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Toxicoproteomic identification of TiO_2 nanoparticle-induced protein expression changes in mouse brain

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A proteomic analysis of the proteins in mouse brain that were differentially expressed in response to TiO_2 nanoparticles was conducted to better understand the molecular mechanism of TiO_2 nanoparticle-induced brain toxicity at the protein level. A total of 990 proteins from mouse brain were resolved by two-dimensional gel electrophoresis. A comparative proteomic analysis revealed that the expression levels of 11 proteins were changed by more than 2-fold in response to TiO_2 nanoparticles: eight proteins were upregulated and three were downregulated by TiO_2 nanoparticles. In addition, the activities of several antioxidative enzymes and acetylcholine esterase were reduced in TiO_2 nanoparticle-exposed mouse brain. The protein profile alterations seem to be due to an indirect effect of TiO_2 nanoparticles, because TiO_2 nanoparticles were not detected in the brain in this investigation.

Keywords: proteomics; TiO_2 nanoparticles; mouse; brain

Introduction

Nanoparticles have been manufactured from many different metals for diverse applications. In particular, titanium dioxide (TiO_2) nanoparticles are an important nanomaterial used in industry (Borm et al. 2006). Nanoparticles of TiO_2 have been used in ultraviolet sunscreen blocks and in self-cleaning and anti-microbial materials owing to their photochemical characteristics. They have also been used as photocatalysts in the cosmetics and pharmaceutical industries, including applications in photodynamic therapy, food production and environmental decontamination of air, soil and water (Kaida et al. 2004; Choi et al. 2006). Although we benefit from the unusual characteristics of TiO_2 nanoparticles, they pose a safety risk to our health and environment. Recent data have indicated that the toxicity of TiO_2 nanoparticles may be related to their surface area, and not to their weight (Warheit et al. 2007; Park et al. 2008).

TiO_2 nanoparticles increase hydrogen peroxide and nitric oxide production in human bronchial epithelial cells and induce micronuclei formation and apoptosis in Syrian hamster embryo fibroblasts (Park et al. 2009). The cytotoxicity and reactive oxygen species generation induced by TiO_2 nanoparticles caused oxidative DNA damage. Moreover, TiO_2 nanoparticles caused mutations resulting in cell death or proliferative disorders due to a loss of cell cycle control (Kiss et al. 2008).

TiO_2 nanoparticles were absorbed and distributed to the lungs, lymph nodes, liver, red blood cells and kidneys (Liu et al. 2009). However, there remain some inconsistent results regarding the uptake and translocation of the nanoparticles to the brain (Johnston et al. 2009). Moreover, there is little information on the mechanism of action of TiO_2 nanoparticles and the molecular pathogenesis of their toxic effects in the brain with regard to protein expression levels. Therefore, the aim of this work is to identify, via a proteomic approach, the proteins that are differentially expressed in mouse brain following exposure to TiO_2 nanoparticles.

Materials and methods

Animals and TiO_2 nanoparticle exposure

Seven-week-old male ICR mice, weighing 19–20 g, were obtained from Orient Bio (Seoul, Korea). The mice were acclimated for 2 days at $25 \pm 2°C$ and with a normal day/night cycle before starting the experiment. Commercial TiO_2 nanoparticles (Sigma-Aldrich, St. Louis, MO), consisting of the anatase crystallographic form with an average diameter <25 nm, were mixed with $1\times$ PBS. To avoid aggregation, the suspensions were ultrasonicated for 10 min in sealed sterile tubes (Son et al. 2007; Shim and Om 2008).

The male ICR mice were divided into a control and a sample group ($n = 6$ for each). The sample group was exposed by intraperitoneal (i.p.) injection with 2.5 mg of TiO_2 nanoparticles in 0.2 mL of endotoxin-free normal PBS (Park et al. 2008). At 30 min after

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injection, TiO_2 nanoparticles were instilled nasally in the sample group (Chen et al. 2006). After 7 days, the animals were anaesthetized with ether, and the brains were collected and stored at $-70°C$.

