Titanium Dioxide Nanoparticles Induce Cognitive Dysfunction of Rats with Endogenous Protective Mechanism of Increased miR-21-5p

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Research

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Abstract

Background

Titanium dioxide nanoparticles (TiO_2 NPs) cause nerve cell damage and central nervous system dysfunction has been well known. However, most of the cognitive deficits occurs before the observable damage to nerve cells and the regulatory mechanism is largely unknown. Mitochondria and energy metabolism are the targets of many neurotoxic substances.

Results

Here, we showed that TiO_2 NPs exposure reduced the mitochondrial membrane potential (ΔΨm) and ATP content, which is the important cause of cognitive deficits, and upregulated microRNA-21-5p (miR-21-5p) levels in rat neuron cells. Upregulated miR-21-5p promoted phosphorylation of c-Jun, consequently promoting transcription of *VDAC1* (encoding voltage-dependent anion channel 1), which in turn preserved the ΔΨm and ATP content of neuron cells.

Conclusions

Mechanistically, upregulated VDAC1 was attributed to TiO_2 NPs-induced phosphorylation of c-Jun (Ser73), which is predicted to be a transcription factor of *VDAC1*, while upregulation of the ΔΨm and ATP content was caused by the increased oxygen consumption rate induced by VDAC1. The results of our present study revealed an endogenous self-protection mechanism in the process of TiO_2 NPs-induced cognitive deficits.

1. Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) have high chemical stability, and excellent optical properties, thus are used widely in various fields, such as cosmetics production, food processing, paint manufacturing, etc. [1]. TiO_2 has been approved by the US Food and Drug Administration for food production as a color additive many years ago [2, 3]. It has been reported that over 93% of the TiO_2 in gum comprises TiO_2 NPs [2]. TiO_2 NPs also has been found in many foods that claim not to contain TiO_2 NPs, and previous tests have shown up to 9 g/kg of food [4]. There is increasing awareness of the health risks caused by the absorption of TiO_2 NPs into the human body. The central nervous system (CNS) is one of the important targets for TiO_2 NPs [5]. The digestive system is an important way for TiO_2 NPs to enter the human body. After entering the blood through the digestive tract, TiO_2 NPs can enter the central nervous system through the blood-brain barrier due to its small size [6, 7], where they damage nerve cell function by promoting inflammation, activating oxidative stress, and destroying cell membrane [8].

It has been reported that TiO_2 NPs induced inflammatory injury in SH-SY5Y cells (Cells derived from human bone marrow neuroblastoma) in a dose-dependent manner [5]. *In vivo*, TiO_2 NPs entering the

central nervous system (CNS) can lead to mitochondrial dysfunction and decrease of mitochondrial membrane potential (ΔΨm) [9]. In the CNS, the energy metabolism level determines the brain's ability to perform its normal functions, such as learning and memory [10, 11]. It has been reported that TiO_2 NPs could cause ΔΨm loss, hippocampal damage, and impairment of spatial recognition memory in mice, which indicated that TiO_2 NPs promote apoptosis of hippocampal neurons through the mitochondrial pathway [12]. So far, dysfunction of the central nervous system is believed to be the result of neuronal cell damage induced by TiO_2 NPs, which promote energy metabolism disorders. However, most of the central nervous system dysfunction occurs before the observable damage to nerve cells. This means that when the damaging effect is observed, the best time to prevent it has been missed. Therefore, revealing the molecular mechanism of TiO_2 NPs-induced energy metabolism disorder will help to prevent the damaging effect of the nervous system earlier.

Our present study defined a novel mechanism of energy metabolism damaged by TiO_2 NPs in neuron cells. Upregulated miR-21-5P, induced by TiO_2 NPs, preserved TiO_2 NPs-induced the decline of ΔΨm and ATP content through increasing oxygen consumption rate (OCR). Upregulation of miR-21-5P promoted transcription of *VDAC1* (encoding voltage-dependent anion channel 1) leading to the upregulation of VDAC1 protein levels, which in turn preserved the OCR, ΔΨm, and ATP content of neuron cells under TiO_2 NPs exposure. This endogenous self-protection mechanism in the process of TiO_2 NPs-induced energy metabolism disorder in neuron cells could improve our understanding of TiO_2 NPs-induced nervous system dysfunction and help us to prevent TiO_2 NPs-induced CNS injury earlier.

2. Materials And Methods

2.1 Animals and treatments

This study was conducted with the approval of the Animal Care and Use Committee of the Tianjin Institute of Environmental and Operational Medicine, China. All rats handling procedures were performed in strict accordance with the guide for the use and care of laboratory animals. All surgery was performed under sodium pentobarbital (1%, 1 mL/100 g weight, i.p.) anesthesia.

Before the experiment, male Wistar rats (180–200 g) were kept in their surroundings for 1 week. The rats were maintained on a 12 h light/dark cycle in constant temperature environment (25 ± 2 °C). Animals were randomly divided into four groups: Control group (treated with 0.5% w/v hydroxypropyl methylcellulose (HPMC)) and three experimental groups (10, 20, or 30 mg/kg body weight (BW) TiO_2 NPs). The TiO_2 NPs (Sigma-Aldrich, St. Louis, MO, USA; 637254) suspensions were given to the rats via intragastric administration every day for 4, 6, or 8 weeks, respectively. Animals were weighed before intragastric administration every day.

The water maze was used to test cognitive function as previously reported [13]. The test was conducted three times a session for 4 consecutive days with an interval of 30 minutes. Software (v3.1.16 Noldus

Information Technology, Wageningen, Netherlands. Statsoft Inc. Oklahoma,USA) was used to track the swimming path in each test.

2.2 Cell culture, Constructs, Reagents, Antibodies, and Quantitative Real-Time PCR (qPCR)

Please refer to the supplementary materials for details

2.3 Mitochondrial membrane potential (ΔΨm)

JC-1 (5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide, Sigma-Aldrich) was used to detect the ΔΨm. Cells were placed in a culture medium containing JC-1 (2.5 μg/mL) for 30 min in a cell incubator. Flow cytometry (FCM) was used to detect the ΔΨm.

