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Molecular mechanism of titanium dioxide nanoparticles-induced oxidative injury in the brain of mice

Yuguan Ze [1], Lei Zheng [1], Xiaoyang Zhao [1], Suxin Gui [1], Xuezi Sang, Junju Su, Ning Guan, Liyuan Zhu, Lei Sheng, Renping Hu, Jie Cheng, Zhe Cheng, Qingqing Sun, Ling Wang, Fashui Hong *

Medical College, Soochow University, Suzhou 215123, People's Republic of China

HIGHLIGHTS

- Exposure to TiO_2 NPs resulted in pathological changes in brain of mice.
- Exposure to TiO_2 NPs led to ROS production, peroxidation of lipid, protein and DNA in brain.
- Exposure to TiO_2 NPs caused the increases of p38, JNK, NF-κB, Nrf-2 and HO-1 expression in brain.
- Activation of HO-1 through the p38-Nrf-2 signaling pathway may modulate TiO_2 NPs-induced oxidative stress in brain.

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ABSTRACT

Numerous studies have demonstrated that the brain is one of the target organs in acute or chronic titanium dioxide (TiO_2) nanoparticles (NPs) toxicity, and oxidative stress plays an important role in this process. However, whether brain oxidative injury responds to TiO_2 NPs by activating the P38-nuclear factor-E2-related factor-2 (Nrf-2) pathway is not fully understood. The present study aimed to examine activation of the P38-Nrf-2 signaling pathway associated with oxidative stress in the mouse brain induced by intranasal administration of TiO_2 NPs for 90 consecutive days. Our findings indicate that TiO_2 NPs caused overproliferation of spongiocytes and hemorrhage in the mouse brain. Furthermore, TiO_2 NPs significantly activated p38, c-Jun N-terminal kinase, nuclear factor kappa B, Nrf-2 and heme oxygenase-1 expression in the brain, which in turn, led to increased production of reactive oxygen species, as well as lipid, protein and DNA peroxidation. These findings suggest that TiO_2 NPs-induced oxidative damage in the mouse brain may occur via the p38-Nrf-2 signaling pathway. Therefore, application of TiO_2 NPs in the environment should be performed with caution.

1. Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) are widely used in a number of applications: as an additive, including as a white pigment in paint, as a food colorant, in sunscreens and in cosmetic creams as well as in the environmental decontamination of air, water, and soil by the destruction of pesticides (Fisher and Egerton, 2001; Kaida et al., 2004; Esterkin et al., 2005; Choi et al., 2006). With the rapid development of nanotechnology, the potential health hazards and environmental impact of manufactured TiO_2 NPs have gained increasing attention.

It has been demonstrated that oxidative stress is one of the most important toxicity mechanisms in the lung (Afaq et al., 1998; Oberdörster et al., 2005; Sun et al., 2012a, 2012b), gill (Federici et al., 2007; Zhang et al., 2007), liver (Wang et al., 2007a,b; Ma et al., 2009; Duan et al., 2010; Cui et al., 2010, 2011, 2012), spleen (Li et al., 2010; Sang et al., 2012), kidney (Chen et al., 2009; Scown et al., 2009; Zhao et al., 2010; Gui et al., 2011) and reproductive system (Zhu et al., 2010; Wang et al., 2011; Gao et al., 2012) in animals following exposure to TiO_2 NPs. Many studies have also shown that TiO_2 NPs can enter the central nervous system (CNS) via the olfactory pathway and damage the brain, i.e. vacuoles in neurons and fatty degeneration, obvious scattered Nissl bodies, large cell somata, irregular appearance of neurons and inflammatory responses (Wang et al., 2007a,b, 2008a,b; Wu et al., 2009). Furthermore, TiO_2 NPs-induced inflammatory responses in the mouse brain were associated with significant increases in tumor necrosis factor alpha, interleukin-1β, specific neurochemicals and lipid peroxidation (Wang et al., 2008b). TiO_2 NPs were also demonstrated to induce microglial activation, thus leading to inflammatory responses in the pre-inflamed brain of mice (Shin et al., 2010). TiO_2 NPs crossed the blood–brain

* Corresponding author. Tel.: +86 0512 61117563; fax: +86 9512 65880103.
E-mail address: Hongfsh_cn@sina.com (F. Hong).
[1] These authors contributed equally to this work.

