for PCA (“JMP import” tab supplementary material) and IPA (“IPA input” tab supplementary material).

The PCA of proteins shows a separation of the control and exposed samples primarily along principal component 2 (PC2, Figure 2). Additionally, a smaller deviation is observed along PC1 where the majority of the exposed and control samples are observed to exhibit positive and negative PC1 values, respectively. One exposed sample is shown to have clustered with the control samples; however, the sample was not excluded because the emPAI scores for 43 out of 58 proteins (74.1%) were within 1 standard deviation of the mean exposed emPAI

score. Another exposed sample appears to be an outlier, but it was not excluded because the emPAI scores for 57 out of 58 proteins (98.3%) were within 1 standard deviation of the mean exposed emPAI score. 38 proteins were shown to contribute to the separation in PC1 and PC2 at LMV values > 0.8 and subsequently, these proteins were used as inputs for IPA. Table 1 shows the top 20 protein inputs based on the highest summed emPAI scores. Complete loadings matrix values of PC1 and PC2 and the calculated loadings matrix vector can be found in supplementary information on the “JMP loadings results” tab.

A t test was also performed on the emPAI scores for the 58 proteins (see supplementary information “JMP import” tab). 7 proteins were found to be significantly different ($p < 0.05$); 6 of these 7 proteins were found to be significant in the separation of the groups by PCA. The 7 significant proteins are: inter-alpha trypsin inhibitor, heavy chain 1 (accession B2RYM3), protein Itih4 (accession D3ZFC6), apolipoprotein A-I (accession P04639), alpha-2-macroglobulin (accession P06238), serine protease inhibitor A3N (accession P09006), serotransferrin (accession P12346), hemopexin (accession P20059). The 7 proteins and their fold differences were used separately as inputs for IPA (see supplementary information “IPA import significant proteins” tab). The resulting biopathway analysis revealed the same top 3 canonical pathways as being significant ($p < 0.05$) as with the 38 proteins from PCA. There were differences in the z scores compared to the IPA of 38 proteins, but none were considered to be significantly up or down regulated (see supplementary information “IPA output significant proteins” tab). The IPA results from the 38 protein inputs represent a more in depth analysis and the remainder of this manuscript will focus on the results for the 38 proteins.

Biopathway analysis

In order to better understand the molecular differences between the two sample groups, a biopathway analysis (see Methods: Informatics above) has been conducted using molecular inputs obtained from the proteomics data. Proteins exhibiting weighted (see Protein comparison above) LMV values > 0.8 from the PCA (38 proteins) were input into IPA to search for interactions with the Ingenuity Knowledge Base molecules (see “IPA input tab” in supplemental information). Input molecules that were found to interact with database molecules were deemed network eligible and a network was generated such that the interactions of the network eligible molecules were maximized. The network was given a score based on the probability of observing the network by chance. The top network generated by IPA as determined by the 38 proteins was given a score of 41. The network is shown in Figure 3 and a list of the network molecules and their relationships can be found in supplemental material on the “Network Relationships” tab.

18 input molecules were found in the network (Figure 3), comprising approximately 1/2 of the molecules in the network. Of the 18 input molecules, 9 are increased in abundance in the exposed samples and 9 are decreased in abundance in the exposed samples. The network was characterized by Cytoscape[51]. There are 37 nodes with no isolated nodes, 26 multi-edge node pairs, and 37 self loops. The network diameter, or the largest distance between two nodes is 6 and the network radius, or the minimum non-zero eccentricities of the nodes is 1. The characteristic path length, or the average shortest path length is 2.842 and the

number of nearest neighbors is 5.892. The number of connected components is 2, indicating strong connectivity; 19 of the 35 molecules in the network (54.3%) have 4 or more connections. The network hubs are Prothrombin (F2) and Interleukin-1 (IL1) with 19 and 18 connections, respectively. IPA functional analysis of this network found the top functions associated to be cardiovascular disease, hematological disease, and organismal injury and abnormalities.