2.4 ATP content

Cold phosphate-buffered saline (PBS) was used to collect cells, and the samples were quickly frozen and preserved in liquid nitrogen. The samples were melted slowly in an ice water bath and vortexed for 10 s. Luciferase driven bioluminescence with an ATP Bioluminescence Assay Kit HS II (Sigma-Aldrich, Cat. No. 11699709001) was used to assay the ATP content.

2.5 Seahorse

The OCR and ECAR were measured as described previously [14] using a Seahorse XFe24 system (Agilent Technologies, Santa Clara, CA, USA).

2.6 Luciferase Reporter Assay

Luciferase reporter assays were conducted as previous reports [15].

2.7 Western Blotting

Western blotting was conducted as described in our previous publication [16].

2.8 Statistical Analysis

SPSS 17.0 software (IBM Corp., Armonk, NY, USA) was used to perform statistical analyses. The differences between two groups were determined using Student's t-test. For three and more groups of data, statistical analysis was performed using analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test or an unpaired two-tailed Student-t-test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1 Upregulation of miR-21-5P expression, mitochondrial dysfunction and cognitive deficits were observed in response to TiO_2 NPs.

As shown in Fig. 1, increased levels of miR-21-5P were detected in prefrontal cortex of rat (Fig. 1A & 1D), primary neuron cells (Fig. 1B & 1E), and SH-SY5Y cells (Fig. 1C & 1F) in response to TiO_2 NPs. The prefrontal cortex requires a large amount of energy, and multiple information processing functions, such as memory, judgment, analysis and thinking, which are involved in preserving fundamental cognitive ability, require energy in the form of ATP. Regulation of the energy balance might be a key factor involved in neuroprotection against TiO_2 NPs. Thus, we determined the ΔΨm and ATP content and found that the ΔΨm and ATP levels decreased markedly in response to TiO_2 NPs in a dose-dependent manner (Fig. 1G & 1H). Water maze test showed that the latency of rats to locate the hidden platform was significantly prolonged after titanium dioxide intervention (Fig. 1I). Meanwhile, the swimming distance of rats at the edge of the pool was also significantly increased (Fig. 1J & 1K). These results suggested that miR-21-5p might be related to mitochondrial function and cognitive deficits under TiO_2 NP treatment.

3.2 MiR-21-5p preserves the ΔΨm and ATP levels of SH-SY5Y cells under TiO_2 NP treatment.

It has been reported that intracellular reactive oxygen species (ROS) can regulate miR-21-mediated pathways [17, 18]; however, regulation by miR-21 of mitochondrial function is rarely reported. To further explore the relationship between miR-21-5p and mitochondrial function under TiO_2 NP intervention, we analyzed the correlation between miR-21-5p and the ΔΨm in SH-SY5Y cells treated with TiO_2 NPs. The results showed that the level of miR-21-5p correlated significantly with the ΔΨm in SH-SY5Y cells treated with TiO_2 NPs (Fig. 2A). There was also a significant positive correlation between the ATP content and mir-21-5p levels in TiO_2 NPs-treated SH-SY5Y cells (Fig. 2B). These results suggested that miR-21-5p has a protective effect on mitochondrial function in SH-SY5Y cells treated with TiO_2 NPs. An miR-21-5p overexpression vector (Fig. 2C) and short hairpin RNA (shRNA) vector to silence miR-21-5p (Fig. 2D) were employed to further evaluate the protective effect of miR-21-5p on mitochondrial function in SH-SY5Y cells treated with TiO_2 NPs. As shown in Fig. 2E, knockdown of miR-21-5p by shmiR-21-5p led to a further decrease in the ΔΨm of TiO_2 NPs-treated SH-SY5Y cells, and miR-21-5p overexpression reversed the decrease in the ΔΨm induced by TiO_2 NPs. Furthermore, miR-21-5p overexpression reversed the decrease in the ATP content induced by TiO_2 NPs in SH-SY5Y cells (Fig. 2F). These results demonstrated that miR-21-5p protects mitochondrial function against damage from TiO_2 NPs in SH-SY5Y cells.

3.3 MiR-21-5p protects the respiratory and glycolytic ability of cells under TiO_2 NPs treatment.

To further assess the effects of miR-21-5p on energy metabolism function in SH-SY5Y cells, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of SH-SY5Y cells were measured by Seahorse XF-24 system (Fig. 3A). All data were normalized to ensure that they were not affected by cell density [14]. Basal, ATP-linked, and maximal respiration were significantly decreased in miR-21-5p-

silenced SH-SY5Y cells (shmiR-21-5p) compared with those in the controls (Fig. 3B). The inhibition of ATP-linked respiration and decline of ATP content mentioned in Fig. 2F showed the same trend. In order to further observe the effect of m on cell glycolysis activity, ECAR induced by oligomycin was employed [19, 20]. A remarkably decrease in ECAR induced by oligomycin was detected in miR-21-5p-silenced SH-SY5Y cells (shmiR-21-5p) compared with that in the controls under TiO_2 NPs treatment. No difference between groups was observed in basal ECAR (Fig. 3C). The OCR to ECAR ratio showed decreased induction of respiratory capacity in miR-21-5p-silenced SH-SY5Y cells (shmiR-21-5p) compared with that in the controls (Fig. 3D). These results indicated that miR-21-5p protects the ability of SH-SY5Y cells to respond to TiO_2 NPs.

