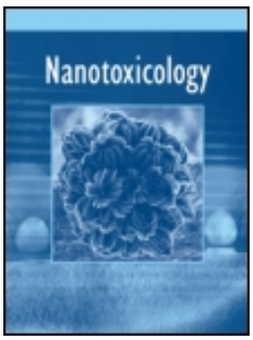

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TiO_2 nanoparticles induce insulin resistance in liver-derived cells both directly and via macrophage activation

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Abstract

Upon exposure, TiO_2 nanoparticles (NPs) have been recovered in internal organs such as the liver, and are proposed to cause cellular/organ dysfunction, particularly in the liver and lungs. We hypothesized that despite being considered "inert" as bulk material, TiO_2 NPs may impair insulin responses in liver-derived cells, either indirectly by inflammatory activation of macrophages, and/or by directly interfering with insulin signaling. Using qRT-PCR and conditioned medium (CM) approaches, we show that exposure to TiO_2 NPs activates macrophages' expression of TNF-α, IL-6, IL-8, IL-1α and IL-1β and the resulting CM induces insulin resistance in Fao cells. Furthermore, direct exposure of Fao cells to TiO_2 results in activation of the stress kinases JNK and p38MAP kinase, and in induction of insulin resistance at the signaling and metabolic levels. Collectively, our findings provide a proof-of-concept for the ability of man-made NPs to induce insulin resistance in liver-derived cells, an endocrine abnormality underlying some of the most common human diseases.

Keywords: Inflammation, insulin resistance, liver, macrophages, TiO_2

Introduction

Nano-sized particles (NPs) are created in many physical processes from erosion to combustion, but are also increasingly being produced and used in a wide range of industrial applications, such as fillers, catalysts, semiconductors, cosmetics, microelectronics and drug carriers (Nel et al. 2006; Donaldson et al. 2001). Due to development and application of nanotechnology and increased pollution, a future increase in environmental load of NPs is projected, triggering the urgent need to evaluate their potential effects on human health. A main feature governing the biological effects of all NPs is their small size and high surface-to-volume ratios, which results in increased uptake and high interaction potency with biological systems (Limbach et al. 2007; Nel et al. 2006). The respiratory system has been most widely studied for its interaction with NPs given that it is the main port of entry for inhaled particles into the human body (Pope & Dockery 2006). Yet, animal and human studies now suggest that NPs can enter the circulation after inhalation exposure or other intake and can accumulate in various internal organs such as the liver, kidney, spleen, heart and brain (Brown et al. 2002; Wang et al. 2007; Kreyling et al. 2002; Oberdorster et al. 2004; Liu et al. 2009). From a mechanistic perspective, NPs deposited within tissues can induce oxidative stress, inflammation and DNA damage that can result in carcinogenesis or cell death, processes implicated in the pathogenesis of respiratory, cardiovascular and, more recently, neuro-degenerative diseases (Oberdorster et al. 2005; Nel et al. 2006; Donaldson et al. 2001; Brook et al. 2004; Onuma et al. 2009; Eom & Choi 2010; Campbell et al. 2005; Fedorovich et al. 2010). Yet, the potential contribution of NPs to the etiology of metabolic/endocrine disorders such as obesity and its closely associated insulin resistance is not well established. This question is of major importance since obesity and its associated morbidities (mainly, type 2 diabetes and cardiovascular diseases) are becoming a major global threat to human health, and while a genetic predisposition underlies these disorders, a clear environmental etiology exists, which currently remains poorly understood. Recent epidemiological and mechanistic studies do suggest that environmental pollutants including ambient particulate matter ($PM_{2.5}$) play a role in the increasing incidence of type 2 diabetes and/or in aggravating the effects of diet-induced obesity (O'Neill et al. 2007; Chen & Schwartz 2008; Pearson et al. 2010). Yet, the potential of a specific manufactured NP with a well-defined size and chemical composition to disrupt

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insulin action in cell types representing this hormone's major metabolic target tissues such as the liver has been scarcely explored.

TiO_2 is widely used in food additives, in sunscreens, in water cleanup technology, in self-cleaning materials, and in white pigments for paints, paper and plastics (Hext et al. 2005). As an ultrafine-sized material, TiO_2 NPs can enter the human body through various routes such as inhalation (respiratory tract), ingestion (gastrointestinal tract), dermal penetration (skin) and injection (blood circulation) (Jin et al. 2008; Oberdorster et al. 2005). While frequently considered as being biologically inert in its bulk form, nano-TiO_2 can accumulate in internal organs including the liver and cause cellular damage (Ma et al. 2009; Wang et al. 2007; Cui et al. 2010a). *In vivo* studies indicate that inhaled or instilled TiO_2 particles can produce reactive oxygen species to a greater extent than the same material in its bulk form (Afaq et al. 1998; Trouiller et al. 2009), and promote transcription of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β (Cui et al. 2010b; Park et al. 2009; Trouiller et al. 2009). Intriguingly, pro-inflammatory cytokines are well-established inducers of insulin resistance and have been implicated in the pathogenesis of obesity and type 2 diabetes (Olefsky & Glass 2010; Shoelson et al. 2006; Nov et al. 2010; Gurgueira et al. 2002; Sun et al. 2009).

In this study we hypothesized that TiO_2 NPs could induce insulin resistance in liver-derived cells, and explored whether this may occur via inflammatory activation of macrophages and/or by direct interaction of the NPs with the liver-derived cells. Our findings provide a proof-of-principle for the possibility that man-made NPs could constitute an additional environmental causative factor in the induction of insulin resistance, a common endocrine abnormality underlying some of the most common human diseases.

Materials and methods

TiO_2 particles specifications

TiO_2 (anatase, Aeroxide TiO2 P25) was obtained from Degussa (Frankfurt, Germany). We used hydrophilic fumed TiO_2 with specified average primary particle size of 21 nm, and with specific BET surface area of 50 ± 15 m^2/g. The material contained >99.95 wt% TiO_2 with some minor impurities (Al_2O_3 content <0.3 wt% ; SiO_2 content <0.2 wt% ; $Fe2O_3$ <0.01 wt%). Such particles were used in similar studies in the past. We further investigated the size distribution of the particles using scanning electron microscopy. The NPs were placed on carbon tape and sputter-coated with 2 nm of chromium (Emitech, K575X). Samples were examined using a Zeiss Ultra 55 field emission scanning electron microscope. Diameter measurements were preformed ($n = 178$) using iTEM analysis program (Olympus). The average ± SD diameter of 178 investigated TiO_2 NPs was 26.4 ± 7.3 nm and the area was 589 ± 327 nm^2.

Preparation of TiO_2 NP suspension

Suspensions of 1 mg/ml TiO_2 were prepared in F12 Coon's or DMEM serum free-medium (SFM), as indicated, and were sonicated in an ultrasonic bath (Elma, Germany) for 15 min immediately before use.