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barrier, accumulated in the mouse brain caused inflammatory cell infiltration, oxidative damage and hippocampal apoptosis, which in turn led to decreased cognitive function in the mouse brain (Hu et al., 2010, 2011; Ma et al., 2010). However, it is not known whether TiO_2 NPs cause activation of the upstream signaling pathway involved in oxidative stress in brain injury.

To determine whether TiO_2 NPs cause activation of the upstream signaling pathway involved in oxidative stress in brain injury, investigations into the P38-nuclear factor-E2-related factor-2 (Nrf-2) pathway are required. It is well known that the mitogen-activated protein (MAP) kinase cascades (i.e. p38 and c-Jun N-terminal kinase (JNK)) are associated with the upstream signaling mechanism responsible for regulating oxidative stress (Kyriakis and Avruch, 2001), and oxidative stress can activate JNKs and p38 MAP kinases involving MAP kinase cascades (Hagemann and Blank, 2001; Takeda et al., 2003; Qadri et al., 2004). Redox-sensitive transcription factors, especially, nuclear factor kappa B (NF-κB) and Nrf-2 have been identified as target transcription factors of TiO_2 NPs toxicity (Ma et al., 2009; Cui et al., 2011). Under oxidative stress, activated NF-κB can initiate the transcription of a variety of genes and the expression of their proteins that function in the immunological and cellular detoxifying defense systems (Janssen et al., 1995; Pinkus et al., 1996), and has been shown to be a transcription factor regulated by intracellular redox status (Sen and Packer, 1996). It has been demonstrated that Nrf2 binds to antioxidant response elements (AREs) and regulates genes involved in protecting cells from oxidative damage (Zhang, 2006; Kensler et al., 2007). For example, the expression of Nrf2-regulated antioxidant genes was decreased in the lungs of mice with cigarette-smoke induced emphysema (Rangasamy et al., 2009). The products of these cytoprotective genes, such as glutathione S-transferase and NAD(P)H: quinine oxidoreductase 1 (Korashy and El-Kadi, 2006), heme oxygenase 1 (HO-1) (Chen et al., 2005) and γ-glutamylcysteine synthetase (Yang et al., 2005), can prevent chemical toxicity and oxidative stress. It is considered to be one of the most important mechanisms by which cells neutralize the effects of various stresses and survive (Jaiswal, 2004). It is known that Nrf2 under normal conditions is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) which functions as a negative regulator and promotes the quick degradation of Nrf2 via the ubiquitin proteasome system (Motohashi and Yamamoto, 2004; Kobayashi et al., 2006). In response to oxidative stress, Nrf2 dissociates from Keap1 and translocates into the nucleus where it forms a heterodimer with macrophage activating factor (Maf), and ultimately activates ARE-dependent gene expression (Sen and Packer, 1996; Kimura et al., 2007). HO-1 catalyzes the first and rate-limited step in the oxidative degradation of heme to carbon monoxide, biliverdin, bilirubin as well as iron, and is highly induced by heme and oxidative stress, and its induction has been shown to increase antioxidant defenses in rats (Morse and Choi, 2002). Therefore, we hypothesized that brain oxidative damage caused by TiO_2 NPs would be associated with activation of Nrf2 and HO-1 in the mouse brain.

The main aim of the present study was to examine whether developmental TiO_2 NPs exposure caused activation of the P38-Nrf-2 signaling pathway associated with oxidative stress in the mouse brain. We also evaluated whether this pathway offered protection against TiO_2 NPs-induced oxidative brain injury.

2. Materials and methods

2.1. Chemicals and preparation

Nanoparticulate anatase TiO_2 was prepared via controlled hydrolysis of titanium tetrabutoxide. Details of the synthesis and characterization of TiO_2 NPs were described in our previous reports (Yang et al., 2002; Hu et al., 2011). The average particle size of powdered TiO_2 NPs suspended in 0.5% w/v hydroxypropylmethylcellulose (HPMC) K4 M solvent after 12 h and 24 h incubation ranged from 5 to 6 nm and the surface area of the sample was 174.8 $m^2 g^{-1}$. The mean hydrodynamic diameter of the TiO_2 NPs in HPMC solvent ranged from 208 to 330 nm (mainly 294 nm), and the zeta potential after 12 h and 24 h incubation was 7.57 mV and 9.28 mV, respectively (Hu et al., 2011). XRD, TEM and DLS findings are available on request.