3.4 VDAC1 is crucial for miR-21-5p in protecting the mitochondria function of SH-SY5Y cells in response to TiO_2 NPs.

The voltage-dependent anion channel 1 (VDAC1) locates in mitochondrial outer membrane, where it regulates protein and metabolite trafficking in and out of the mitochondria [21]. It has also been reported that VDAC has an important relationship with mitochondrial respiration [22]. Thus, we assessed the levels of VDACs, including VDAC1, VDAC2, and VDAC3, in SH-SY5Y cells with or without shmiR-21-5p. As shown in Fig. 4A, VDAC1 and VDAC3 levels decreased markedly in SH-SY5Y cells (shmiR-21-5p) compared with those in SH-SY5Y cells (Nonsense) under TiO_2 NPs treatment. The level of VDAC1 increased significantly in response to TiO_2 NPs treatment, while VDAC3 levels were similar between the groups (Fig. 4B). A *VDAC1* overexpression vector (Fig. 4C) was employed to study the role of VDAC1 in miR-21-mediated protection of respiratory function in response to TiO_2 NPs treatment. *VDAC1* overexpression reversed the lower $\Delta\Psi m$ levels (Fig. 4D) and ATP content (Fig. 4E) induced by shmiR-21-5p under TiO_2 NPs treatment. Similarly, *VDAC1* overexpression increased the basal, ATP-linked, and maximal respiration that had been decreased by shmiR-21-5p in SH-SY5Y cells under TiO_2 NPs treatment (Fig. 4F). These results confirmed that VDAC1 plays an important role in mir-21-5p-mediated protection of cellular respiratory function

3.5 Phosphorylated c-Jun is responsible for *VDAC1* upregulation.

The mRNA levels of *VDAC1* in TiO_2 NPs-treated SH-SY5Y cells were remarkably higher than those in the control group (Fig. 5A). Overexpression of miR-21-5p resulted in a significantly higher level of *VDAC1* mRNA under TiO_2 NPs treatment (Fig. 5B), while knockdown of miR-21-5p by shmiR-21-5p decreased the *VDAC1* mRNA level compared with that in control group (Fig. 5C). These results suggested that miR-21-5p regulates the transcription of *VDAC1*. To study the molecular mechanism by which miR-21-5p regulates *VDAC1* transcription, *VDAC1* promoter constructs Del 1 (-2000 to + 135) and Del 2 (-908 to + 135) (Fig. 5D) were employed to assess promoter-dependent transcriptional activity. The promoter-dependent transcriptional activity of the *VDAC1* promoter construct Del 1 was remarkably higher in TiO_2 NPs-treated SH-SY5Y cells than in the control group, while the activity of *VDAC1* promoter construct Del 2 was not different between the groups (Fig. 5E). Thus, TiO_2 NPs may increase the mRNA levels of *VDAC1* through

the promoter region (−2000 to −908). Next, potential transcription factors were predicted and detected by western blotting. The results indicated that TiO_2 NPs promoted the phosphorylation of c-jun, but had no effect on other transcription factors, including c-ETS1, T3R-β, c-Myb, and NF-AT2 (Fig. 5G). Inhibition of c-Jun phosphorylation prevented the transcription of *VDAC1* mRNA (Fig. 5H) under TiO_2 NPs treatment and led to a further decline in the ΔΨm level (Fig. 5I) and ATP (Fig. 5J) content in SH-SY5Y cells. These results suggested that phosphorylated c-Jun, as a transcription factor of *VDAC1*, promotes the transcription of *VDAC1* mRNA and protects the respiration function in SH-SY5Y cells under TiO_2 NPs treatment. Taken together, our results strongly demonstrated that miR-21-5p upregulation contributes to TiO_2 NPs-induced c-Jun (Ser73) phosphorylation, which, as a transcription factor, leads to VDAC1 transcriptional upregulation, which in preserves the ΔΨm level and ATP content in neuron cells, as summarized in Graphical abstract.

4. Discussion

Titanium dioxide nanoparticles (TiO_2 NPs) have been widely used in various industries, such as chemistry, paint, food, and medicine [23]. It has been reported that TiO_2 NPs induce central nervous system (CNS) dysfunction by accumulating in the brain [24]. In zebrafish, a low dose of TiO_2 NPs (5 μg/L) can lead to the aggregation of TiO_2 NPs in neurons, the increase of apoptosis, and the decrease of spatial recognition [25]. *In vitro*, TiO_2 NPs were also found to induce apoptosis in primary cultured hippocampal neurons [12]. In the process of TiO_2 NPs-induced neuron apoptosis, it was found that TiO_2 NPs could promote the decrease of ΔΨm, the release of cytochrome c, and the increase of caspase-3 level [24]. Those results indicated that mitochondria participate in the apoptosis of neurons induced by TiO_2 NPs. Mitochondria are semi-autonomous organelles responsible for energy production, cell differentiation, cell information transmission, cell growth, and cell cycle [26]. Given their many crucial functions in cell physiology, it is not surprising that mitochondria are involved in, or mediate, cognitive deficits, even before neuronal damage. Although some studies have reported the damaging effect of TiO_2 NPs on mitochondrial function, to date, most studies have focused on the downstream signaling pathway after mitochondrial function damage, such as the activation of the apoptosis pathway or oxidative stress effects. The molecular mechanisms of mitochondrial function regulation under the influence of TiO_2 NPs remain unclear. Understanding these mechanisms is essential to protect CNS function under TiO_2 NPs exposure. In the present study, we demonstrated that TiO_2 NPs are strong positive regulators of miR-21-5p expression, which protects the neuron cells in response to TiO_2 NPs, and that VDAC1 plays an important role in the function of miR-21-5p by preserving oxygen consumption rate, ΔΨm, and the ATP content.