Cell culture

Fao rat hepatoma cells were grown in F12 Coon's modified culture medium. The murine macrophage cell line (J774.1, American Tissue Culture Collection) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 gr/l glucose. Both cell lines were supplemented with 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all obtained from Biological Industries, Beit-Haeemek, Israel).

mRNA extraction and quantitative RT-PCR

J774.1 macrophages were cultured at a density of 1×10^6 cells/well in a six-well plate and incubated for 24 h at 37°C before treatment. Cells were then washed once with DMEM, and incubated with serum-free DMEM supplemented with TiO_2 at different concentrations, as indicated, for 2 h at 37°C. Following 2 h, total mRNA was extracted using the Perfect-Pure RNA Kit (5PRIME, Hamburg, Germany), and first-strand cDNA was generated by Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative amplification of specific cytokine mRNA transcripts was carried out by quantitative real-time PCR using TaqMan probes and performed in StepOnePlus RT-PCR instrument (Applied Biosystems, CA). The following primers were used: IL-1α (Mm00439621_m1); IL-1β (Mm00434228_m1); IL-6 (Mm99999064_m1); IL-8 (CXCL2) (Mm00436450_m1); and TNF-α (Mm00443258_m1). Data were analyzed by ABI PRISM SDS 7000 software (Applied Biosystems, CA). Relative gene expression was normalized to Tata box binding protein (Tbp) (Mm00446973_m1) mRNA. Results are reported ± SEM as fold change over control (cells incubated without TiO_2).

Conditioned medium experiments

J774.1 macrophage cells were grown in 10 cm dishes to 70–75% confluence, after which cells were incubated with 5 ml serum-free DMEM supplemented with or without (control) 100 μg/ml TiO_2 for 2 h at 37°C. Following 2 h, the conditioned medium (CM) was collected from either control or TiO_2-treated plates and subjected to two sequential centrifugations, the first, 450 × g, 5 min, to remove floating cells and cellular debris, followed by the second, 20,800 × g, 15 min, at 25°C to remove excess TiO_2. After centrifugations, supernatant (CM) derived from control or TiO_2-treated macrophages was placed onto Fao cells (1 ml/well in a six-well plate). Fao cells were exposed to CM for 6 h, and then stimulated for 10 min with 100 nM insulin, as indicated. Plates were then washed three times with ice-cold PBS, and stored at -80°C until analyzed for insulin signaling.

Direct exposure of Fao cells to TiO_2 NPs

Fao cells were grown at ~80% confluence in six-well plates (typically 24 h after seeding). Cells were rinsed and incubated with 50 and 200 μg TiO_2 diluted in 1 ml of F12 Coon's SFM for 2 h at 37°C. Control cells were incubated only with 1 ml of F12 Coon's SFM. Cells were then stimulated with 100 nM insulin for 10 min or left untreated, as indicated. Finally, plates were thoroughly rinsed three times with ice-cold PBS, and stored at -80°C until analyzed.

Cell lysates and Western blot analysis

After treatments, Fao cells were scrapped in ice-cold lysis buffer containing 25 mMTris HCl, pH 7.5; 25 mM NaCl; 0.5 mM EGTA; 10 mM NaF; 1% (vol/vol) Triton-X-100; 0.05% (vol/vol) SDS; 0.5% (vol/vol) DOC; 80 mM β-glycerophosphate; 10 mM sodium pyrophosphate; 10 mM sodium orthovanadate; and inhibitors (a 1:1000 dilution of protease inhibitor mixture; Sigma). Insoluble material was removed by 15-min centrifugation (12,000 × g) at 4°C, and the supernatant collected. Protein concentration was determined using the Bio-Rad Bradford method procedure (Munich, Germany). Protein samples were resolved on 8% SDS-PAGE and were Western blotted with the following antibodies, as indicated: monoclonal anti-phosphotyrosine (4G10) antibodies (1:4000 dilution), polyclonal anti-insulin receptor substrate (IRS)1, IRS2 were and used at 1:1000 dilution (all purchased from Millipore, Billerica, MA); polyclonal anti-p85α (1:1000), anti-Jun kinase (JNK) (1:1000) and anti-p38 (1:500) antibodies were obtained from Santa Cruz Biotechnology (Santa-Cruz, California); polyclonal anti-pSer21/9 glycogen synthase kinase (GSK)3β, anti-GSK3β, anti-phospho-JNK and anti-phospho-p38 antibodies were purchased from Cell Signaling and used at 1:1000 dilution. Quantification of the immunoblots was done using the NIH Image 1.62b7.

Immunoprecipitation

Cell lysates (300 μg protein) were incubated with anti-IRS1 or anti-IRS2 antibodies (Millipore, Billerica, MA) overnight at 4°C. A total of 50 μl of protein A-Sepharose beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added for 2 h at 4°C. Immunocomplexes were then washed three times with lysis buffer, and re-suspended in 50 μl 1x Laemmli sample buffer. Immunocomplexes were resolved by 8% SDS-PAGE, transferred to nitrocellulose membranes and then Western blotted with the indicated antibodies.

Glycogen synthesis

Glycogen synthesis was measured in six-well plates by assessing the incorporation of U-^{14}C-glucose into glycogen, as we previously described (Bloch-Damti et al. 2006). Briefly, after treatments, Fao cells were washed three times in PBS and incubated with PBS containing 0.1 mM (U-^{14}C) glucose (2 μCi /well) in the absence or presence of 100 nmol/liter insulin for 1 h. Cells were then washed, homogenized and glycogen precipitated on a Whatman (Middlesex, UK) paper in 70% ethanol.

Statistical analysis

Data are presented as the mean ± SEM. Statistical significance was assessed using Student's t test when two groups were compared, and with ANOVA with a Dunnett's post-hoc test when multiple groups were compared. $P \leq 0.05$ was set as the threshold for significance.