2.2. Animals and treatment

80 CD-1 (ICR) male mice (24 ± 2 g) were purchased from the Animal Center of Soochow University (China). All mice were housed in stainless steel cages in a ventilated animal room. Room temperature of the housing facility was maintained at 24 ± 2 °C with a relative humidity of 60 ± 10% and a 12-h light/dark cycle. Distilled water and sterilized food were available *ad libitum*. Prior to dosing, the mice were acclimated to this environment for 5 d. All animals were handled in accordance with the guidelines and protocols approved by the Care and Use of Animals Committee of Soochow University (China). All procedures used in the animal experiments conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH, 1996).

For the experiments, the mice were randomly divided into four groups ($N = 20$), including a control group (treated with 0.5% w/v HPMC) and three experimental groups (2.5, 5, and 10 mg kg^{-1} body weight [BW] TiO_2 NPs). About the dose selection in this study, we consulted the report of World Health Organization in 1969. According to the report, LD50 of TiO_2 for rats is larger than 12 000 mg/kg BW after oral administration. In addition, the quantity of TiO_2 nanoparticles does not exceed 1% by weight of the food according to the Federal Regulations of US Government. In the present study, we selected 2.5, 5, and 10 mg kg^{-1} BW nano-TiO_2 exposed to mice by intranasally administration every day. They were equal to about 0.15–0.7 g nano-TiO_2 of 60–70 kg body weight for humans with such exposure, which were relatively safe doses. The mice were weighed, and the TiO_2 NPs suspensions were administered intranasally every day for 90 d. Symptoms and/or mortality were observed and carefully recorded each day during the 90-d period.

2.3. Preparation of brain

Following 90 d of TiO_2 NPs administration, all mice were weighed and then sacrificed after being anesthetized using ether. The brains were excised, rinsed in phosphate buffered saline (PBS), and quickly frozen at −80 °C.

2.4. Titanium content analysis

Brains were thawed, and approximately 0.1 g of brain was weighed, digested and analyzed for titanium content. Inductively coupled plasma-mass spectrometry (ICP-MS, Thermo Elemental X7, Thermo Electron Co., Finland) was used to analyze the titanium concentration in the samples. The data were expressed as nanograms per gram fresh tissue.

2.5. Histopathological examination of brain

For the pathologic studies, all histopathologic examinations were performed using standard laboratory procedures. The brains were embedded in paraffin blocks, then sliced (5 μm thick) and placed onto glass slides. After hematoxylin–eosin (HE) staining, the stained sections were evaluated by a histopathologist unaware

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Table 1
Real time PCR primer pairs. PCR primers used in the gene expression analysis.

Gene name	Description	Primer sequence	Primer size (bp)
Refer-actin	mactin f	5′-GAGACCTTCAACACCCCAGC-3′	
	mactin r	5′-ATGTCACGCACGATTTCCC-3′	263
p38	mp38 F	5′-GGAGAAGATGCTCGTTTTGGA-3′	
	mp38 R	5′-TTGGTCAAGGGGTGGTGG-3′	211
jnk	mjnk F	5′-TCTCCAGCACCCATACATCAA-3′	
	mjnk R	5′-TCCTCCAAATCCATTACCTCC-3′	150
nf-κb	mnf-κb F	5′-GGTGGAGGCATGTTCGGTA-3′	
	mnf-κb R	5′-TGACCCCTGCGTTGGATT-3′	142
nfr2	mnf-κb F	5′-CTTCCATTTACGGAGACCCACC-3′	
	mnf-κb R	5′-GGATTCACGCATAGGAGCACTG-3′	176
HO-1	mHO-1 F	5′-GACAGAAGAGGCTAAGACCGC-3′	
	mHO-1 R	5′-TGGAGGAGCGGTGTCTGG-3′	213

of the treatments, using an optical microscope (Nikon U-III Multipoint Sensor System, Japan).

2.6. Expression levels of oxidative stress cytokines

The level of mRNA expression of the oxidative stress-related cytokines (i.e., p38, JNK, Nrf-2, NF-κB, and HO-1) in the mouse brain were determined using real-time quantitative RT polymerase chain reaction (RT-PCR) (Ke and Chen, 2000; Liu and Saint, 2002; Livak and Schmittgen, 2001). Synthesized cDNA was used for the real-time PCR by employing primers that were designed using Primer Express Software according to the software guidelines (Table 1). The gene expression analysis and experimental system evaluation were performed according to the standard curve and quantitation reports. To determine p38, JNK, Nrf-2, NF-κB, and HO-1 levels in the mouse brain, enzyme linked immunosorbent assays (ELISA) were performed using commercial kits which were selective for each respective protein (R&D Systems, USA) following the manufacturer's instructions. The absorbance was measured on a microplate reader at 450 nm (Varioskan Flash, Thermo Electron, Finland), and the concentration of p38, JNK, Nrf-2, NF-κB, and HO-1 was calculated from a standard curve for each sample.