Canonical pathways were generated based on the 38 input proteins. The most significant pathways, those with p values <0.05, are found in Figure 4. The top 3 canonical pathways based on p value were found to be acute phase response signaling, farnesoid X receptor/retinoid x receptor (FXR/RXR) activation, and liver x receptor/retinoid X receptor (LXR/RXR) activation. A z score for the pathway is determined from the predicted up or down regulation of a molecule. Those that are predicted to be up regulated are given a +1 (N₊) and those that are predicted to be down regulated are given a -1 (N₋). The N₊ and N₋ are summed to give N. The z score is then calculated by equation 3 [52]:

$$Z = \frac{x}{\sigma_x} = \frac{N}{\sqrt{N}}, \quad (3)$$

where a z score of $|z| \geq 2$ is considered significant. Acute phase response signaling pathway was found to have a positive z score, or is up regulated, and the LXR/RXR activation pathway to have a negative z score, or is down regulated. However, neither canonical pathway was found to be significantly changed ($|z| < 2$). Biopathway analysis implicated the top 3 biological functions based on the p value as synthesis of reactive oxygen species, coagulation of blood, and damage of liver. Synthesis of reaction oxygen species has a negative z score (down regulated), but the associated z score is not large enough to be considered significantly down regulated. The other 2 biological functions did not have associated z scores. Furthermore, there were no other biological functions with z scores that were considered significant. The top toxicological functions based on p value were damage of liver, proximal tubular toxicity, and injury of liver. There are no associated z scores with the toxicological functions. The associated molecules, p-values, and z scores for each pathway and function mentioned above are listed in Table 2. See supplementary information for complete bioanalysis data on the “IPA output” tab. We will focus the remainder of our discussion on the canonical pathways found to be most significant from our 38 input proteins.

LXR/RXR activation plays a role in regulating lipid metabolism as well as the catabolism of cholesterol to bile acids. FXR/RXR activation regulates many metabolic processes including the regulation of bile, lipids, and glucose and is often complementary and reciprocal to LXR/RXR activation. It is well known that changes in lipid and glucose metabolism are associated with many diseases including type 2 diabetes, obesity, and cardiovascular disease [53]. The p values of these pathways suggest that these pathways are significant in relation to TiO₂ NP exposure; however, our data does not indicate these pathways as being significantly up or down regulated.

Other researchers have studied the effect of NP exposure on lipid and cholesterol levels. Bourdon and coworkers exposed C57BL/6 mice to carbon NPs via intratracheal instillation and analyzed the high density lipoprotein (HDL), low density lipoprotein (LDL), and cholesterol levels at 1, 3, and 28 days post exposure[54]. They found a significant decrease in plasma HDL at 3 and 28 days post exposure, an increase in plasma LDL at 28 days post exposure, and a marginal increase in hepatic cholesterol at 28 days post exposure. Another research group investigated the effect of nickel hydroxide NP via whole body inhalation exposure for 5 hours per day, 5 days per week for a duration of 1 week or 5 months on apoprotein E-deficient mice[55]. In the examination of plasma LDL and cholesterol concentrations, there was found to be no significant difference between exposed and control samples despite the marked increase in atherosclerosis. Additionally, Hu and coworkers exposed apoprotein E-deficient mice to TiO₂ NPs at 3 different exposure levels via intratracheal instillation for 6 weeks[56]. The plasma HDL levels were decreased in the high exposure group compared to control and the plasma organic lipid ratios were increased in the high and medium exposure groups relative to the control. The medium and high exposure groups also showed a significant increase in the progression of atherosclerosis.

Although it cannot be discounted that the aforementioned studies have differences in the type of NP exposure and model organism used, these studies suggest that lipid and cholesterol metabolism are affected as a result of NP exposure. The biopathway analysis of our data indicated that the LXR/RXR activation and FXR/RXR activation pathways are significantly related to the proteins found to contribute to exposed and control sample differences as elucidated by PCA. The results of the previous studies also suggest that significant differences in these pathways at later time points relative to exposure may also be observed. We plan to examine the metabolites, including lipids and cholesterol, and proteins at multiple time points post exposure in order to determine if there is a significant up or down regulation in the LXR/RXR activation and FXR/RXR activation pathways as a result of TiO₂ NP exposure.