Results

We first considered the possibility that TiO_2 NPs could induce insulin resistance in liver derived cell *indirectly*, that is, via activation of macrophages. These could recapitulate either circulating monocytes that reach the liver, and/or Kuppfer cells, resident liver macrophages that are in close proximity to hepatocytes. To this end, we employed a CM cell culture paradigm, in which J774.1 macrophages were incubated for 2 h with different concentrations of TiO_2 NPs. To first determine the capacity of these specific NPs to activate macrophages, RNA was extracted at the end of the incubation period, reverse-transcribed to cDNA and quantitative real-time PCR was performed to measure the relative mRNA expression of several inflammatory cytokines. Though with varied robustness and dose-dependency, TiO_2 clearly induced expression of IL-1α, IL-1β, IL-6, IL-8 (KC) and TNF-α, by 3- to 33-fold at the highest TiO_2 concentration measured compared to control cells (Figure 1). Similar results

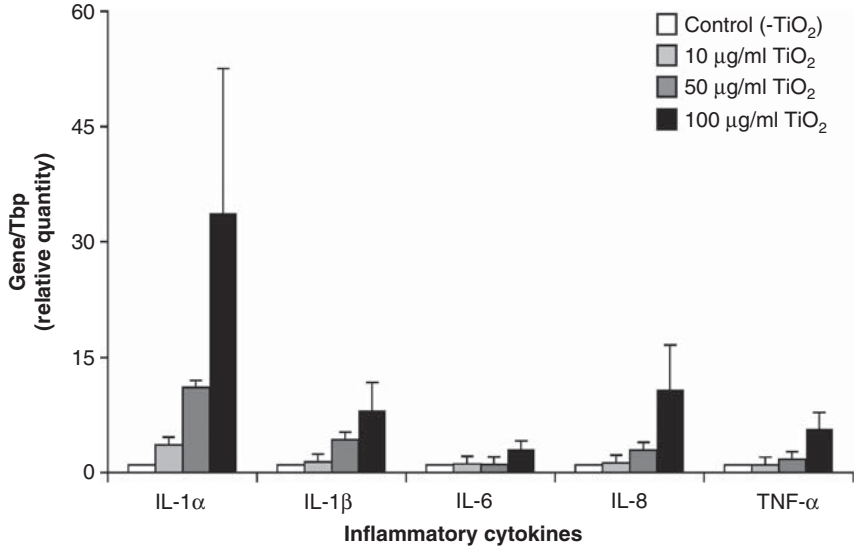

Figure 1. TiO_2 NPs up-regulate mRNA expression of inflammatory cytokines in macrophages. J774.1 cells were incubated with the indicated concentrations of TiO_2 for 2 h at 37°C. RNA was then extracted from cells, reverse transcribed into cDNA and the mRNA expression levels of the indicated inflammatory cytokines were measured by qRT-PCR (TaqMan procedure). Results were normalized to the quantity of Tata binding protein (Tbp) mRNA. Values are the mean ± SEM of four independent experiments each carried out in duplicates.

were obtained with a different macrophage cell line (Raw264, data not shown).

To test the potential biological significance of this proposed inflammatory activation of macrophages by TiO_2 NPs, growth medium obtained from TiO_2-treated macrophages was placed on Fao hepatoma cells for 6 h. Fao cells were washed, and treated with or without 100 nM insulin for 10 min (Figure 2A). Insulin stimulation of Fao cells pre-exposed to CM from control macrophages greatly increased the tyrosine phosphorylation of a band of ~180 kDa corresponding to the insulin receptor substrate proteins IRS1 and IRS2 (Figure 2B). Similarly, a robust increase could be seen in the Ser phosphorylation of GSK3β (Figure 2C), a kinase downstream the signaling cascades emanating from the insulin receptor, which participates in the stimulation of glycogen synthesis by insulin, a major metabolic response to this hormone in liver cells. Both signaling responses to insulin stimulation were significantly attenuated in hepatoma cells that were pre-incubated with medium from TiO_2-treated macrophages, without affecting the total expression of either IRS1, IRS2 or GSK3β (Figure 2). This finding is consistent with the well-established capacity of various inflammatory cytokines to inhibit insulin signaling (Olefsky & Glass 2010; Shoelson et al. 2006; Nov et al. 2010), and confirms the biological significance of the expression data shown in Figure 1.

Next, given reports suggesting that some NPs, including TiO_2, could cross biological barriers and were found *in vivo* in various tissues including the liver (Ma et al. 2009; Wang et al. 2007; Cui et al. 2010a; Brain et al. 2009; Liu et al. 2009), we entertained the possibility that TiO_2 NPs could *directly* affect insulin response in liver-derived cells. Fao cells were incubated with 0, 50 or 200 μg/ml TiO_2 NPs for 2 h, and stimulated without or with 100 nM insulin for 10 min. Insulin-stimulated tyrosine phosphorylation of the IRS-corresponding band decreased dose-dependently, reaching a significant attenuation ($p < 0.001$) for the higher TiO_2 concentration, in which lower total protein levels,