Sample preparation

Brain tissues were solubilized using tip-probe sonication in modified lysis buffer (7 M urea, 2 M thiourea, 4.5% w/v CHAPS, 40 mM Tris, 100 mM DTE) containing a protease inhibitor cocktail. The solubilized samples were clarified by centrifugation at $12,000 \times g$ for 50 min at 15°C. The supernatant fractions were transferred to new tubes, divided into aliquots, and stored at $-70°C$ until analysis (Shim and Lee 2008; Kim and Lee 2009).

Two-dimensional gel electrophoresis (2-DE)

Isoelectric focusing (IEF) was performed with IPG Drystrips on nonlinear pH gradient 3–10 (24 cm) on IPGphore (all GE Healthcare). Protein was solubilized in rehydration buffer (7 M urea, 2 M thiourea, 4.5% w/v CHAPS, 40 mM Tris, 100 mM DTE, 0.25% IPG buffer). Sample loading on IPG strips with a pH gradient from 3 to 10 was performed with a total volume of 450 μL followed by focusing to a total of 84 kVh at 20°C. After isoelectric focusing, the strips were soaked twice for 20 min each in equilibration buffer (6 M urea, 2% w/v SDS, 20% v/v glycerol, 5 mM tributylphosphine, 2.5% acrylamide, 0.01% bromophenol blue, 0.375 M Tris-HCl, pH 8.8). The second-dimension SDS-PAGE was performed in 8–16% gradient polyacrylamide gels without a stacking gel, at 10°C for 2 h at 5 mA/gel and then at 18 mA/gel (Bartkowiak et al. 2009; Choi et al. 2009; Jeon et al. 2011; Park et al. 2010). Protein bands were visualized by staining with Coomassie Brilliant Blue G-250. The stained gels were scanned on a flatbed scanner (PowerLook 1100; UMAX, Fremont, CA), and the data were analyzed using ImageMaster 2D Platinum 6.0 software (GE Healthcare, Fairfield, CT).

LC-MS/MS

The two-dimensional liquid chromatography mass spectroscopy (LC-MS/MS) system used in this study consisted of an autosampler, two capillary high-performance liquid chromatography (HPLC) pumps, and an LCQ Deca XP Plus ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA) with an electrospray ionization source (AMR, Tokyo, Japan). Two-dimensional HPLC was performed using a combination of SCX chromatography (Microtrap SCH, 1 mm × 8 mm × 12 μm; Michrom BioResources, Auburn, CA) with reversed-phase HPLC (MagicC18, 0.2 mm × 50 mm × 5 μm; Michrom Bio Resources).

Peptides were detected using an electrospray ionization ion trap mass spectrometer (LCQ Deca XP; Thermo-Finnigan, Rockford IL) in positive ion mode, with a spray voltage of 2.5 kV and spray temperature of 150°C. The LCQ Deca XP automatically sets the collision energy in LC-MS/MS mode. After acquiring full-scan mass spectra, three LC-MS/MS scans were acquired for the next three most intense ions, using dynamic exclusion (Yamashita et al. 2005).

Data analysis

Proteins were identified using the Turbo SEQUEST algorithm in the BioWorks 3.1 software package and the NCBI database (NIH, Bethesda, MD). The identified peptides were further evaluated using the charge state versus the cross-correlation number (Xcorr). The Xcorr criteria for peptide evaluation were >2.0 for singly charged ions, >2.5 for doubly charged ions, and >3.7 for triply charged ions. Only the best-matched peptides were adopted (Yamashita et al. 2005).

Western blotting

Brain tissues were homogenized in ice-cold lysis buffer for 30 min at 4°C and then centrifuged at $12,000 \times g$ for 30 min. Total proteins were denatured and resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, incubated overnight with individual antibody at 4°C and then with horseradish peroxidase-conjugated anti-mouse IgG for 1 h. The membranes were visualized using the ECL plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ).