LXR/RXR activation pathway also plays a role in the regulation of inflammation. Acute phase response signaling pathway is a rapid inflammatory response that can be initiated by tissue injury or trauma. It is interesting to note that there is a positive z score (up regulation) associated with acute phase response signaling, and a negative z score (down regulation) associated with LXR/RXR activation, but neither is considered significant. Others have observed an up regulation of the acute phase response signaling pathway in response to TiO₂ NP exposure via gene expression analysis of mouse lung tissue[35, 36]. Husian and coworkers examined differentially expressed genes from mouse lung tissue after intratracheal instillation of low, medium, and high concentrations of TiO₂ NPs at 1, 3, and 28 days post exposure. Biopathway analysis of the differentially expressed genes revealed a significant up regulation in the acute phase response signaling and LXR/RXR activation pathways at 1 day post exposure and at all doses[36]. In a separate paper, Saber and coworkers examined the BALF for inflammatory cells in these same mice. The increase in the inflammatory cells found in BALF correlated to an increase in the up regulation of acute phase response signaling and LXR/RXR activation pathways, except at the low dose concentration. The low dose exposure compared to the control did not show a significant difference in the inflammatory cell concentration in BALF at any time point[32]; however,

the genes associated with the inflammatory response were found to be perturbed indicating that inflammatory signaling has been initiated, but without a measurable change in the inflammatory cells[36]. Halappanavar and coworkers also noted an up regulation of the acute phase response pathway as a result of gene expression analysis of mouse lung tissue and an increase in inflammatory cells in BALF as a result of whole body inhalation of TiO₂ NPs [35]. While there are noted differences in these studies and ours, such as the species used, amounts of TiO₂ NPs exposure, and tissue examined, parallels can be drawn from these studies to our results. We hypothesize that while acute phase response signaling and LXR/RXR activation pathways are observed to be highly associated with the input proteins from plasma, a significant up regulation in these pathways should be observed in vasculature and lung tissues. Future comparative proteomic studies of plasma, vasculature, and lung tissue will be performed to test this hypothesis.

As with the previous studies noted time dependent changes in lipid and cholesterol metabolism [54-56], it would be also prudent to examine different time points post exposure to determine when, or if, a significant up regulation pathway(s) related to the inflammatory response are observed in plasma. Demonstrated by the work of Gustafsson and coworkers, the BALF inflammatory cell concentrations of TiO₂ exposed rats via intratracheal instillation were examined at 1, 2, 8, 16, 30, and 90 days post exposure[30]. The authors demonstrated an increase in the inflammatory cells peaking at approximately 16 days post exposure. This work suggests that although an analysis of plasma in the present study demonstrated a non-significant up regulation of the acute phase response at 24 hours post exposure, additional time points are needed in order to observe changes in the regulation of the inflammatory response.

Conclusion

In order to determine the molecular pathways involved after pulmonary exposure to TiO₂ NPs, comparative proteomic analyses was performed. 38 proteins were identified as being able to distinguish between the two sample cohorts. Ingenuity Pathway Analysis of the 38 proteins indicated the significant pathways associated with these molecules to be acute phase response signaling, LXR/RXR activation, and FXR/RXR activation. The acute phase response signaling pathways was found to be up regulated and the LXR/RXR activation pathway was found to be down regulated, although neither significantly. The top 3 significant biological functions were synthesis of reactive oxygen species, coagulation of blood, and damage of liver. Synthesis of reactive oxygen species was found to be down regulated (negative z score), but this biological function was not significantly down regulated.

We have planned further studies to examine larger cohorts and perform metabolomic and proteomic analyses of plasma, vascular, and lung tissue. Furthermore, we will examine different amounts of NP exposure to examine the differences in molecular response and will examine responses at time points from hours to days post exposure to determine molecular response changes over time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Physiological examination of vasculature tissue of rats exposed to TiO₂ nanoparticles (NPs) has shown microvasculature dysfunction resulting from pulmonary inflammation. This study begins to elucidate the molecular mechanisms responsible for microvasculature dysfunction by examining relative differential abundances in plasma proteins through bottom-up proteomic strategies. Principal component analyses of the protein differences indicated 38 proteins which contribute the most to the separation of the two groups along principal component 1 and principal component 2. Biopathway analysis of the 38 proteins showed 13 pathways which are significantly different ($p < 0.05$) as a result of TiO₂ exposure, but none of the pathways were found to be significantly up or down regulated ($z > |2|$). This work lays the foundation for future proteomic studies to further examine plasma and tissue samples from rats exposed to TiO₂ NPs.