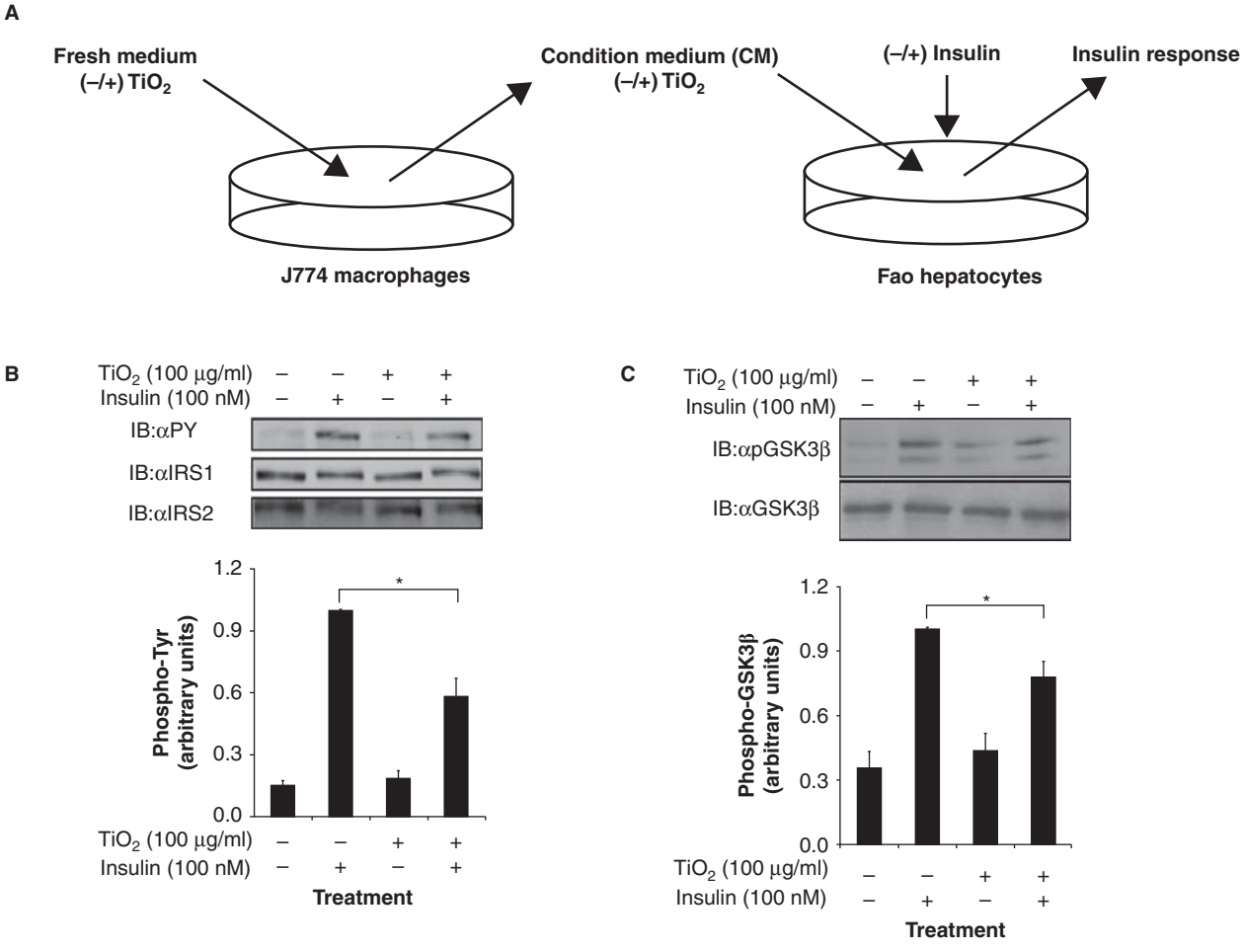

Figure 2. Exposure of Fao cells to conditioned medium (CM) derived from TiO_2-treated macrophages inhibits insulin signaling. (A) J774.1 murine macrophage cells were incubated with serum-free medium in the absence (control) or presence of 100 μg/ml TiO_2 NPs for 2 h. Following 2 h, CM obtained from control or TiO_2-treated macrophages was collected and placed on hepatoma cells (Fao) for 6 h. Fao cells were then stimulated with 100 nM insulin for 10 min or left untreated, as indicated, and analyzed for insulin signaling. (B) Fao cells were extracted, as detailed in the article, and aliquots of total cell extract (20 μg) were resolved by means of SDS-PAGE and immunoblotted (IB) with anti-phospho-tyrosine (PY), anti-IRS1 or anti-IRS2 antibodies. Similarly, cell lysates were immunoblotted with antibodies directed to phosphorylated and total GSK3β (C). Western blots of a representative experiment are displayed. Densitometry analyses of IRS protein tyrosine phosphorylation (B), as well as of GSK3β serine phosphorylation (C) are presented. In each experiment, the insulin-stimulated cells were assigned an arbitrary unit of 1, and values are the mean ± SEM of five independent experiments. *$p \leq 0.05$, compared with insulin-stimulated Fao cells incubated with CM derived from control macrophages.

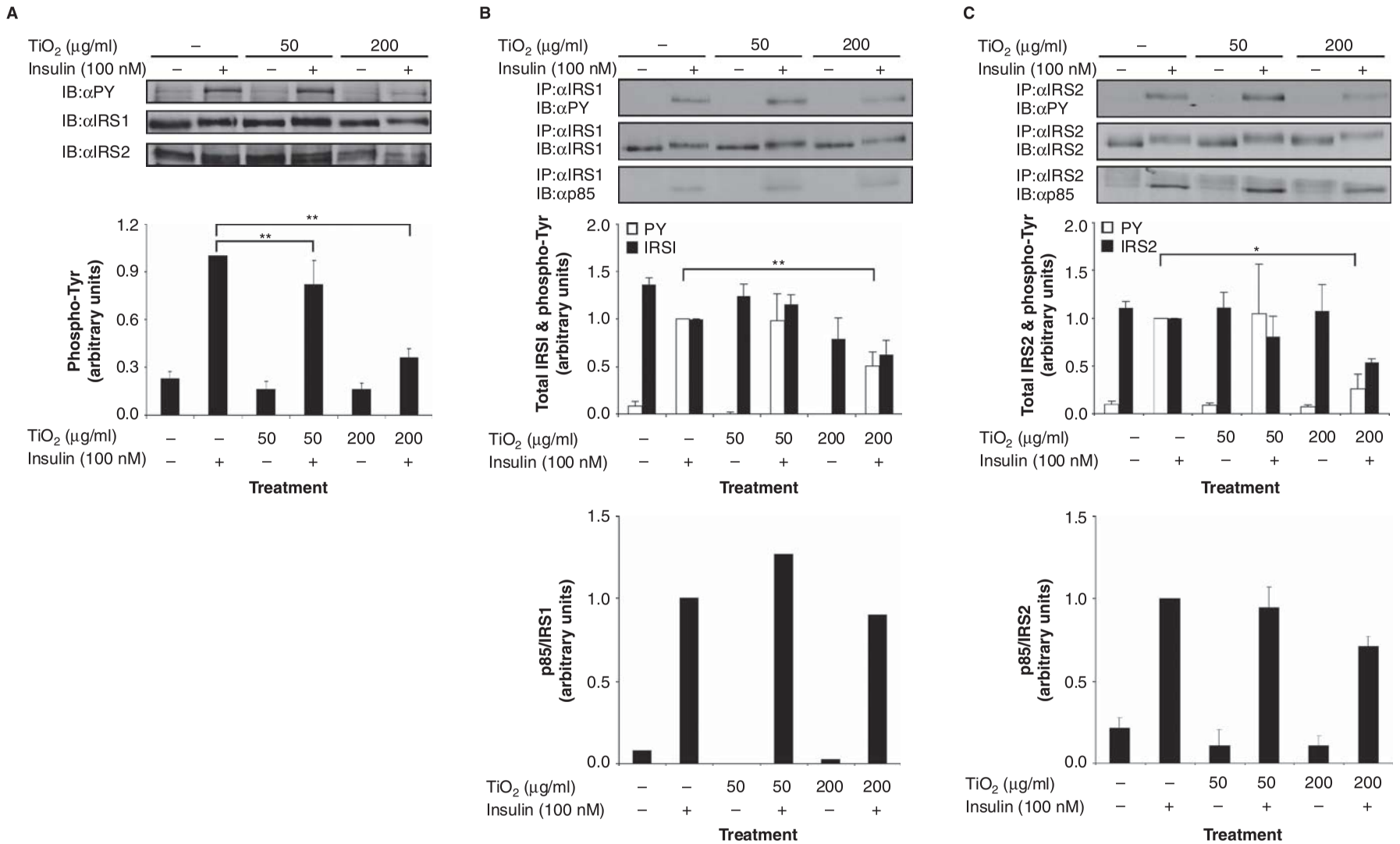

Figure 3. Direct exposure of Fao cells to TiO_2 NPs decreases the amount of insulin-stimulated tyrosine-phosphorylated IRS proteins, partly due to lower IRS2 protein content. Fao cells were incubated with serum-free medium supplemented with 0, 50 or 200 μg/ml of TiO_2 NPs for 2 h. Cells were then stimulated with 100 nM insulin for 10 min or left untreated, as indicated. Total cell extracts (20 μg) (A) or protein samples (300 μg) subjected to immunoprecipitation (IP) with anti-IRS1 (B) or anti-IRS2 antibodies (C) were resolved by SDS-PAGE and immunoblotted (IB) with anti-PY, anti-IRS1, anti-IRS2 (A, B, C) and with anti-p85 antibodies (B, C), as indicated. Representative blots and densitometry values (mean ± SEM) of at least three experiments are presented. *$p \leq 0.05$; **$p \leq 0.01$, compared with untreated cells stimulated with insulin.