Antioxidative enzyme assays

Antioxidative enzyme assays were performed as in our earlier reports (Lee and Shin 2003). Catalase (CAT) activity was determined spectrophotometrically by measuring the decrease of absorbance at 240 nm due to H_2O_2 decomposition. Superoxide dismutase (SOD) activity was determined using the xanthine/xanthine oxidase/nitroblue tetrazolium system. Inhibition of cytochrome *c* reduction by SOD was measured by the reduction of nitroblue tetrazolium. Glutathione peroxidase (GPX) was determined by the method of Paglia and Valentine (1967). All determinations are expressed as the mean ± S.E. of three separate experiments. Acetylcholine esterase activity was evaluated by measuring the product of its reaction with the substrate acetylthiocholine iodide (ATC). The product,

thiocholine, was evaluated according to the method of Ellman with some modifications (Ellman et al. 1961).

Results

Proteomic analysis

To study the TiO_2 nanoparticle-induced alterations of protein expression in mouse brain, proteomic analysis was performed using high-resolution 2-DE. Figure 1 shows the results of 2-DE of proteins from mouse brain, following exposure to TiO_2 nanoparticles. More than 990 protein spots, with isoelectric points between 3 and 10 and relative molecular masses between 6.5 and 205 kDa, were detected by 2-DE followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). A comparison of the 2-DE protein patterns from brains with and without TiO_2 nanoparticle treatment allowed the identification of 11 protein spots that differed in expression level by more than 2-fold between the basal and treated conditions (Table 1). Of these, eight proteins were upregulated and three were downregulated (Figure 2). The upregulated proteins included a mitochondrial inner membrane protein, heat shock protein, coronin-1A, guanine nucleotide-binding protein, apolipoprotein A-1, hemoglobin beta, lactoylglutathione lyase and cytochrome