2.7. Oxidative stress assay

Assays of reactive oxygen species (ROS; O_2^- and H_2O_2) production, malondialdehyde (MDA), carbonyl content and 8-hydroxydeoxyguanosine (8-OHdG) levels in brain tissues were carried out using commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's instructions.

2.8. Statistical analysis

All results are expressed as means ± standard error (SE). Significant differences were examined by Dunnett's pair-wise multiple comparison *t*-test using SPSS 19 software (USA). A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Titanium content

Titanium content in the mouse brain was detected and shown in Fig. 1. With increased exposure doses, significant titanium accumulation was observed ($p \ll 0.01$), but titanium content from unexposed mice was not detectable.

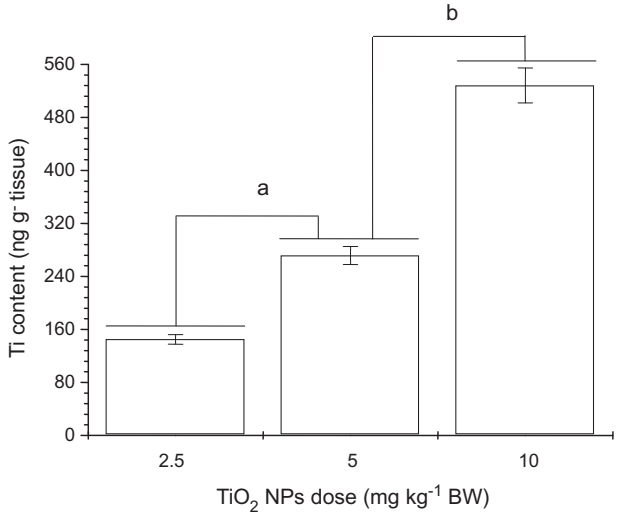

Fig. 1. Titanium contents in the brain of ICR female mice following intranasal administration of TiO_2 NPs for 90 consecutive days. Letters indicate significant differences between groups ($P < 0.05$). Values represent means ± SE ($N = 5$).

3.2. Brain histopathological observations

During administration, animals were all at growth state. The daily behaviors such as eating, drinking and activity in the TiO_2 NPs-treated groups were as normal as the control group. Brain histopathological changes, however, are observed in Fig. 2. Overproliferation of spongiocytes in the mouse brain (Fig. 2b and c) was observed in the 2.5 and 5 mg kg^{-1} BW TiO_2 NPs-treated groups, as compared with unexposed mice. However, in the 10 mg kg^{-1} BW TiO_2 NPs-treated group, overproliferation of spongiocytes and severe hemorrhage in the brain were observed (Fig. 2d). Thus, these results showed that exposure to higher doses of TiO_2 NPs caused severe brain injury. This may be ascribed to ROS generation, which was confirmed by further assays of ROS production in the brain.

3.3. Oxidative stress

High levels of ROS within a cell have a number of direct and indirect consequences on cell signaling pathways and may lead to organ damage. To establish whether ROS levels were elevated in mouse brain injury, we measured ROS generation rate and levels of lipid, protein and DNA peroxidation. As shown in Table 2, mice