particularly of IRS2, could also be observed (Figure 3A). Intriguingly, immunoprecipitation of IRS1 or IRS2 followed by immunoblotting with anti-phosphotyrosine antibodies suggested that the majority of the diminution in insulin-stimulated tyrosine phosphorylation of IRSs could be attributed to IRS2 (Figure 3C), the major IRS isoform in liver cells. This diminution is further reflected by a reduction in binding of the p85 regulatory subunit of phosphatidylinositol-3-OH kinase (PI(3)K), (Figures 3B and C), which binds to phosphorylated tyrosine residues of IRS proteins following insulin stimulation (Saltiel & Kahn 2001). Further downstream the insulin-signaling cascade, there was a definite dose-dependent diminution in the capacity of insulin to stimulate the Ser phosphorylation of GSK3β (Figure 4A). This reached statistical significance ($p < 0.05$) for the higher concentration of TiO_2, largely corresponding to the phosphotyrosine results. Importantly, consistent with the effect of TiO_2 NPs on GSK3β, these supposedly inert NPs dose-dependently diminished insulin-stimulated glycogen synthesis, as measured by the rate of incorporation of radio-labeled glucose to glycogen (Figure 4B).

It was verified that the insulin resistance observed is a consequence of the cellular response to TiO_2 NPs and not a result of direct interaction of insulin with the NPs (NP-insulin interaction could potentially induce loss of the hormone's bioactivity). To that end, insulin was pre-incubated in medium containing 200 μg/ml TiO_2 NPs. NPs were then sedimented by centrifugation, and the insulin-containing supernatant was used to stimulate Fao cells. We observed no discernable loss of bioactivity of insulin that was pre-incubated with TiO_2 NPs, as detected by the ability of the hormone to stimulate tyrosine phosphorylation of IRS proteins or serine phosphorylation of GSK3 (data not shown).

The molecular basis for cellular insulin resistance - the impaired capacity of cells or tissues to respond to some or all of the metabolic actions of insulin, still remains an enigma. Yet, a large body of evidence implicates in this process the activation of "stress kinases" – enzymes activated by various intracellular or extracellular stresses. These in turn may act upon insulin signaling molecules, rendering them less responsive/sensitive to insulin stimulation. Intriguingly, the Mitogen Activated Protein (MAP) kinases p38 MAPK and c-Jun activated kinases (JNK) have been shown to be stimulated in different cell types by various environmental factors, including NPs. In addition to various "stresses", these kinases are also mildly activated in many cell types by mitogens and growth factors, including insulin. We therefore set to determine if TiO_2 NPs at concentrations that induce insulin resistance in liver cells activate these kinases. Increasing concentrations of TiO_2 clearly activated these two kinases as evidenced by increased dual-phosphorylation on their activatory TGY motif, reflected by the signal obtained with anti-p-p38 or anti-pJNK antibodies (Figure 5). Interestingly, in some (though not all) individual experiments, the small insulin-induced increase in phosphorylation of p38 MAPK and of JNK seemed to be retained after NPs exposure (Figure 5). This result suggests the possibility that TiO_2 NPs can directly and selectively interfere with the metabolic signaling of insulin, while attenuating less the mitogenic/stress-related functions of the hormone.

Discussion

Due to their small size and high surface-to-volume ratio, NPs can interact with tissues and organs more robustly than the same materials in bulk form. This has led to a body of

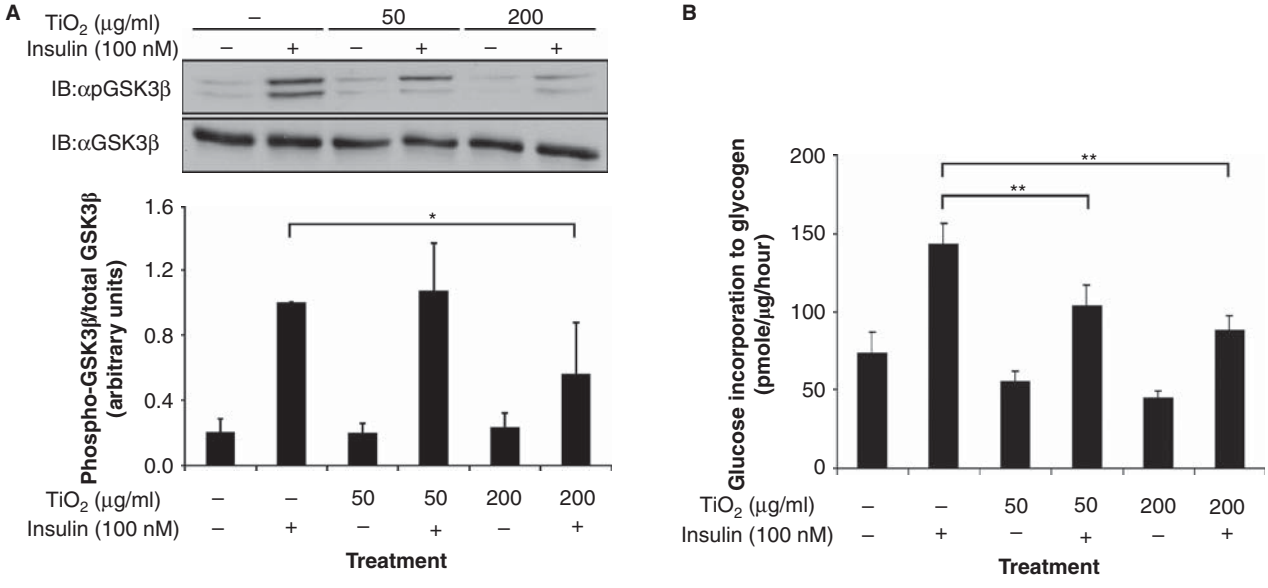

Figure 4. Direct exposure of Fao cells to TiO_2 NPs leads to insulin resistance and impaired glycogen synthesis. Fao cells were pre-incubated with indicated concentrations of TiO_2 NPs, followed by insulin stimulation, as described in Figure 3. To assess impairment in insulin signaling, total cell extracts (20 μg) were immunoblotted (IB) with antibodies against phospho-, as well as total GSK3β (A). Band densities were quantified by densitometry. Results were normalized to total GSK3 protein content and are depicted as mean ± SEM of five independent experiments. Representative blots are shown. (B) Glycogen synthesis was assessed as depicted in the article. Results (mean ± SEM) of two experiments carried out in triplicates are shown. *$p \leq 0.05$; **$p \leq 0.01$, compared with untreated cells stimulated with insulin.