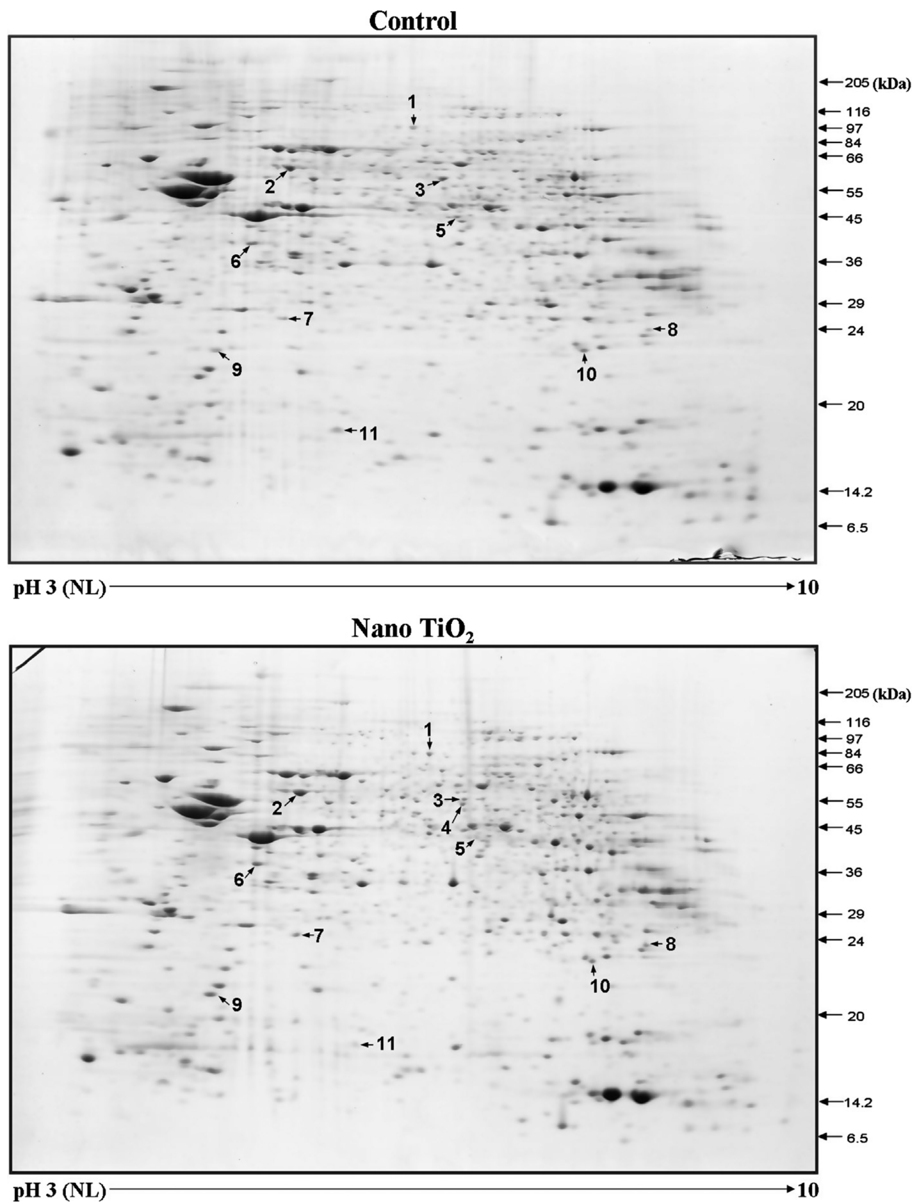

Figure 1. Representative two-dimensional gel electrophoretic profiles of mouse brain by TiO_2 nanoparticle treatment. Proteins on 2-DE gel were visualized by coomassie brilliant blue staining.

Table 1. Identifications of altered proteins from mouse brain following TiO_2 nanoparticle treatment.

No.	Identified Protein	Annotation	Score	Cov%	MW	Change
1	Isoform 1 of mitochondrial inner membrane protien	IPI00228150.1	260.34	42.54	83899.5	↗
2	Isoform 1 of 60kDa heat shock protien, mitochondrial precursor	IPI00308885.6	260.33	52.01	60955.1	↗
3	T-complex protien 1 subunit beta	IPI00320217.9	270.33	63.18	57476.9	↘
4	Coronin-1A	IPI00222600.4	80.30	24.73	50988.9	↗
5	Septin-5	IPI00416280.3	88.26	27.57	42875.8	↘
6	Guanine nucleotide-binding protien G(o) subunit alpha 2	IPI00115546.4	118.29	39.27	40036.2	↗
7	Apolipoprotien A-1 precursor	IPI00121209.1	106.23	39.77	30587.3	↗
8	Hemoglobin, beta adult major chain	IPI00110658.1	70.40	64.08	15202.2	↗
9	Lactoylglutathione lyase	IPI00321734.7	90.31	54.89	20809.5	↗
10	Cytochrome b-c1 complex subunit	IPI00133240.1	80.35	38.32	29367.4	↗
11	Carbonic anhydrase 2	IPI00121534.11	20.23	12.70	29032.3	↘

b-c1 complex. The downregulated proteins were t-complex protein 1, septin-5 and carbonic anhydrase 2. These results were reproducible in three independent experiments.

Validation by Western blotting

To confirm the upregulation of heat shock protein and hemoglobin beta expression by TiO_2, detected via proteomics, we performed Western blotting analysis using specific antibodies (Figure 3). The results indicated that heat shock protein and hemoglobin beta were expressed at considerably lower levels in normal brain cells, but were present at markedly elevated levels after TiO_2 nanoparticle treatment. The result indicates that Western blotting data were well consistent with the proteomic data.

Enzyme assays

The activities of antioxidative enzymes, named superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), and acetylcholine esterase were measured in TiO_2 nanopaerticle-exposed mice as shown in Table 2. The activities of SOD, CAT and GPX in the brain were notably reduced after TiO_2 nanoparticle exposure. Moreover, the acetylcholine esterase activity, one of the indirect markers for brain damage, was also reduced in TiO_2 nanoparticle-exposed mice. Acetylcholine esterase metabolizes acetylcholine to choline and acetyl CoA.

Discussion

Proteomics, a powerful tool for analyzing the expressed genome, has been used successfully to examine changes in global protein profiles in order to select specific biomarkers at the protein level (Blackford et al. 1997; Xiao et al. 2003). While gene expression can be analyzed by measuring mRNA levels, numerous studies have revealed that the abundance of a protein is not strongly correlated with the level of the corresponding mRNA. Thus, proteomic analysis was performed using high-resolution two-dimensional electrophoresis (2-DE) and mass spectrometry to identify differentially expressed proteins in mouse brain after treatment with TiO_2 nanoparticles.