MiR-21 is considered an oncogenic molecular due to inhibiting the expression of phosphatases, which regulate the signaling pathways such as AKT and MAPK in cells [27, 28]. In non-cancer cells, miR-21 usually acts as a protector [29, 30, 31]. Although microRNA-21 (miR-21) is believed to be related to the regulation of mitochondrial energy metabolism [32, 33, 34, 35], the effect of TiO_2 NPs on miR-21 has not

been reported. In the present study, we discovered that the level of miR-21-5p was upregulated in primary neuron cells, SH-SY5Y cells, and rat prefrontal cortex under TiO_2 NPs treatment in a dose- and time-dependent manner (Fig. 1A – 1F). Considering that TiO_2 NPs also led to a decrease in the ΔΨm (Fig. 1G) and ATP content (Fig. 1H), miR-21-5p seemed to be a cause or result of mitochondrial dysfunction under TiO_2 NPs treatment. To confirm the function of miR-21-5p, the correlation of the miR-21-5p level with the ΔΨm or ATP content of SH-SY5Y cells treated with TiO_2 NPs was analyzed, and SH-SY5Y cells (miR-21) and SH-SY5Y cells (shmiR-21) were employed (Fig. 2C &2D). Under TiO_2 NPs treatment, there was a significant correlation between miR-21-5p and the ΔΨm (Fig. 2A) or the ATP content (Fig. 2B). Knockdown of miR-21-5p resulted in a significant decrease in the ΔΨm and ATP content of SH-SY5Y cells under TiO_2 NPs treatment. Overexpression of miR-21-5p resulted a significant increase in the ΔΨm and ATP content of SH-SY5Y cells under TiO_2 NPs treatment (Fig. 2E & 2F). Our results indicated that the increase of miR-21-5p expression induced by TiO_2 NPs is an important protective mechanism to preserve the ΔΨm and ATP content in neuron cells.

In the process of mitochondrial electron transport, the electrochemical potential energy is stored in the inner membrane of mitochondria. On both sides of the inner membrane, the asymmetric distribution of proton and other ion concentration will form the ΔΨm, which is the core driving oxidative phosphorylation to produce ATP [36]. The oxygen consumption rate (OCR) is the basis of mitochondrial oxidative phosphorylation and ATP synthesis [14, 37]. In our study, OCR was measured using the Seahorse system in SH-SY5Y cells (shmiR-21) and SH-SY5Y cells (Nonsense) under TiO_2 NPs treatment. Basal, ATP-linked, and maximal respiration were remarkably declined in SH-SY5Y cells (shmiR-21) compared with those in SH-SY5Y cells (Nonsense) (Fig. 3B). This inhibition in ATP-linked respiration corresponded with the decreased ATP content in SH-SY5Y cells (shmiR-21) (Fig. 2F). Thus the damaged OCR induced by TiO_2 NPs was protected by mir-21-5p. Although glycogen depletion, caused by uncontrolled glycolysis, leads to cognitive impairment [38], glycolysis is the main source of energy supply for anoxic neurons. Moreover, previous studies suggested that lactate, a glycolytic product, plays an important role in memory processing [14]. Our results showed that miR-21 knockdown reduced the extracellular acidification rate (ECAR) induced by oligomycin under TiO_2 NPs treatment (Fig. 3C). This suggested that glycolysis is also one of the ways by which miR-21 protects neurons under TiO_2 NPs treatment.

Voltage-dependent ion channels are involved in the process of energy metabolism [39]. Here, we determined the expression levels of VDACs, and found that VDAC1 expression was regulated by miR-21 (Fig. 4A) as well as by TiO_2 NPs (Fig. 4B). VDAC1plays an important role in glycolysis and oxidative phosphorylation [40]. High levels of VDAC1 were demonstrated in the brains of patients with Alzheimer's disease and in amyloid precursor protein transgenic mice [41]. Some studies have found that Vdac1 overexpression is related to the release of cytochrome c [40]. However, in the present study, VDAC1 overexpression reversed the reduction of the ΔΨm (Fig. 4D) and ATP content (Fig. 4E) induced by miR-21 knockdown in SH-SY5Y cells under TiO_2 NPs treatment. Basal, ATP-linked, and maximal respiration also increased significantly in SH-SY5Y cells (VDAC1, shmiR-21) compared with those in control SH-SY5Y

cells (Vector, shmiR-21) (Fig. 4F). Therefore, we speculated that TiO_2 NPs would upregulate the VDAC1 via miR-21 accumulation, which, as an endogenous protection mechanism, consequently preserves the OCR, ΔΨm, and ATP content.

Up to now, no mechanism of titanium dioxide regulating VDAC has been reported. To explore how miR-21 regulates VDAC1 under TiO_2 NPs treatment, we detected the *VDAC1* mRNA level in SH-SY5Y, SH-SY5Y (miR-21), and SH-SY5Y (shmiR-21) cells with TiO_2 NPs treatment (Fig. 5A – 5C). Thus, miR-21 might upregulate the VDAC1 by promoting its mRNA transcription under TiO_2 NPs treatment. To verify this speculation, the promoter-dependent transcriptional activities of *VDAC1* were detected in SH-SY5Y cells with or without TiO_2 NPs treatment. The results indicated that *VDAC1* promoter transcription factor binding sites (-908 to -2000) were responsible for *VDAC1* mRNA upregulation in TiO_2 NPs-treated cells (Fig. 5D & 5E). Although studies have reported that the transcriptional regulation of *VDAC1* plays an important role in different cell models [42, 43, 44], the mechanism of *VDAC1* transcription regulation is still unclear, especially under the intervention of TiO_2 NPs. Bioinformatic methods predicted transcription factor binding sites and then the levels of the predicted transcription factors using western blotting in cells with or without TiO_2 NPs treatment (Fig. 5G). The level of phosphorylated (P)-c-Jun (Ser73) was upregulated markedly in TiO_2 NPstreated SH-SY5Y cells compared with that in the control cells. Furthermore, inhibition of c-Jun phosphorylation reversed the upregulation of VDAC1 induced by TiO_2 NPs and decreased the ΔΨm and ATP content in SH-SY5Y cells. Based on these results, we proposed a previously unreported mechanism of *VDAC1* transcriptional regulation and the protective role of VDAC1 protein in neuron cells in response to TiO_2 NPs.