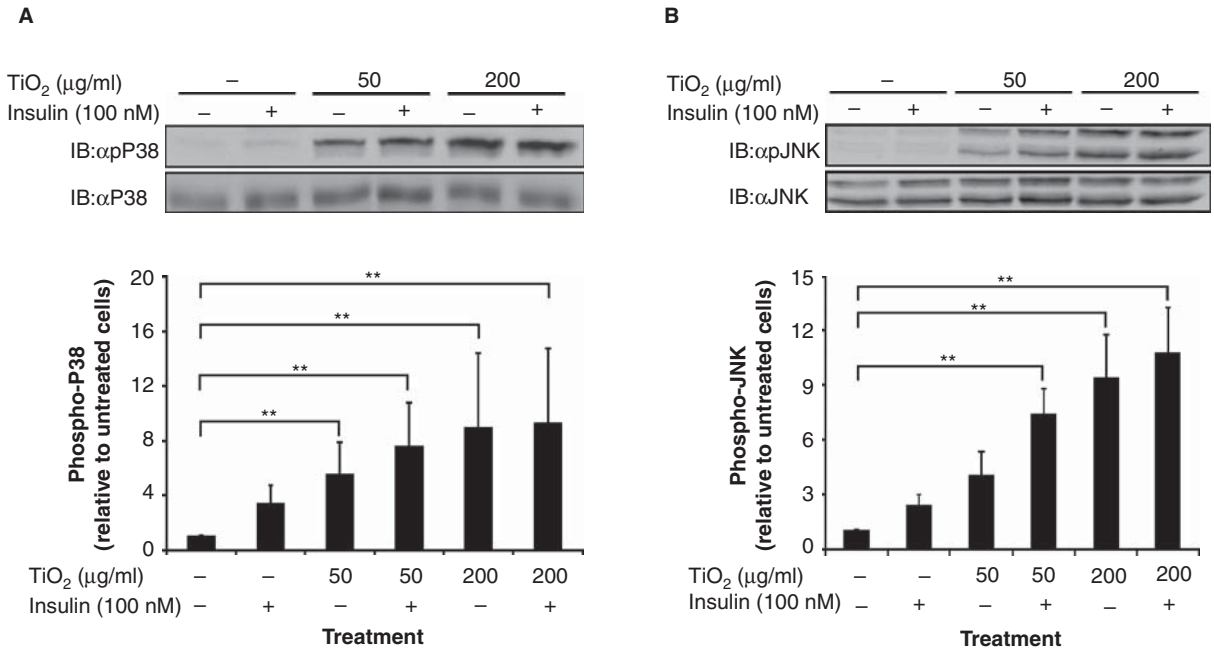

Figure 5. TiO_2-induced insulin resistance is associated with activation of stress kinases. Fao cells were treated with indicated concentrations of TiO_2 NPs, and stimulated with 100 nM insulin, as described in Figure 3. Aliquots of cell extract (20 μg) were resolved by means of SDS–PAGE and subjected to Western blot analysis using antibodies against phosphorylated- and total p38 (A), or phosphorylated- and total JNK (B). Densitometry analyses of phospho-p38 (A) and phospho-JNK (B) are displayed. Values are mean ± SEM of at least five independent experiments. Western blots of a representative experiment are displayed. $^{**}p \leq 0.01$, compared with control untreated cells.

research that identified possible biological effect of various NPs, such as induction of oxidative stress, mitochondrial ROS generation and damage, as well as activation of inflammatory cascades. Although TiO_2 is widely considered to be inert and is used abundantly in diverse products in the working and living environment, possible toxic effects of TiO_2 in its NP form have been reported. Former studies have shown that nano-TiO_2 can accumulate in the liver after direct oral administration (Wang et al. 2007) and induce inflammation (Cui et al. 2010b) and oxidative damage or interact with DNA (Li et al. 2009) by covalent binding between nano-anatase TiO_2 and DNA. In large doses, TiO_2 accumulation can lead to hepatocyte necrosis, apoptosis and hepatic fibrosis. In the present study we addressed possible TiO_2 NPs effects on liver cells beyond previously studied cytotoxicity. In particular, given the central role that the liver exerts in whole-body metabolic and endocrine regulation, we asked whether TiO_2 NPs could exert metabolic/endocrine effects on liver-derived cells. Using *in vitro* approaches that enable assessment of biological effects on a specific cell type, we found that exposure to TiO_2 NPs can impair insulin signaling towards glycogen synthesis, a major insulin-regulated pathway in the liver. This could be demonstrated both via a mediatory role of macrophages, in which TiO_2 NPs induced a transcriptional activation of pro-inflammatory genes, and directly by interfering in liver cells at specific steps in insulin's intracellular signal transduction cascades.

In vitro systems such as those we have utilized herein are inherently artificial, and clearly the (patho)physiological relevance of the findings of this study will require additional confirmation. Although it is hard to "translate" levels of *in vivo* exposure to *in vitro* systems, several exposure studies suggest that human TiO_2 NPs occupational exposure could be within the bulk range of exposure level that had measurable systemic effects in mice (Liao et al. 2009), that is, at the low mg/Kg body weight. Serum levels of IL-6 and TNF-α were elevated in mice following a single intra-tracheal dose of TiO_2 NPs (Park et al. 2009), consistent with our findings of transcriptional activation of these, and additional pro-inflammatory cytokines genes. The strengths of our *in vitro* approach are hence in its ability to elucidate the effects of TiO_2 NPs on one cell-type at a time and to unravel its effects on cell-cell interaction, a situation that is difficult to investigate in whole tissue and *in vivo* systems. Indeed, we could demonstrate that the mechanisms for TiO_2 NPs-induced insulin resistance in the liver could include: *(i) macrophage-mediated inflammation:* various ambient and industrial NPs have been shown to activate macrophages and inflammatory cytokines such as TNF-α, IL-6 and IL-1 that have been demonstrated to induce insulin resistance in liver cells, and were implicated in the induction of hepatic insulin resistance *in vivo* (Arkan et al. 2005; Hotamisligil et al. 1993; Klover et al. 2003). Here the connection between NP-activated macrophages and insulin-resistant liver cells is made using a conditioned medium approach, suggesting that either in the lungs (alveolar macrophages), the circulation (monocytes) or within the liver (Kuppfer cells), NPs could target cells of macrophage lineage and result in hepatocyte insulin resistance. *(ii) Direct interruption of insulin signaling in liver cells:* TiO_2 NPs impaired early insulin signaling, particularly at the level of insulin receptor substrate proteins. IRS2, likely the dominant metabolic IRS isoform in the liver, was less phosphorylated on tyrosine residues. This resulted in diminished interaction with the p85 subunit of PI(3)k, an enzyme generating the lipid messenger phosphatidylinositol 3,4,5 trisphosphate (PIP3),