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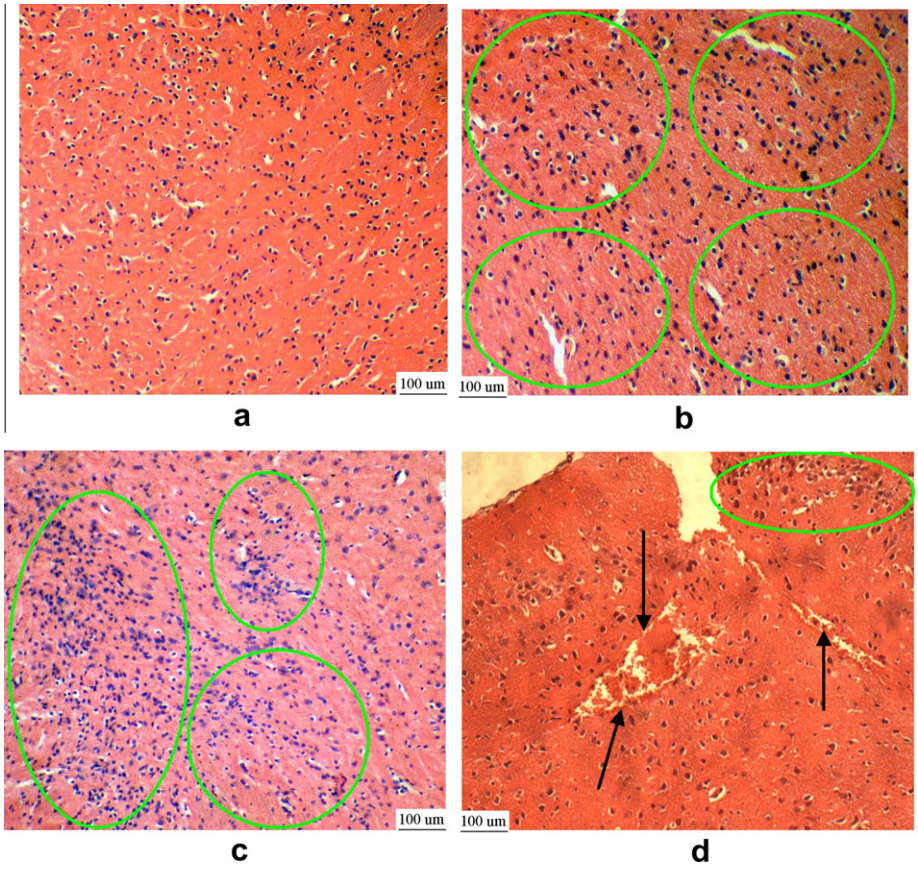

Fig. 2. Histopathology of ICR mouse brain tissue following intranasal administration of TiO_2 NPs for 90 consecutive days. (a) Control, (b) 2.5 mg kg^{-1} BW TiO_2 NPs, (c) 5 mg kg^{-1} BW TiO_2 NPs and (d) 10 mg kg^{-1} BW TiO_2 NPs. Green cycle indicates spongiocyte proliferation; Black arrows indicate hemorrhage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Oxidative stress of brain of mice after intranasal administration of TiO_2 NPs for 90 consecutive days.

Oxidative stress	TiO_2 NPs (mg kg^{-1} BW)			
	0	2.5	5	10
$O_2^{\cdot-}$ (nmolmg^{-} protein min)	34.10 ± 1.71a	57.70 ± 2.88b	86.01 ± 4.30c	104.91 ± 5.25d
H_2O_2 (nmol mg^{-} prot min)	48.75 ± 2.44a	64.88 ± 3.24b	102.61 ± 5.13c	130.59 ± 6.53d
MDA (μmol g^{-} tissue)	24.72 ± 1.24a	31.74 ± 1.59b	41.91 ± 2.10c	53.27 ± 2.66d
Carbonyl (μmol mg^{-} prot)	0.43 ± 0.02a	0.49 ± 0.02a	0.56 ± 0.03b	0.73 ± 0.04c
8-OHdG (mg g^{-} tissue)	0.35 ± 0.02a	0.41 ± 0.02a	0.53 ± 0.03b	0.70 ± 0.04c

Letters indicate significant differences between groups ($P < 0.05$). Values represent means ± SE ($N = 5$).

treated with 2.5–10 mg kg^{-1} BW TiO_2 NPs for 90 consecutive days showed significantly increased levels of $O_2^{\cdot-}$ and H_2O_2, and increased MDA, protein carbonyl and 8-OHdg contents in the brain as compared to untreated mice ($P < 0.05$ or 0.01). The effects of TiO_2 NPs on ROS generation, and lipid, protein and DNA peroxidation levels were dose-dependent. Oxidative damage in the mouse brain caused by exposure to TiO_2 NPs may be related to activation of the upstream signaling pathway responsible for regulating oxidative stress, which was confirmed by further assays of the activation of the P38-Nrf-2 signaling pathway in mouse brain.