Highlights

- 38 plasma proteins contributed most to the separation in PCA.
- Biopathway analysis revealed 13 pathways as significantly different between groups.
- None of the biopathways were found to be significantly up or down regulated.
- Future studies will examine plasma and tissue at multiple time points and doses.

Figure 1.

A. The top 19 protein locations, totaling 500 hits, along with the counts for all identified proteins in control and exposed samples. **B.** The top 18 protein functions totaling 308 hits for all identified proteins in control and exposed samples.

Figure 2.

PCA scatter plot, generated in JMP, is derived from the emPAI scores of the 58 proteins identified by at least 2 unique peptides and found in 3 or more samples. Control samples are shown in red and exposed samples are shown in blue. Sample regions are shown as semi-transparent shading of the correlating color.

Figure 3.

Network 1 from IPA showing the inter-relationships between relevant proteins. Solid lines indicate direct relationships and dashed lines indicate indirect relationships. Loops represent feedback systems. The inter-relationships shown are known from the IPA database. The 38 input molecules are highlighted in green (increased in abundance) and red (decreased in abundance). The network hubs are Prothrombin (F2) and Interleukin-1 (IL1).

Figure 4.

IPA canonical pathways generated from input molecules. The bars indicate the p-value of the pathway; blue bars indicate a negative z-score (down regulation of the pathway) and orange bars indicate a positive z-score (up regulation of the pathway). The orange boxes indicate the ratio of the number of input molecules to total number of molecules in the pathway.

Table 1

Protein inputs for Ingenuity Pathway Analysis

Accession ^a	Description ^b	Symbol ^c	Fold difference ^d	Control Ave $\pm$ Std ^e	Exposed Ave $\pm$ Std ^e	Sequence Coverage ^f	Weighted Vector Score ^g
P20059 ^h	Hemopexin	Hpx	1.48	3.96 $\pm$ 1.12	5.84 $\pm$ 1.18	19.13 - 56.74%	1.65
P09006 ^h	Serine protease inhibitor A3N	Serpina3n	-2.88	3.52 $\pm$ 0.67	1.22 $\pm$ 0.67	8.37 - 43.54%	1.64
P14046	Alpha-1-inhibitor 3	Ali3	-1.89	4.45 $\pm$ 1.25	2.36 $\pm$ 2.25	21.87 - 46.24%	1.56
P14480	Fibrinogen beta chain	Fgb	-1.74	2.61 $\pm$ 1.19	1.50 $\pm$ 0.95	5.64 - 35.70%	1.50
G3V9J1	Protein LOC297568	LOC297568	**	**	1.96 $\pm$ 2.50	21.94 - 42.92%	1.49
F1LN83	Fetuin-B	Fetub	-1.27	2.80 $\pm$ 0.63	2.21 $\pm$ 1.22	15.34 - 44.18%	1.43
F1M983	Protein Cfh	Cfh	-1.65	0.62 $\pm$ 0.41	0.37 $\pm$ 0.38	2.76 - 14.34%	1.41
P04639 ^h	Apolipoprotein A-I	Apoa1	1.58	7.60 $\pm$ 2.61	12.0 $\pm$ 4.20	30.50 - 62.55%	1.36
G3V843	Coagulation factor II, isoform CRA_a	F2	-2.63	0.34 $\pm$ 0.43	0.13 $\pm$ 0.34	4.03 - 12.48%	1.35
H6X2W5	C-reactive protein, pentraxin-related	Crp	1.12	3.15 $\pm$ 0.84	3.54 $\pm$ 2.88	16.09 - 24.78%	1.33
G3V615	Complement factor B, isoform CRA_b	Cfb	-1.24	0.91 $\pm$ 0.81	0.73 $\pm$ 0.70	8.39 - 26.21%	1.32
P02770	Serum albumin	Alb	1.42	2.29 $\pm$ 1.12	3.24 $\pm$ 1.14	5.92 - 50.00%	1.30
B2RYM3 ^h	Inter-alpha trypsin inhibitor, heavy chain 1	Itih1	-1.92	0.77 $\pm$ 0.11	0.40 $\pm$ 0.33	3.32 - 13.05 %	1.30
P55159	Serum paraoxonase/arylesterase 1	Pon1	*	0.51 $\pm$ 0.65	*	14.37 - 22.82%	1.30
P12346 ^h	Serotransferrin	Tf	1.35	4.29 $\pm$ 1.29	5.82 $\pm$ 1.17	15.62 - 47.74%	1.23
D3ZFC6 ^h	Protein Itih4	Itih4	-2.89	1.52 $\pm$ 0.89	0.52 $\pm$ 0.52	2.58 - 21.35 %	1.18
P05544	Serine protease inhibitor A3L	Serpina3l	1.56	2.94 $\pm$ 2.83	4.60 $\pm$ 2.25	27.36 - 60.29%	1.17
F1M4L8	Complement component-3/alpha-2-macroglobulin	Cpand8	-1.03	1.20 $\pm$ 0.85	1.17 $\pm$ 0.82	11.74 - 18.13%	1.17
G3V9R9	Afamin, isoform CRA_c	Afm	1.37	1.93 $\pm$ 0.63	2.65 $\pm$ 0.83	5.26 - 41.12%	1.16
P01026	Complement C3	C3	1.02	3.90 $\pm$ 0.53	4.00 $\pm$ 0.79	22.49 - 47.29%	1.12