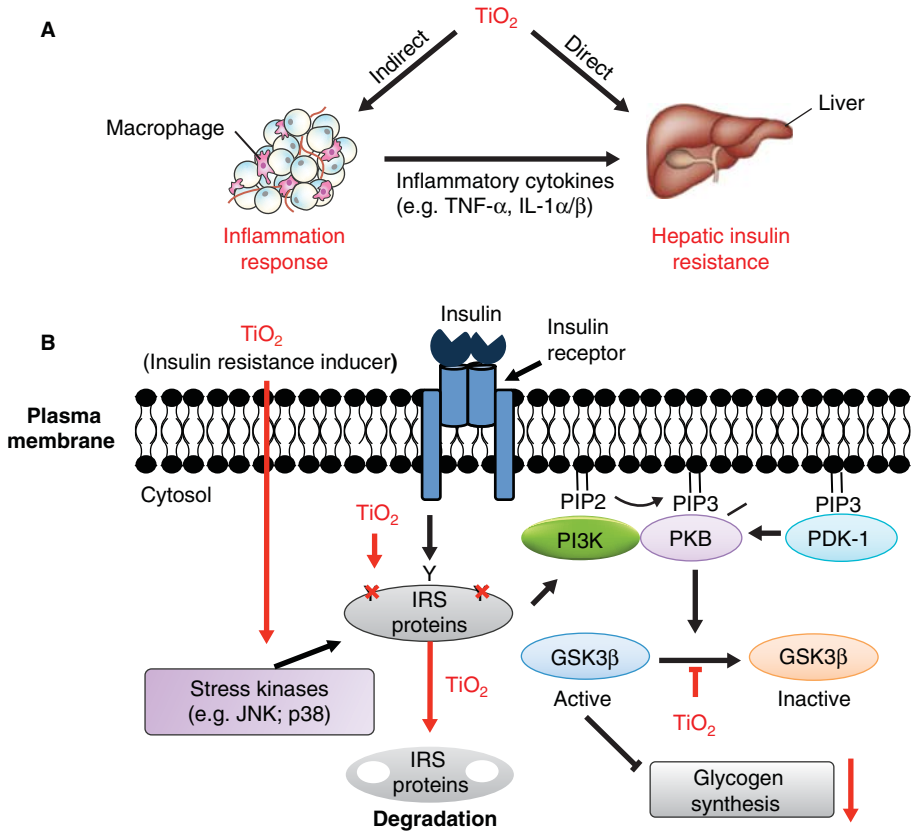

Figure 6. TiO_2 NPs are potential inducers of hepatic insulin resistance via a direct effect and/or mediated by macrophages, a model. (A) TiO_2 can directly inhibit hepatic insulin signaling and induce insulin resistance in hepatocytes. TiO_2 can also attenuate insulin signaling, indirectly, by activation of macrophages, which secrete inflammatory cytokines that act in anendocrine/paracrine fashion to induce insulin resistance in liver-derived cells. (B) At the molecular level, we show that TiO_2 NPs decrease tyrosine phosphorylation of IRS proteins and impair downstream insulin signaling. This is manifested by a reduction in serine phosphorylation of GSK3β and impairment of glycogen synthesis. In addition, TiO_2 activates stress kinases, such as JNK and p38, which are known to phosphorylate IRS proteins on its serine residues, potentially resulting in a negative feedback loop on insulin signaling, leading to insulin resistance.

which is required for most (if not all) of insulin's metabolic effects (Figure 6). The molecular mechanism remains to be elucidated, but appears to involve a decrease in IRS2 protein content, possibly reflecting enhancement of insulin-induced protein degradation. Downstream the insulin signaling cascade, insulin-stimulated phosphorylation of GSK3β, a step required for the stimulation of glycogen synthesis, was impaired following exposure to TiO_2 NPs. Our current analysis cannot sort if the diminution of this more-distal step in the insulin signaling cascade represents a second target for NPs, or is a consequence of the upstream insulin signaling defect at the level of IRS2. Nevertheless, the finding that insulin-stimulated glycogen synthesis was affected by exposure to TiO_2 NPs provides proof-of-concept for the ability of TiO_2 NPs to induce insulin resistance, which is essentially a metabolic rather than a signaling phenomenon.

A major paradigm in the induction of impaired insulin action at the level of IRS proteins is that increased phosphorylation on serine residues, the consequence of activation of Ser/Thr IRS kinases and/or decreased de-phosphorylation, impinges on the capacity of these proteins to undergo insulin-induced phosphorylation on tyrosine residues that is required for signal propagation (Paz et al. 1997). Phosphorylation of IRSs on Ser residues may render them worse substrates for the insulin receptor kinase, but may also alter cellular localization and target these proteins to degradation, resulting in their decreased content (Greene et al. 2003), as was seen here. Furthermore, various kinases have been implicated in the increased Ser (and resulting diminished tyrosine) phosphorylation of IRSs. Here we show that JNK and p38MAP kinase are activated in liver-derived cells by TiO_2 NPs, and at least the former kinase is widely implicated as an IRS serine kinase causing impaired insulin action (Boura-Halfon & Zick 2009; Lee et al. 2003). The mechanism for TiO_2 NPs-induced activation of JNK remains to be explored, but this stress kinase, along with p38MAP kinase, are well-known to respond to inflammatory signals and to reactive oxygen species, which have been shown to be induced by various NPs, including TiO_2 (Olefsky & Glass 2010; Brown et al. 2004).

In conclusion, utilizing an *in vitro* system we could demonstrate induction of insulin resistance specifically in liver-derived cells by a manufactured NP that is widely used and frequently considered inert. If proven relevant to human level of exposure, this study provides potential mechanistic pathways by which manufactured NP could induce hepatocyte insulin resistance, a leading public health problem.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Afaq F, Abidi P, Matin R & Rahman Q. 1998. Cytotoxicity, pro-oxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed to ultrafine titanium dioxide. J Appl Toxicol 18:307–312.

Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, et al. 2005. IKK-beta links inflammation to obesity-induced insulin resistance. Nat Med 11:191–198.

Bloch-Damti A, Potashnik R, Gual P, Le Marchand-Brustel Y, Tanti JF, Rudich A, Bashan N. 2006. Differential effects of IRS1 phosphorylated on Ser307 or Ser632 in the induction of insulin resistance by oxidative stress. Diabetologia 49:2463–2473.

Boura-Halfon S, Zick Y. 2009. Phosphorylation of IRS proteins, insulin action, and insulin resistance. Am J Physiol Endocrinol Metab 296: E581–591.