Spot 1, determined to be a mitochondrial inner membrane protein (mitofilin), was upregulated by TiO_2 nanoparticles. This protein controls mitochondrial cristae morphology and is essential for normal mitochondrial function (John et al. 2005). A recent study of cortical brain samples from fetuses with Down's syndrome showed a significant reduction in mitofilin. Although the precise physiological role of this protein is unclear, its upregulation suggests that it is involved in enhancing mitochondrial function (Myung et al. 2003).

The heat shock protein 60 (HSP60) level increased following TiO_2 nanoparticle exposure. HSP60 is a mitochondrial chaperone protein involved in mediating the proper folding and assembly of mitochondrial proteins, especially in response to oxidative stressors. Additionally, HSP60 has been proposed to be an antiapoptotic protein (Lin et al. 2001). The loss of HSP60 function leads to increased misfolding and aggregation of proteins, as well as increased vulnerability to oxidative stress. The increased HSP60 level induced by TiO_2 nanoparticles may be linked to an important defense mechanism that protects the mouse brain from deleterious stress conditions by preventing irreversible protein aggregation (Boyd-Kimball et al. 2005).

Septins are evolutionarily conserved proteins that have an essential function in cytokinesis and also participate in apoptosis, vesicular transport, cytoskeletal dynamics and cell polarity. Interestingly, septins are abundant in the central nervous system, which is composed largely of postmitotic neurons. Septins are associated with many neurological diseases, including Parkinson's and Alzheimer's diseases (Tsang et al.