3.4. Activation of the P38-Nrf-2 signaling pathway

To confirm whether TiO_2 NPs exposure activated the oxidative stress-upstream signaling pathway, including p38, JNK, NF-κB, Nrf-2 and HO-1, in TiO_2 NPs-induced brain injury, real-time quantitative RT-PCR and ELISA were used to detect alterations in the oxidative stress-related genes and their proteins levels in TiO_2 NPs-treated mice (Figs. 3 and 4). Figs. 3 and 4 indicate that exposure to TiO_2 NPs significantly induced the expression of p38, JNK, NF-κB, Nrf-2 and HO-1 in the treated mouse brain ($P < 0.05$ or 0.01), which was consistent with the trends observed on the histological photomicrograph of brain sections and oxidative stress in exposed mice.

4. Discussion

The results of the present study indicate that the TiO_2 NPs accumulation in the mouse brain was observed (Fig. 1), which resulted significant overproliferation of spongiocytes and hemorrhage of brain after intranasal administration of TiO_2 NPs for 90 consecutive days (Fig. 2), which may indirectly or directly disturb the homeostasis of trace elements, neurotransmitters and enzymes in the brain. Following administration by injection for 14 consecutive

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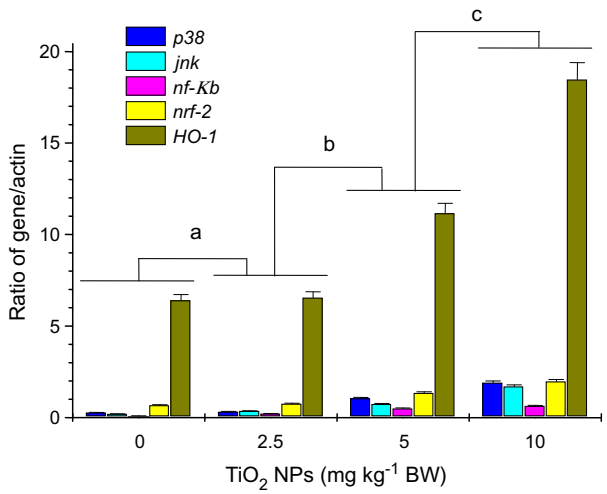

Fig. 3. Effect of TiO_2 NPs on the amplification of oxidative stress gene mRNA in the mouse brain as shown by real-time PCR analysis following intranasal administration of TiO_2 NPs for 90 consecutive days. Letters indicate significant differences between groups ($P < 0.05$). Values represent means ± SE ($N = 5$).

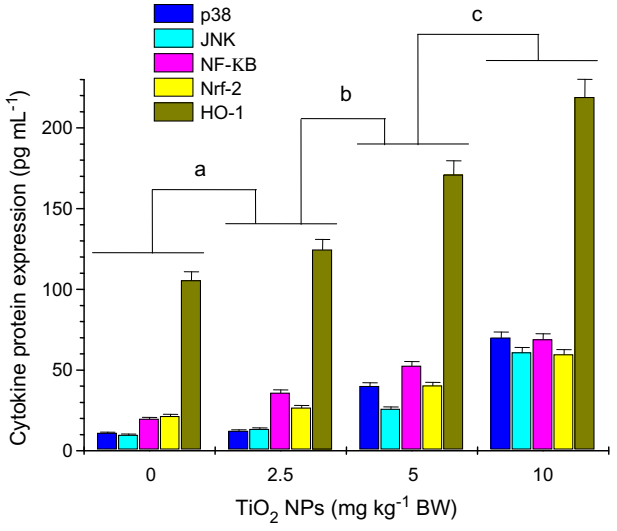

Fig. 4. Effect of TiO_2 NPs on the oxidative stress protein level in the mouse brain as shown by ELISA following intranasal administration of TiO_2 NPs for 90 consecutive days. Letters indicate significant differences between groups ($P < 0.05$). Values represent means ± SE ($N = 5$).

days and by intragastric administration for 90 consecutive days, TiO_2 NPs caused inflammatory cell infiltration, hippocampal apoptosis and oxidative stress in the mouse brain and hippocampus (Ma et al., 2010; Hu et al., 2011). The present study suggests that brain injury in mice may be triggered by TiO_2 NPs-induced oxidative stress and activation of oxidative stress-genes which resulted in brain lesions.