Brain JC, Donaghey T, Molina R. 2009. Biologic responses to nanomaterials depend on exposure, clearance, and material characteristics. Nanotoxicology 3:174–180.

Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, et al. 2004. Air pollution and cardiovascular disease: a statement for healthcare professionals from the expert panel on population and prevention science of the american heart association. Circulation 109:2655–2671.

Brown DM, Donaldson K, Borm PJ, Schins RP, Dehnhardt M, Gilmour P, et al. 2004. Calcium and ROS-mediated activation of transcription factors and TNF-alpha cytokine gene expression in macrophages exposed to ultrafine particles. Am J Physiol Lung Cell Mol Physiol 286:L344–353.

Brown JS, Zeman KL, Bennett WD. 2002. Ultrafine particle deposition and clearance in the healthy and obstructed lung. Am J Respir Crit Care Med 166:1240–1247.

Campbell A, Oldham M, Becaria A, Bondy SC, Meacher D, Sioutas C, et al. 2005. Particulate matter in polluted air may increase biomarkers of inflammation in mouse brain. Neurotoxicology 26:133–140.

Chen JC, Schwartz J. 2008. Metabolic syndrome and inflammatory responses to long-term particulate air pollutants. Environ Health Perspect 116:612–617.

Cui Y, Gong X, Duan Y, Li N, Hu R, Liu H, et al. 2010a. Hepatocyte apoptosis and its molecular mechanisms in mice caused by titanium dioxide nanoparticles. J Hazard Mater 183:874–880.

Cui Y, Liu H, Zhou M, Duan Y, Li N, Gong X, et al. 2010b. Signaling pathway of inflammatory responses in the mouse liver caused by TiO2 nanoparticles. J Biomed Mater Res A 96:221–229.

Donaldson K, Stone V, Clouter A, Renwick L, MacNee W. 2001. Ultrafine particles. Occup Environ Med 58:211–216, 199.

Eom HJ, Choi J. 2010. p38 MAPK activation, DNA damage, cell cycle arrest and apoptosis as mechanisms of toxicity of silver nanoparticles in Jurkat T cells. Environ Sci Technol 44:8337–8342.

Fedorovich SV, Alekseenko AV, Waseem TV. 2010. Are synapses targets of nanoparticles? Biochem Soc Trans 38:536–538.

Greene MW, Sakaue H, Wang L, Alessi DR, Roth RA. 2003. Modulation of insulin-stimulated degradation of human insulin receptor substrate-1 by Serine 312 phosphorylation. J Biol Chem 278:8199–8211.

Gurgueira SA, Lawrence J, Coull B, Murthy GG, Gonzalez-Flecha B. 2002. Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. Environ Health Perspect 110:749–755.

Hext PM, Tomenson JA, Thompson P. 2005. Titanium dioxide: inhalation toxicology and epidemiology. Ann Occup Hyg 49:461–472.

Hotamisligil GS, Shargill NS, Spiegelman BM. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259:87–91.

Jin CY, Zhu BS, Wang XF, Lu QH. 2008. Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells. Chem Res Toxicol 21:1871–1877.

Klover PJ, Zimmers TA, Koniaris LG, Mooney RA. 2003. Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice. Diabetes 52:2784–2789.

Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, et al. 2002. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. J Toxicol Environ Health A 65:1513–1530.

Lee YH, Giraud J, Davis RJ, White MF. 2003. c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. J Biol Chem 278:2896–2902.

Li N, Ma L, Wang J, Zheng L, Liu J, Duan Y, et al. 2009. Interaction between Nano-Anatase TiO(2) and Liver DNA from Mice In Vivo. Nanoscale Res Lett 5:108–115.

Liao CM, Chiang YH, Chio CP. 2009. Assessing the airborne titanium dioxide nanoparticle-related exposure hazard at workplace. J Hazard Mater 162:57–65.

Limbach LK, Wick P, Manser P, Grass RN, Bruinink A, Stark WJ. 2007. Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. Environ Sci Technol 41:4158–4163.

Liu H, Ma L, Zhao J, Liu J, Yan J, Ruan J, Hong F. 2009. Biochemical toxicity of nano-anatase TiO2 particles in mice. Biol Trace Elem Res 129:170–180.

Ma L, Zhao J, Wang J, Liu J, Duan Y, Liu H, et al. 2009. The acute liver injury in mice caused by Nano-Anatase TiO(2). Nanoscale Res Lett 4:1275–1285.

Nel A, Xia T, Madler L, Li N. 2006. Toxic potential of materials at the nanolevel. Science 311:622–627.

Nov O, Kohl A, Lewis EC, Bashan N, Dvir I, Ben-Shlomo S, et al. 2010. Interleukin-1beta may mediate insulin resistance in liver-derived cells in response to adipocyte inflammation. Endocrinology 151:4247–4256.

Oberdorster G, Oberdorster E, Oberdorster J. 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113:823–839.

Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C. 2004. Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol 16:437–445.

Olefsky JM, Glass CK. 2010. Macrophages, inflammation, and insulin resistance. Annu Rev Physiol 72:219–246.

O'Neill MS, Veves A, Sarnat JA, Zanobetti A, Gold DR, Economides PA, et al. 2007. Air pollution and inflammation in type 2 diabetes: a mechanism for susceptibility. Occup Environ Med 64:373–379.

Onuma K, Sato Y, Ogawara S, Shirasawa N, Kobayashi M, Yoshitake J, et al. 2009. Nano-scaled particles of titanium dioxide convert benign mouse fibrosarcoma cells into aggressive tumor cells. Am J Pathol 175:2171–2183.

Park EJ, Yoon J, Choi K, Yi J, Park K. 2009. Induction of chronic inflammation in mice treated with titanium dioxide nanoparticles by intratracheal instillation. Toxicology 260:37–46.

Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y. 1997. A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. J Biol Chem 272:29911–29918.

Pearson JF, Bachireddy C, Shyamprasad S, Goldfine AB, Brownstein JS. 2010. Association between fine particulate matter and diabetes prevalence in the U.S. Diabetes Care 33:2196–2201.

Pope CA 3rd, Dockery DW. 2006. Health effects of fine particulate air pollution: lines that connect. J Air Waste Manag Assoc 56:709–742.

Saltiel AR, Kahn CR. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414:799–806.

Shoelson SE, Lee J, Goldfine AB. 2006. Inflammation and insulin resistance. J Clin Invest 116:1793–1801.

Sun Q, Yue P, Deiuliis JA, Lumeng CN, Kampfrath T, Mikolaj MB, et al. 2009. Ambient air pollution exaggerates adipose inflammation and insulin resistance in a mouse model of diet-induced obesity. Circulation 119:538–546.

Trouiller B, Reliene R, Westbrook A, Solaimani P, Schiestl RH. 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Res 69:8784–8789.

Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, et al. 2007. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol Lett 168:176–185.