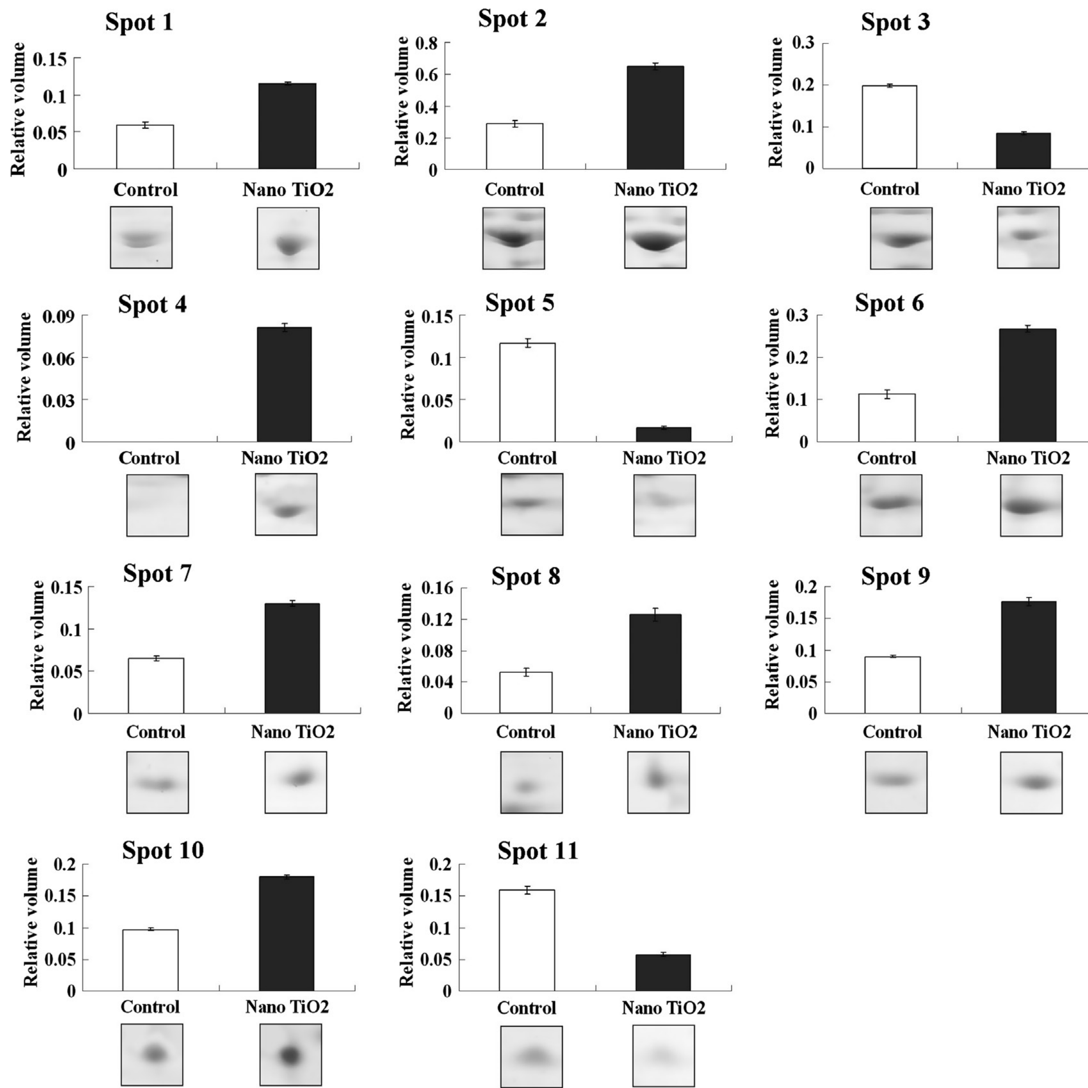

Figure 2. Up- and down-regulated proteins from mouse brain following TiO_2 nanoparticle treatment. Protein expression levels were determined by relative intensity using image analysis. The data on all the bar charts represent means ±SEM from three individual 2DE gels in each experimental group.

2008). Septin 5 (spot 5) was found to be downregulated by TiO_2 nanoparticles. Septin 5, a parkin substrate, is a vesicle- and membrane-associated protein that significantly inhibits exocytosis (Son et al. 2005).

Guanine nucleotide-binding protein (spot 6), which showed increased expression with TiO_2 nanoparticle treatment, is integral to one of the most prevalent signaling systems in mammalian cells, regulating

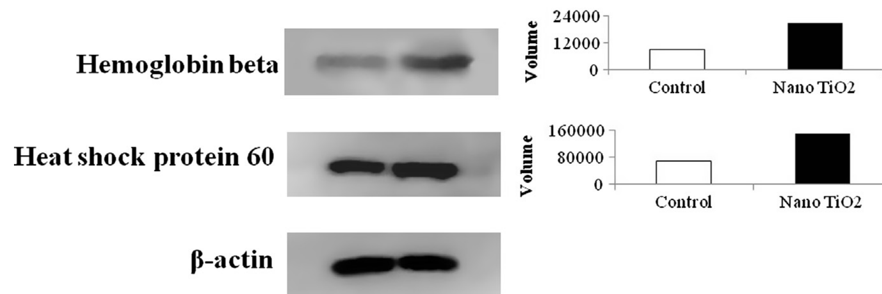

Figure 3. Western blot analysis of protein expression of the hemoglobin beta and heat shock protein 60 in mouse brain by TiO_2 nanoparticle treatment. Band volumes in the western blots were normalized against β-action.

Table 2. The activities of antioxidative enzymes and acetylcholine esterase of mouse brain after TiO_2 nanoparticle treatment. Relative activity refers to comparison with the control.

Enzyme	Relative activity (%)
Superoxide dismutase	39.17 ± 4.84
Catalase	78.71 ± 1.18
Glutathione peroxidase	68.09 ± 3
Acetylcholine esterase	62.5 ± 2.13

functions as diverse as sensory perception, cell growth and hormonal regulation (Roberts and Waelbroeck 2004).

Apolipoprotein A-1, the main apolipoprotein in the central nervous system (Ito et al. 2006), is present in a high-density lipoprotein complex in the cerebrospinal fluid and is thought to contribute to intercellular cholesterol transport in the brain (Dietschy and Turley 2001). Some studies have proposed that systemically circulating apolipoprotein A-1 is transported across the blood–brain barrier (Ito et al. 2004). Apolipoprotein A-1 (spot 7) was upregulated in TiO_2 nanoparticle-treated mouse brain.