In summary (Graphical abstract), our results defined a novel effect of TiO_2 NPs on miR-21-5p upregulation, which would inhibit the expression of an as yet unidentified phosphatase, leading to increased or maintained phosphorylation of c-Jun. This active form of c-Jun would increase the transcriptional activity of *VDAC1* leading to the upregulation of VDAC1 protein levels, an increased OCR, elevation of the ΔΨm, and preservation of the ATP content. These findings demonstrated the protective of miR-21 in TiO_2 NPs-induced neuron cells, providing novel insights into the multiple levels of VDAC1 protein regulation and revealed an endogenous neuroprotective mechanism in response to TiO_2 NPs.

5. Conclusion

TiO_2 is widely used in the processing of food, medicine, and cosmetics. Most products claim to contain TiO_2 NPs free. However, TiO_2 NPs has been found in a variety of products. In the present study, we found that TiO_2 NPs leads to cognitive decline by declining the oxygen consumption rate and ATP level of neurons. Downregulated VDAC1 is responsible for declined energy metabolism induced by TiO_2 NPs. Up-regulation of miR-21-5p may be one of the endogenous protective mechanisms of TiO_2 NPs injury by preserving energy metabolism of neurons. This study provides new evidence for TiO_2 NPs-induced nervous system injury.

6. Declarations

Ethics approval and consent to participate

This study was compliant with the Declaration of Helsinki Guidelines and was approved by the Committee on the Ethics of Animal Experiments of the Tianjin Institute of Environmental and Operational Medicine.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design: Xinxing Wang. Acquisition of data (acquired and managed patients, provided facilities, etc.): Chao Li, Fei Wei, and Xiujie Gao, Xiaoming Wang. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, and computational analysis): Chengfeng Shen, and Tie Han. Writing, review, and/or revision of the manuscript: Chao Li, Xinxing Wang, and Tie Han. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Fei Wei, Chao Li, and Xinxing Wang. Study supervision: Xinxing Wang, Tie Han and Zhaoli Chen.