List of top 20 proteins identified by PCA used as inputs for IPA. Where **a** – Uniprot protein Accession number, **b** – Uniprot protein name/description, **c** – Uniprot protein symbol, **d** – the ratio of the average emPAI score for the exposed samples to the average emPAI score for control samples, **e** – the average and standard deviation of the normalized emPAI scores multiplied by a scaling factor of 100, **f** – sequence coverage ranges are reported except where all samples had the same sequence coverage, **g** – the weighted vector score calculated by equation 2, and **h** – proteins found to be significantly different (p < 0.05) by t test.

* indicates the protein was not detected in the exposed samples.

** indicates the protein was not detected in the control sample.

Table 2

Top statistically significant results from Ingenuity Pathway Analysis

Ingenuity Canonical Pathways	P value	Z score	Molecules
Acute Phase Response Signaling	2.00E-28	0.302	HPX, C3, APOH, C9, AHSG, SERPINA3, FGG, F2, ALB, APOA1, TF, ITIH4, CRP, CFB, SERPINA1, FGB, FGA
FXR/RXR Activation	8.94E-24	-	APOE, HPX, APOB, C3, APOH, FETUB, AHSG, C9, ALB, APOA1, TF, ITIH4, SERPINA1, FGA
LXR/RXR Activation	5.01E-22	-0.277	APOE, HPX, APOB, C3, APOH, AHSG, C9, ALB, APOA1, TF, ITIH4, SERPINA1, FGA

Biofunctions - Categories	Diseases or Functions	P value	Z score	Molecules
Free Radical Scavenging	Synthesis of Reactive Oxygen Species	1.34E-14	-1.161	APOE, ALB, APOA1, C3, TF, ITIH4, CRP, CFB, SERPINA3, CFH, SERPINA4, F2
Hematological System Development and Function, Organismal Functions	Coagulation of Blood	3.44E-11	-	APOE, C3, APOH, CRP, C9, FGA, F2
Gastrointestinal Disease, Hepatic System Disease, Organismal Injury and Abnormalities	Damage of Liver	5.34E-08	-	ALB, SERPINA1, CFH, SERPINA4, F2

Tox Functions - Categories	Diseases or Functions	P value	Molecules
Liver Damage	Damage of Liver	5.34E-08	ALB, SERPINA1, CFH, SERPINA4, F2
Renal Damage, Renal Tubule Injury	Proximal Tubular Toxicity	1.52E-05	HPX, ALB, FGB, FGA
Liver Damage	Injury of Liver	5.34E-05	SERPINA1, CFH, F2

Top statistically significant canonical pathways, biological functions, and toxicological functions as determined from the 38 proteins input into Ingenuity Pathway Analysis.