ROS were one of the triggers involved in brain damage. In order to demonstrate whether exposure to TiO_2 NPs following intranasal administration caused oxidative stress in the mouse brain, we assayed the levels of ROS accumulation, and lipid, protein and DNA peroxidation. The results showed overproduction of ROS (such as $O_2^{\cdot-}$ and H_2O_2) and high levels of lipid, protein and DNA peroxidation in the mouse brain treated with TiO_2 NPs (Table 2). These findings prove that TiO_2 NPs exposure resulted in oxidative stress in the mouse brain, consistent with brain injury. Previous studies have also suggested that TiO_2 NPs exposure can increase ROS production and lipid peroxidation levels in mouse spleen, liver, and kidney (Li et al., 2010; Liu et al., 2010; Zhao et al., 2010). ROS are generated following acute TiO_2 NPs overload and play an important role in the progression of organ injury (Li et al., 2010; Liu et al., 2010; ; Ma et al., 2010; Zhao et al., 2010; Hu et al., 2011). Here, we speculate that TiO_2 NPs may displace other Fenton metals from their binding sites, which in turn, induce ROS formation (Choi and Alam, 1996; Kaida et al., 2004; Long et al., 2006). Another mechanism by which TiO_2 NPs can generate ROS is by decreasing the activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, or intracellular levels of antioxidants such as glutathione (Li et al., 2010; Liu et al., 2010; Zhao et al., 2010; Ma et al., 2010; Hu et al., 2011). As ROS act as second messengers in intracellular signaling cascades (Long et al., 2007), the increase in ROS caused by TiO_2 NPs may be associated with activation of the P38-Nrf-2 signaling pathway in brain injury.

To further prove that activation of the upstream signaling pathway is associated with oxidative stress due to exposure to TiO_2 NPs, we investigated MAP kinase signal transduction and subsequent transcription factor activation, which showed high levels of p38 and JNK expression following exposure to TiO_2 NPs (Figs. 3 and 4). In response to oxidative stress caused by TiO_2 NPs, the p38 and JNK MAP kinase signaling pathway was activated in the mouse brain. In this study, TiO_2 NPs-induced NF-κB activation was observed (Figs. 3 and 4). NF-κB is the major stress response transcription factor, and has been reported to respond to a wide variety of environment stressors. Nrf-2 is a critical regulator of the cellular antioxidant response and xenobiotic metabolism, and its accumulation in the nucleus is an essential signaling step for its function as a transcription factor (Valko et al., 2006). In the present study, treatment with TiO_2 NPs resulted in a significant increase in Nrf-2 gene and protein expression in mouse brain (Figs. 3 and 4). The expression of a wide array of antioxidant and detoxification genes is positively regulated by the ARE-driven sequence, a cis-acting regulatory element that is bound by Nrf-2. Thus, Nrf-2 may serve as a master regulator of ARE-driven cellular defense systems against oxidative stress (Nguyen et al., 2004). These findings show that the high level of Nrf-2 expression may be a cellular adaptive response to TiO_2 NPs toxicity. A possible mechanism for Nrf-2 activation by TiO_2 NPs may be that existing Nrf-2 escaped ubiquitination and proteasomal degradation, resulting in increased stability of this transcription factor in the cytoplasm and its subsequent nuclear translocation. Whether TiO_2 NPs also interact with Nrf-2 and/or Keap 1 requires further study.

To further reveal the possible downstream genes through which p38-Nrf-2 activation following exposure to TiO_2 NPs may protect against oxidative stress, the expression of HO-1, whose promoters contain an ARE, was determined in TiO_2 NPs-treated mice. HO-1 has antioxidant capacity and therefore acts as a potent anti-inflammatory protein during oxidative injury (Lee et al., 2005). Our data show that TiO_2 NPs exposure induced a marked increase in HO-1 level (Figs. 3 and 4). These findings indicate that HO-1 may be partly responsible for the protective effect of p38-Nrf-2 against TiO_2 NPs-induced oxidative injury in the mouse brain. The increase in HO-1 expression in the mouse brain following exposure to TiO_2 NPs may be regulated via p38 MAP kinase and the Nrf-2 signal transduction pathway, and act as a cellular adaptive response to oxidative stress.

5. Conclusion

This study showed that TiO_2 NPs exposure following intranasal administration caused activation of HO-1 through the p38-Nrf-2

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signaling pathway, which in turn, led to ROS accumulation and oxidation of lipids, proteins and DNA, and triggered brain injury in mice. Our findings suggest the need for caution in workers and consumers when handling nanomaterials.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.01.094.

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