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Figures

Figure 1

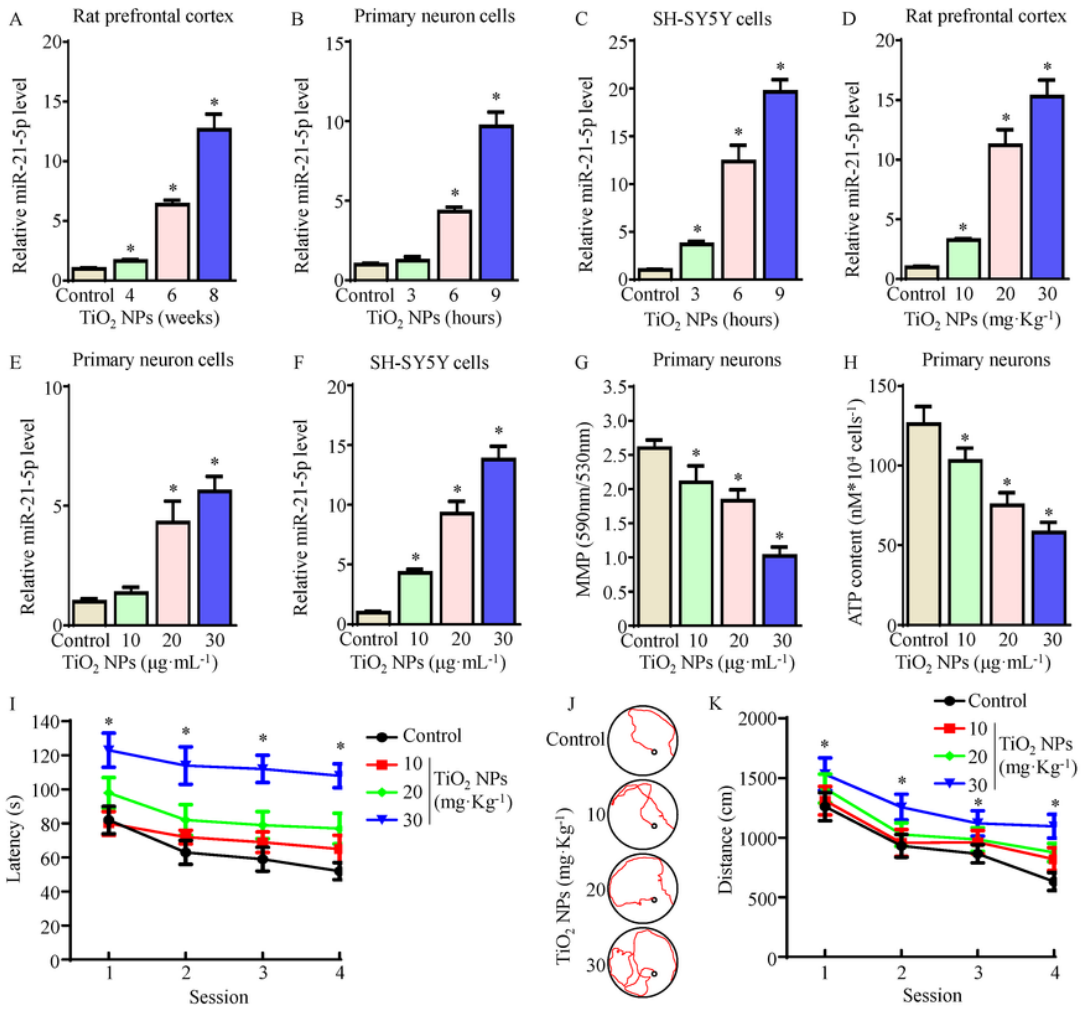

Figure 1

TiO2 NPs led to cognitive deficits, mitochondrial dysfunction, and upregulation of miR-21-5P. (A) Wistar rats (six rats in each group) were exposed to 20 mg·Kg-1 TiO2 NPs for 4, 6, or 8 weeks. The extracts of prefrontal cortex tissue were subjected to RT-PCR to determine miR-21-5P expression. Gapdh was used as an internal reference. (B-C) Primary neuron cells or SH-SY5 cells were seeded into wells of 6-well plates and cultured until the cell density reached 80-90%. The cells were then exposed to 20 μg·mL-1 TiO2 NPs for 3, 6, or 9 hours. The extracts were subjected to RT-PCR to determine miR-21-5P expression. Gapdh/GAPDH was used as an internal reference. (D) Wistar rats (six rats in each group) were exposed to 10, 20, or 30 mg·Kg-1 TiO2 NPs for 8 weeks. The extracts of prefrontal cortex tissue were subjected to RT-PCR to determine miR-21-5P expression. Gapdh was used as an internal reference. (E-F) Primary neuron cells or SH-SY5 cells were exposed to 10, 20, or 30 μg·mL-1 TiO2 NPs for 9 hours. The extracts were subjected to RT-PCR to determine miR-21-5P expression. Gapdh/GAPDH was used as an internal reference. (G) Primary neuron cells were treated with 10, 20, or 30 μg·mL-1 TiO2 NPs for 9 hours. Changes in the mean JC-1 fluorescence ratio (590 nm/530 nm) of cells were detected to indicate the ΔΨm. (H) Primary neuron cells were treated with 10, 20, or 30 μg·mL-1 TiO2 NPs for 9 hours. The cell extracts were

subjected to HPLC to determine the ATP levels. (I-K) Wistar rats (six rats in each group) were exposed to 10, 20, or 30 mg·Kg-1 TiO2 NPs for 8 weeks and sent to water maze test. The symbol (*) indicates a significant increase in comparison with that of the control (P < 0.05).

Figure 2

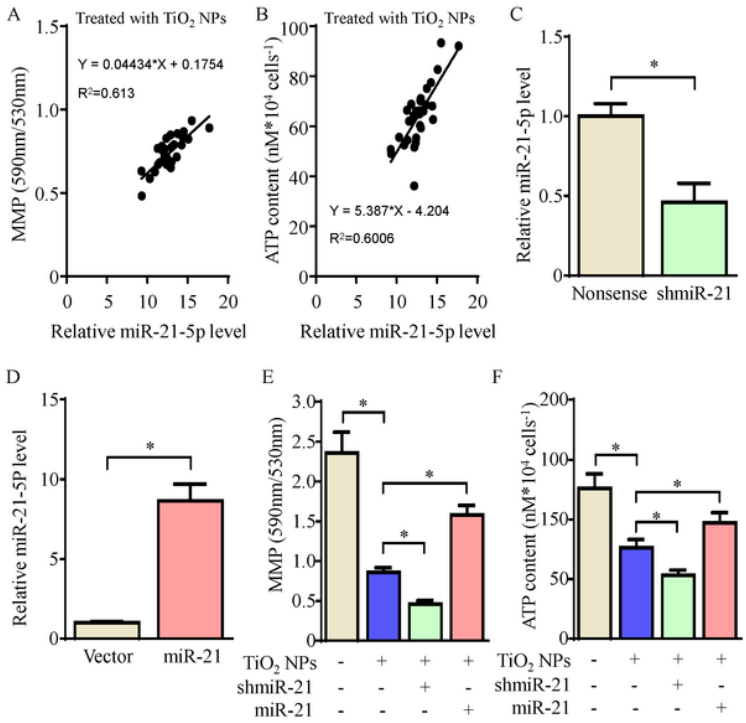

Figure 2

MiR-21-5p preserved the mitochondrial membrane potential (ΔΨm) and ATP levels of SH-SY5Y cells under TiO2 NP treatment. (A) SH-SY5 cells were seeded into wells of 6-well plates and cultured until the cell density reached 80–90%. The cells were then exposed to 20 μg·mL-1 TiO2 NPs for 9 hours. Some of the cells were collected for ΔΨm detection and some for RT-PCR in the same group. (B) SH-SY5 cells were seeded into wells of 6-well plates and cultured until the cell density reached 80–90%. The cells were then exposed to 20 μg·mL-1 TiO2 NPs for 9 hours. Some of the cells were collected for ATP detection and some for RT-PCR in the same group. (C) MiR-21-5p knockdown was identified in SH-SY5 cells (shmiR-21-5p) in comparison with control SH-SY5 cells (Nonsense). The symbol (*) indicates a significant decrease in comparison with that of the nonsense group (P < 0.05). (D) MiR-21-5p overexpression was identified in SH-SY5 cells (miR-21-5p) in comparison with control SH-SY5 cells (Vector). The symbol (*) indicates a significant increase in comparison with that of the vector group (P < 0.05). (E) SH-SY5 cells, SH-SY5 cells

(shmiR-21-5p), or SH-SY5 cells (miR-21-5p) were treated with or without 20 μg·mL-1 TiO2 NPs for 9 hours. The cells were then subjected to ΔΨm detection. The symbol (*) indicates a significant change in comparison between marked groups (P < 0.05). (F) SH-SY5 cells, SH-SY5 cells (shmiR-21-5p), or SH-SY5 cells (miR-21-5p) were treated with or without 20 μg·mL-1 TiO2 NPs for 9 hours. The cells were then subjected to ATP detection. The symbol (*) indicates a significant change in comparison between marked groups (P < 0.05).