Alpha and beta chains of hemoglobin in brain are known to be the precursors of numerous bioactive peptides, some of which are the hemorphins, neokyotorphin and the hemopressins (Gelman et al. 2010). Moreover, hemoglobin expression in mouse brain was suggested to be upregulated in response to ischemia and mutation, and the hemoglobin-derived peptides could be endogenous signaling molecules within the central nervous system, which could bind to CB1 cannabinoid receptors. Moreover, hemoglobin-induced cytotoxicity, caspase activation and oxidative stress were also reported (Gelman et al. 2010). Upregulation of hemoglobin beta (spot 8) was found in TiO_2 nanoparticle-exposed mouse brain.

Exposure to TiO_2 nanoparticles increased the expression of the cytochrome *b-c1* complex (spot 10), which is the most widely occurring electron transfer complex capable of energy transduction. Cytochrome *b-c1* complexes are found in the plasma membranes of phylogenetically diverse photosynthetic bacteria, and in the inner mitochondrial membrane of all eukaryotic cells. In all of these species, the *b-c1* complex transfers electrons from a low-potential quinol to a higher-potential *c*-type cytochrome and links this electron transfer to proton translocation (Trumpower 1990). Thus, upregulation of the cytochrome *b-c1* complex may enhance energy production in response to TiO_2 nanoparticle-induced toxicity.

The expression of carbonic anhydrase II (spot 11) was reduced with exposure to TiO_2 nanoparticles. Carbonic anhydrase isozyme II (CAII), a cytosolic enzyme, is highly expressed in most organs; in brain, it is located primarily in oligodendrocytes (Haapasalo et al. 2007). This enzyme catalyzes the reversible reaction involving the hydration of carbon dioxide and dehydration of carbonic acid. A recent study has revealed that CAII expression was regulated in endothelial cells of melanoma neovessels and in esophageal, renal and lung cancer (Yoshiura et al. 2005). CAII is also expressed in several brain cancers, including oligodendrogliomas and astrocytomas. The downregulation of CAII by TiO_2 nanoparticle treatment suggests that it may have an important functional role in brain tumor metabolism (Haapasalo et al. 2007).

We observed the intracellular localization of TiO_2 using light microscopy, to examine whether TiO_2 nanoparticles enter and accumulate in the mouse brain. However, TiO_2 nanoparticles were not observed in mouse brains exposed to TiO_2 nanoparticles for up to 7 days (data not shown), although recent studies have addressed the neurotoxicity of TiO_2 nanoparticles, which can pass through the blood–brain barrier (Wang et al. 2007, 2008). Some reports about transport of nanoparticles to the brain are often inconsistent. Actually TiO_2 was retained in the liver, spleen, kidney and lung, not in the brain, when the biodistributions of different-sized TiO_2 were systematically examined (Johnston et al. 2009). The translocation rate of nanoparticles to the brain has been known to be very low, probably due to the very tight blood–brain barrier. Moreover, the in vivo and in vitro response to nanoparticles exhibited great diversity, with regard to nanoparticle sizes, aggregates, surface charge, composition and crystal form as well as cell type or organ target, exposure time and exposure route (Johnston et al. 2009; Sohaebuddin et al. 2010). Accordingly, TiO_2 nanoparticles seem to affect the protein profile indirectly in the brain, because we could not detect TiO_2 nanoparticles in the brain under the experimental conditions used here. The investigations into the toxicity of TiO_2 nanoparticle via blood–brain barrier transport should be a focus of future experiment.

Various antioxidative enzymes were notably reduced in TiO_2-exposed mouse brain, probably suggesting a severe oxidative stress state through reactive oxygen species accumulation. Moreover, the reduction of acetylcholine esterase activity by TiO_2 might suggest an increase in cholinergic activity by raising the acetylcholine level, and the elevated acetylcholine continuously influences the receptor to cause cholinergic nerve excitability in the TiO_2 nanoparticle-exposed brain (Ma et al. 2010).

Our results identify proteins associated with brain neurotoxicity and provide a basis for understanding how nanoparticles might indirectly influence protein biosynthesis and enzyme activities in brain.

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