Figure 3

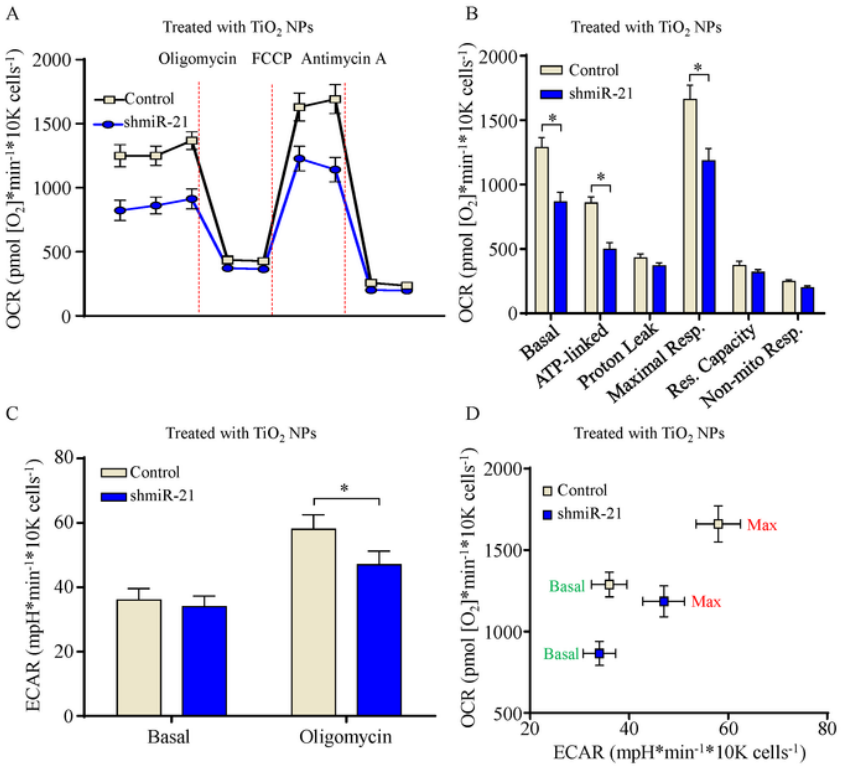

Figure 3

MiR-21-5p knockdown decreased maximal respiration in SH-SY5 cells treated with TiO2 NPs. (A) SH-SY5 cells (Control) or SH-SY5 cells (shmiR-21-5p) were treated with 20 μg·mL-1 TiO2 NPs for 9 hours and then subjected to Seahorse system analysis to determine the oxygen consumption rate (OCR). (B) Quantitative analysis of (A). The symbol (*) indicates a significant decrease in comparison with that of the control (P < 0.05). (C) SH-SY5 cells (Control) or SH-SY5 cells (shmiR-21-5p) were treated with 20 μg·mL-1 TiO2 NPs for 9 hours and then subjected to Seahorse system analysis to determine oligomycin-induced extracellular acidification rate (ECAR). (D) Basal and maximal OCR to ECAR ratios were calculated to

indicate the induction of a response to FCCP in SH-SY5 cells (shmiR-21-5p) compared with that in the control.

Figure 4

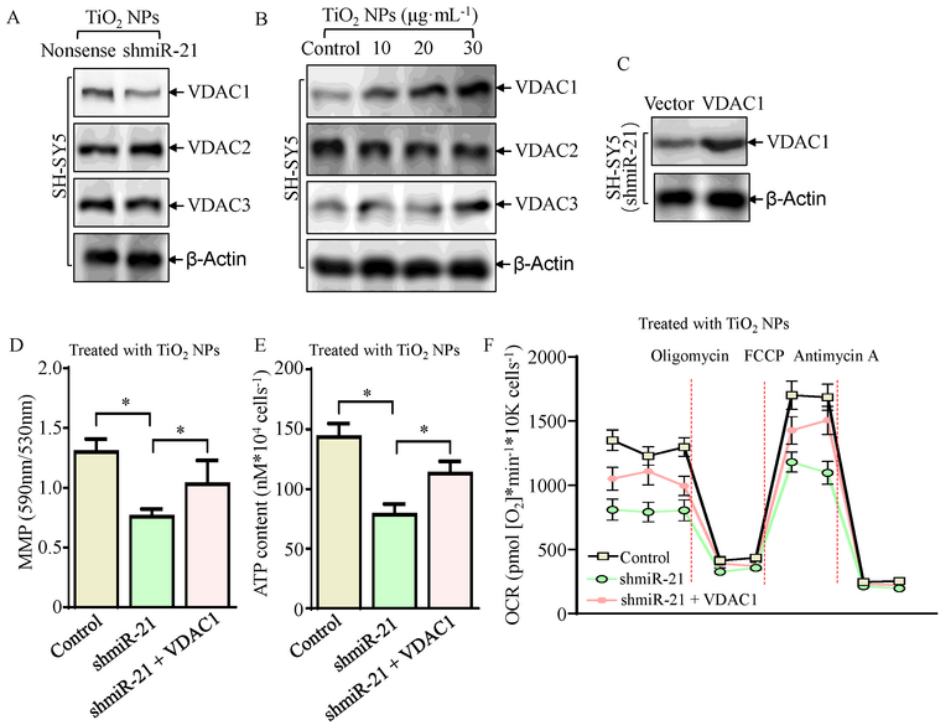

Figure 4

VDAC1 was crucial for miR-21-5p to protect the mitochondria function of TiO2 NP-treated SH-SY5 cells. (A) SH-SY5 cells (Nonsense) and SH-SY5 cells (shmiR-21-5p) were treated with 20 μg·mL-1 TiO2 NPs for 9 hours. The cell extracts were subjected to western blotting to determine the VDAC1, VDAC2, and VDAC3 levels. β-Actin was used as a protein loading control. (B) SH-SY5 cells were exposed to 10, 20, or 30 μg·mL-1 TiO2 NPs for 9 hours. The cell extracts were subjected to western blotting to determine the VDAC1, VDAC2, and VDAC3 levels. β-Actin was used as a protein loading control. (C) VDAC1 overexpression was identified in SH-SY5 cells (shmiR-21, VDAC1) in comparison with control SH-SY5 cells (shmiR-21, vector). (D) SH-SY5 cells (Nonsense), SH-SY5 cells (shmiR-21), and SH-SY5 cells (shmiR-21, VDAC1) were treated with 20 μg·mL-1 TiO2 NPs for 9 hours. Changes in the mean JC-1 fluorescence ratio (590 nm/530 nm) of cells were detected to indicate the ΔΨm levels. (E) SH-SY5 cells (Nonsense), SH-SY5 cells (shmiR-21-5p), and SH-SY5 cells (shmiR-21-5p, VDAC1) were treated with 20 μg·mL-1 TiO2 NPs for 9 hours. The cell extracts were subjected to HPLC to determine the ATP levels. (F) SH-SY5 cells

(Nonsense), SH-SY5 cells (shmiR-21-5p), and SH-SY5 cells (shmiR-21-5p, VDAC1) were treated with 20 μg·mL-1 TiO2 NPs for 9 hours. The cells were analyzed using the Seahorse system to determine the OCR.

Figure 5

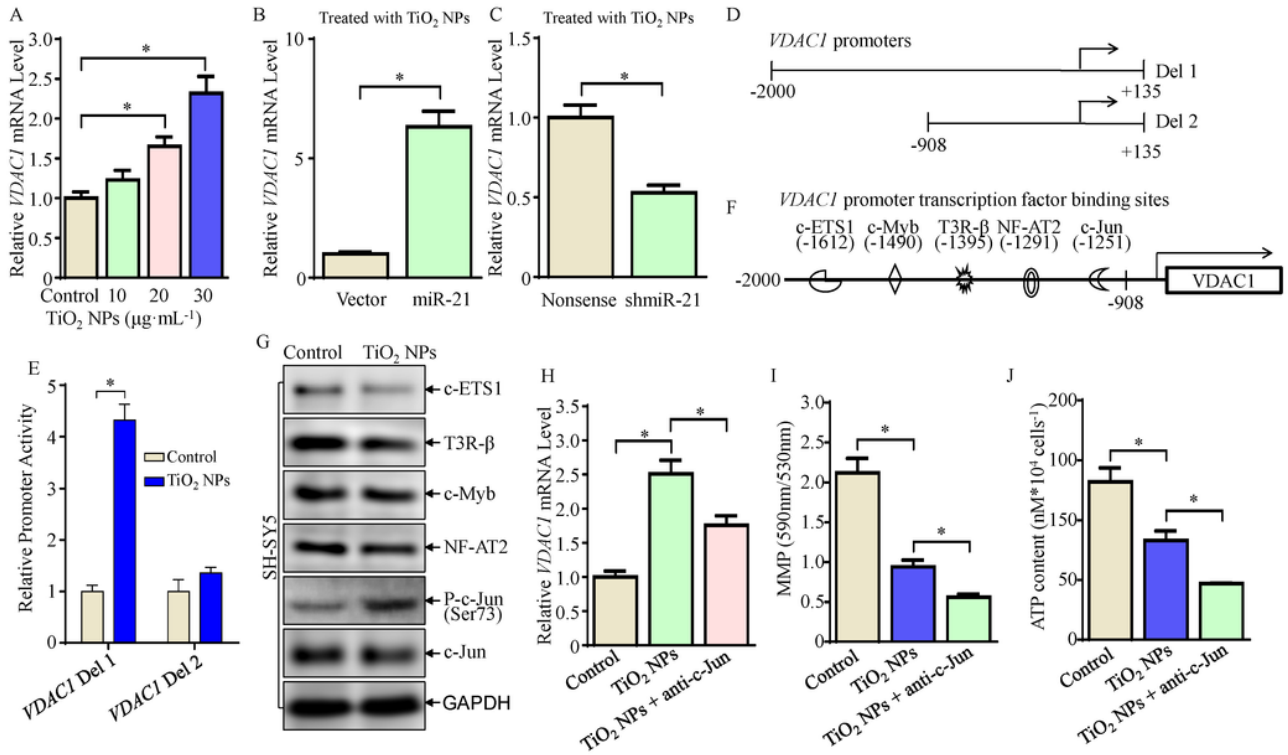

Figure 5

Phosphorylated c-Jun was responsible for upregulation of VDAC1 in TiO2 NP-treated SH-SY5 cells. (A) SH-SY5 cells were exposed to 10, 20, or 30 μg·mL-1 TiO2 NPs for 9 hours. The cell extracts were subjected to RT-PCR to determine VDAC1 mRNA expression. GAPDH was used as an internal reference. (B) SH-SY5 cells (Vector) and SH-SY5 cells (miR-21-5p) were treated with 20 μg·mL-1 TiO2 NPs for 9 hours. The cell extracts were subjected to RT-PCR to determine VDAC1 mRNA expression. GAPDH was used as an internal reference. (C) SH-SY5 cells (Nonsense) and SH-SY5 cells (shmiR-21-5p) were treated with 20 μg·mL-1 TiO2 NPs for 9 hours. The cell extracts were subjected to RT-PCR to determine VDAC1 mRNA expression. GAPDH was used as an internal reference. The symbol (*) indicates a significant increase in comparison with that of the control ($P < 0.05$). (D) Schematic illustration of the construction of the VDAC1 promoter-driven luciferase reporter constructs. (E) The VDAC1 promoter-driven luciferase reporter constructs co-transfected with TK into H9c2 cells, with or without exposure to 20 μg·mL-1 TiO2 NPs for 9 hours, respectively. The luciferase activity of the transfectants was evaluated after 24 h, and

the results are presented as relative VDAC1 promoter activity by normalization to TK and pGL3, respectively. The symbol (*) indicates a significant increase in comparison with the that of the control ($P < 0.05$). (F) Potential transcription factor binding sites in the VDAC1 promoter region (-2000 ~ -908) were analyzed using the TRANSFAC 8.3 engine online. (G) SH-SY5 cells were treated with 20 μg·mL-1 TiO_2 NPs for 9 hours. The cell extracts were analyzed for the levels of potential transcription factors of VDAC1 using western blotting. (H-J) SH-SY5 cells and SH-SY5 cells (anti-c-Jun) were treated with 1% O_2 for 6 hours. The cell extracts were analyzed for the levels of VDAC1 mRNA and ATP content. Changes in the mean JC-1 fluorescence ratio (590 nm/530 nm) of cells were detected to indicate the $\Delta\Psi m$ levels.

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